The Beatability-aiding Effect of *Aspergillus niger* Crude Cellulase on Bleached Simao Pine Kraft Pulp and its Mechanism of Action

Zheng-Jian Zhang,[a,*] Yun-Zhi Chen,[a] Hui-Ren Hu,[b] and Yi-Zhou Sang [c]

Bleached simao pine kraft pulp was treated with *Aspergillus niger* crude cellulase produced by submerged fermentation using the pulp as the carbon source. The effects of the cellulase on the pulp beatability and mechanical properties were studied. Fourier transform infrared spectroscopy (FT-IR) was performed to study the effect of cellulase treatment on the cellulose crystallinity index. The fiber morphology difference before and after treatment was also revealed by atomic force microscope observation. Compared to the control pulp, the beating time of the cellulase-treated pulp with the dosage of 7 u/g could be reduced from 360 s to 260 s under the same beating degree of 48°SR, which indicates a savings of about 28% beating energy consumption. The cellulase treatment had negative impacts on the pulp's mechanical properties. The cellulase preferentially adsorbed on the fine surface. The cellulose amorphous region was easier to treat with cellulase than the crystalline region. Atomic force microscope images demonstrated that the primary wall of fibers was peeled off and the S_1 layer of fibers came to the surface after the cellulase treatment.

Keywords: Aspergillus niger; Cellulase; Beatability; Action mechanism; Bleached simao pine kraft pulp

*Contact information: a: College of Packaging and Printing Engineering, Tianjin University of Science and Technology, Tianjin, 300222, P.R. China; b: Tianjin Key Laboratory of Pulp & Paper, College of Material Science and Chemical Engineering, Tianjin University of Science and Technology, Tianjin, 300457, P.R. China; c: Department of Chemical and Biological Engineering, The University of British Columbia, 2360 East Mall, Vancouver, BC, V6T 1Z3, Canada; *Corresponding author: zhangzj@tust.edu.cn*

INTRODUCTION

The kraft pulp fibers made from fast-growing trees usually have high coarseness and are not easy to beat or refine to obtain their desired properties (Chen 2003). For the development of required pulp properties, beating requires 15% to 18% of the total electrical energy required for producing paper from wood (Bhardwaj *et al.* 1996). In order to expand the use of these fast-growing trees, cellulose fibers as raw materials in modern papermaking industry, new technologies, and methodologies need to be developed to deal with the pulp beatability problem and the related energy consumption in the beating process.

In order to produce desired results, there are at least four ways that fibers can be modified, namely genetic modification, chemical modification (Sang and Xiao 2009), enzymatic modification, and mechanical modification (Baum 2002). As one part of fiber engineering, modification of cellulose fibers by enzymatic treatment aims at decreasing energy consumption in the production of pulp and increasing beatability of chemical pulps or improvement of fiber properties (Bajpai 1999). The improved understanding of the enzyme-fiber interaction and the continuous evolution of the properties and

capabilities of the enzyme make it possible to achieve these goals during the beating stage.

The application of enzymes to modify wood pulp is not new. Diehm (1942) first reported that hemicellulase enzymes from *Bacillus* and various *Aspergillus* could aid in the beating of wood pulps by increasing the rate of fiber hydration. The response of unbleached and bleached radiate pine slabwood to treatment with different enzymes was assessed and quantified (Kibblewhite and Clark 1996). The results from their work concluded that unbleached slabwood fibers of high coarseness with thick cell walls show the greatest response to enzyme modification. Garcia *et al.* (2002) investigated the effect of two different cellulases on drainability (Schopper–Riegler method). The final conclusion was that treatment with cellulases could lead to significant savings of energy during beating. Cellulase acts on the surface and inner layers of the cellulose fiber in an efficient enough way that allows for the production of special paper with reduced energy consumption (Karmakar and Ray 2011).

Cellulases are produced by several microorganisms including bacteria, actinomycetes, and fungi, but the latter are of great interest because they excrete their enzymes extracellularly (Gomathi *et al.* 2012). *Trichoderma reesei* is the most efficient producer of endo- and exo-glucanases, but does not excrete a sufficient amount of β-glucosidase, for which *Aspergillus niger* strains are known to be good producers (Bansal *et al.* 2012).

