Application of a Water Jet System to the Pretreatment of Cellulose

Yuka Watanabe[1], Shinichi Kitamura[1], Kazunori Kawasaki[2], Tomoki Kato[2], Koichi Uegaki[2], Kota Ogura[3], and Kazuhiko Ishikawa[1,2,*]

1 National Institute of Advanced Industrial Science and Technology (AIST),
Biomass Technology Research Center,
3-11-32, Kagamiyama, Higashi-hiroshima, Hiroshima 739-0046, Japan

2 National Institute of Advanced Industrial Science and Technology (AIST),
Health Research Institute,
1-18-31, Midorigaoka, Ikeda, Osaka 563-8577, Japan

3 Sugino Machine Limited,
2410, Hongo, Uozu, Toyama, 937-8511, Japan

Key Words

water jet, biomass, cellulose, atomization, nanofiber

ABSTRACT

Plant cellulose is the most abundant organic compound on earth. Technologies for producing cellulose fiber or improving the enzymatic saccharification of cellulose hold the key to biomass applications. A technology for atomizing biomass without strong acid catalysis remains to be developed. The water jet is a well-known device used in machines (e.g., washing machines, cutters, and mills) that use high-pressure water. In this study, we examined whether a water jet system could be used to atomize crystalline cellulose, which comprises approximately 50% of plant biomass. The Star Burst System manufactured by Sugino Machine Limited (Sugino Machine; Toyama, Japan) is a unique atomization machine that uses a water jet to atomize materials and thereby places lower stress on the environment. After treatment with this system, the crystalline cellulose was converted into a gel-like form. High-angular annular dark-field scanning transmission electron microscopy showed that the cellulose fibers had been converted from a solid crystalline into a matrix of cellulose nanofibers. In addition, our results show that this system can improve the saccharification efficiency of cellulases by more than three-fold. Hence, the Star Burst System provides a new and mild pretreatment system for processing biomass materials.

INTRODUCTION

As the primary component of plant biomass, cellulose is the most abundant organic compound on earth and has the potential to be a long-term sustainable resource for energy use and chemicals. The challenge for scientists is to access this biopolymer and convert it into useful materials or into fermentable sugars. An environmentally low-impact pretreatment procedure for producing cellulose fibers or improving the enzymatic saccharification of cellulose represents a key technology for the application of cellulose-containing biomass. A number of pretreatment procedures have been developed[1-4]. Among these methods, sulfuric acid treatment[3], steam explosion[5], hot-compressed water (HCW) treatment[6-8], lime pretreatment[9,10], and ammonia pretreatment[11] are known to be effective methods. However, most of them have individual disadvantages in their application. HCW is one of the most effective pretreatment processes for the application of biomass and can remove hemicelluloses and lignins from the biomass without the use of chemicals. Mechanical pulverization and ball milling (BM) methods[12-14] also seem promising for the pretreatment of cellulose-containing biomass. In particular, the treatment of materials with mechanical pulverization or BM after HCW has been shown to improve the enzymatic accessibility of the biomass and the production of cellulose

nanofibers[2]. Because HCW and BM do not use chemicals, it is anticipated that a method combining these 2 methods will be employed easily for industrial use. However, these treatments consist of many steps and are difficult to scale-up for mass production. Furthermore, the final concentration of the biomass product from this system has been limited to less than 10%[2].

Technologies that can economically convert biomass resources into commercially viable materials will be important for the application of biomass. The water jet is a well-known device that is used in machines (e.g., washing machines, cutters, and mills) that use high-pressure water. Recently, the Sugino Machine was developed for the atomization of materials by using a high-pressure water jet system. This system places low stress on the environment and provides a new technology for wet atomizing ceramics, stones, crystal, organisms, etc. In this study, we examined whether this system could be applied to the pretreatment of crystalline cellulose.

