Improvement of cellulase production in cultures of *Acremonium cellulolyticus* using pretreated waste milk pack with cellulase targeting for biorefinery

著者	Park Enoch Y., Naruse Kazuya, Kato Tatsuya
journal or publication title	Bioresource Technology
volume	102
number	10
page range	6120-6127
year	2011-05
出版者	Elsevier
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URL	http://hdl.handle.net/10297/5803

doi: 10.1016/j.biortech.2011.02.063

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2 **Improvement of cellulase production in cultures of**

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4 **with cellulase targeting for biorefinery**

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ABSTRACT

Cellulase production in cultures of *Acremonium cellulolyticus* was significantly improved by using waste milk pack (MP) that had been pretreated with cellulase. When MP cellulose pretreated with cellulase (3 FPU/g MP) for 12 h was used as the sole carbon source for *A. cellulolyticus* culture in a 3-L fermentor, the cellulase activity was 16 FPU/ml. This was 25-fold higher (0.67 FPU/ml) compared with untreated MP cellulose and was comparable to that achieved with pure cellulose (Solka Floc). As the pretreatment progressed, roughness on the surface of untreated MP cellulose became to be smooth, but development of fissures on the surface of pretreated MP cellulose was observed. Cellulase pretreatment of MP increased both the accessibility of *A. cellulolyticus* to the surface and number of adsorption sites of cellulase on the surface of MP cellulose, leading to improved cellulase production in the *A. cellulolyticus*.

Keywords: Waste milk pack, Cellulase; *Acremonium cellulolyticus*; Waste paper; Biorefinery

1. Introduction

Cellulase production is the most important step in the commercial production of ethanol and other chemicals from renewable cellulosic materials. To date, many potential cellulase producers have been isolated (Atanasova et al., 2010) and used to produce cellulase (Fujii et al., 2009) in submerged culture processes. However, cellulase producers require the relatively expensive cellulose powder as the sole carbon source, and this hinders the industrial application of cellulase in bioconversion processes. To overcome this problem, the use of waste agro-biomass as a carbon source for the production of cellulase has been suggested due to its lower cost (Krishna C, 1999; de Lima et al., 2005; Sukumaran et al., 2009). Agricultural residues present in abundance, such as corn stover (Yao et al., 2010), wheat straw (Emtiazi and Nahvi, 2000), rice straw (Sun et al., 2008), bagasse (Adsul et al., 2004), and lignocellulose (Hendriks et al., 2009), have been used for cellulase production. These raw materials are cheaper, but pretreatment is generally required to improve their utilizability as carbon sources for the cellulase producer, which increases the costs considerably. The cellulolytic biomass has to be hydrolyzed to reducing sugars by cellulolytic enzymes or acids. Cost-efficient acids can be used as hydrolysis agents, but these are not environmentally friendly because the process requires high temperatures and the disposal of acid wastes is a problem. Thus, improving the process to produce cost-efficient cellulolytic enzymes from microorganisms is an important objective in the biorefining of cellulosic biomass.

Waste paper is a cellulolytic biomass that has been targeted for recycling. In Japan, approximately 30.6 million tons of paper is produced and consumed each year. The importance given to recycling has resulted in increased public awareness such that in

2008, 75.1% of the annual paper production was collected and 61.8% was reused (<http://www.jpap.gr.jp/states/used-paper/index.html>). When paper materials are recycled, they are usually converted into lower grade paper products, for example, office paper is converted to magazine paper and cardboard is converted to sanitary products. As the paper is recycled further, the length of the cellulose fibers decrease. This shortening of cellulose fibers reduces the paper quality; therefore, the maximum ratio of paper-to-paper recycling is considered to be 65%. Thirty-five percent of all paper is not fit for recycling and is disposed of as paper sludge, which is incinerated or landfilled without reuse.

