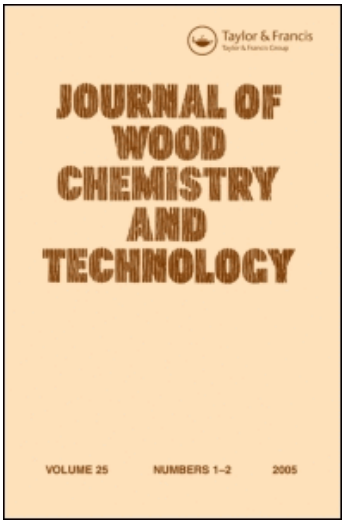

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Study on Direct Delignification with Laccase/Xylanase System

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Abstract: Direct degradation of lignin with laccase/xylanase system (LXS) instead of the expensive laccase/mediator system (LMS) was investigated. The optimal treatment conditions with LXS were determined. The lignin degrading ability and the physical properties of enzyme treated pulps were compared between LXS and LMS. The results indicated that the optimum treatment conditions of pine kraft pulp with LXS were pH 4.2, temperature 45°, pulp consistency 3%, time 3 h, laccase dosage 10 IU/g. LXS has as strong an ability to delignify with good selectivity as LMS. The strength of pulp was obviously enhanced by LMS treatment to the same extent as LMX when compared with the control at 30 ^{0}SR. Thus, LXS can entirely replace expensive and complicated LMS for bio-delignification application.

Keywords: Bio-delignification, laccase/mediator system, laccase/xylanase system, lignin

INTRODUCTION

Lignin is the second most abundant natural macromolecule material on earth. It is well known for its complicated chemical structure and its resistance to biodegradation. Only a few kinds of micro-organisms, belonging to basidiomycetes, in nature can degrade lignin. White-rot fungi are known to effectively degrade lignin and transform complicated lignin polymers into CO_2 and H_2O.[1,2] White-rot fungus degrades lignin by secreting several extracellular enzymes, among which laccase is significantly important. However,

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laccase along has limited action on lignin. Laccase needs a mediator as well as oxygen to be an effective lignin degrader.[3,4] Many mediators have been discovered but all of them are too expensive for any industrial application. This article brings forward the notion of using a white-rot fungus to produce a novel laccase/xylanase system that can effectively degrade lignin without a mediator. The laccase/xylanase system is produced by controlling culture conditions of the white-rot fungus. This novel system is different from the simple mixed enzyme system of laccase and xylanase, which still needs mediator such as 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) for effective delignification.[5] In addition, those two enzymes are usually produced from different strains (xylanase are usually produced from mould). They had their own optimal operating conditions, and therefore the blend of the enzymes may not give good application results.[5]

MATERIALS AND METHODS

Materials

Unbleached mason pine (*Pinus massoniana*) kraft pulp of kappa number around 30, brightness 25.9% SBD was obtained as dried pulp sheet from Qingshan Papermaking Mill in Fujian Province.

Laccase/Mediator System (LMS)

The purified laccase in LMS system was provided by Novozyme Co. and its enzyme activity was 150 IU/ml after dilution. Violuric acid as mediator was obtained from Sigma Co.

Laccase/Xylanase System (LXS)

Laccase/xylanase system was directly produced by controlling the culture conditions of white-rot fungus (*Lentinus lepidueus*). The optimum conditions were: soluble starch as carbon source, protein peptone as nitrogen source, culture temperature 30°C, and time 7 days. The enzyme activities of laccase, xylanase, and cellulase were 730 IU/ml, 4.49 IU/ml, and 0.231 IU/ml, respectively. The activity of lignin peroxidases or Mn- peroxidases was not found.

Methods

Enzyme Treatment

The pulp was treated with laccase in plastic bags. The treatments were carried out at different pH, temperature, pulp consistency, time, and enzyme dosage.

After enzyme treatments, liquid was separated from the treated pulp by filtration for analyses of absorbance value and reducing sugars in the filtrate. The treated pulp was washed with tap water and squeezed to remove water. Then the pulp was dispersed to determine pulp properties. The control pulp was treated under the same conditions as those of the enzyme treated pulp but without adding any enzyme to it. When LXS was used,no mediator was added. But when LMS was applied, then pulp was treated with violuric acid (dosage 0.5% based on oven dried weight of pulp) in the presence of oxygen (pressure 0.3MPa).