The choice of inducer substrate is one of the main factors influencing cellulase production and the use of lignocellulosic substrates, in place of other commercial inducers such as carboxymethyl cellulose or lactose that can contribute to the specificity of the enzymatic pool and improve hydrolysis yields (Cunha *et al.* 2012).

In this work, the modification of bleached simao pine kraft pulp with the crude cellulase produced from the strain of *Aspergillus niger* using the pulp as the carbon source (inducer substrate) was investigated. The key objectives of the work were to evaluate the potential energy savings of the cellulase treatment during beating and its action mechanism.

Effects of the cellulase dosage on the pulp beatability and mechanical properties were studied. Regarding action mechanism analysis, mean fiber length and mean fiber width were measured by Fiber Tester 912, the cellulose crystallinity index was evaluated by Fourier transform infrared spectroscopy (FT-IR), and the fiber surface morphology change was also demonstrated by atomic force microscope (AFM) observation.

EXPERIMENTAL

Materials

The bleached simao pine kraft pulp was supplied by Yunnan Yunjing Forest & Paper Co., Ltd., China. *Aspergillus niger* used for cellulase production was obtained from Tianjin University of Science and Technology, Tianjin, China. All other chemicals used in the experiment were analytical-grade products and purchased from commercial sources in China.

Methods

Cellulase production

The optimum submerged fermentation conditions of *Aspergillus niger* for cellulase production are shown in the Table 1. The crude cellulase produced by *Aspergillus niger* under these conditions was separated for further experiments.

Table 1. Optimum Submerged Fermentation Conditions of *Aspergillus niger* for Cellulase Production

Bleached simao pine kraft pulp dosage (%)	Vogels liquid dosage (%)	Microelement liquid dosage (%)	Bran dosage (%)	Strain seed dosage (%)	Tween-80 dosage (%)
1	4	0.2	1	10	0.2
Strain seed age (h)	Initial pH value	Temperature (^{o}C)	Rotation speed (r/min)	Culture time (h)	
36	5-6	30	150	120	

Note: One hundred milliliter Vogels liquid was prepared from 6.25 g $Na_3C_6H_5O_7.2H_2O$, 12.5 g KH_2PO_4, 5 g NH_4NO_3, 1 g $MgSO_4$, and 0.5 g $CaCl_2$ using distilled water. The formulation of microelement liquid was 0.025 g/mL $FeSO_4$, 0.0098 g/mL $MnSO_4$, 0.0083 g/mL $ZnCl_2$, 0.01 g/mL $CoCl_2$, and 0.00375 g/mL HCl.

Cellulase activities measurement

A filter paper assay was used to estimate total cellulase activity in the crude enzyme preparation (Ghose 1987) and is expressed as filter paper units (FPU). Endoglucanase activity (Cx) was determined with carboxymethyl cellulose as the substrate following procedure in Ghose (1987). The procedures for the measurement of exonucleases activity (C1) and β-glucanase activity (Cb) followed those described in reference (Liu 2002).

One FPU, C1, Cx, or Cb activity unit is defined as one microgram reducing sugar produced by 1 mL enzyme liquid per minute using filter paper, absorbent cotton, carboxymethylcellulose sodium (CMC), or salicin as the substrate, respectively, under the conditions of 50 °C and a pH of 4.8. Based on this definition, the unit for the cellulase activity was written as u/mL. The production amount of reducing sugar was measured by the phenol-sulfuric acid method (Zhao *et al.* 2006).

Pulp treatment and beatability measurement

Thirty grams oven dry (o.d.) of pulp was diluted to a consistency of 4% and adjusted to the required pH value 4.8 by the addition of dilute sulfuric aid (H_2SO_4) and warmed to the desired temperature 50 ^{o}C before the addition of cellulase. After the cellulase treatment, pulp was filtered using a Buchner funnel connected to a laboratory vacuum pump. Then the pulp was thoroughly washed by distilled-deionized water to remove the cellulase and the beatability test was performed.