RESULTS AND DISCUSSION

Star Burst System using a water jet

Sugino Machine has constructed a unique wet atomizing system (Star Burst System; SBS) using super high pressure water jet system. The SBS is a system that can provide a fluid at pressures as high as 245 MPa. The SBS divides pressurized fluid into 2 channels and allows each fluid to collide in a chamber. After leaving the chamber, the water merges into single flow again. This arrangement creates a cross-collision for the atomization, emulsification, and dispersion of materials. Figure 1 shows the schematic illustration of the SBS. This system includes (1) a raw material tank, (2) 2 intensifiers, (3) an atomizing chamber, (4) a pair of nozzles disposed in the chamber, (5) a heat exchanger, and a conduit providing fluid communication between the components. A suspension containing materials is fed from the tank into the intensifiers where it is compressed and discharged under a super high pressure from a pair of diamond-shaped nozzles to form a pair of water jets that collide against each other in the chamber and that result in the atomization of the material. The solution of atomized material is delivered to the heat exchanger where it is cooled and fed back into the raw material tank. The procedure is repeated according to the desired number of atomizing cycles.

Application of SBS to crystalline cellulose

Crystalline cellulose comprises approximately 50% of the biomass from plants. To examine the potential effect of the SBS on biomass materials, we applied the SBS to crystalline cellulose powder (Ceolus PH102, Asahi Kasei Chemicals Corporation, Tokyo, Japan) suspended in water (1% w/v). The crystalline cellulose suspension was then fed to the pump

from the tank (Figure 1). Under the super high pressure (245 MPa), the suspension was atomized by the SBS. After this treatment, the suspension containing the crystalline cellulose was converted into a gel-like form that exhibited remarkably high viscosity. Figure 2 shows images of the cellulose powder suspended in water before (Figure 2A-1) and after (Figure 2A-2) 10 cycles of the SBS treatment. However, the gel-like cellulose could be obtained after 5 treatment cycles. Moreover, the viscosity of the cellulose depended on the number of treatment cycles (data not shown). Using the SBS, we were able to atomize the crystallized cellulose solution at concentrations of up to 15% (w/v). This gel-like cellulose is stable and shows no degradation for several months at room temperature. Furthermore, we were able to prepare a transparent cellulose film (Figure 2B) from the gel-like cellulose (Figure 2A-2) by drying it at room temperature.

Influence of the SBS treatment on saccharification by cellulase

We examined the influence of SBS treatment on the saccharification of crystalline cellulose (Ceolus) by cellulase under 2 different temperature conditions (50°C and 85°C). In the reaction at 50°C, the treated cellulose suspension was hydrolyzed by a mesophilic cellulase cocktail (cellulase from *Trichoderma reesei* ATCC 26921 [Sigma-Aldrich Corporation, St. Louis, MO, USA] and cellobiase from *Aspergillus niger* [Novozyme 188] [Sigma]). The mixture ratio was 1 (*Trichoderma reesei*):1 (Novozyme 188) (v/v). The FPU of the cellulase activity of the cocktail was estimated to be 132 u/ml at pH 5.5 and 50°C[15]. For the reaction at 85°C, we prepared 2 types of hyperthermophilic cellulases from *Pyrococcus* spp[16,17]. One of the cellulases is an endo-type cellulase (EGPh, family 5) from *P. horikoshii*[18], whereas the other one is a beta-glucosidase (BGLPf, family 1) from *P. furiosus*[16]. The hyperthermophilic cellulase cocktail was prepared as a mixture of the obtained enzymes (2.5 μM EGPh and 0.625 μM BGLPf).

Figure 3 shows the results of the enzymatic saccharification of the treated cellulose suspension. The amount of glucose produced from the saccharification reaction was measured using a glucose assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan), as described in the Materials and Methods. For both the temperatures, the efficiency of the cellulose saccharification was improved by the SBS treatment and increased according to the number of treatment cycles.

After 2 days of cellulose saccharification by these enzymes, transparent solutions were obtained for the 2 different temperature conditions. Complete saccharification was observed for the mesophilic cellulases but not for the hyperthermophilic cellulases. Complete saccharification can be carried out using endo-type cellulase (EG), beta- glucosidase (BGL), and cellobiohydrolase (CBH). The mesophilic cellulase cocktail contains the above three types of

cellulases. Without CBH in the hyperthermophilic cellulase cocktail, a complete saccharification of the cellulose could not occur. Therefore, the transparent solution observed for the hyperthermophilic cellulases (Figure 2A-3) seemed to contain both glucose and small particle of crystalline cellulose. The effect of the SBS on the saccharification reaction by the mesophilic system was smaller than that on the hyperthermophilic one (Figure 3). The reactivity of CBH toward the cellulose did not seem to be influenced by SBS largely. The treatment of SBS seemed primarily to increase the accessibility of the cellulose for EG.

