EFFECT OF REFINING ON DELIGNIFICATION WITH A LACCASE /XYLANASE TREATMENT

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Previous research has demonstrated that a laccase/xylanase system (LXS) from white-rot fungus (*Lentinus lepideus*) has the same ability to delignify as a laccase/mediator system (LMS). In order to enhance delignification ability of LXS treatment, the effect of refining on delignification with LXS treatment of Masson pine (*Pinus massoniana*) pulp was investigated by refining the pulp in a PFI mill to different revolutions (14,000/21,000/35,000/42,000/56,000) prior to LXS treatment (enzyme dosage 5/10/15IU/g o.d. pulp). The results indicated that both kappa number and yield of the LXS treated pulp decreased with increasing refining. A substantial delignification without severe yield loss could be achieved by moderate refining prior to LXS treatment. The SEM images suggested that refining increased the accessibility of the material to enzymes and thereby enhanced the delignification ability of LXS. The small cellulase activity detected in the LXS had little effect on the viscosity of the treated pulp even at high enzyme dosage.

Keywords: Delignification; Laccase/xylanase system; Masson pine; Refining

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INTRODUCTION

Masson pine is an important fast-growing wood. It is a high-quality papermaking raw material, producing high strength pulp because of its long fiber (Wang 1998; You *et al.* 1996). Like other softwood species, Masson pine is more difficult to delignify than hardwood species due to its lignin structure and higher lignin content. For softwood, about 10% of the lignin is left in the bleachable-grade pulp for further delignification in the bleach plant. In order to reduce the pollutant load in bleach plant effluents, many prebleaching delignification processes have been investigated. Among them, oxygen delignification and prebleaching with xylanase have been successfully implemented in many pulp mills worldwide. The main advantage of the xylanase prebleaching process over oxygen delignification is that it can be implemented without a major capital investment. Other prebleaching technologies that have been widely studied are delignification with a laccase/mediator system (LMS) (Bourbonnais and Paice 1996; Bajpai *et al.* 1996), a methylsyringate laccase mediator system (Martínez-Ruiz *et al.* 2009), or in the presence of 1-hydroxybenzotriazol (HBT) as mediator (Valls *et al.* 2009; Roncero and Valls 2009) Unfortunately, the requirement for an expensive mediator precludes it from practical application. In our previous report, we have demonstrated that the laccase/xylanase system (LXS) isolated from white-rot fungus (*Lentinus lepideus*) has the same delignification ability as a laccase/mediator system (LMS) (You *et al.* 2008). This finding is unusual and surprising, as a synthetic system using commercial laccase and xylanase proved to be ineffective (You *et al.* 2009a,b). While the reason for the efficacy of the

LXS system needs to be investigated, the finding provides a potential inexpensive prebleaching system for practical applications.

Mechanochemistry is an emerging interdisciplinary subject; it uses mechanical force to steer chemical reactions along pathways that are unattainable by conventional processing (Hickenboth *et al.* 2007). This new method has attracted attention for various potential high-tech applications in recent years (Hickenboth *et al.* 2007; Beyer and Clausen-Schaumann 2005; Chen *et al.* 2001). The related devices include high-energy mills such as a mixing mill, vibration mill, colloid mill, centrifugal grinding, *etc.* (Baláž 2008; Yuan and Sun 2009). Conventionally, the laboratory PFI mill is used for refining bleached pulp. The fiber flexibility and handsheet performance will be improved after PFI refining. In the process, PFI milling causes fibrillation of the fibers and breaks off more fines (Kerekes 2005; Chakraborty *et al.* 2007). The principle of PFI milling is similar to the powder mechano-chemistry process. The combination of mechanical refining with enzyme treatment has been motivated by a need to improve delignification and bleachability in subsequent bleaching stages. The feasibility of treating raw materials with enzyme was examined in our previous study. In particular we investigated materials such as wheat straw pulp treated with recombinant xylanase (You *et al.* 2010) and the effect of prior mechanical refining on biobleaching of wheat straw pulp with Laccase/xylanase treatment (Lian *et al.* 2012). The results indicated that for wheat straw pulp, the LXS treatment after the mechanochemical process could save 28.6% effective usage of chlorine in the subsequent hypochlorite bleaching process, compared with the traditional bio-bleaching. In this report, a new application of mechnochemistry will be tried in biobleaching. We will demonstrate that the delignification ability of LXS system can be further enhanced by refining the Masson pine pulp prior to LXS treatment.