One of the aggressive utilizations of waste paper and paper sludge is to use it as an alternative carbon source for cellulase production. Waste paper is a good carbon source for cellulase production by microorganisms because waste paper is composed of delignified biomass. Toyama et al. (2002) tried to isolate cellulase hyperproducers of *Trichoderma reesei* for the utilization of waste paper. They used 1% (w/v) paper powder medium for the 1st screening and found that the hydrolytic activity of the selected strain was 3.5-fold higher than that of the original strain. Waste paper hydrolysate was also reported to induce cellulase in cultures of *T. reesei* (Chen and Wayman, 1991). Ju and Afolabi (1999) showed that the enzymatic hydrolysate of waste paper induced cellulase activity in a continuous culture of *T. reesei*, and they attributed this to the higher concentrations of oligomer-inducing intermediates in the waste paper hydrolysate. Similarly, paper sludge is a good carbon source for cellulase production. Paper mill sludge was used for cellulase production with mixed cultures of *T. reesei* and *Aspergillus niger* (Maheshwari et al., 1994). However, cellulase production from paper sludge was low compared to that obtained when pure cellulose was used as the carbon

source. The reason for this was that paper sludge contains impurities such as clay and several kinds of metal ions. To improve the cellulase production rate, the paper sludge was pretreated with ammonium hydroxide or hydrogen peroxide. This resulted in the production of 2.4 FPU/ml of cellulase from the *T. reesei* culture, which was half of the value obtained when steam-exploded wood was used as the substrate (Shin et al., 2000). Prasetyo et al. (2010b) optimized culture condition in the culture of *A. cellulolyticus* using untreated waste paper sludge as the carbon source, and achieved 9.31 FPU/ml of cellulase activity at the flask scale and 10.96 FPU/ml in a 3-L fermentor. However, cellulase production from paper sludge was only approximately 60% of that obtained when pure cellulose was used as the carbon source (Ikeda et al., 2007).

In Japan, about 251,000 tons of paper packs were used as containers for food, beverages, and milk in 2008, which accounted for 0.7% of the total paper consumption (<http://www.yokankyo.jp/cat02.html>). Paper packs are made from conifers and coated with polyethylene film, and 70% of these are used as milk containers. Due to their good safety, light weight, easy handling, and renewability, the usage of paper pack has increased every year by approximately 1%. In Japan, the recycling ratio of paper packs has increased every year and was reportedly 32% in 2008 (<http://www.yokankyo.jp/cat02.html>). Eighty-five percent of the recovered paper packs was reused as sanitary products, despite the fact that this material was composed of high-quality cellulose. One way to reuse paper packs is to produce cellulase for the saccharification of cellulosic biomass to reducing sugars. These sugars can then be converted (for example, by fermentation) to value-added bioproducts such as ethanol or other chemicals (Scott et al., 1994; Wayman et al., 1993).

Filamentous fungi, typically *Trichoderma reesei*, have been used for industrial cellulase production due to their ability to produce extracellular protein in high amounts. Unfortunately, the amount of β -glucosidase secreted by *T. reesei* is insufficient for an efficient saccharification (Sternberg et al., 1977). *Acremonium cellulolyticus*, which was isolated in 1987 (Yamanobe et al., 1987) and subsequently engineered to enhance its performance, produces both cellulase and β -glucosidase in addition to carbomethyl cellulose-hydrolyzing enzyme (CMCase) and small amounts of xylanase, β -1,3-glucanase, and amylase. This microorganism has been used as an alternative cellulase producer (Ikeda et al., 2007; Park et al., 2002; Prasetyo et al., 2010a). The *A. cellulolyticus* strain uses Solka Floc (SF), which is composed of 100% cellulose and contains 70%–80% crystalline cellulose and 20%–30% amorphous cellulose, as the carbon source for cellulase production. However, SF is an expensive carbon source, and this is an obstacle in the industrial production of cellulase from *A. cellulolyticus*.

There are no specific reports on cellulase production from MP cellulose. Waste MP is very clean, pure, and delignified cellulose, but it has been reused only as a sanitary product. In this study, we developed a method for utilizing MP cellulose as a carbon source for producing cellulase from *A. cellulolyticus*, which is a value-added bioproduct. MP was first pretreated with cellulase and then used as the carbon source for an *A. cellulolyticus* culture. This pretreated MP cellulose improved significantly the production of cellulase, which was similar to the cellulase activity level obtained with SF as the carbon source. The physicochemical and morphological changes of cellulase-pretreated MP were investigated.

