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Effect of *Bacillus circulans* D1 Thermostable Xylanase on Biobleaching of Eucalyptus Kraft Pulp

Daniela A. Bocchini, Valquiria B. Damiano, Eleni Gomes, and Roberto Da Silva*

Departmento de Quimica de Geociencias,
Laboratório de Bioquímica dos Processos e Microbiologia Aplicada,
IBILCE—UNESP, Rua Cristovão Colombo 2265,
São José do Rio Preto, SP, CEP 15054-000, Brazil,
E-mail: dasilva@qeg.ibilce.unesp.br

Abstract

The alkalophilic *Bacillus circulans* D1 was isolated from decayed wood. It produced high levels of extracellular cellulase-free xylanase. The enzyme was thermally stable up to 60°C, with an optimal hydrolysis temperature of 70°C. It was stable over a wide pH range (5.5—10.5), with an optimum pH at 5.5 and 80% of its activity at pH 9.0. This cellulase-free xylanase preparation was used to biobleach kraft pulp. Enzymatic treatment of kraft pulp decreased chlorine dioxide use by 23 and 37% to obtain the same kappa number (κ number) and brightness, respectively. Separation on Sephadex G-50 isolated three fractions with xylanase activity with distinct molecular weights.

Index Entries: *Bacillus circulans*; biobleaching; kraft pulp; thermophilic; xylanase.

Introduction

After cellulose, hemicelluloses are the most abundant organic materials found on Earth and are the main polysaccharides of plant cell walls. They are closely associated with cellulose in plant tissues and account for 40–45% of the dry weight of hardwood and softwood. Xylan is the main constituent of hemicellulose from wood, and it accounts for >90% of the hemicellulose in kraft pulp from hardwood, and about 50% of hemicelluloses in softwood pulps *(1)*. There is evidence that xylan binds to lignin by

*Author to whom all correspondence and reprint requests should be addressed.

covalent bounds, and that hydrogen bonds and Van der Wall's forces bind xylan to the cellulose chain *(2)*.

In recent years, there has been increasing interest in applying xylanolytic enzymes, mainly endoxylanases, to the pulp bleaching process. After cooking wood chips under alkaline conditions (kraft process), about 90% of lignin is removed and about 10% remains, encrusting the cellulose and hemicellulose fractions. Traditional bleaching using chlorine-based compounds effectively removes residual lignin, but it is very harmful to the environment. Many researchers are investigating environmentally clean bleaching processes using chemical or biotechnological approaches *(3,4)*. The efficacy of xylanase in biobleaching has been demonstrated in many scientific reports. These reports conclude that xylanase depolymerizes xylan, opening the polymeric cellulose-hemicellulose-lignin matrix, releasing lignin-bound fragments, and facilitating the chemical removal of lignin during bleaching *(4)*. In addition, these enzymes improve paper qualities, such as brightness and viscosity *(5–9)*. Over the last decade, a number of microbial enzymes have been assessed for potential application in the paper and pulp industry *(3–30)*. Bleaching occurs under high temperature and alkalinity and the enzymes must tolerate these conditions.

Bacillus circulans D1, an alkalophilic and thermophilic bacterial strain, was isolated in our laboratory, from decayed wood. It shows high xylanase production. The crude xylanase produced by this microorganism is thermostable and cellulase free. Our goal was to employ the crude xylanase produced by *B. circulans* D1 as a booster in chlorine-based biobleaching of *Eucalyptus* kraft pulp, and to analyze its effectiveness on reducing chlorine consumption.