The reference pulps were incubated in the same conditions as cellulase-treated pulps prior to beating except the condition without enzyme addition. Beating of pulps (at 10% pulp consistency and a beating intensity of 3.33 N/mm) was done in a PFI mill (Norway pulp and paper institute, Norway) at room temperature. Beating degree, wet weight, and water retention value (WRV) were measured in accordance with relevant TAPPI standard methods.

Hand-sheet making and measurement of mechanical properties

Handsheets were made from each pulp sample to measure the mechanical properties according to TAPPI T 205 om 88. Afterwards, the handsheets were kept in a conditioning room specified in TAPPI T 402 om 93. The tensile and tear strengths were measured in accordance with TAPPI T 494 om 88 and T 414 om 88, respectively, using Lorentzen and Wettre (L&W) tensile and tear strength testers (Kista, Sweden).

Action mechanism analysis

In order to explore the action mechanism of cellulase treatment for the beatability aid effect, the pulps were only treated by *Aspergillus niger* crude cellulase and not subject to PFI beating, and the effects of cellulase on mean fiber length, mean fiber width, cellulose crystalline degree, as well as the fiber morphology were studied. Mean fiber length and mean fiber width were measured using a Fiber Tester 912 (Lorentzen & Wettre Company, Sweden).

The FT-IR was taken to study the change of cellulose crystallinity before and after the cellulase treatment. FT-IR analysis of untreated fibers and treated fibers were performed at ambient temperature using a VECTOR 22 (Bruker Optik *GmbH*., Germany) FT-IR spectrometer in transmission mode.

To distinguish the surface morphology of fibers upon cellulase treatment, JSPM-5200 AFM (Japan Electron Optics Laboratory *Co., Ltd.*, Japan) was employed. All the probes were exposed to an ultraviolet light for more than 8 h to remove any potential organic contaminants prior to use.

RESULTS AND DISCUSSION

Measurement of the Cellulase Activities

In order to have a high measurement accuracy of cellulase activities, the optical density (OD) value should be controlled in the range of 0.3 to 0.5. In order to obtain the actual cellulase activity, serial dilution experiments were carried out at a temperature of 50 °C and a pH value of 4.8. Table 2 presents the results under different diluted multiples.

Table 2. Measurement of the Cellulase Activities

Diluted multiple	0	2	4	6	8	10
FPU OD value	-	-	0.460	-	0.278	-
FPU (u/mL)	-	-	43.57	-	52.75	-
Cx OD value	-	0.576	0.509	-	0.339	-
Cx (u/mL)	-	109.07	246.35	-	257.10	-
C1 OD value	-	-	0.390	-	-	-
C1 (u/mL)	-	-	36.96	-	-	-
Cb OD value	-	-	0.714	0.507	0.358	0.276
Cb (u/mL)	-	-	250.31	267.11	271.47	281.84
Note: One FPU, C1, Cx or Cb activity unit is defined as one microgram reducing sugar produced by 1 ml cellulase liquid per minute using filter paper, absorbent cotton, CMC or salicin as substrate respectively under the conditions of 50°C and pH 4.8. Based on this definition, the unit for the cellulase activity was written as u/mL.						

When the diluted multiple was 4, FPU and C1 OD values were 0.460 and 0.390, respectively, which is in the range of 0.3 to 0.5, and the FPU and C1 activities were 43.57 u/mL and 36.96 u/mL, respectively. For the Cx and Cb activities, when the diluted multiple was 8, the OD values were 0.339 and 0.358, respectively, which corresponds to the activities at 257.10 u/mL and 271.47 u/mL.

Effect of Cellulase Treatment on Beatability

In order to improve the beatability of bleached simao pine kraft pulp, cellulase treatment was applied before carrying out beating. In this paper, the applied cellulase dosage was expressed as u/g (FPU) enzyme on o.d. pulp. The cellulase treatment conditions were as follows: pulp concentration 4%, pH 4.8, temperature 50 ^{o}C, and reaction time 1 h. The results obtained from both the control (no cellulase added) and the cellulase-treated pulps are shown in Table 3. The effects of cellulase on beatability were evaluated by beating degree, wet weight, and WRV.