Laccase Activity Assay

Laccase activity using ABTS was determined by the method described by Bourbonnais and Paice.[6] Reaction mixtures contained in a total volume of 3.0 ml: 0.1mol sodium acetate buffer (pH 4.5), 0.03% (W/V) ABTS, and 0.1ml culture supernatant. Oxidation of ABTS was measured by monitoring the increase in absorbance at 415 nm over an initial 10-min period. One Unit of enzyme activity is defined as the amount of laccase needed to oxidize 1 μmol of ABTS per minute.

Reducing Sugar Assay

Reducing sugars in the filtrate were determined by DNS Method.[7]

Determination of UV Absorbance

Several drops of CCl_3COOH were added to the filtrate, adjusted to pH 9, and centrifuged. The supernatant was diluted 10 times and absorbance value was measured at 280 nm with UV-751 spectrometer (Shanghai spectrum instrument Co.) The absorbance value reflects the relative amount of lignin dissolved in the filtrate.

Chemical Composition Assay

Holocellulose, Klason lignin, benzene-alcohol extractive, and pentosan content of the treated pulps were determined according to the China Standard Methods for the Papermaking Industry (G B/T2677). Acid soluble lignin was determined with UV-spectrophotometer.[8] The delignification selectivity of enzyme treatment was described by the ratio of holocellulose content to Klason lignin content.[9]

Determination of Pulp Properties

The yield, brightness, and strength of pulp were determined according to GB/T2677. Pulp was refined in PFI mill to around 30 SR and the basis weight of hand sheets is 60 g/m^2.

Determination of Crystallinity

A small amount of control and enzyme-treated pulps were made into tablet and then crystallinity was determined with model DMAX-3B X-ray diffractometer. Detecting conditions were: tube pressure: 30 KV, tube currency: 20 mA, Cu-target. The following formula was used to calculate relative crystallinity:

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

where, I_{002} is maximum intensity of diffraction angle of 002 crystal lattice; I_{AM} is the scatter intensity of amorphous background diffraction when $2\theta = 18^\circ$.

Determination of SEM

SEM images of both control and enzyme treated pulps were taken with model SEM-505 electron microscope.

RESULTS AND DISCUSSION

The Optimal Conditions of LXS Treatment

The key for any kinds of enzyme treatments is that pulp should be treated under optimal conditions. This article studied the parameters that influence the effect of LXS enzyme treatment by detecting the absorbance value of dissolved lignin and reducing sugar content in the filtrate. The results can be seen in Figures 1–4 and Table 1.

Most enzymes have preferred pH ranges where they attain their best activity. High enzyme activity and reaction rate can be achieved only when pH is set at optimal level. As shown in Figure 1, the absorbance value reached highest level and the reducing sugar content was relatively low when pH was set at 4.2. Consequently, the optimal pH for LXS enzyme treatment was 4.2, because the LXS was mainly used for lignin degradation.

The catalytic reaction of enzyme also depends on the treatment temperature, thus there is a suitable temperature for enzyme treatment. Results in Figure 2 indicated that the treatment effect became better as the temperature rose in a certain range. The absorbance value and reducing sugar content gradually increased with increasing of temperature. However, the absorbance

Table 1. Effect of enzyme dosage on delignification and properties of pulps

Enzyme dosage /IU·g^{-1}	0	5	10	15	20
Absorbance value	0.383	0.551	0.586	0.596	0.618
Reducing sugar/%	0.159	0.162	0.171	0.174	0.179
Burst index/kpa·m^2·g^{-1}	2.1	2.2	2.8	3.1	3.2
Breaking length/km	3.6	4.4	4.5	4.6	4.7
Tear index /mN·m^2·g^{-1}	12.8	14.3	15.6	15.9	16.1
Yield/%	99.4	98.0	97.8	97.0	95.3
Beating degree/^{0}SR	31.0	30.5	31.4	31.2	30.0

Enzyme treatment conditions: pH 4.2, temperature 45°C, pulp consistency 3%, time 3 h.

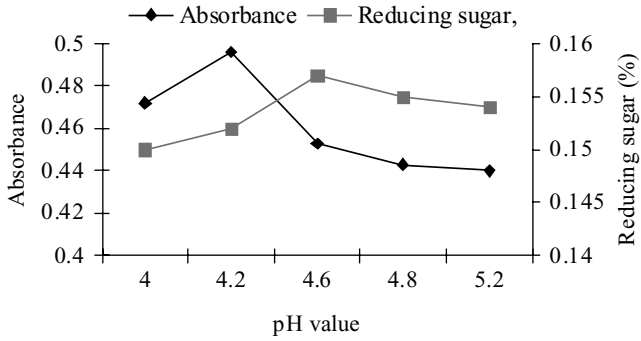


Figure 1. Effect of pH on delignification. (Enzyme treatment conditions: temperature 45°C, pulp consistency 3%, time 2 h, laccase dosage 10 IUg^{-1}.)