EXPERIMENTAL

Materials

Unbleached Masson pine (Pinus massoniana) pulp

Unbleached kraft pulp (UP), with Kappa number of 28.9, a brightness of 25.8% ISO, and a viscosity of 1025.3 mL/g was obtained as dried pulp sheet from Qingshan Papermaking Mill in Fujian Province, China.

Laccase/Xylanase system (LXS)

A laccase/xylanase system was directly produced by controlling the culture conditions of the white-rot fungus (*Lentinus lepideus*). The fungus was grown in a Petri dish on a potato culture medium for 7 days. Enzymes were produced by inoculating the fungus in 50 mL of culture medium (carbon source: starch, 20 g/L; nitrogen source: protein gel, 2 g/L). The flask was placed in a shaker incubator at 29 ^{o}C and 170 rpm for 7 days. Laccase activities were determined by oxidation of ABTS (You *et al.* 2008). The enzyme activities of laccase, xylanase, and cellulose were 156 IU/mL, 4.28 I U/mL, and 0.152 IU/mL, respectively. The activity of lignin peroxidases or Mn- peroxidases was not found. The xylanase activities were determined by reference literature (Bailey *et al.* 1992). The cellulase activities were determined by International standard (Ghose 1987).

Methods

Refining

The pulp (20 g, O.D.) was refined in a PFI mill (model PL11, Xianyang Electromechanical Company, Shanxi). Refining conditions were: a pulp consistency of 10%, a refining pressure of 3.34 N/mm, and a gap between the working surfaces of 0.2 mm. Refining degrees were determined by the Schopper-Riegler tester (Machine Factory of the Shaanxi University of Science and Technology, according to ISO 5267-1:1999, Pulps-Determination of drainability- Part1: Schopper-Riegler method).

Enzyme treatment

The pulp (20 g, O.D.) was treated with LXS in acetate/acid-sodium acetate buffer (0.1 M, pH 4.2) medium in plastic bags. The treatments were carried out at different enzyme dosage under the same conditions (pH 4.2, temperature 45 ^{o}C, pulp consistency 3%, time 3 h). After enzyme treatment, pulp solution was divided into two parts. One part was the pulp; it was washed with water, and then dispersed to determine pulp properties. The other part was effluent, which was collected directly and subjected to analysis. The control pulp (CP) was treated under the same conditions as those of the enzyme-treated pulp but without adding any enzyme.

Pulp sample codes are presented in Table 1.

Table 1. Pulp Treatment

NO	Sample code	Conditions
1	UP	Unbleached Masson pine (*Pinus massoniana*) pulp
2	RP	Pulp with prior mechanical pulping and no treatment
3	CP	Pulp with prior mechanical pulping and treatment(no enzymatic)
4	LXSP	Pulp without mechanical pulping + enzymatic treatment
5	RLXSP	Pulp with prior mechanical pulping + enzymatic treatment

Kappa number determination

The kappa number of pulp was measured after alkali extraction. The alkali extraction was carried out under the following conditions: pulp consistency 10%, alkali dosage 1%, temperature 70 ℃ and time 90 min. The alkali-extracted pulp was washed, and the kappa number was determined according to T236 cm-85 (Kappa number of pulp).

Determination of delignification

The delignification was calculated by following formula,

$$\text{Delignification} = (K_{CP}-K_{RLXSP})/K_{CP} \quad (1)$$

where, K_{CP} is Kappa number of the control pulp, and K_{RLXSP} is Kappa number of the RLXSP at different revolutions.

