Biobleaching Effects of Crude Xylanase from *Streptomyces griseorubens* LH-3 on Eucalyptus Kraft Pulp

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In this work, a xylanase-producing strain, *Streptomyces griseorubens* LH-3, was cultured, and the crude xylanase was prepared. Analysis of its enzymatic properties revealed that the crude xylanase possessed good thermal stability at temperatures below 60 ^{o}C, exhibited a wide pH range from 4.0 to 9.0, and was cellulase-free. This crude enzyme was used to treat eucalyptus kraft pulp, and the release of chromophores was the highest at the dosage of 20 IU g^{-1} dry pulp. Compared with the untreated group, biobleaching of eucalyptus kraft pulp with this enzyme increased the brightness of the pulp by 12.9% and reduced the Kappa number by 27.4%. Biobleaching of eucalyptus kraft pulp with this enzyme obtained the same final pulp brightness compared with that of the control; however, hydrogen peroxide consumption was reduced by 17% and the yield and viscosity of the pulp was increased by 1.47% and 1.53%, respectively. This crude xylanase has promising potential for industrial applications.

Keywords: Xylanase; Biobleaching; Viscosity of pulp; Eucalyptus kraft pulp

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INTRODUCTION

Kraft pulp has excellent paper-making performance and meets the requirements for manufacturing many types of paper. Thus, it has been widely applied in a variety of industries and fields. Kraft pulping is a process for the conversion of wood into pulp using hydrogen sulfide and hydroxyl ions. At the end of the kraft process, about 90% of the lignin contained in the wood has been removed. The remaining 10% represents modified lignin, which is responsible for the characteristic brown coloration associated with kraft pulp (Bajpai and Bajpai 1992). To obtain a bright pulp, the remaining lignin must be removed. This has been achieved through chemical bleaching, which involves the use of chemicals such as chlorine gas, chlorine dioxide, and alkalis to dissolve lignin in pulp and to release the chromophoric groups and other colored substances by completely destroying the lignin structure (Patel *et al.* 1993). In this way, bright pulp can be achieved. However, during such a chemical bleaching process, large amounts of organic chlorine compounds are generated. These compounds are toxic, mutagenic, persistent, and bio-accumulated in the environment, causing numerous harmful disturbances in biological systems. Thus, there is a motivation to replace or supplement traditional bleaching with environmentally compatible procedures (Ziaie-Shirkolaee *et al.* 2008).

One environmentally friendly technique is the use of xylanases in the biobleaching of pulps. Viikari *et al.* (1986) reported the first study on the role of xylanase in the biobleaching of pulp. Since then, many studies on biobleaching with xylanases have been reported (Manimaran *et al.* 2009; Garg *et al.* 2011), and a number of xylanase products have been developed. With the xylanase biobleaching technique, the pulp is usually treated with xylanase before chemical bleaching (Martin-Sampedro *et al.* 2012). Xylanases catalyze the hydrolysis of reprecipitated xylan located on the surface of microfibrils. This facilitates pulp bleaching and lowers chemical consumption, thereby reducing the discharge of toxic organo–chlorine compounds into the environment. Using xylanase from *Aspergillus niger* in the biobleaching of eucalyptus pulp, Khonzue *et al.* (2011) reduced chemical consumption by 20% to achieve the same final pulp brightness compared with the control. Using xylanase from *Bacillus* sp. XTR-10 in the biobleaching of kraft pulp, Saleem *et al.* (2009) achieved a 15% saving in the chemicals required to obtain the same final pulp brightness, comparable with the control.

While chemical bleaching can increase paper brightness, it also causes serious hydrolysis of the cellulose components and reduces the yield and viscosity of the pulp. Viscosity reduction is not desirable because this property is related to the degree of cellulose polymerization and to paper strength (Paice *et al.* 1988). Current studies on xylanase biobleaching have mainly focused on reduction of the consumption of bleaching chemicals (Garg *et al.* 2011; Manimaran *et al.* 2009; Khandeparkar and Bhosle 2007; Li *et al.* 2005); very little attention has been given to the effects of xylanase on the yield and viscosity of pulp (Kantelinen *et al.* 1993; Xu *et al.* 2013). In the present study, a fermentation of *S. griseorubens* LH-3 was conducted, the crude xylanase extract was prepared, and the enzymatic properties of the crude xylanase were studied. The goal was to employ this crude xylanase as a booster in the biobleaching of eucalyptus kraft pulp, to evaluate its effectiveness in reducing chemical consumption, and then to analyze any improvements in the yield and viscosity of the pulp.