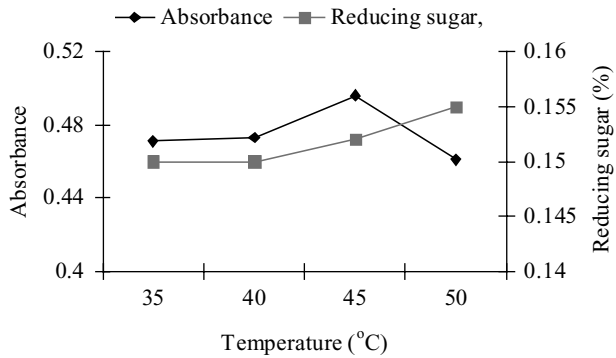


Figure 2. Effect of temperature on delignification. (Enzyme treatment conditions: pH 4.2, pulp consistency 3%, time 2 h, laccase dosage 10 IUg^{-1}.)

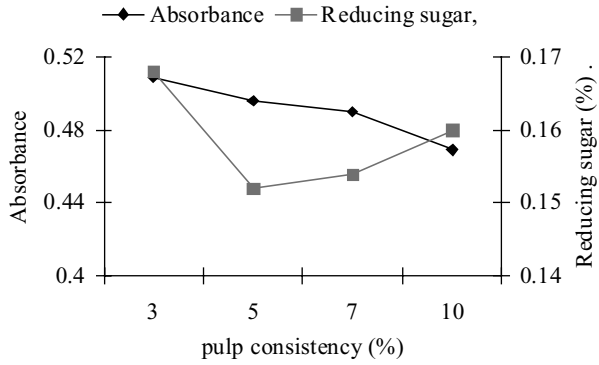


Figure 3. Effect of pulp consistency on delignification. (Enzyme treatment conditions: pH 4.2, temperature 45°C, time 2 h, laccase dosage 10 IUg^{-1}.)

value reached the maximal level when the temperature reached 45°C, above which the content of dissolved lignin decreased, due presumably to the low laccase enzyme activity. It is interesting to note that xylanase apparently has higher optimal temperature than laccase.

The consistency of pulp affects the reaction rate of enzyme. The higher the consistency of pulp, the higher the concentration of enzyme, which should be beneficial for delignification. On the other hand, the high consistency of pulp will lead to uneven reaction owing to the difficulty for enzyme and pulp to mix effectively. As shown in Figure 3, with the increasing concentration of pulp, the absorbance value and reducing sugar content reduced. When pulp consistency was fixed at 3%, both the absorbance value and reducing sugar

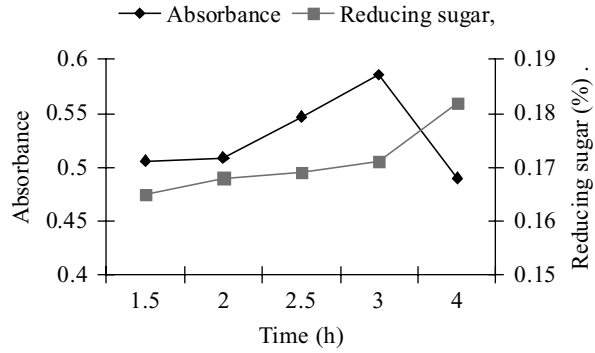


Figure 4. Effect of time on delignification. (Enzyme treatment conditions:pH 4.2, temperature 45°C, pulp consistency 3%, laccase dosage 10 IUg^{-1}.)

content reached maximum, which meant LXS treatment should be operated at low pulp consistency.

Reaction time is another important factor for enzyme treatment. The lignin cannot be sufficiently degraded if the reaction time is too short. But if the reaction time were too long, it would lead to high energy consumption and low production capacity. Figure 4 showed that the absorbance value increased as the time of enzyme treatment prolonged. When the reaction time was set at 3 h, the absorbance value reached maximum and then decreased beyond 3 h. It maybe explained that the dissolved lignin was absorbed back to pulp.