Reducing sugar assay

Reducing sugar in the filtrate was determined using the dinitrosalicylic acid (DNS) Method (Miller 1959). Effluent was collected and filtered using a micro filter syringe 2.0 mL of filtered effluent was taken and mixed thoroughly with 3.0 mL of 1% w/v solution

of 3,5-dinitrosalicylic acid in a test tube using a vortex mixer. The test tube was then placed in boiling water bath for 10 min. Absorbance of resulting solution was recorded at 550 nm in a UV-751 spectrophotometer (Shanghai Spectrum Instrument Limited Company, China) after cooling. Reducing sugar content was determined according to the standard line of xylose and was based on oven-dried weight of pulp (Chen 2002).

Determination of UV absorbance

The filtrate from enzymatic hydrolysis was boiled to denature the enzymes, cooled and centrifuged. The supernatant was diluted 10 times, and absorbance value was measured at 280 nm with UV-751 spectrometer. The absorbance value reflects the relative amount of lignin dissolved in the filtrate.

Determination of pulp properties

The beating degree, yield of pulp, and brightness were measured by related TAPPI test methods. Viscosity of pulp was analyzed according to ISO 5351/1.

Determination of cellulose crystallinity

Small amounts of control and enzyme-treated pulp were made into tablets, and then crystallinity was determined by model DX-2000 X-ray diffractometer (Dandong Fangyuan Instrument Limited Company, Liaoning). Detecting conditions were: a tube pressure 30 KV, a tube currency 20 mA, and a Cu-target. The following formula was used to calculate relative crystallinity,

$$\text{Relative crystallinity} = (I_{002}\text{-}I_{AM})/I_{002} \quad (2)$$

where I_{002} is the maximum intensity of the diffraction angle of 002 crystal lattice, and I_{AM} is the scattering intensity of the amorphous background diffraction when $2\theta = 18°$.

SEM observation

The morphology of the untreated and treated pulps was imaged with a Fei Quanta 200 the scanning electron microscopy (SEM), provided by Philips.

RESULTS AND DISCUSSION

Effect of Refining on Delignification Ability of LXS Treatment

The previous research demonstrated that the laccase/xylanase system (LXS) from white-rot fungus (*Lentinus lepideus*) had the same ability to delignify as a laccase/ mediator system (LMS) (You *et al.* 2008). In order to further improve delignification, the pulps were refined in a PFI mill. Then they were treated with 10 IU of enzymes (based on laccase/g of pulp) under the optimum treatment conditions established previously for Masson pine pulp (pH 4.2, temperature 45 ^{o}C, pulp consistency 3%, time 3 h). The results are shown in Table 2.

As can be seen in Table 2, the kappa number of the control pulp (CP) dropped from 28.9 to 27.2, and the yield dropped to 98.2%, resulting presumably from leaching of residual lignin and hemicelluloses in pulp. Enzyme treatment of the pulp resulted in a 10% delignification and a small yield loss without refining. The kappa number and yield of enzyme-treated pulp gradually decreased. During the course of refining the primary

wall and the S1 layers were removed, exposing the S2 layer to the surface. More importantly, the cell wall was delaminated and cut to some extent. All these increased the accessibility of enzymes to the cell wall, resulting in a higher degree of delignification and a high yield loss. The kappa number of pulp was reduced from 27.2 to 21.8, representing an increase in delignification by 20% when refining to 21,000 revolutions. This was twice the degree of delignification by LXS as compared with pulp without refining. As the refining increased beyond 35,000 revolutions, the decrease in kappa number became negligible, whereas the yield loss became severe. This high yield loss at high refining degree may be caused by the creation of a large amount of fines that were lost during the fiber processing. Contrary to kappa number and pulp yield, refining appeared to have little effect on either viscosity or brightness of the pulp. The results clearly indicate that the small amount of cellulase activity in the LXS has little effect on the degree of polymerization of cellulose.