The beating degree after cellulase treatment increased steadily with an increasing dosage of cellulase (Table 3). When 7 u/g of cellulase was applied, a beating degree of 40°SR was observed, which is an increase of 16°SR compared to the control sample. In these experiments, the further increase of the beating degree is caused by the cellulase action, which damages the cell wall of the pulp fibers and improves the beatability of pulp. Within the same beating time, the cellulase-treated pulp had higher beating degree compared to that of the untreated sample. This means that in order to achieve the same beating degree, the cellulase-treated sample needs less time and thus a lower energy input. Beating treatment resulted in a slightly lower wet weight value than the control sample. The increase of WRV was observed when the cellulase was used. The highest WRV value of 1.76 g/g was achieved at a cellulase dosage of 9 u/g.

Table 3. Effect of Cellulase Treatment on Beatability

Cellulase dosage (u/g)	Beating degree (°SR)	Wet weight (g)	WRV (g/g)
0	24	11.33	1.42
1	27	10.28	1.48
3	33	9.19	1.53
5	38	8.06	1.61
7	40	5.80	1.68
9	46	5.68	1.76
Note: Beating time was 4 minutes.			

Effect of Cellulase Treatment on the Mechanical Properties and Beating Time Pulp fibers

The beating degree of the control sample and the cellulase-treated samples was controlled around 48°SR for the investigation of the effect of cellulase on the pulp's mechanical properties. The physical properties of pulp that were monitored are tabulated in Table 4.

When the cellulase dosage was lower than 3 u/g, the mechanical properties of cellulase-treated pulp decreased slightly, while significant decreases in mechanical properties were observed when the cellulase dosage exceeded 3 u/g. This can be explained by the fact that the strength of a single fiber is damaged during the cellulase treatment and the fiber is shortened after beating, resulting in lower strength properties.

Table 4. Effect of Cellulase Dosage on the Mechanical Properties and Beating Time of Pulp Fibers

Cellulase dosage (u/g)	Tensile index (N.m/g)	Stretch (%)	Tensile energy absorption index (mJ/g)	Burst index (kPa.m^2/g)	Tear index (mN.m^2/g)	Zero tensile index (N.m/g)	Beating time (s)
0	72.73	2.74	1330	5.08	12.48	141.08	360
1	70.25	2.60	1250	4.83	10.04	140.71	350
3	66.58	2.58	1140	4.65	10.15	140.24	325
5	61.50	2.52	1080	4.10	9.10	130.59	285
7	59.08	2.25	901	3.71	9.07	128.29	260
9	58.06	2.22	896	3.74	8.78	126.66	240
Note: Beating degree was 48°SR.							

Tear index decreased due to the cutting action of cellulase on the fiber of pulp. Compared to the control pulp, the beating time of cellulase-treated pulp with a dosage of 7 u/g could be reduced from 360 s to 260 s under the same beating degree 48°SR. This corresponds to a savings of about 28% beating energy consumption.

Mean Fiber Length and Mean Fiber Width Analysis

The effect of cellulase on mean fiber length and mean fiber width is shown in Fig. 1. Figure 2 shows the effect of the cellulase dosage on the fiber fraction content of different lengths.