2. Materials and Methods

2.1. Cellulose materials used

Ten types of cellulose materials (cellulose type I) from different manufacturers were used in this study to investigate cellulase production (Table 1). Waste MP was collected from local supermarkets in Shizuoka city (Japan). MP is made from conifers and coated with polyethylene film. SF (CAS #9004-34-6; International Fiber Corp., New York, USA) was used as the carbon source for the preculture of *A. cellulolyticus*. SF is a fine white powder that is used as an industrial filtration material. It is composed of 100% cellulose, of which approximately 70%–80% is crystalline cellulose and 20%–30% is amorphous cellulose.

2.2. Pretreatment of MP with cellulase

Collected waste MP was autoclaved at 121°C for 20 min in distilled water, and the polyethylene films were peeled off. The material was then dried at 60°C in a dry oven for 2 d. Dried MP (water content, 10% (w/v)) was crushed to prepare cellulose powder using the Crusher system (WB-1, Osaka Chem. Co. Ltd., Osaka, Japan) at 25000 rpm for 10 s. MPs were pretreated in both shake flasks and a 3-L fermentor. In the flask-scale prepretreatments, sterilized 500-ml Erlenmeyer flasks containing 50 ml of medium (consisting of 5 g/l KH_2PO_4 (pH 4.5) and 2.5 g MP cellulose powder) were incubated at 35°C. Pretreatment of the substrate was initiated by the addition of Acremozyme (Meiji Seika Kaisha, Ltd., Tokyo). The amount of enzyme used (3–15 FPU/g substrate) and pretreatment time (4–20 h) were decided in advance. Reactions were conducted using a reciprocal shaker at 120 strokes per min (spm) at 35°C. After the reaction, the reaction mixture was autoclaved at 121°C for 20 min to deactivate

cellulase and then filtered through filter paper (Whatman No. 1). The filter cake (pretreated MP cellulose) was washed twice with distilled water and dried at 60°C for 2 d. The dried pretreated MP cellulose was used as the sole carbon source for *A. cellulolyticus* cultures to produce cellulase. In the 3-L fermentor system, 75 g of MP cellulose powder was added to 1500 ml of medium containing 5 g/l of KH₂PO₄ (pH 4.5), and this was incubated at 35°C and 600 rpm. Pretreatment of MP was initiated by the addition of cellulase. The amounts of enzyme used were 3 and 15 FPU/g MP cellulose, and the pretreatment times were 12 and 6 h, respectively. The procedures after the pretreatment step were the same as those used in the shake-flask scale pretreatments.

2.3. Microorganisms and media

The microorganism used in this study was *A. cellulolyticus* C-1 (Ferm P-18508) provided by Tsukishima Kikai Co. Ltd. (Tokyo). This is a hyperproducer mutant of the original strain *A. cellulolyticus* Y-94 (Yamanobe et al., 1987). *A. cellulolyticus* produces crystalline cellulose-hydrolyzing enzyme (FPase), β -glucosidase, and CMCase. It also produces various types of other polysaccharide-hydrolyzing enzymes such as xylanase, β -1,3-glucanase, and amylase (Nihira et al., 2001; 2003). The strain was seeded on potato dextrose agar slants and kept at 30°C for 3–5 d until the colonies had grown. The colonies on dextrose agar slant was then stored at 4°C in a refrigerator. The colour of the colonies was brown to reddish brown, and they had short white hyphae without spores. Prior to cultivation, *A. cellulolyticus* was seeded on SF agar slants, incubated at 28°C for 3.5 days until the colonies had grown, and then stored at 4°C. The preculture medium contained 40 g/l SF powder, 24 g/l KH₂PO₄, 1 ml/l Tween 80, 5 g/l (NH₄)₂SO₄, 4.7 g/l potassium tartrate, 1.2 g/l MgSO₄·7H₂O, 0.01 g/l ZnSO₄·7H₂O, 0.01

g/l $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$, 0.01 g/l $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, and 2 g/l urea (pH 4.5). The cellulase production medium contained 50 g/l MP cellulose, 24 g/l KH_2PO_4 , 1 ml/l Tween 80, 5 g/l $(\text{NH}_4)_2\text{SO}_4$, 4.7 g/l potassium tartrate, 1.2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/l $\text{MnSO}_4 \cdot 6\text{H}_2\text{O}$, 0.01 g/l $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$, and 4 g/l urea (pH 4.5).