Materials and Methods

Media and Screening of Microorganisms

Nutrient medium was prepared as described by Horikoshi *(31)*, containing xylan (10 g/L), beef extract (10 g/L), peptone (10 g/L), NaCl (10 g/L), and KH_2PO_4 (1 g/L), and Na_2CO_3 (5 g/L) (separately sterilized). For the solid nutrient medium, 15 g/L of agar was added. To screen for xylanolytic and alkalophilic microorganisms, the nutrient medium was used with dried corn straw strips, as a carbon source replacing xylan. The corn straw strips were placed vertically into tubes containing nutrient medium. Samples of soil, agricultural wastes, and decayed wood were collected (approx 0.5 g of each sample) and put into flasks containing 1.5 mL of nutrient medium. In the laboratory, these samples were transferred to tubes containing screening medium and incubated at 50°C for 120 h. After incubation, 1.0 mL of the contents from the tubes, with degraded straws trips, was diluted in 5.0 mL of sterile distilled water and one loop was inoculated on plates containing solid nutrient medium. The plates were maintained at 50°C, and then the developed colonies were transferred to slant tubes with solid nutrient medium.

Microorganism and Growth Conditions

B. circulans D1 was isolated from decaying wood and was identified by Fundacao Andre Tosello, Campinas, SP, Brazil. For enzyme production, the *B. circulans* D1 was grown in alkaline medium (pH 9.0) except using 2% corn straw, powdered to a diameter of 1.0 mm, as the carbon source. Twenty milliliters of the medium, in 125-mL Erlenmeyer flasks, was inoculated with 10^7 cells/mL and incubated at 45°C with shaking at 150 rpm. After 48 h, the bacteria were harvested by centrifuging at 12,000*g* at 4°C for 20 min. The cell-free solution was used as crude enzyme solution.

Determination of the enzymatic activity

Enzyme activity was expressed in international units (IU). Xylanase (1,4-β-D-xylan xylanhydrolase, EC 3.2.1.8) activity, carboxymethyl cellulase (endo-1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4), and Avicelase (exo-1,4-β-D-cellobiohydrolase, EC 3.2.1.91) were assayed by incubating 0.1 mL of appropriately diluted enzyme with 0.9 mL of a solution containing 0.5% of the respective substrate (xylan [Birchwood; Sigma, St. Louis, MO], carboxymethyl cellulose [C5678; Sigma], and avicel [Merck]) in 0.1 *M* acetate buffer, pH 5.5. After incubating at 60°C for 10 min, the reducing substances released were assayed by dinitrosalicylic acid as described by Miller *(13)*. Controls were prepared with 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, by using a xylose or glucose standard curve.

Effect of Temperature and pH on Xylanase Activity and Stability

Xylanase activity was measured between 45 and 80°C. Thermal stability (without substrate) was also estimated by maintaining the enzyme for 1 h at the same temperatures. After cooling, the residual activities were estimated under the standard condition. The effect of pH on xylanase activity was tested from 3.5 to 10.5. pH stability (without substrate) was estimated by maintaining the enzymes for 24 h in the same pH range and then determining the residual activities under the standard condition.

Fractionation of Xylanase

The crude enzyme was precipitated by adding ethanol to make a 70% (v/v) solution; the precipitate was collected by centrifugation. The concentrated crude enzyme (12 mL), containing a total of 601 IU, was applied to a Sephadex G-50 column that had been equilibrated with 20 m*M* acetate buffer, pH 5.0, and was eluted with the same buffer.

Products of Xylan Hydrolysis

Birchwood xylan hydrolysates were examined by descending paper chromatography, as described by Trevelyn et al. *(32)*.

Enzymatic Treatment of Pulp

Eucalyptus kraft pulp was courteously donated by Champion Paper and Cellulose from Mogi Guaçu, SP, Brazil. It had an initial κ number (milliliters of 0.1 *N* potassium permanganate consumed by 1 g of dry wood chemical pulp under specific conditions) of 13.85 and brightness of 37.0 ISO. The unbleached pulp was washed twice with distilled water to remove residual brown liquor. Crude enzyme was applied to the pulp at a concentration of 13.5 IU/g of dried pulp. The enzymatic treatment was made in plastic bags at a slurry concentration of 10%. The pH of this mixture was adjusted to 5.5 by adding glacial acetic acid, and then the mixture was homogenized and the plastic bags were maintained at 60°C for 4 h. Then, the plastic bags were cooled in cold water for a few minutes, and the pulp was washed twice with distilled water and filtered in a Buchner funnel.