X-ray analysis of the cellulose

The effect of the SBS treatment on the crystallinity of the cellulose was examined with X-ray diffractometry using a Rigaku RINT-TTR III diffractometer (Figure 4). The crystallinity was determined and analyzed from the charts by using a previously described method[19]. The curve (a) in Figure 4 corresponding to the untreated cellulose showed a crystallinity of approximately 70%. The curves (b and c) (Figure 4) corresponding to cellulose treated by 1 and 10 cycles of the SBS treatment, respectively, showed a slight decrease in the crystallinity of the cellulose. Moreover, the decrease in the crystallinity depended on the SBS cycle number. However, after 10 treatment cycles, the crystallinity of cellulose remained largely unchanged. These results indicate that the detailed crystalline structure of the cellulose was affected by the SBS treatment, but that the main crystalline structure was not influenced by it.

Electron microscopic observation

The morphology of the untreated and SBS-treated cellulose suspension was examined with the quick-freeze and deep-etch replication method. Figure 5 shows the high-contrast images obtained with high-angular annular dark-field scanning transmission electron microscopy (HAADF-STEM). Thick fibers of at least 1 μm width were observed in the non-treated samples (Figure 5A and C). In contrast, the SBS-treated cellulose showed fine cellulose fibers of ~25 nm width (Figure 5B and D), indicating the efficient production of cellulose nanofibers with the SBS processing.

Three-dimensional images of the SBS-treated cellulose were reconstructed by electron tomography (Figure 6). The cellulose nanofibers appeared to be associated with each other and to form loose, three-dimensional networks. The results indicate that the SBS processing had converted the cellulose from a solid crystalline to a matrix of cellulose nanofibers, consistent with the gel-like properties of the treated suspension. The increased surface area of the three-dimensional networks of cellulose nanofibers would offer an advantage in the efficiency of enzymatic reactions relative to that of the non-treated thick cellulose fibers.

CONCLUSION

The SBS made by Sugino Machine is a unique system for atomizing materials. With the SBS, we were able to atomize crystalline cellulose at concentrations of up to 15% (w/v). The crystalline cellulose was converted into a gel-like form; moreover, nanofibrillated cellulose was prepared by this system. Fine cellulose nanofibers (25 nm in width) were observed in the gel-like cellulose by using electron microscopy. Furthermore, this system can control the crystal structure of the cellulose and improve the saccharification efficiency of cellulose by cellulases by more than three-fold. The energy consumed by the SBS is estimated to be 18.5 kWh for kg of crystalline cellulose. In addition, one has to atomize the materials to a particle size of less 0.1 mm by using a cutter or conventional mills before processing the material with the SBS. The energy consumed by the mill is estimated to be less than 2 kWh for 1 kg of the materials. To apply the SBS for the treatment of biomass, it is necessary to estimate the energy consumed and the products (cellulose fiber, fermentable sugars, etc.) generated from the biomass. Because of the high energy consumption, SBS is not yet applicable to the production of bio-ethanol. Originally, SBS was developed to atomize many types of hard material, including steel, ceramics, stone, etc. Therefore, the SBS contains many devices for processing hard materials consuming high energy consumption. For the SBS, atomizing the biomass is much easier than atomizing certain minerals. We are now improving the SBS for use with biomass materials. By improving the intensifier, the check valves, and the chamber, it should be possible to decrease energy consumption greatly. This chemical-free system will be applicable for the production of fine nanofibrillated cellulose. This system also provides a new mild treatment system for many types of biomass. Studies on self-saccharification or self-processing plants are in progress. The SBS will be proposed for use in the new treatment system for plants.

MATERIALS AND METHODS

Materials

Micronized crystal cellulose powder KC flock W-50GK (average particle size 45 μm) (KCF) was supplied by Nippon Paper Chemicals (Tokyo, Japan). And Micronized crystalline cellulose powder Ceolus PH-102 (average particle size 90μm) (Ceolus) was supplied by Asahi Kasei Chemicals Corporation (Tokyo, Japan). As a conventional mesophilic cellulases, commercial cellulase product derived from fungus *Tricoderma reesei* ATCC 26921 and beta-glucosidase product derived from *Aspergillus niger* were purchased from SIGMA (St. Louis, MO). For the other chemicals, reagent grade were used.