Enzyme dosage is a key factor for enzyme treatment. Increasing the dosage of enzyme will lead to high cost. Results in Table 1 show the influence of enzyme dosage (based on laccase) on delignification and pulp properties when LXS was applied. As shown in Table 1, the higher the enzyme dosage, the higher the dissolved lignin and the reducing sugar content in the filtrate. At the mean time, the physical properties of the pulp improved while the pulp yield decreased as the enzyme dosage increased. However, these effects appeared to level off when dosage went above 10 IU/g. Thus, it was concluded that 10 IU/g was the optimal dosage for LXS treatment process.

There was little cellulase activity contained in the LXS system. The results in Table 1 show that the strength of the LXS-treated pulp was not adversely affected by the presence of a small amount of cellulase activity. In fact, the strength of LXS-treated pulp was substantially better than that of control pulp, which is in agreement with an earlier study.[10] When enzyme dosage was set at 10 IU/g, the burst index, breaking length, and tear index of enzyme-treated pulp increased by 33.3%, 25.0%, and 21.9%, respectively, as compared with those of the control pulp. The probable causes of these strength improvements by LXS treatment will be discussed later.

Comparison of Delignification Ability of LXS, LMS, and Laccase

Laccase can oxidize phenolic compounds and reduce molecular oxygen into water. However, because of the low redox potential (300–800 mv), laccase cannot directly degrade non-phenolic structural units,[11] which account for 90% of lignin structure in wood and 50–70% of units in the residual lignin in unbleached kraft pulps. It needs some low-molecular weight compounds as mediator to transfer electron in the process of redox reaction. With the help of these redox mediators, laccase will have more powerful catalytic-oxidative ability. In this study, we compared delignification ability of laccase (L), LMS, and LXS , all under the optimal treatment conditions for LXS treatment: pH 4.2, temperature 45°C, pulp consistency 3%, and time 3 h. The results are listed in Table 2.

As can be seen in Table 2, considerable amount of lignin and reducing sugars leached into the filtrate in the control experiment without enzyme addition.

Table 2. Comparison of LXS with L and LMS in delignification ability

Enzyme treated pulp	Enzyme dosage/IU·g^{-1}	0	5	10	15	20
L	Absorbance value	0.383	0.386	0.413	0.437	0.493
	Reducing sugar/%	0.159	0.160	0.160	0.161	0.162
LMS	Absorbance value	0.383	0.558	0.569	0.590	0.608
	Reducing sugar/%	0.159	0.159	0.160	0.161	0.164
LXS	Absorbance value	0.383	0.551	0.586	0.596	0.618
	Reducing sugar/%	0.159	0.162	0.171	0.174	0.179

As expected, without the presence of xylanase, both L and LMS were not able to cause dissolution of reducing sugars in the filtrate, whereas in the LXS, the reducing sugar content of the filtrate increased with the increasing dosage of enzyme. Both LMS and LXS are much more effective in degrading lignin than L. For example, the increase in the absorption values of LMS and LXS was more than 6 times larger than L at 10 IU/g. These results indicated that laccase need oxygen and mediator to degrade lignin, but mediator is not required in the presence of xylanase in LXS system.

Chemical Composition of Pulp after Enzyme Treatment

Chemical composition of pulp before and after enzyme treatment is listed in Table 3. These pulps were treated with two different enzyme systems under the optimal conditions using a laccase dosage of 20 IU/g. Especial noteworthy is that the lignin content of pulps decreased greatly, the delignification rate reached 27% and 28%, respectively, after LMS and LXS treatments. The delignification selectivity, described as the ratio of holocellulose content to Klason lignin content, is higher for the enzyme-treated pulps than that of the original, indicating that more lignin and fewer carbohydrates are dissolved during the enzyme treatment. In addition, small amounts of pentosan were dissolved

Table 3. Chemical composition before and after enzyme treatment

Pulp	Original	LMS treatment	LXS treatment
Benzene alcohol extractive%	0.4	0.2	0.3
Klason lignin content/%	5.7	4.3	4.2
Acid soluble lignin content/%	1.0	0.6	0.6
Pentosan content/%	7.2	6.6	6.2
Holocellulose content/%	93.0	92.8	92.9
Delignification rate/%		26.9	28.4
Delignification selectivity		18.9	19.4

after both LMS and LXS treatments, slightly more pentosan being dissolved by LXS than by LMS. The dissolution of pentosan in LMS may be due to the delignification of LCC (lignin-carbohydrate complex), which released some low molecular weight xylan to the filtrate. In the LXS, xylan may be released to the filtrate by both delignification of LCC and the action of xylanase in LSX system. It is interesting to note that the results of lignin and pentosan contents after enzyme treatments in Table 3 are consistent with those in Table 2.