EXPERIMENTAL

Materials

S. griseorubens LH-3 was initially isolated from decaying tree roots at The Agricultural Farm of Guangxi University, China, and preserved at The Institute of Food and Fermentation Engineering of Guangxi University. Unbleached eucalyptus kraft pulp was provided by Hainan Jinhai Pulp & Paper (APP). Birchwood xylan and carboxymethyl cellulase (CMCase) were purchased from Sigma (St. Louis, MO, USA).

Culture Conditions

S. griseorubens LH-3 strain was cultured and activated with the seed culture medium (1.0 g of bagasse semi-cellulose, 1.0 g of peptone, 0.4 g of NaCl, 0.05 g of $MgSO_4$, and 0.05g of K_2HPO_4 per liter, pH 8.0) at 37 ^oC with shaking for 24 h. About 10% of the activated seed culture was inoculated into xylanase-producing fermentation media (30.0 g of bagasse semi-cellulose, 10.0 g of KNO_3, 0.5 g of KH_2PO_4, and 0.5 g of $MgSO_4$ per liter, pH 8.0) and incubated in an incubating shaker at 37 ^oC with shaking at 160 rpm for 96 h.

Enzyme Assays

Enzyme activity was expressed in international units (IU). Xylanase activity and CMCase were assayed by incubating 0.2 mL of appropriately diluted enzyme with 1.8 mL of a solution containing 1% of the respective substrate (xylan and carboxymethyl cellulose) in acetate buffer (pH 6.0). After incubating at 50 ^{o}C for 15 min, the reducing substances released were assayed by dinitrosalicylic acid as described by Miller (1959). Controls were prepared with the enzyme added after boiling. The definition of 1 IU of activity toward the substrate just mentioned was 1 μmol of xylose or glucose equivalent released/min under the stated assay conditions, using either a xylose or glucose standard curve.

Effect of pH on the Activity and Stability of Crude Xylanase

The effect of pH on xylanase activity was tested from 3.0 to 10.0 at 50 ^{o}C. The following four buffers were used to achieve the defined pH ranges: 50 mM sodium acetate buffer (pH 3.0–6.0), 50 mM phosphate buffer (pH 6.0–8.0), 50 mM Tris–HCl buffer (pH 8.0–9.0), and 50 mM glycinesodium hydroxide buffer (pH 9.0–10.0). The pH stability (without substrate) was estimated by maintaining the enzymes for 60 min at 50 ^{o}C in the same pH range, and then the residual activity was determined under the standard conditions.

Effect of Temperature on the Activity and Stability of Crude Xylanase

Xylanase activity was measured at 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, and 85 ^{o}C, under the optimal pH. Thermal stability (without substrate) was estimated by maintaining the enzyme for 60 min at 30, 40, 50, 60, 70, and 80 ^{o}C, under the optimal pH. After cooling, the residual activity was estimated under standard conditions.

Biobleaching of Pulp with Xylanase (X) and Hydrogen Peroxide (P)

Twenty grams of unbleached dry eucalyptus kraft pulp was put in polyethylene bags and maintained at 10% consistency and pH 8.0. Samples were separately treated with different doses of crude xylanase, varying from 0 to 50 IU g^{-1} dry pulp, and mixed well by hand and then left for 60 min at 60 ^{o}C. After completion of the reactions, 2 L of distilled water was added, and the pulps were filtered. The compositions of the filtered solutions and the yield of the pulp were determined.

Pulps, untreated and treated with enzyme, were fully washed and bleached with hydrogen peroxide (H_2O_2) under the reaction conditions: 3% H_2O_2, 3% NaOH, 0.2% Diethylene triamine pentacetate acid (DTPA), 0.5% $MgSO_4$, and 20 g of dry pulp at a concentration of 10%, at 60 ^{o}C for 60 min. After bleaching, the pulp was washed thoroughly and the physical and chemical properties of the pulp were investigated.

Single-Stage Bleaching of Pulp with Hydrogen Peroxide (P)

H_2O_2 was used as the bleaching agent (Ferrer *et al.* 2011). The pulp was bleached with H_2O_2 at concentrations of 3%, 3.2%, 3.4%, 3.6%, 3.8%, and 4.0% under the same bleaching conditions as mentioned above. After bleaching, the pulp was washed thoroughly and the physical and chemical properties were investigated.

Analytical Methods

The filtrates were analyzed for the release of reducing sugars by the DNS method (Miller 1959) at a wavelength of 540 nm and for chromophores by measuring absorbance at 237, 273, 280, and 465 nm, respectively (Patel *et al.* 1993).