Chemical Bleaching

Pulps, treated and untreated with enzyme, were bleached with chlorine dioxide (ClO_2) followed by extraction with NaOH(E) (DE bleaching stages). The amount of ClO_2 used in the bleaching was determined by calculating the concentration of active chlorine on the aqueous solution of ClO_2 and varied around the kappa (κ) factor recommended (κ factor is a standard quantity, determined experimentally, used in studies comparing bleaching processes and represents the percentage of active chlorine divided by the κ number of the pulp: κ number × κ factor = % of ClO_2), which under typical industrial conditions is 0.26. The samples contained 10.0 g of oven-dried pulp and ClO_2, at a 10% slurry concentration. Each sample was homogenized, maintained in a bath at 60°C for exactly 30 min, cooled, and then washed with distilled water in a Büchner funnel. The alkaline extraction was made with pulp at 10% concentration, at 70°C for 1 h and NaOH final concentration of 1.6%. Afterward, the samples were washed with distilled water and filtered in a Buchner funnel.

Pulp Properties

Pulp properties were investigated according to the Standard Methods of the Technical Association of the Pulp and Paper Industry (TAPPI Standard Methods). For determination of κ number, the microKappa methodology was used, by reacting pulp samples with acidified potassium permanganate, as described in TAPPI protocol T-236 OM-85. After delignification, the pulp was pressed and transformed in dried sheets, at room temperature, for viscosity and brightness experiments. Viscosity was evaluated by dissolving pulps in cupriethylenodiamine and by measuring the viscosity with an Ostwald viscosimeter, as described in TAPPI protocol T-230 OM-82. The brightness of the paper sheets was measured by reflectance at 457 nm with an Elrepho 2000 instrument, according to TAPPI protocol T-452 OM-87.

Table 1
Characteristics of Treated and Untreated Kraft Pulp

	Untreated pulp			Pulp treated with xylanase		
	κ number	Brightness (ISO)	Viscosity (cP)	κ number	Brightness (ISO)	Viscosity (cP)
Before bleaching	13.85	37.00	60	12.80	41.00	61.5
After bleaching	2.70	56.50	32	1.70	60.00	35

Pulps were submitted to bleaching under typical conditions: κ factor of 0.26 (0.36 g of ClO_2/10 g dry wt pulp) and 1.6% NaOH extraction.

Results and Discussion

Properties of Crude Xylanase

The crude enzyme solution was analyzed for cellulase activity, but it was free of this enzyme. The optimum pH and temperature for xylanase activity were 5.5 and 70°C, respectively. The thermal stability of the crude enzyme was measured, in the absence of substrate, from 20 to 80°C for 1 h. This enzyme loses activity when incubated above 60°C. The crude xylanase was stable in the pH range of 5.5.–10.5 after incubating in different buffers for 24 h. These values agree with those reported for xylanases produced by other *Bacillus* strains *(23–25)*.

Effect of Enzymatic Treatment and Chemical Bleaching on Pulp

Table 1 shows the results from enzymatically treating and bleaching with ClO_2. The initial k number of the pulp was 13.85, and it was reduced after enzymatic treatment to 12.80. In addition to this reduction in k number, pulp brightness increased by about 4 U.

The pulps, treated and untreated with xylanase, were submitted to DE bleaching sequence, with varying ClO_2 concentrations. For xylanase treatment, investigators have used only one ClO_2 concentration, but the chlorine concentration used after enzyme treatment may influence the results. To analyze this point, bleaching was performed with different ClO_2 concentrations. As shown in Fig. 1, the κ number of the pulp treated with xylanase decreased quickly (from 3.5 to 1.7) until it reached the value of 1.70 IU (0.36 g of ClO_2), and then it slowed down, tending to stabilize at about 1.0–1.5 IU. From this result we can suppose that there is a ClO_2 concentration at which the majority of the delignification reaction occurs and that an additional increase in chlorine concentration will not significantly decrease κ number. This reaction occurs probably because there are few xylan-lignin associations at this stage of κ number. In our study, according to Table 1 and Fig. 1, at a κ factor of 0.26 (0.36 g of ClO_2), the range of delignification was 80 (13.5–2.70) and 88% (13.5–1.7) for untreated pulp and enzyme-treated pulp, respectively. This is important in order to reduce