Preparation hyperthermophilic cellulases

The hyperthermophilic endo-cellulase (EGPh, Gene ID: PH1171)[18] from *Pyrococcus horikoshii* and beta-glucosidase (BGLPf, Gene ID: PF0073)[20] from *Pyrococcus furiosus* were used in this study. EGPh and BGLPf were prepared and purified by the methods as follows[11]. The recombinant enzymes were expressed in *E. coli* BL21(DE3) cells (Novagen, Madison, Wisconsin) from the T7 promoter of pET11a (Novagen). Cell cultures were grown at 37°C in Luria Broth with 100 mg/mL ampicillin until OD_{600} reached 0.8, and IPTG was added to a final concentration of 0.1 mM for the protein induction. The both enzymes were purified by ammonium sulfate fractionation after heat treatment (30 min at 80°C) and eluted through a HiTrapQ anion exchange column. After confirming the purity of the proteins using SDS-PAGE, the protein concentration of EGPh, BGLPf was determined from UV absorbance at 280 nm, using 136270 and 133850 as the molar extinction coefficient calculated from their protein sequences, respectively.

Cellulose atomized by Star Burst System (SBS)

Approximately 200 g of cellulose micronized powder was suspended in 49 times weight of distilled water assisted by a powerful stirring device and placed in the feed tank of the Star Burst System HJP-25080, which was used for suspension jet collision. The aqueous cellulose suspension from the feed tank was injected from small nozzles at high speed. The machine automatically permits repeated super high-pressure collision treatments and our samples received 1 to 20 collisions (cycles).

Enzyme saccharification assay

The cellulase enzyme activity toward the cellulose substrate was examined in 20 mM sodium acetate buffer (pH 5.5) at 50°C (conventional mesophilic enzyme) and 85°C (hyperthermophilic enzyme). The enzyme reaction was carried out by adding the enzyme solution to the 1% Ceolus suspension (20 mM sodium acetate buffer, pH 5.5). The determination of glucose released from cellulose by enzymes was carried out by Glucose assay kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan).

Electron microscopy

The quick-freeze and deep-etch replicas were prepared as described[21]. The samples of cellulose in water with and without the SBS-treatment were quickly frozen by the metal-contact method using liquid helium. The frozen materials were freeze-fractured and deep-etched, and then the exposed surfaces were rotary-shadowed with platinum/carbon at an angle of 25° by using a freeze-replica apparatus (BAF400D, Balzers). The procedure did not include any sample

drying processes that might cause re-aggregation of dispersed fine fibers, accurately replicating the structure of the samples dispersed in water.

The replicas were observed with HAADF-STEM using Tecnai G2 F20 (FEI Co.) operated at 200 kV. The observation by STEM has an advantage in obtaining images with higher contrast than that by the conventional transmission electron microscopy. Here, the three-dimensional images of the cellulose nanofibers were reconstructed by HAADF-STEM tomography[22]. HAADF-STEM images of tilt series were collected over an angular range of ±70° in 1° steps. The software used for reconstruction of three-dimensional images was Inspect3D (FEI Co.), and that used for visualization by volume rendering was Avizo5 (MCS).

ACKNOWLEDGEMENTS

This work is supported by New Energy and Industrial Technology Development Organization (NEDO), and we thank Dr.Minoru Hayashida (Sugino Machine Limited) for X-ray analysis of the cellulose, and Dr.Yumiko Mishima and Ms.Yuka Maeno (Biomass Technology Research Center, National Institute of Advanced Industrial Science and Technology) for useful comments and valuable discussion.