After being washed, the pulp was evaluated for Kappa number (T236cm-85), brightness (ISO) (T452om-02), and viscosity (T230om-04) as per TAPPI test methods (Anonymous 2000–2001).

RESULTS AND DISCUSSION

Xylanase Production by *S. griseorubens* LH-3

The culture medium of *S. griseorubens* LH-3 was collected 96 h after culturing and centrifuged at 8000 rpm at 4 ^{o}C for 10 min. The supernatant was used as the crude enzyme extract for the characterization of enzymatic properties and the biobleaching of the pulp. The xylanase activity in this crude extract was determined to be 135 U/mL, but no cellulase activity was detected. The optimum temperature and pH for xylanase activity were 65 ^{o}C and 6.0, as shown in Fig. 1 (A and C), respectively. The thermal stability of the crude enzyme was measured, in the absence of substrate, from 30 to 80 ^{o}C for 60 min, and 80% of xylanase activity was maintained by incubation of the reaction solution at 60 ^{o}C for 60 min (Fig. 1B). The crude xylanase was stable in the pH range of 4.0 to 9.0 after incubating in different buffers for 60 min (Fig. 1D).

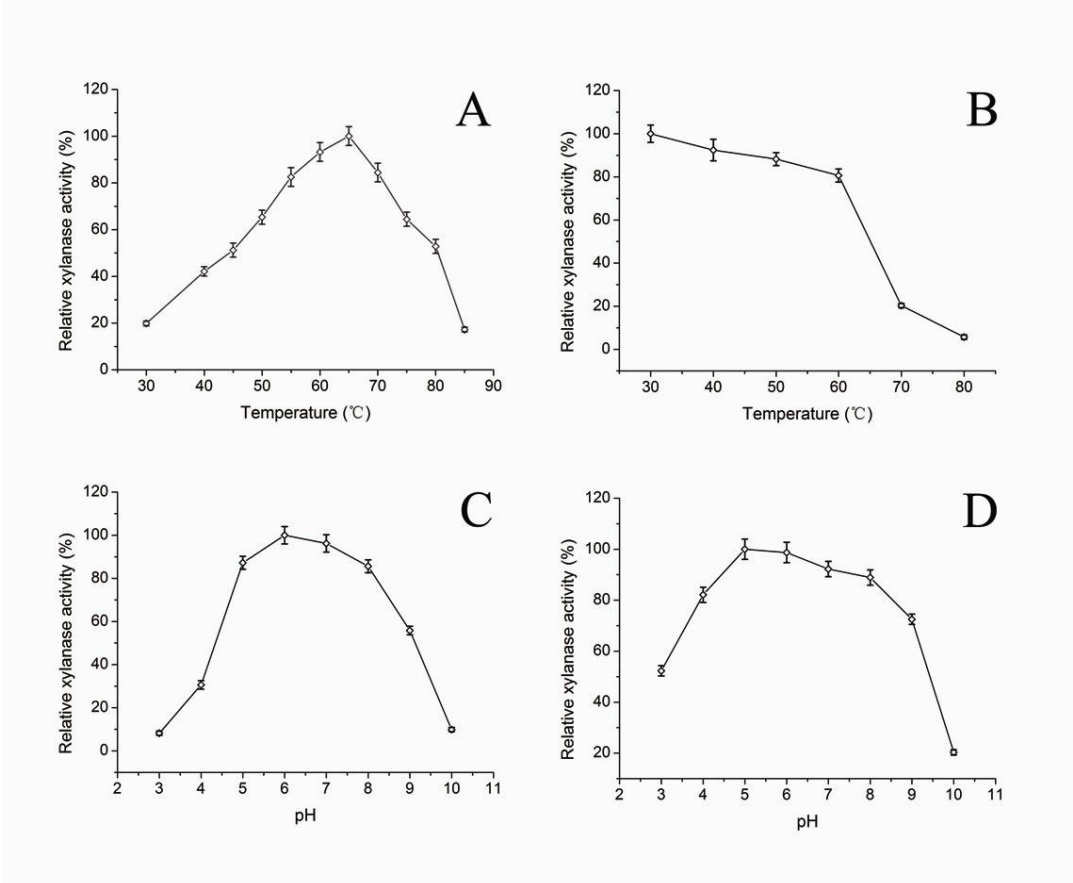

Fig. 1. Effect of temperature on the activity (A) and thermostability (B), and effect of pH on the activity(C) and stability (D) of crude xylanase form *S. griseorubens* LH-3

Commercial enzymes used in the paper and pulp industry have pH and temperature optima ranging from 3 to 8 and 30 °C to 75 °C, respectively (Viikari *et al.* 1994). The crude xylanase produced by *S. griseorubens* LH-3 showed the pH and thermal stability to be within these limits; therefore, it can be used for pulp bleaching.