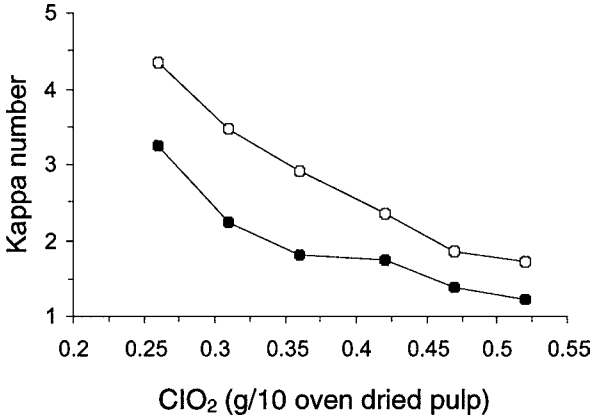

Fig. 1. κ Number of kraft pulp (—●—) treated and (—○—) untreated with xylanase from *B. circulans* D1. Data are mean of two replicates.

the bleaching process cost and discharge of chlorinated organic compounds. The enzymatic treatment of pulp resulted in significant chlorine savings. If we consider, e.g., the κ number of 2.7, this number was already obtained with a κ factor of 0.17 (0.23 g of ClO_2/10 g of dried pulp) in the pulp treated with enzyme. To reach this value in the untreated pulp, it was necessary to employ a κ factor of 0.22 (0.3 g of ClO_2/10 g of dried pulp). The enzyme decreased by 23% the amount of ClO_2 used for pulp delignification. Many scientific articles have reported a decrease in the active chlorine rate in relation to the κ number, varying from 20 to 40% *(12)*.

Enzymatic treatment of the pulp increased brightness by approx 4 IU compared to untreated pulp (Fig. 2). The results indicate that for a given brightness, e.g., 56.5, a κ factor of 0.25 (0.35 g of ClO_2/10 g of dried pulp) would be needed for the untreated pulp, compared with 0.16 (0.22 g of ClO_2/10 g of dried pulp) in the pulp treated with xylanase. This is 37% less ClO_2 used to reach the same brightness. Using xylanase from *Streptomyces thermoviolaceus* in the biobleaching of kraft pulp, Garg et al. *(22)* observed a 30–35% saving in the chlorine required to obtain pulp brightness, comparable with the control. Using xylanase of *B. circulans* AB 16 in biobleaching of eucalyptus pulp, Dhillon et al. *(8)* reduced chlorine consumption by 20% to achieve the same final pulp brightness compared with the control.

The pulp treated with enzyme had more viscosity than the untreated pulp. This was likely owing to the selective enzymatic hydrolysis of low molecular weight pulp xylans that probably contributed to a smaller average viscosity *(8,20)*. This parameter indicates that the crude enzyme did not disrupt fiber, and thus loss of paper properties *(8,10)*. A slight increase in viscosity was also obtained by Dhillon et al. *(8)* using xylanase from *B. circulans* AB 16, and by Buchert et al. *(21)* using xylanase from *Trichoderma reesei*. Viscosity reduction is not desirable because this property is related to the degree of cellulose polymerization and to paper strength.

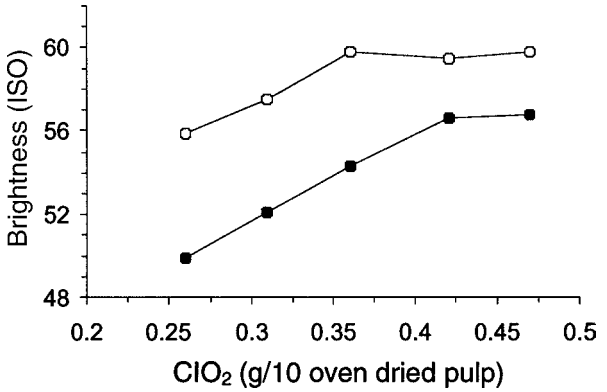

Fig. 2. Brightness of kraft pulp (—○—) treated and (—●—) untreated with xylanase from *B. circulans* D1. Data are mean of seven replicates.