2.4. Cellulase production in flask and fermentor cultures

In the flask cultures, 4 colonies were inoculated in a 500-ml Erlenmeyer flask containing 50 ml preculture medium. The preculture was incubated for 60 h at 28°C and 220 rpm in a rotary shaker (TB50R, Takasaki Kagaku Co., Kawaguchi, Japan). Cellulase production in flasks was initiated by adding 2.5 ml (5%) of the preculture to a 500-ml Erlenmeyer flask containing 50 ml of production medium. The cultures were incubated for 5 days at 28°C and 220 rpm on a rotary shaker (Takasaki Kagaku). Cultures were triplicated and average values were presented with standard deviation.

Cellulase was also produced in a 3-L jar fermentor equipped with Labo-controller (MDL-80, Marubishi, Tokyo Japan). The working volume was 1.5 L. Distilled water was used to dissolve all the components of the production medium, and the system was sterilized at 121°C for 20 min. An appropriate amount of urea was added to 50 ml of distilled water, and the solution was filtered through 0.45- μm filter. Urea was added to the fermentor prior to inoculation. Inoculum (150 ml) was added to the fermentor that contained 1350 ml of production medium. The broth was cultured at 28°C, and the pH value was controlled at 4.5 using 2 N NaOH and 2 N H_2SO_4 . Air was supplied at 1.5 volume of air per volume of medium per minute (vvm) at an agitation rate of 500 rpm. An antifoam agent (Silicone 72, KM-70, Shin-Etsu Chemical Co. Ltd.,

Japan) was added in small amounts as required. Each experiment was carried out in duplicate; the data are presented as averages.

2.5. Analytical methods

The total cellulase activity was determined by the standard IUPAC procedure using Whatman No. 1 filter paper, and the activity was expressed in filter paper units (FPU) (Ghose, 1987). The FPU unit is based on the International Unit (IU), in which the absolute amount of glucose in the FPU assay at a critical dilution is 2 mg for 0.5 ml of a critical enzyme concentration in 60 min. The protein concentration in the supernatant was determined by the Lowry method using a spectrophotometer (UV-1800, Shimadzu, Kyoto). The concentration of reducing sugars in the medium was determined by the dinitrosalicylic acid (DNS) method. Due to the difficulty in separating the mycelium from the medium, the cell weight was measured by the inner nucleic acid (INA) method, and the dry cell weight (DCW) was calculated by the following two equations: $\text{INA concentration (g/l)} = 1.72 \times \text{absorbance}$ and $\text{DCW (g/l)} = 16.565 \times \text{INA}$ (Ikeda et al., 2007). The absorbance was measured spectrophotometrically at a wavelength of 260 nm.

2.6. Degree of polymerization (DP) and crystallinity of cellulose

The DP of cellulose was determined by the viscosity method (Sobue and Migita, 1958) based on the following relationship: $\text{DP} = [\eta]/162$, where 162 is molecular weight of D-glucose anhydrous and $[\eta]$ is the intrinsic viscosity of the solution. The intrinsic viscosity was calculated by interpolation using the specific viscosity and

concentration. All experiments were performed at 25°C using an Ostwald capillary viscometer and ethyl acetate as the solvent.

Cellulose crystallinity was determined by X-ray diffractometry (Rigaku AFC-6R, Tokyo, Japan). Samples were scanned from $2\theta = 2^\circ$ to 70° with a step size of 0.01° . The determination time was 1 s/ 0.02° . The crystallinity index (CI) was defined as follows (Segal et al., 1959):

$$CI = (I_{22.5} - I_{18.5})/I_{22.5} \times 100$$

where $I_{22.5}$ and $I_{18.5}$ are the intensities of diffraction at $2\theta = 22.5^\circ$ and $2\theta = 18.5^\circ$, respectively.

2.7. Length and width of pretreated MP cellulose fibers

The length and width of the fibers in the MS cellulose powder were measured using the Fiber Quality Analyzer system (FQA, OpTest Equip. Inc. Hawkesbury, ON, Canada). The fiber length and fiber width were in the ranges of 0.07–10 mm and 0.2–10 mm, respectively. Fibers with widths in the range of 0.07–0.2 mm were not considered in the width measurements.