The lack of brightness increase with decreasing of the kappa number was unexpected. Two reasons may explain this. Firstly, pulp refining lowers scattering coefficients of pulp, leading to lower pulp brightness. Secondly, laccase may oxidize residual lignin and hence increase its absorption coefficient. Both would cause decreased brightness that offsets the increase due to the lowered lignin content.

Table 2. Effect of Refining Revolutions on Delignification with LXS Treatment

PFI revolutions	Beating degree (°SR)	Kappa number	Delignification* (%)	Brightness (% ISO)	Viscosity (ml/g)	Yield (%)
CP	13.0	27.2	0	26.5	1022	98.2
0	12.5	24.4	10.3	26.9	998	97.7
14000	15.7	23.0	15.4	26.6	1022	96.8
21000	17.5	21.8	19.9	26.5	1043	96.2
35000	21.1	21.5	21.0	26.5	1051	95.6
42000	25.5	21.2	22.1	26.5	1018	92.3
56000	36.5	20.0	26.5	26.4	1018	90.4

* based on the control pulp (CP)

As shown in Table 2, excess refining beyond 21,000 revolutions resulted in large yield losses without much delignification. Furthermore, excess refining consumed energy and may cause difficulty in pulp washing in the subsequent bleaching operation. For these reasons, we adapted 21,000 revolutions as a standard refining condition for the subsequent studies.

Effect of Enzyme Dosage and Refining on Delignification

The effect of LXS dosage and refining on delignification was compared under the same refining conditions (21,000 revolutions in PFI). Figures 1 through 6 show the results for Kappa number, viscosity, brightness, and yield of the treated pulps as well as the contents the dissolved lignin and the reducing sugars in the filtrate of the enzyme treatment.

Figure 1 shows the effect of different enzyme dosage on kappa number of pulp. In both cases, the kappa number decreased rapidly at low enzyme dosage and gradually leveled off with increasing of enzyme dosage. It was also clear from Fig. 1 that refining greatly improved delignification by the enzymes. For refined pulp (RLXSP), the delignification already reached a higher value even at the LXS dosage of 5 IU/g.

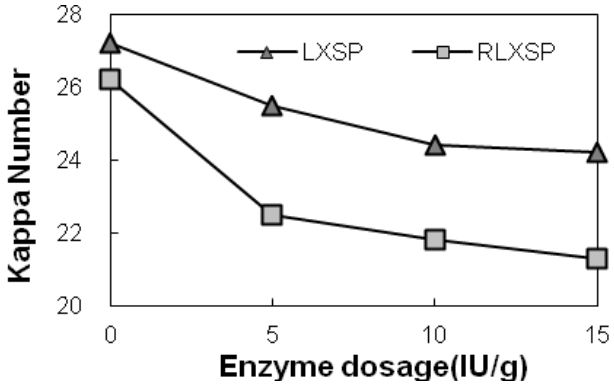


Fig. 1. Effect of LXS dosage on kappa number

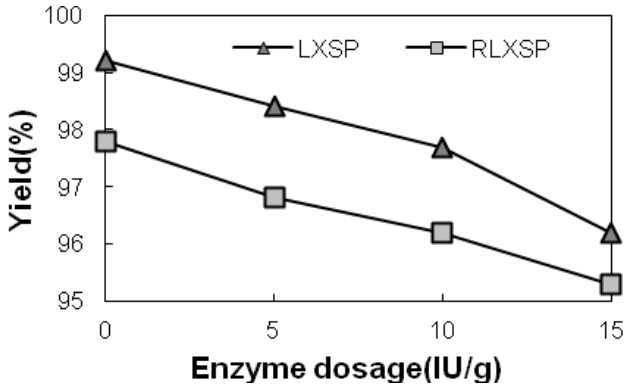


Fig. 2. Effect of LXS dosage on yield before and after refining

There was little improvement in lignin removal by further increasing of enzyme dosage. On the other hand, in the case of LXS treatment of pulp without refining (LXSP), the kappa number continued to drop until the dosage of 10 IU/g before leveling off. Even with 10 IU/g, the extent of delignification was much lower than the pulp (RLXSP) treated with 5 IU/g.