REFERENCE

1. Kurabi, A.; Berlin, A.; Gilkes, N.; Kilburn, D.; Bura, R.; Robinson, J.; Markov, A.; Skomarovsky, A.; Gusakov, A.; Okunev, O.; Sinitsyn, A.; Gregg, D.; Xie, D.; Saddler, J. Appl Biochem Biotechnol 2005, 121-124, 219-230.
2. Lee, S. H.; Chang, F.; Inoue, S.; Endo, T. Bioresour Technol 2010, 101, 7218-7223.
3. Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y. Y.; Holtzapple, M.; Ladisch, M. Bioresour Technol 2005, 96, 673-686.
4. Pan, X.; Xie, D.; Gilkes, N.; Gregg, D. J.; Saddler, J. N. Appl Biochem Biotechnol 2005, 121-124, 1069-1079.
5. Christopher J. Biermann, T. P. S., Gary D. Mcginnia. Journal of Wood Chemistry and Technology 1984, 4, 111 – 128.
6. H.F. Hörmeyer, W. S., G. Bonn and, O. Bobleter. Holzforschung 1988, 42, 95–98.
7. Negro, M. J.; Manzanares, P.; Ballesteros, I.; Oliva, J. M.; Cabanas, A.; Ballesteros, M. Appl Biochem Biotechnol 2003, 105 -108, 87-100.
8. Kazumasa, S. K., SUDO. Sadao,NAGASAWA. Mitsuro,ISHIHARA. Mokuzai Gakkaishi 1983, 29.
9. Parajo, J. C.; Alonso, J. L.; Santos, V. Appl Biochem Biotechnol 1996, 56, 289-299.
10. Vincent S. Chang, W. E. K., Barry Burr, Mark T. Holtzapple. Biotechnology Letters 2001, 23, 1327-1333.
11. Gao, D.; Uppugundla, N.; Chundawat, S. P.; Yu, X.; Hermanson, S.; Gowda, K.; Brumm, P.; Mead, D.; Balan, V.; Dale, B. E. Biotechnol Biofuels 2011, 4, 5.
12. Yoshikuni, T. N., Tanaka. Seung-Hwan Lee. Takashi,Endo. Biotechnology and Bioengineering 2008, 99, 75-85.
13. Einosuke, M. F., YAKU. Ryutaro, TANAKA. Tetsuo, KOSHIJIMA. Mokuzai Gakkaishi 1982, 28, 122-128.
14. Mary, M. L., Hontz. John,Nystrom. Biotechnology and Bioengineering 1974, 16, 1471-1493.
15. GHOSE, T. K. Pure and Applied Chemistry 1987, 59, 257-268
16. Kim, H. W.; Ishikawa, K. J Microbiol Biotechnol 2010, 20, 889-892.
17. Kim, H. W.; Ishikawa, K. Proteins 2010, 78, 496-500.
18. Ando, S.; Ishida, H.; Kosugi, Y.; Ishikawa, K. Appl Environ Microbiol 2002, 68, 430-433.
19. Akira Kai , P. X. Polymer Journal 1990, 22, 955-961.
20. Bauer, M. W.; Bylina, E. J.; Swanson, R. V.; Kelly, R. M. J Biol Chem 1996, 271, 23749-23755.

21. Heuser, J. Methods Cell Biol 1981, 22, 97-122.
22. Kubel, C.; Voigt, A.; Schoenmakers, R.; Otten, M.; Su, D.; Lee, T. C.; Carlsson, A.; Bradley, J. Microsc Microanal 2005, 11, 378-400.