Other than slightly lower pentosan content, the holocellulose content did not change by either enzyme treatments suggesting that cellulose was not damaged, which is in agreement with no pulp strength damage and no change in cellulose crystallinity after enzyme treatment. Finally, after LMS and LXS treatment, the benzene-alcohol extractives decreased by 50.0% and 25.0%, respectively, this should be beneficial to the subsequent bleaching process.

Determination of Crystallinity

Crystallinity is the percentage of crystallization region in cellulose structure. As long as the crystallinity increases, the tensile strength, elastic modulus, hardness, density, and dimensional stability of fiber will also increase.[12] In this research, crystallinity was determined by X-ray diffraction method. Table 4 and Figure 5 showed the results of crystallinity after enzyme treatment.

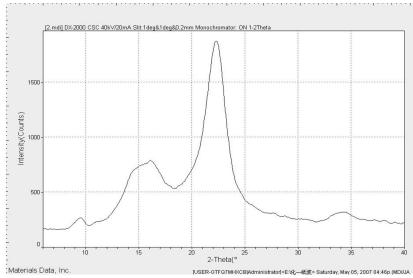
As shown in Table 4 and Figure 5, the crystal structure and the crystallinity of cellulose did not change after LXS and LMS treatment. These results indicated that the small cellulase activity found in LXS had little effect on cellulose. Therefore, the pulp could maintain their crystallinity after enzyme treatment and it also explained why the pulp strength did not reduce after enzyme treatment.

SEM Observation

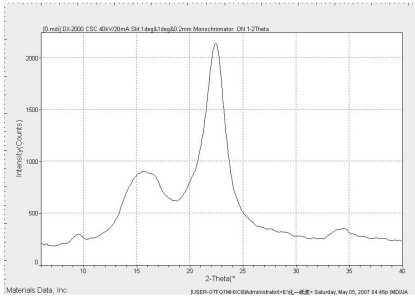
Change in microstructure of fibers was observed before and after enzyme treatment using SEM. Figure 6 is the SEM images of surface and cross-section of the pulp treated with LXS and LMS system under optimal conditions. As can be clearly seen from Figure 6, the surface and cross-section of original pulp was smooth and undamaged, whereas the two enzyme-treated pulps had rough fiber surface and had many tiny holes and cracks in the cross-section of cell wall, presumably resulting from dissolution of lignin. Comparing the

Table 4. Crystallinity before and after enzyme treatment

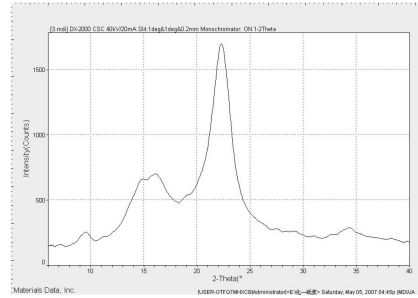
Pulp	Original	LMS treatment	LXS treatment
Crystallinity %	67	68	68



a. original pulp



b LMS treated pulp



c LXS treated pulp

Figure 5. Crystallinity of pulps before and after enzyme treatments.

images of the two different enzyme-treated pulps, more holes and cracks were found in LXS-treated pulp. This may be due to the fact that LXS contained xylanase that could degrade xylan and LCC structure in fiber surface and cell wall.[13] The change of fiber structure indicated that enzyme treatment could enhance permeability of bleaching chemical and dissolved lignin in subsequent bleaching operation, resulting in reduced chemical dosage and effluent pollution load.

Determination of Pulp Physical Strength

When pulp is treated with a different enzyme system, the pulp properties will be varied due to different selectivity of the enzyme system. This research compared the physical properties of pulps treated under optimal conditions and the results are listed in Table 5.