2.8. Scanning electron microscopy (SEM)

The MP samples were oven-dried at 60°C overnight. They were placed on black tape, coated with gold, and observed under a scanning electron microscope (JEOL, JSM-6300, Tokyo, Japan) with a JX A-6400 system (ADS + OM). Images were recorded at 3 different magnifications (50×, 2000×, and 20000×).

3. Results and discussion

3.1. Effect of the degree of polymerization (DP) and crystallinity of cellulose on cellulase production

The DP and CI of 10 different types of cellulose were measured before use. The DP varied in the range 70–850, while the CI varied in the range 0.59–0.91 (Fig. 1A and B). Cellulase production was examined in flask cultures. The cellulase activity increased up to a DP of 400 but decreased when the DP was higher than 400 (Fig. 1A). In the case of CI, the cellulase activity was high when cellulose with low CI was used, but the activity was low when the CI was higher than 0.75 (Fig. 1B). This indicates that the DP and CI of cellulose affected cellulase production in the *A. cellulolyticus* culture. SF was the best carbon source for *A. cellulolyticus* culture. The use of cellulose of vegetable origin as the carbon source did not induce cellulase production even though the CI (0.63) and DP (below 100) were low. Celluloses (nos. 4 and 6 in Fig. 1 and Table 1) with high CI and low DP produced cellulase, but the values were less than 50% of those obtained with SF. Pulps (nos. 9 and 10 in Fig. 1 and Table 1) from conifers (MP) and hardwood with high CI and high DP showed significantly different cellulase activity. Hardwood pulp produced 4 FPU/ml of cellulase, but MP could not produce cellulase in *A. cellulolyticus* culture, even though both materials contain a similar type of cellulose. The DP and CI of hardwood pulp were 850 and 0.87, respectively, while the corresponding values of MP (conifer pulp) were 700 and 0.8, respectively (Fig. 1A and B).

3.2. Pretreatment of MP with cellulase

The DP and CI were measured after pretreatment of MP cellulose with cellulase (15 FPU/g MP). The CI of untreated MP cellulose was 0.75, but the value increased proportionally with the pretreatment time. After pretreatment for 10 h, the CI reached 0.82 (Fig. 2). This reflects the fact that cellulase hydrolysed amorphous cellulose faster than crystalline cellulose. On the other hand, the DP of untreated MP cellulose was 700, but this decreased to 550 after pretreatment for 10 h (Fig. 2). This indicates that MP cellulose was degraded as the hydrolysis by cellulase progressed.

In our investigations, the DP of MP cellulose was similar to that of SF, but the CI increased; these changes might not induce cellulase production. In the case of saccharification by cellulase, the hydrolytic yield was proportional to the DP, but decreased when the CI was higher than 0.8 (data not shown). With regard to cellulase production, we did not find an exact correlation between DP and cellulase production or between CI and cellulase production.

3.3. Length and width of the pretreated MP cellulose fibers

After pretreating MP cellulose with cellulase (15 FPU/g MP) for 0–10 h, the width and length of the pretreated MP cellulose fibers were measured, as shown in Fig. 3. There was no significant change in the fiber width from 0 h to 8 h of pretreatment (Fig. 3A), but the fiber length gradually decreased (Fig. 3B). The distribution of the fiber length after 0 and 4 h of pretreatment was similar, but after 8 h of pretreatment, the distribution shifted to a shorter range. Based on these data, the average width and length of the fiber were calculated (Fig. 4). The fiber width was in the range 24 μm –27 μm and was unaffected by cellulase pretreatment (Fig. 4A) even when the pretreatment time was extended to 24 h (data not shown). On the other hand, the average fiber length

decreased gradually from 1.9 mm to 0.8 mm as the pretreatment time increased from 4 h to 6 h, and it finally reached 0.4 mm after pretreatment for 10 h (Fig. 4B). Thus, the fiber width was unaffected by cellulase pretreatment, but the fiber length decreased by one-fourth.

With respect to the fiber width, fibers longer than 0.07 mm and shorter than 0.2 mm were excluded from the fiber width measurement. If small fibers included from this measurement present during pretreatment, the specific surface area of MP cellulose must be increased further, which might increase the accessibility of *A. cellulolyticus* to the surface of MP cellulose.