The effect of enzyme dosage and refining on pulp yield is shown in Fig. 2. As can be seen, a small yield loss, about 1.5%, occurred as a result of pulp refining. The enzymatic treatment was less efficient than it seems to be in the figure. This was probably due to the fact that fines created during refining were lost. Thus, refined pulp had lower yield than the pulp without refining at all enzyme dosage levels. While the decrease in pulp kappa number leveled off with increasing enzyme dosage (Fig. 1), the pulp yield continued to decrease with increasing enzyme dosage. This continuous loss of yield may be due at least partly to the presence of a small xylanase activity in LXS. The cellulose activities detected in the LXS probably were too small to cause yield loss, since the viscosity of the pulp was unaffected by LXS treatment. By comparing Figs. 1 and 2, it was clear that the optimal enzyme dosages were 5 IU/g for the refined pulp and 10 IU/g for pulp without refining. Even at 10 IU/g, the delignification of pulp without refining was less than that of the refined pulp at 5 IU/g.

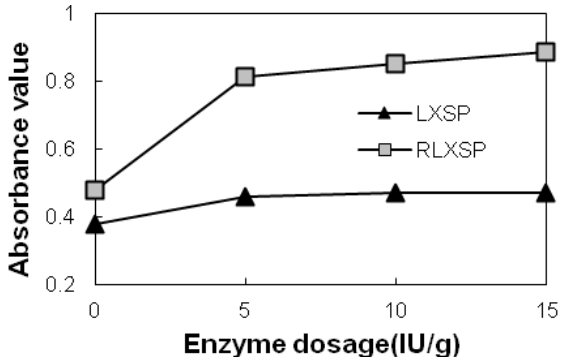


Fig. 3. Effect of enzyme dosage on absorbance value at 280 nm

Laccase oxidation of residual lignin resulted in the dissolution of some lignin in the filtrate of LXS treatment. The amount of the dissolved lignin in the filtrate could be estimated by the UV absorbance at 280 nm, as shown in Fig. 3. As could be seen, after LXS treatment the refined pulp gave much high absorbance at 280 nm than the pulp without refining. These results were in good agreement with the kappa number reduction as shown in Fig. 1.

In addition to laccase, the LXS contains xylanase and a small cellulase activity. Reducing sugar content in the filtrate was determined, and results are shown in Fig. 4. Without refining, the reducing sugar content of the filtrate increased only slightly with increasing enzyme dosage. On the other hand, the reducing sugar content increased substantially with increasing enzyme dosage after refining. Since pulp viscosity was unaffected by LXS treatment (Table 1 and Fig. 5), the increase in reducing sugar content must be due to the activity of xylanase. Thus, refining increased the accessibility not only to laccase but also to xylanase.