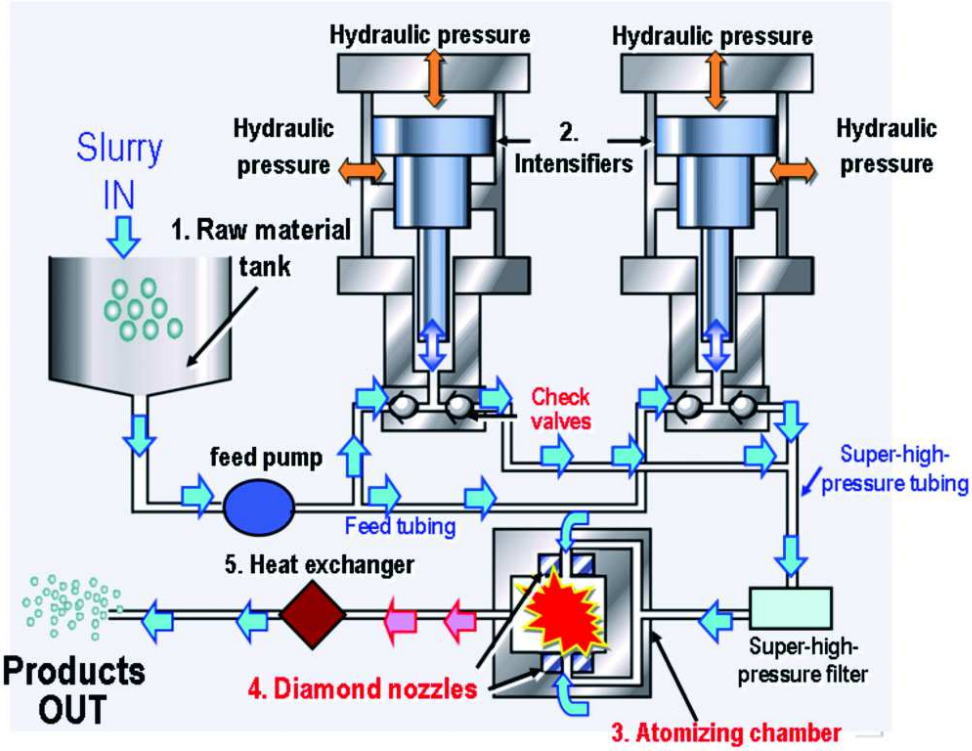

Figure 1 Illustration of SBS.
86x65mm (300 x 300 DPI)

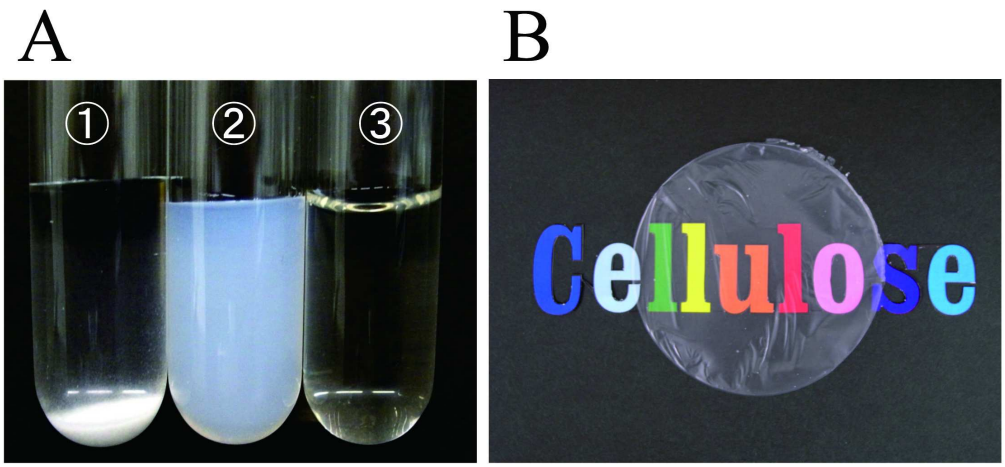

Figure 2 Crystalline cellulose in water and cellulose film.
A-1, 1% of crystalline cellulose (Ceolus) in water; A-2, 1% of gel-like Ceolus prepared from (A-1) by 10 cycles treatment of SBS; A-3, 1% of Ceolus prepared from (A-2) hydrolyzed by hyperthermophilic cellulase for 24 hours; B, cellulose film prepared from the gel-like cellulose (A-2).

187x91mm (300 x 300 DPI)