3.4. Topological changes in MP cellulose

Because the width of MP cellulose fibers was unchanged even after pretreatment with cellulase, we examined the surface changes in MP cellulose. For this purpose, SEM images of pretreated MP cellulose were recorded. The length of untreated cellulose fibers was long, but decreased and shortened with the progress of pretreatment time (supplementary file 1A). The untreated sample showed the roughness of the surface of MP cellulose (0 h in supplementary file 1B), but the surface became gradually to be smooth as the cellulase pretreatment time increased (6 h and 10 h in supplementary file 1B). After 6 and 10 h of pretreatment, fissures were observed on the surface of MP cellulose fiber (6 h and 10 h in supplementary file 1C). The fissures were in the range of 10-200 nm in width from SEM pictures (10 h in supplementary file 1C). Development of fissures on the surface of pretreated MP cellulose also increased the accessibility of *A. cellulolyticus* to the surface of MP cellulose. We hypothesize that this might be one of the reasons for the improved cellulase production.

3.5. Effect of cellulase pretreatment of MP cellulose on cellulase production in *A. cellulolyticus* cultures

MP cellulose pretreated with cellulase (15 FPU/g MP) was used as the carbon source for *A. cellulolyticus* cultures in flasks. When untreated MP cellulose was used, the cellulase activity after 96 h of culture was only 0.19 FPU/ml, but when cellulase-pretreated MP cellulose was used, the cellulase production increased significantly. The activities in 96-h cultures using MP celluloses pretreated for 6 h, 8 h, and 10 h were 13.3, 14.0, and 12.9 FPU/ml, respectively (Fig. 5). The specific cellulase activity significantly improved upon pretreatment of MP cellulose, and it increased from 0.01 FPU/mg protein in untreated MP to 0.60 FPU/mg protein after 8 h of pretreatment. When MP cellulose was pretreated with cellulase for 8 h and used as the carbon source in the *A. cellulolyticus* culture, the cellulase activity and specific activity dramatically improved and were found to be 74-fold and 60-fold higher, respectively, than those of untreated MP cellulose. After cellulase pretreatment of MP cellulose, the MP cellulose hydrolysate contained glucose, xylose, arabinose, and cellobiose. When these hydrolysates were used as the carbon source, cellulase activity was not detected (data not shown). Therefore, monosaccharides from the hydrolysis of MP cellulose did not induce cellulase in the culture of *A. cellulolyticus*.

The effect of pretreatment of MP cellulose with cellulase was confirmed in a 3-L fermentor. When MP cellulose was pretreated with cellulase (3 FPU/g MP) for 12 h, the cellulase activity increased to 16.0 FPU/ml with a specific activity of 0.67 FPU/mg protein after a culture time of 84 h (Fig. 6A and D). When pretreatment was carried out with 15 FPU/g MP for 6 h, the maximum cellulase activity was 12.6 FPU/ml after 96 h

of culture. However, when untreated MP cellulose was used as the carbon source in the culture, the cellulase activity was 0.73 FPU/ml with a specific activity of 0.11 FPU/mg protein after 96 h of culture (Fig. 6A and D). The cellulase production rates increased to 4.56 and 3.60 FPU/ml/d, respectively, after pretreatment of MP with cellulase. The pretreated conditions were 3 FPU/g MP for 12 h and 15 FPU/g MP for 6 h. These results were 20-fold and 25-fold higher than those obtained with untreated MP cellulose. The reason why cellulase production was low when untreated MP cellulose was used might be due to difficulties in cell growth. Until 60 h of culture, the dry cell weight of *A. cellulolyticus* was almost zero (Fig. 6B). The protein concentrations obtained with pretreated MP celluloses were higher than 20 g/l, but when untreated MP cellulose was used, the value was 6 g/l (Fig. 6C).

Similarly, newspaper, office paper, and hardwood pulp were pretreated with cellulase (15 FPU/g sample) for 6 h and used as the carbon source. In the case of newspaper and office paper, the cellulase activity increased by 1.7-fold and 1.3-fold, respectively, in comparison with those of the untreated samples (Table 2). However, in the case of hardwood pulp, the cellulase activity decreased drastically. Cell growth in a culture containing pretreated hardwood pulp was similar to that in the untreated material, and the protein concentration was almost 2-fold higher than that of the untreated material (data not shown). This suggests that cellulase-inducing components of hardwood pulp might be removed by the pretreatment process.