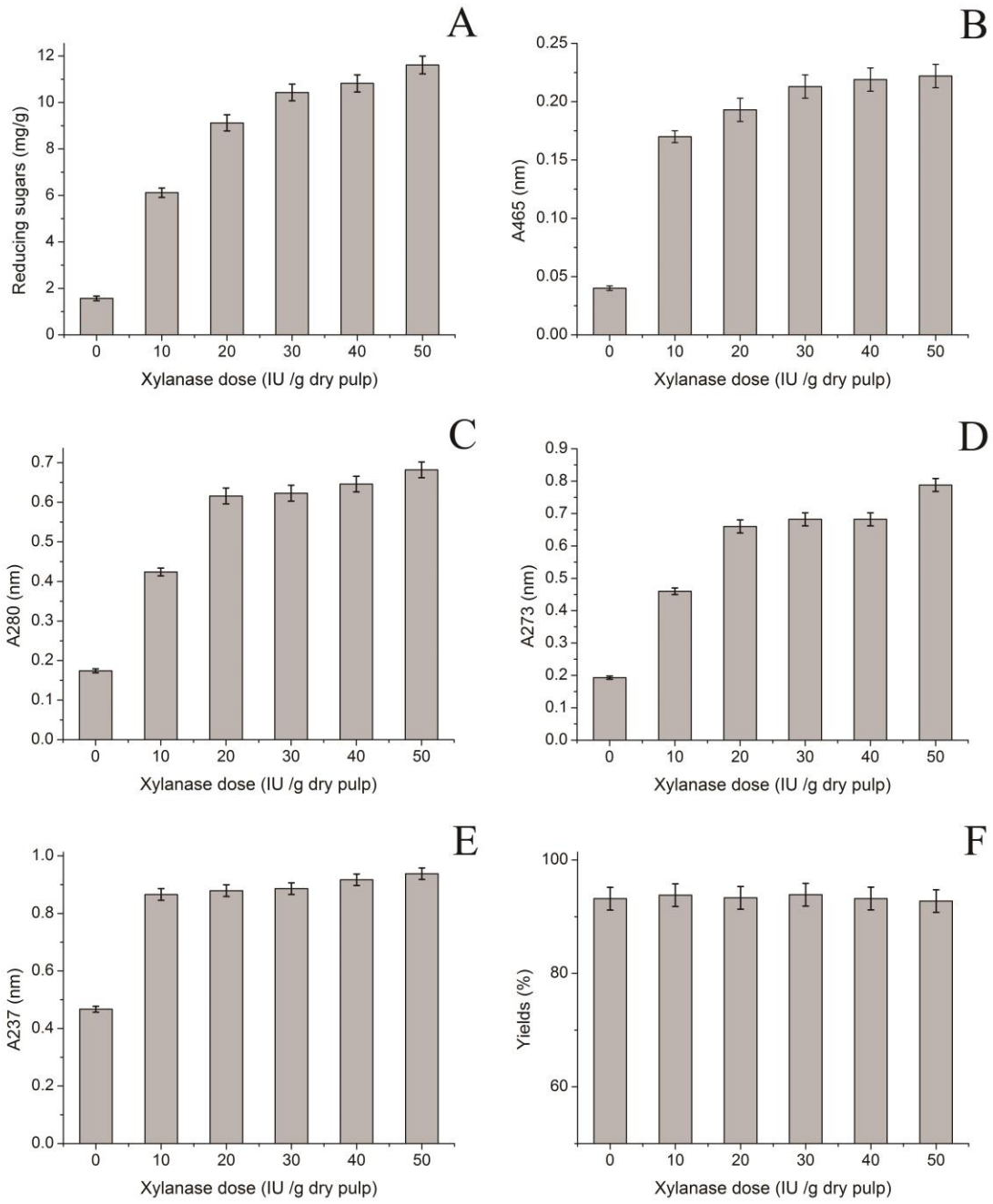

Fig. 2. Effects of xylanase dose on reducing sugar released (A), and colored substances (B–E), and yields (F) of eucalyptus kraft pulps treated with *S. griseorubens* LH-3 xylanase preparations. All the experiments were performed at pH 8.0 and at 10% of pulp consistency, with a retention time of 60 min at 60 °C.