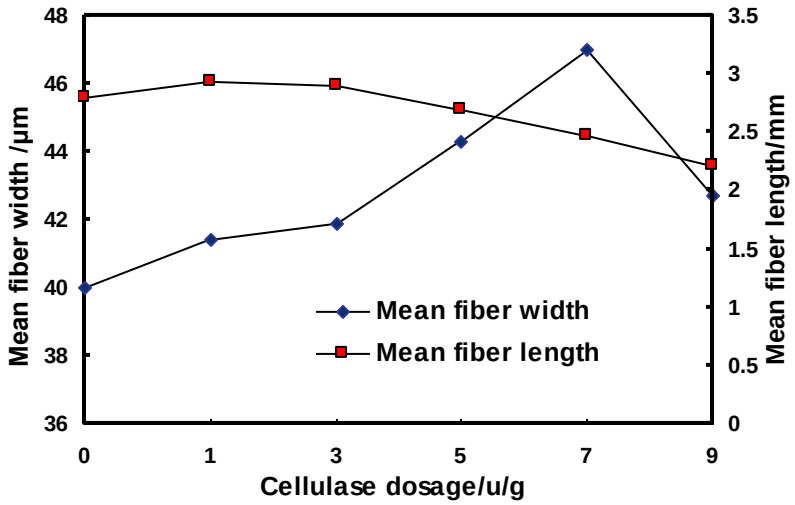

Fig. 1. Effect of cellulase dosage on the mean fiber length and mean fiber width

When the cellulase dosage was lower than 1 u/g, the mean fiber length increased slightly and the content of fiber fraction with fiber length lower than 3 mm decreased. This is because the fines have a higher specific surface area compared to long fibers; the cellulase preferentially adsorbed on the fine's surface and acted on them. After the cellulase treatment, the fraction of fines was hydrolyzed. This caused an increase in mean fiber length. When increasing the cellulase dosage beyond 1 u/g, the content of fiber fraction (longer than 3 mm) decreased and the mean fiber length was reduced below that of the control sample due to the cutting action of cellulase. The mean fiber width

increased steadily after cellulase treatment in the dosage range of 0 to 7 u/g. These changes indicated enhanced external fibrillation. When the cellulase dosage was beyond 7 u/g, the mean fiber width decreased significantly due to the peel off effect under the condition of excess cellulase dosage.

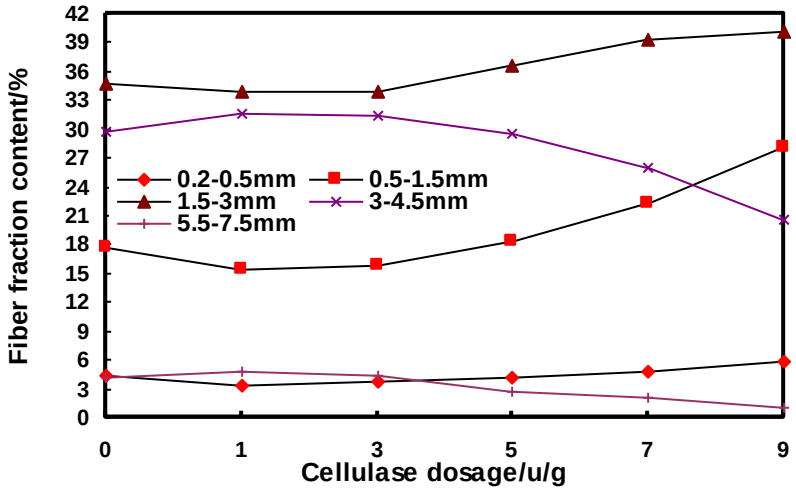

Fig. 2. Effect of cellulase dosage on the fiber fraction content

Cellulose Crystallinity Index Analysis

The IR crystallinity index of cellulose (Nelson and O'Connor 1964; Kataoka and Kondo 1998) was evaluated as the intensity ratio between IR absorptions at 1427 cm^{-1} and 895 cm^{-1}, which are assigned to CH_2 bending mode (Liang and Marchessault 1959) and deformation of anomeric CH (Blackwell *et al.* 1970). Figure 3 presents the effect of cellulase dosage on cellulose crystallinity index.

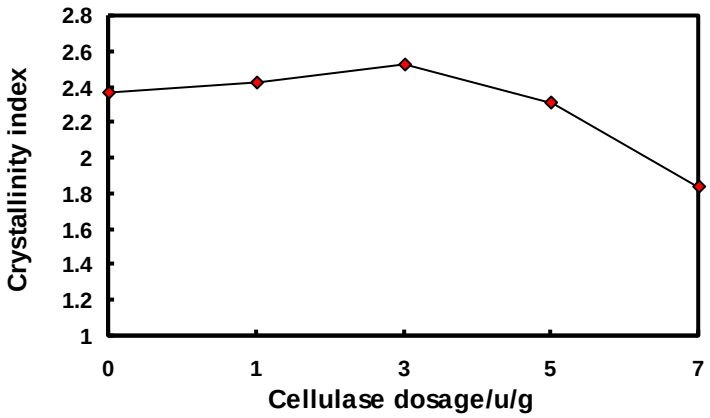

Fig. 3. Effect of cellulase dosage on cellulose crystallinity index

As can be seen from Figure 3, when the cellulase dosage was lower than 3 u/g, the cellulose crystallinity index increased. This is because the structure of the cellulose amorphous region is unconsolidated and is easier to treat with cellulase than the crystal region. With the further increase of cellulase dosage beyond 3 u/g, the cellulose