Our results showed that it was possible to significantly improve cellulase production by pretreating MP with cellulase. Using 3 FPU/g MP of cellulase, we obtained 16 FPU/ml of cellulase in 84 h. Using SF as the carbon source, the maximum

cellulase activity was 17.4 FPU/ml (Ikeda et al., 2007). Therefore, the cellulase activity from waste MP was comparable to that obtained from pure cellulose (SF). When 50 g MP cellulose/l was used as the carbon source, 150 FPU of cellulase/l was required for pretreatment, but 1.6×10^4 FPU of cellulase was produced (107 fold). When 15 FPU/g MP was used, 750 FPU of cellulase was used for pretreatment and 1.3×10^4 FPU was produced (17 fold). Therefore, when less cellulase is used for the pretreatment of MP cellulose, the cellulase production efficiency is higher.

In this study, the reason why cellulase production was improved by pretreatment with cellulase is not known, but following factors are considered. The types of cellulose crystals present may be important for cellulase production from *A. cellulolyticus*. Enzymatic hydrolysis of cellulose II proceeds much faster than that of cellulose I (Wada et al., 2010); thus, the hydrolysis of cellulose I can be accelerated via its conversion to cellulose II. However, with respect to cellulase production from *A. cellulolyticus* culture, there was no definite correlation between cellulase activity and cellulose I or II. In our experience, In the case of cellulose I, cellulose I produced cellulase from the *A. cellulolyticus* culture (Fig. 1) but cellulose II did not (data not shown). Transition of the cellulose type during pretreatment of office paper with cellulase was measured by X-ray diffractometry analysis, and no transition from cellulose I to cellulose II or from cellulose II to cellulose I was observed (data not shown). This indicates that pretreatment with cellulase did not change the crystal structure.

One possible explanation is that it depends on the accessibility of *A. cellulolyticus* to cellulose because it is expected that *A. cellulolyticus* mycelia would access the cellulose surface and induce cellulase to assimilate cellulose and release glucose, which is

required for growth. Pretreatment of MP cellulose with cellulase caused to development of fissures on the surface of MP, which increased the accessibility of *A. cellulolyticus* mycelia to the surface of MP cellulose, leading to cellulase induction for cell growth and improved cellulase production. In several studies on *T. reesei*, it was reported that crystalline cellulose may have possible adsorption sites for cellulases (Henrissat et al., 1988; Jamal et al., 2004). Similarly, pretreatment of MP by cellulase may increase the number of possible adsorption sites of cellulases on the MP cellulose surface, leading to increased cellulase production in *A. cellulolyticus*. Thus, the results of this study showed that cellulase could be produced from MP cellulose in cultures of *A. cellulolyticus* and that pretreatment with cellulase resulted in improved cellulase production, its levels of 16 FPU/ml, similar to those obtained with pure cellulose.

4. Conclusions

Cellulase production is a key step in biorefining in the production of second-generation bioethanol as an alternative fuel (Santos et al., 2010). When *A. cellulolyticus* was cultured in pretreated MP cellulose with 3 FPU/g MP for 12 h, the cellulase activity significantly increased to 16 FPU/ml in a 3-L fermentor. This was 25-fold higher than the activity achieved with untreated MP cellulose. This result would be useful to bioconvert MP cellulose to the high-value product cellulase because cellulase production is a key step in biorefining, in the application of simultaneous saccharification and fermentation to generate ethanol fuel.

Acknowledgements

We thank Mr. Hiroyuki Fukazawa of Shizuoka Industrial Research Institute for measuring the length and width of the pretreated MP cellulose fibers. We also thank Dr.

Vipin Kumar Deo of Shizuoka University for manuscript editing. This study was supported by a Comprehensive Support Programs for Creation of Regional Innovation in Japan Science and Technology Agency.