Pretreatment of Pulp with Xylanase (X)

The pulp was treated with different doses of the crude xylanase. Analyses of the resulting filtrates revealed that, compared with those of the control group, the amounts of reducing sugars (OD_{540}) and chromophores released (measured at 237, 273, 280, and 465 nm, respectively) in enzyme-treated solutions were significantly increased, and those continuously increased with increases in enzyme doses. However, when the enzyme dose reached 20 IU g^{-1} dry pulp, this increase slowed down (Fig. 2, A–E). The treatment of pulp with xylanase can release reducing sugars, chromophoric groups (OD 465 nm), and other phenolic substances with OD at 280 nm, 273 nm, and 237 nm, and the amounts of these released substances were increased with increasing enzyme doses. When the amount of enzyme reached saturation, further increases in the amount of enzyme did not cause further increases in the amount of released substances (Khandeparkar and Bhosle 2007). Similar phenomena have also been reported by others. For example, Goluguri *et al.* (2012) treated pulp with xylanase from *T. basicola* (MTCC-1467) and observed that the amount of reducing sugars and chromophore released increased with increasing enzyme doses and reached maximal values when the enzyme dosage was increased to 20 IU g^{-1} dry pulp.

It was observed that enzyme-treated pulps did not cause significant reduction in yield (Fig. 2F). The crude xylanase from *S. griseorubens* LH-3 was cellulase-free; thus, it did not result in a significant reduction in the yield of pulp due to the degradation of the cellulose components by cellulase. Gübitz *et al.* (1997) reported that due to the presence of cellulase in the fungal xylanase extracts, the treatment of pulp with this enzyme extract resulted in a 16% loss of the yield, whereas Manimaran *et al.* (2009) reported that treatment of bagasse pulp with cellulase-free xylanase extract yielded a loss of only 2.5%.

Biobleaching of Eucalyptus Kraft Pulp with Xylanase and Hydrogen Peroxide (XP)

Table 1. Effects of Xylanase Doses on Fiber Properties of Eucalyptus Kraft Pulps Treated with *S. griseorubens* LH-3 Xylanase followed by Bleaching with 3% Hydrogen Peroxide

Enzyme Dosage (IU g^{-1} Dry Pulp)	Brightness (% ISO)	Increase in Brightness (%)	Kappa Number	Decrease in Kappa Number (%)	Yields (%)	Viscosity (mL/g)
0	55.1±1.25	0	7.67±0.15	0	91.00±1.38	723.9±2.68
10	60.0±1.23	8.9	6.48±0.13	18.4	90.30±1.37	711.2±2.44
20	62.2±1.22	12.9	6.02±0.17	27.4	89.69±1.35	710.3±2.38
30	61.8±1.22	12.1	6.13±0.15	25.1	88.81±1.26	708.2±2.47
40	61.2±1.24	11.1	6.03±0.19	27.2	85.05±1.35	702.3±2.48
50	61.5±1.25	11.6	6.15±0.19	24.7	86.10±1.45	710.1±2.39

All experiments were performed at 10% pulp consistency and retention time of 60 min at 60 °C.

The benefits obtained with xylanase are generally dependent on the chemical bleaching sequence used (Birijlall *et al.* 2011; Viikari *et al.* 1994). One-stage hydrogen

peroxide delignification is a practical method of evaluating the effects of enzymes in bleaching (Martin-Sampedro *et al.* 2012). The pulp was treated with different doses of crude xylanase extract and then bleached with 3% H_2O_2. The results are shown in Table 1. Compared with those in the control groups, the brightness of the pulp was significantly increased in the enzyme-treated group. When the enzyme dose reached 20 IU/g dry pulp, the brightness was increased by 12.9% and reached the maximal value (62.2%), as compared with that of the control group. Further increase in enzyme doses did not bring about significant increases in brightness. The yield, viscosity, and Kappa number of the enzyme-treated group were all significantly reduced. The Kappa number was reduced by 1.5 units and by 27.4%. Saleem *et al.* (2009) reported that treatment of wood kraft pulp with xylanase from thermophilic *Bacillus* sp. XTR-10 increased the brightness by 25.94% and reduced the Kappa number by 16.2%. Our results indicated that the treatment of eucalyptus kraft pulp with crude xylanase from *S. griseorubens* LH-3 enhanced the brightness and reduced the Kappa number of the pulp.