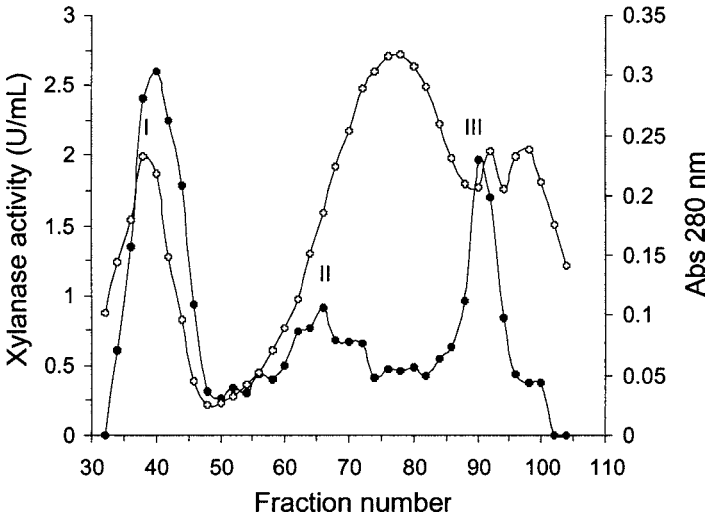

Fig. 3. Fractioning of *B. circulans* D1 xylanase on Sephadex G-50. (—●—) Xylanase activity; (—○—) 280-nm absorbance.

Fractions of Xylanases on Crude Enzymatic Extract

The crude precipitated enzyme was applied to a Sephadex G-50 columm to separate it from pigments and other enzymes present in the crude enzymatic extract. The column separated the crude enzyme into three xylanase fractions (Fig. 3) designated xylanases I, II, and III, representing distinct molecular weights of enzymes. Silva et al. *(26)* cited gel filtration as an initial step of xylanase purification. Khanna et al. *(27)* also used Sephadex G-50, and elution of the xylanase resulted in one peak of xylanase activity.

Multiplicity in xylanases has been shown in other *Bacillus* species *(28)*. Our result on xylanase partial purification is in disagreement with that observed by Nakamura et al. *(28)*, in which *B. circulans* AB 16 displayed two isoforms of xylanases after one multistage purification procedure. Additional assays on *B. circulans* D1 xylanase purification are being performed in order to obtain more accurate results. Xylanases I–III were concentrated with a Centricon concentrator with a 10-kDa cutoff and then characterized regarding optimum activity at different pH and temperature values and to assess thermal and pH stability. Xylanases I–III showed higher activity at pH near 5.5, 6.5–7.0, and 5.5–6.5, respectively. The optimal temperatures for xylan hydrolysis were 65°C for xylanase I and III and about 65–70°C for xylanase II. At 80°C, >67% of the activity of xylanase III was retained, whereas at the same temperature, the remaining activities of xylanases I and II were about 37 and 44%, respectively.

Products of Xylan Hydrolysis

When using the crude enzyme and the enzymatic fractions collected after chromatography on Sephadex G-50, the main products of xylan hydrolysis identified by paper chromatography (data not shown) were xylotetraose and xylotriose. Xylose was not observed as a hydrolysis product, which suggests the presence of only endoxylanases in the enzymatic extract produced by *B. circulans* D1. Our results are in good agreement with Nakamura et al. *(28)*, Kang et al. *(29)*, and Breccia et al. *(30)*, who have cited similar results.

Conclusion

The extracellular cellulase-free xylanase produced by alkalophilic *B. circulans* D1 exhibited desirable properties such as activity at elevated temperature, and alkali and thermal stability, which are advantageous for application in the pulp and paper industry. Application of xylanase to the kraft pulp significantly reduced the requirement of oxidizing ClO_2 in the bleaching process. The crude xylanase displayed action of endoxylanase and was separated in three fractions of distinct molecular weight.

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