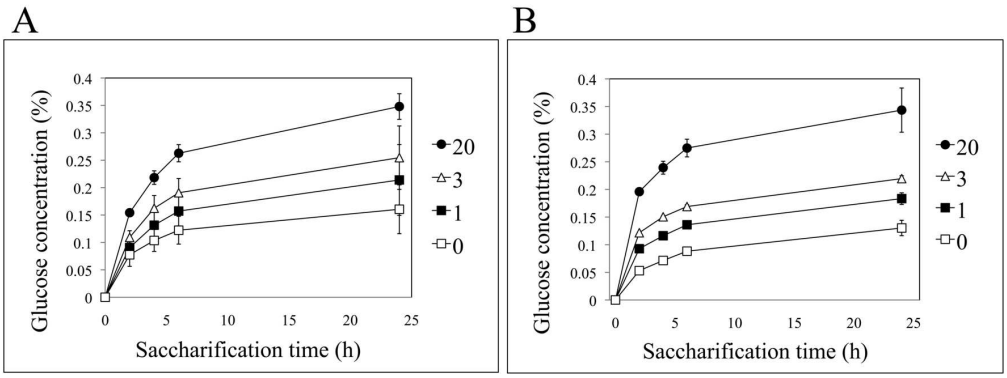

Figure 3 Enzymatic saccharification efficiency toward cellulose by SBS treatment. 2% of cellulose (Ceolus) suspension in water was treated SBS and 1% of the treated Ceolus suspension (20 mM sodium acetate buffer, pH 5.5) was used as the substrate. A, The mesophilic cellulase cocktail from *Trichoderma reesei* and Novozyme 188 with the ratio 1 (*Trichoderma reesei*) : 1 (Novozyme 188) (v/v) was prepared. 1 μl of the prepared cocktail enzyme was added to the 2ml of 1% of Ceolus substrate solution. The reaction was carried out at 50°C. B, 150μl of the hyperthermophilic enzyme mixture (2.5 μM EGPh; and 0.625 μM BGLPf) was added to the 2ml of 1% of Ceolus substrate solution. The reaction was carried out at 85°C. The cycle number of SBS are 0 (□), 1(■), 3 (△), and 20 (●).

74x27mm (600 x 600 DPI)

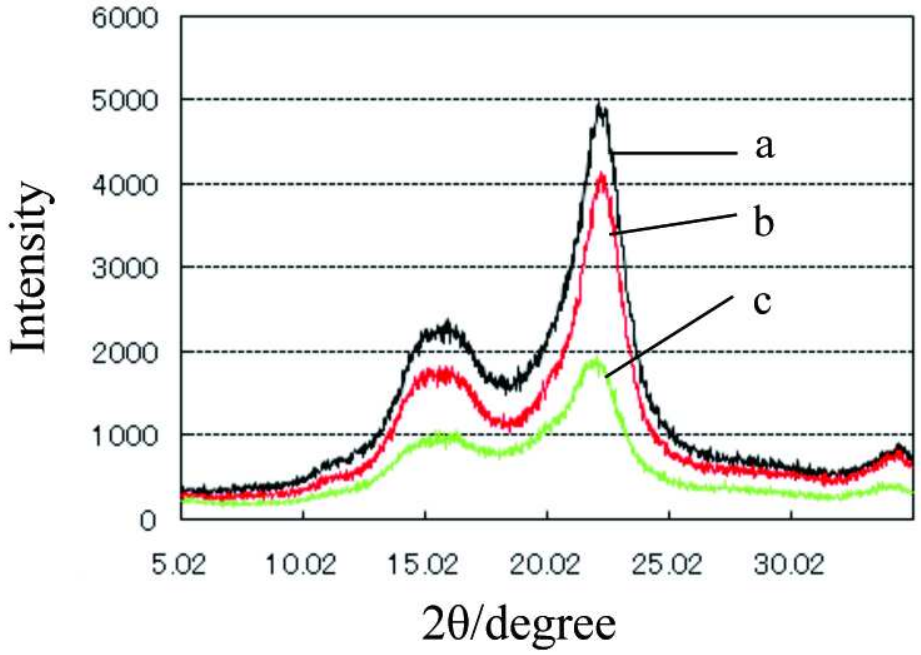

Figure 4 X ray analysis diagram of crystalline cellulose (Ceolus) of untreated (a) (0 cycle) and after treated 1(b) and 10 cycle(c) by SBS.
67x50mm (300 x 300 DPI)