To reach 62% ISO brightness through bleaching with H_2O_2, enzymatic treatment of kraft pulp was performed. This treatment reduced the consumption of H_2O_2 by 17% and increased the yield and viscosity of pulp by 1.47% and 1.53%, respectively (Table 2).

Table 2. Effect of Enzyme Treatment on Pulp Biobleaching

Bleaching Process	Brightness (% ISO)	H_2O_2 (%)	Decrease in H_2O_2 (%)	Yield (%)	Increase in Yield (%)	Viscosity (ml/g)	Increase in Viscosity (%)
XP [a]	62.2±1.22	3	17	89.69 ±1.35	1.47	710.3 ±2.38	1.53
P [b]	62.0±1.42	3.60	0	87.56 ±1.31	0	697.5 ±2.11	0

[a] Pulp treatment with xylanase (20 IU /g dry pulp) followed by bleaching with hydrogen peroxide
[b] Pulp bleaching with hydrogen peroxide only
All experiments were performed at 10% pulp consistency and a retention time of 60 min at 60 °C.

Table 3. Comparison of Reduction in Chemical Consumption Obtained by *S. griseorubens* LH-3 Xylanase with Other Bacterial Xylanase Treatments

Microorganism	Type of Pulp	Chemical Reduction (%)	Reference
S. griseorubens LH-3	Eucalyptus	17	
Aspergillus niger	Eucalyptus	20	Khonzue *et al.* 2011
Bacillus stearothermophilus SDX	Wheat straw	20	Garg *et al.* 2011
Bacillus sp. XTR-10	Wood kraft	15	Saleem *et al.* 2009
Thermomyces lanuginosus SSBP	Bagasse	18	Manimaran *et al.* 2009
Arthrobacter sp. MTCC 5214	Kraft	29	Khandeparkar and Bhosle 2007
Thermomyces lanuginosus CBS 288.54	Wheat straw	28.3	Li *et al.* 2005

It has been reported that treatment of pulp with xylanase from different sources could reduce the consumption of chemicals by 10% to 30% (Table 3). However, there have been few reports about the effectiveness of enzymatic biobleaching on the yield and viscosity of pulp. Paice *et al.* (1988) treated hardwood kraft pulp with xylanase and then with chemical bleaching, achieving 80% of ISO brightness and 15.5 mPas viscosity, whereas the viscosity of the group without enzymatic treatment was 15.1 mPas Khandeparkar and Bhosle (2007) treated kraft pulp with xylanase prepared from *Arthrobacter* sp. MTCC 5214 and then with chemical bleaching, achieving 79% of ISO brightness and found that the viscosity of the enzyme-treated pulp was 4.18 P., whereas the viscosity of the untreated pulp was 4.17 P. These reports are consistent with our experimental results, showing that enzymatic biobleaching can improve pulp viscosity. However, no research on its mechanism has been reported. Biobleaching with *S. griseorubens* LH-3 xylanase increased both yield and viscosity of eucalyptus kraft pulp. There may be two reasons for this. The first reason is the reduction of the consumption of chemicals, which, in turn, reduces the degradation of cellulose components. The second is due to the specific hydrolysis of the xylan component of the lignin–carbohydrate complexes (LCC) (Viikari *et al.* 1994) by this cellulose-free xylanase. Hydrolysis of xylan leads to the exposure of lignin within interior cellulose layers and accelerates the release of lignin during the subsequent chemical bleaching, thus reducing the damage to cellulose. Kantelinen *et al.* (1993) investigated enzymatic bleaching and found that the average molecular mass of lignin extracted from enzymatically treated pulp was markedly higher than that of reference pulp with no enzymatic pretreatment. These results indicate that enzymatic hydrolysis of the xylan components of pulp can accelerate the release of lignin and thus reduce the damage to cellulose.

CONCLUSIONS

1. Biobleaching of eucalyptus kraft pulp with crude xylanase from *Streptomyces griseorubens* LH-3 not only significantly increased brightness and reduced Kappa number, but also effectively reduced chemical consumption, and it especially increased the yield and viscosity of the pulp. This crude enzyme extract could have promising potential for industrial applications.
2. There may be two reasons for the increased yield and viscosity of eucalyptus kraft pulp by treatment with crude xylanase from *S. griseorubens* LH-3. The first reason is the reduction of the consumption of chemicals. The second is due to the specific hydrolysis of the xylan component of the lignin–carbohydrate complexes (LCC). Future studies could be conducted to further illuminate the mechanism of this xylanase extract on the biobleaching of pulp.

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