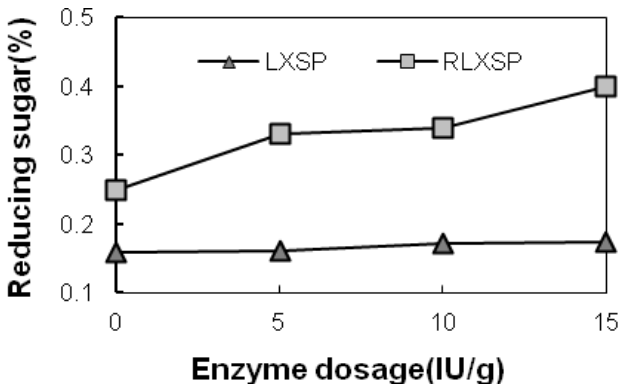


Fig. 4 Effect of enzyme dosage on reducing sugar content in the filtrate

The viscosity of pulp after LXS treatment is shown in Fig. 5. The viscosity remained constant with increasing enzyme dosage for both pulps before and after refining. This may be due to the fact that fines created during refining were lost. Thus, refined pulp had more long fiber than the pulp without refining, and the result was an increase in the viscosity. Together with results in Table 1, it was clearly indicated that the small amount of cellulase activity detected in the LXS had little effect on the viscosity of pulp whether it was with or without refining. The brightness of pulp was not affected by LXS treatment.

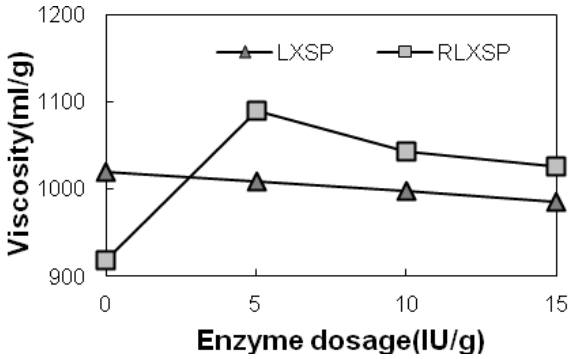


Fig. 5. Effect of enzyme dosage on pulp viscosity

Effect of LXS Treatment and Refining on Crystallinity of Cellulose

X-ray diffraction was used to determine the crystallinity of cellulose. Table 3 shows that LXS treatment had little effect on crystallinity of pulp without refining. On the other hand, refining slightly lowered the crystallinity, presumably due to the creation of fines during the refining. But after LXS treatment (RLXSP), the crystallinity was raised to the same level as LXSP. The increase in crystallinity was likely due to the loss of fines of the refined pulp during the LXS treatment.

Table 3. Crystallinity of the Treated Pulp

Pulp code	OP	LXSP	RP	RLXSP
Crystallinity %	67	68	63	69

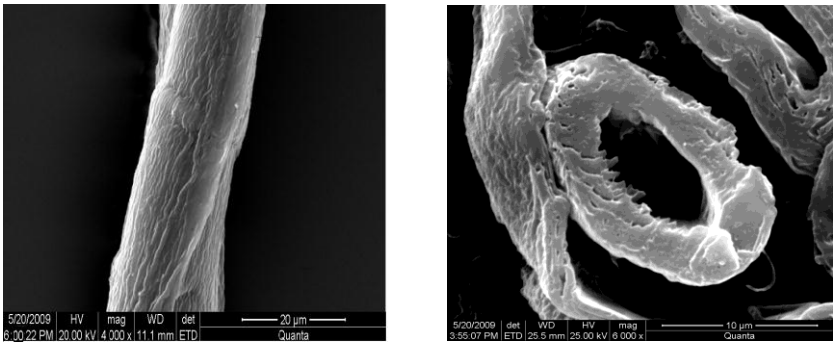
SEM of Fiber Morphology with Treated Pulp

SEM images were used to help observe the changes of the microstructure of Masson pine pulps. Figure 6 shows the SEM images of surface and cross-section of the LXS treated pulp before and after refining.

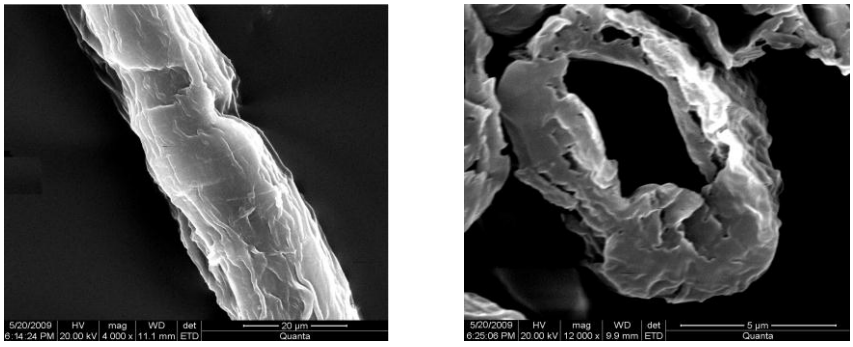
Without refining, the fiber surface and cross-section of original pulp were smooth and solid-like (6a), whereas the enzyme-treated pulp had a rough fiber surface and, in the cross section of the cell wall, tiny holes and cracks, presumably resulting from dissolution of lignin and xylanase by LXS (6b).