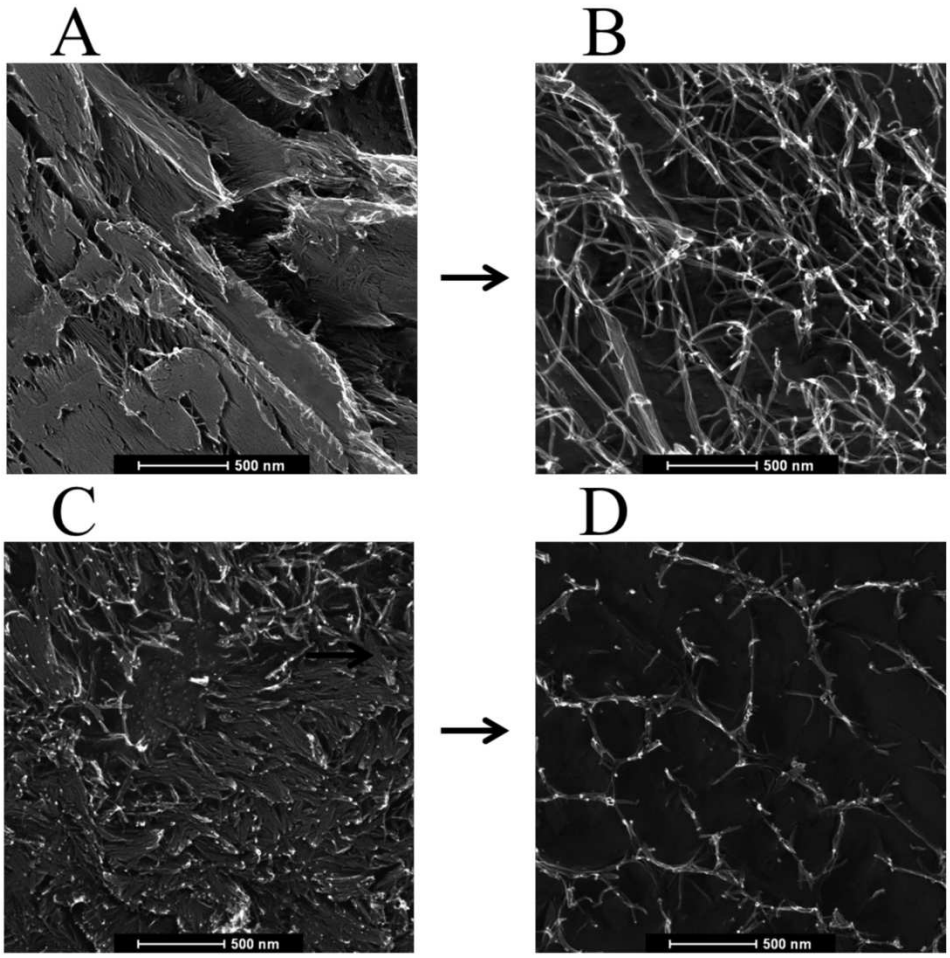

Figure 5 Representative images of cellulose in water before and after the SBS-processing observed using the HAADF-STEM and quick-freeze and deep-etch replication.
The thick fibers of micrometer-size (A: KCF, C: Ceolus) was converted to nanofibers (B: KCF with 10 cycles of SBS-treatment, D: Ceolus with 20 cycles of SBS-treatment).

97x95mm (300 x 300 DPI)

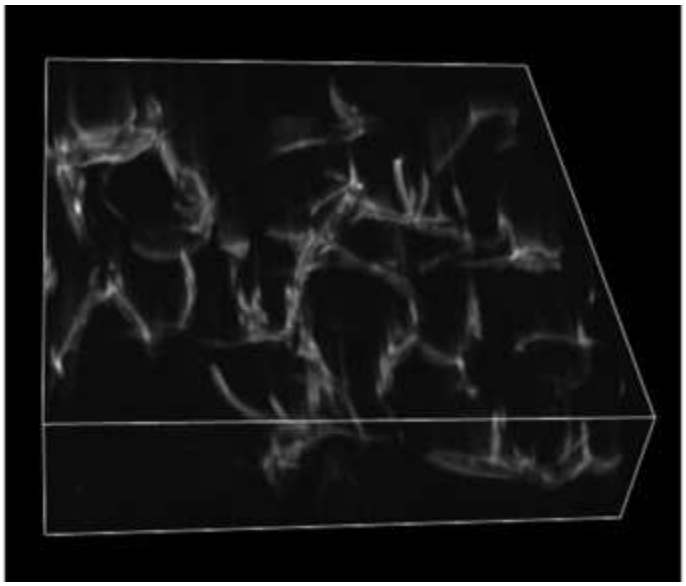

Figure 6 A representative three-dimensional image of cellulose nanofibers in water reconstructed by HAADF-STEM tomography.
The sample was Ceolus treated with 20 cycles of the SBS processing. The visualized volume was 1,350 x 1,350 x 360 nm.

32x24mm (300 x 300 DPI)