As can be seen in Table 5, compared with those of the control pulp, the burst index, breaking length, and tear index of the two enzyme-treated pulps increased substantially. The increase is most likely due to the increased flexibility of the enzyme-treated pulps as a result of delignification (Table 4) and delamination of cell wall (Figure 6). It is especially noteworthy that the two enzyme-treated pulps had almost identical physical properties, chemical

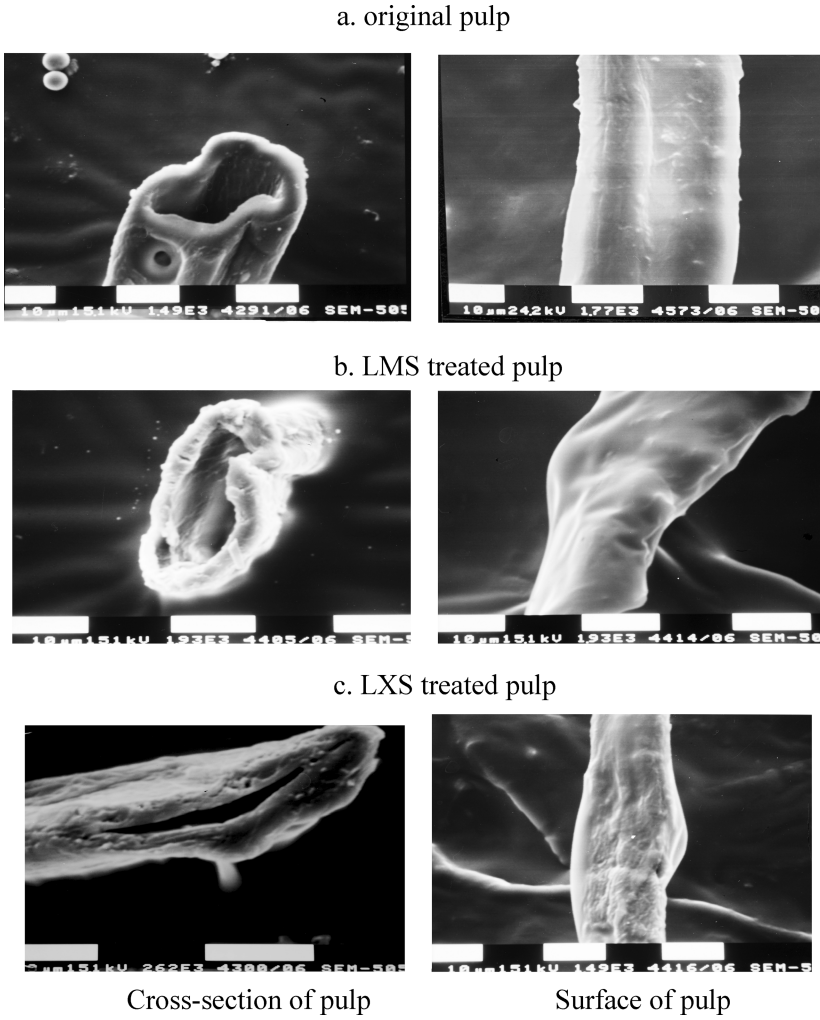


Figure 6. SEM images of the surface and the cross-section of original and enzyme treated pulps.

composition, crystallinity, degree of delignification, and delignification selectivity (Table 3). These results clearly indicate that LXS could entirely replace LMS in the delignification process and that in the presence of xylanase, laccase requires no costly mediator for delignification. As to the reasons why LXS could degrade lignin directly, it might be due to the fact that xylanase has the ability to degrade LCC, which helped the catalysis process of laccase. Surprisingly, the brightness was not improved after enzyme treatment in spite of some delignification. Whether or not the LXS treatment is beneficial to the pulp

Table 5. Effect of enzyme treated pulp on properties of pulps

Pulp	Original	LMS treated	LXS treated
Burst index/kpa·m^2·g^{-1}	2.1	2.7	2.8
Breaking length/km	3.3	4.5	4.5
Tear index/mN·m^2·g^{-1}	12.0	15.3	15.6
Yield/m^2	100	97.8	97.8
Brightness/% SBD	25.4	25.5	25.6
Beating degree/^{0}SR	30.6	31.2	31.4

bleachability and pulp strength of the fully bleached pulp is currently under investigation in our laboratory.

CONCLUSIONS

1. The optimum treatment conditions of LXS were pH 4.2, temperature 45°C, pulp consistency 3%, time 3 h, and enzyme dosage 10 IU/g.
2. Both LXS and LMS system have better ability to delignify pulp than laccase alone.
3. The LXS- and LMS-treated pulps have identical physical properties, chemical composition, crystallinity, degree of delignification, and delignification selectivity, indicating that LXS has the same ability to delignify as LMS. Apparently, in the presence of xylanase, mediator is not requires for laccase to delignify. Thus, the LXS system could entirely replace the costly and complicated LMS in biopulping and biobleaching.
4. Both LXS- and LMS-treated pulps have higher pulp strength properties than the original pulp, presumably due to the delignification and delamination.

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