crystallinity index decreased gradually. This can be explained by the fact that the cellulose crystalline region is also damaged under the higher cellulase dosage, resulting in lower cellulose crystallinity index.

Fiber Morphology Observed by AFM

Figure 4 shows AFM images of virgin fibers and the fibers treated with the cellulase. Clearly, the surface morphology of fibers changed significantly after the cellulase treatment.

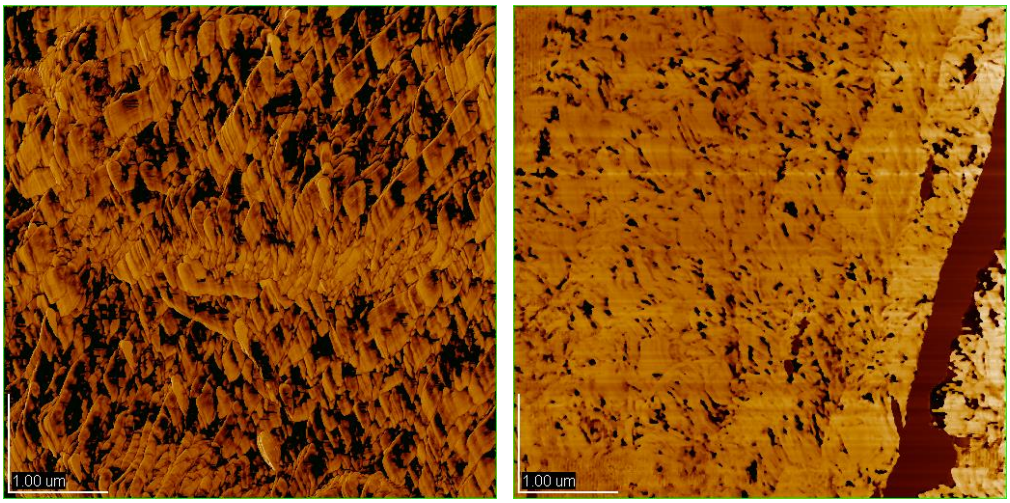

Fig. 4. AFM images of fiber before (left) and after (right) cellulase treatment with 7 u/g (5μm×5μm)

AFM images show that before treatment, the fiber surface was completely covered by the primary layer in which the microfibirls presented an irregular reticulated arrangement. This means the kraft pulping process did not damage the primary layer and left it on the outer surface. This is the reason why the bleached simao pine kraft pulp is not easy to beat. After cellulase treatment, the microfibrils on the surface of the treated fiber appeared to be clearer than the virgin fiber, indicating the primary layer of the fiber cell wall was peeled off by the cellulase and the first layer of secondary cell wall of fiber (S1 layer) came to the surface. The microfibrils showed a cross spiral arrangement with vertical direction to the fiber axle (Fig. 4). This is beneficial for the beating process that follows, as confirmed by the improved beatability.

CONCLUSIONS

1. Treating bleached simao pine kraft pulp with a 7 u/g dosage of cellulose before the beating stage improved the pulp beatability and decreased the energy consumption of papermaking by 28% to reach the same beating degree of 48°SR. With an increased dosage of cellulase, the pulp mechanical properties worsened under the same beating degree.
2. The cellulase preferentially adsorbed on the surfaces of fines rather than on those of long fibers. The structure of the cellulose amorphous region was more easily attacked (or treated) by cellulase than the crystalline region.

3. AFM images demonstrated that the primary layer covered the fiber surface of the controlled pulp. After the cellulase treatment, the primary layer of fiber cell wall was peeled off and the S1 layer came to the surface.

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