Refining of pulp clearly caused delamination and swelling of fiber, as shown in Fig. 6 by comparing images 6(c) and 6(a). The increased accessibility to LXS treatment could result in further delamination and swelling of fiber, as shown in Fig. 6(d). Also, the morphologies are consistent with the previously reported effects on morphologies of wheat straw pulp with laccase/xylanase treatment (Lian *et al.* 2012). These observations supported the results that more lignin and xylan were dissolved by LXS treatment after refining.

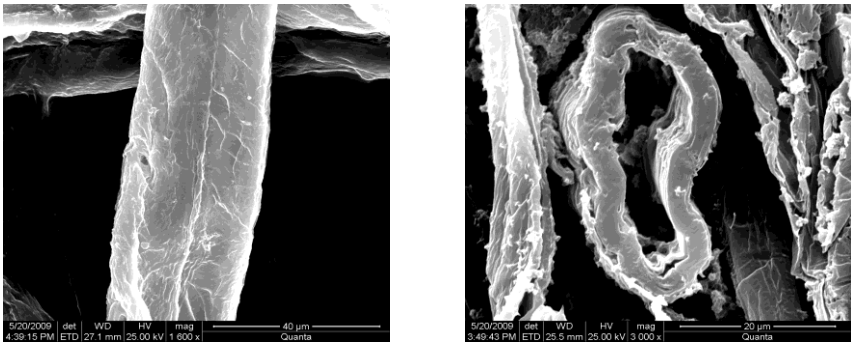
The increased delamination and swelling as a result of LXS treatment after refining may also enhance permeability of bleaching chemicals and dissolved lignin in subsequent bleaching operations, resulting probably in a reduced bleaching chemical dosage and effluent pollution load.



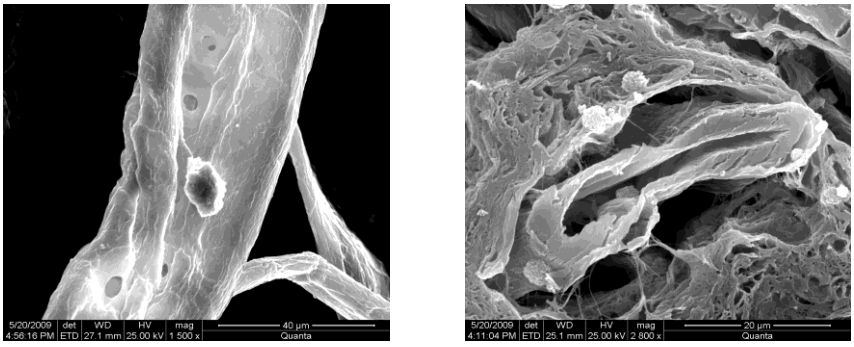
(a) UP ----surface and cross section of fiber



(b) LXSP ---surface and cross section of fiber



(c) RP---surface and cross section of fiber



(d) RLXSP ---surface and cross section of fiber

Fig. 6. SEM images of the treated and untreated pulp

CONCLUSIONS

1. The delignification ability of the laccase/xylanase system (LXS) can be further enhanced by refining the Masson pine pulp prior to LXS treatment.

2. Both delignification and yield loss increased with increasing refining revolutions.

3. The small cellulase activity in LXS had no effect on the viscosity of the treated pulp.

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