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481 production for bioconversion of corn stover – Feasibility evaluation. Bioresource
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483

484 Table 1. Used celluloses for cellulase production in the culture of *A. cellulolyticus* in
485 flask

No.	Origin	Manufacturer	Note
1	Vegetable	Fluka Chemie, Buchs, Switzerland	Microcrystalline for chromatography
2	Not specified	Sigma-Aldrich Co., St. Louis, MO, USA	Microcrystalline
3	Not specified	Alfa Aesar, Karlsruhe, Germany	Microcrystalline
4	Filter paper	Advantec Toyo Kaisha, Ltd., Tokyo, Japan	Microcrystalline
5	Not specified	Merck & Co., Inc., Whitehouse St., NJ, USA	Microcrystalline for chromatography
6	Cotton	Funakoshi Co., Tokyo, Japan	Microcrystalline for thin layer chromatography
7	Pulp	Nacalai Tesque, Inc., Kyoto, Japan	Cellulose powder of a and b cellulose
8	Not specified	International Fiber Co., New York, USA	Fine white powder used for an industrial filtration material
9	Conifers pulp	Collected at super markets (Shizuoka, Japan)	Milk pack
10	Hardwood pulp	Provided by Tomoegawa Co., Ltd., Tokyo, Japan	Lignin removed pulp
11	Glucose	Wako Pure Chem. Ind. Ltd., Osaka, Japan	Powder

486

Table 2. Comparison of cellulase production with untreated and pretreated newspaper and office paper in the culture of *A. cellulolyticus* in flask

Cellulose	Activity ^a	Untreated sample	Pretreated sample with cellulase ^b
Newspaper	Cellulase activity (FPU/ml)	1.05	1.86
	Specific activity (FPU/mg protein)	0.26	0.34
Office paper	Cellulase activity (FPU/ml)	4.35	5.68
	Specific activity (FPU/mg protein)	0.46	0.65
Hardwood pulp	Cellulase activity (FPU/ml)	3.20	0.10
	Specific activity (FPU/mg protein)	0.43	9.10×10^{-3}

^a Activity was measured at 96 h culture.

^b Sample was pretreated with 15 FPU/g sample of cellulase for 6 h.

Legends for figures

Fig. 1. Effect of DP (A) and CI (B) on cellulase production in the culture of *A.*

cellulolyticus. Ten types of cellulose were used for measurement of DP and CI. All cultures were carried out in flask for 96 h. Numbers beside symbols indicate sample number shown in Table 1. Bars indicate standard deviation of three different flask experiments. Dotted lines show trend of effect of DP and CI on cellulase production.

Fig. 2. DP and CI of pretreated MP cellulose with cellulase. MP cellulose was pretreated with 15 FPU/g MP cellulose of cellulase for 0-10 h. Symbols: Closed triangles and dotted line, CI; closed circle and bold line, DP.

Fig. 3. Distributions of width (A) and length (B) of pretreated MP cellulose fibers. The MP cellulose was pretreated with 15 FPU/g MP cellulose of cellulase for 0 h, 4 h and 8 h. Cellulose fibers in the ranges of 0.07-10 mm in length and in the range of 0.2-10 mm in width were measured. Fibers, longer than 0.07 mm and shorter than 0.2 mm were excluded in this measurement.

Fig. 4. Average width (A) and length (B) of pretreated MP cellulose fibers. The MP cellulose was pretreated with 15 FPU/g MP cellulose of cellulase for 0 -10 h.

Fig. 5. Effect of pretreated MP cellulose with cellulase on cellulase production in the culture of *A. cellulolyticus* in flask for 96 h. The MP cellulose was pretreated with 15 FPU/g MP cellulose of cellulase for 6 h. Symbols: black bars, cellulase activity; open circles, specific enzyme activity.

Fig. 6. Cellulase activity (A), dry cell weight (B), protein concentration (C), and specific enzyme activity (D) in the culture of *A. cellulolyticus* using untreated and pretreated MP cellulose. The MP cellulose was pretreated with 15 FPU/g MP cellulose for 6 h (closed circles) and 3 FPU/g MP cellulose for 12 h (closed triangles). For control experiment, untreated MP cellulose (open circles) was also used for the carbon source.

A

B

Supplementary File 1 . SEM pictures of pretreated MP celluloses. The MP cellulose was pretreated with 15 FPU/g MP cellulose of cellulase for 0 h, 2 h, 6 h, and 10 h. Samples were magnified 50 times (A), 2000 times (B) and 20000 times (C). Arrows in B and C indicate roughness and fissures on the surface of MP cellulose, respectively. Bars in A, B, and C denote 500 μm, 20 μm, and 2 μm, respectively.