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Application of Xylanases from Amazon Forest Fungal Species in Bleaching of Eucalyptus Kraft Pulps

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ABSTRACT

Crude xylanase preparations from Penicillium corylophilum, Aspergillus niger *and* Trichoderma longibrachiatum *were used to treat Eucalyptus kraft pulp, prior to chlorine dioxide and alkaline bleaching sequences. The enzyme pretreatment improved brightness and delignification of non-delignified and oxygen-bleached samples of eucalyptus kraft pulp. Xylanase preparations from* T. longibrachiatum *and* P. corylophilum *were more effective to reduce pulp kappa number. A small reduction in viscosity was obtained when the oxygen-bleached pulp was treated with xylanase preparation from A. niger. For all enzyme samples, the best release of chromophoric material from the pulp was at 237 nm. The enzyme preparation from* P. corylophilum *was responsible for the highest release of reducing sugar at a dosage interval of 10-20 IU/g dry weight pulp. Scanning electron microscopy studies of oxygen-bleached pulp after xylanase treatment revealed morphological changes, including holes, cracks, filament forming and peeling.*

Key words*:* Xylan; xylanase; kraft pulp; pulp bleaching

INTRODUCTION

Xylan is the major hemicellulose component of the pulp fiber from hardwood (Viikari et al. 1990). Studies performed by Dahlman et al. (2003) showed that the surface of fiber pulps from softwood and hardwood have higher molecular masses xylan polymers and lower frequency of uronic acid side groups. Xylanases from a wide variety of fungal species have been reported to have potential application in the pulping and bleaching processes (Salles et al. 2000; Subramaniyan and Prema 2002; Viikari et al. 1990). The use of xylanases in prebleaching of cellulose pulp has become an alternative approach in eliminating chlorine in bleaching and reducing chlorinated organic compounds in bleach plant effluents, reduce the kappa number (residual lignin content in the pulp) and increase the brightness of the pulp (Filho 1998; Subramaniyan and Prema 2002; Techapun et al. 2003). They can be applied in elementary chlorine and chlorine dioxide containing bleaching sequences, as well as in combination with oxygen, ozone and hydrogen peroxide (Filho 1998; Techapun et al. 2003; Viikari et al. 1990; 1994). Xylanase removes the

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xylan portion in pulp selectively without affecting cellulose. The enzymatic hydrolysis of reprecipitated xylan on the surface of the fibers renders the fiber structure more permeable for lignin extraction (Filho 1998; Subramaniyan and Prema 2002). In a previous paper (Medeiros et al. 2003), ten fungal species from the Amazon forest were isolated, identified and evaluated for their capacity to produce xylan-degrading enzyme activity during growth in liquid medium containing oat spelt xylan as the carbon source. The best producing strains of β-xylanase activity were *Penicillium corylophilum*, *Aspergillus niger* and *Trichoderma longibrachiatum*. Xylanase activity from *A. niger* was the most thermostable of the three enzyme samples. The xylanase preparations were not active against filter paper and carboxymethyl cellulose as substrates. The aim of this work was to analysis the xylanase activity of crude extract obtained from cultures of *Penicillium corylophilum*, *Aspergillus niger* and *Trichoderma longibrachiatum* as alternative in kraft pulp bleaching.

MATERIALS AND METHODS

Reagents

Oat spelt xylan and carboxymethyl cellulose were purchased from Sigma Chemical Co. (St. Louis, MO).

Organism and enzyme production

Trichoderma longibrachiatum, *Penicillium corylophilum* and *Aspergillus niger* were isolated from decomposed wood in the natural forest reserve of INPA (National Research Institute of Amazonia, Brazil) and purified as described before (Medeiros et al. 2003). They were maintained in BDA medium at 28^{O}C. For production of xylanase activity, the fungi were cultivated in Erlenmeyer flasks containing 1.0% (w/v) oat spelt xylan in 300 ml of minimal medium (0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_47H_2O$, 0.1% $(NH_4)_2SO_4$) supplemented with 0.06% yeast extract. Flasks were inoculated with a suspension of 1.0 x 10^7/ml spores from routine subcultures. Cultures were grown at pH 7.0 for 5 days at 28^{O}C with shaking at 100 rpm. Subsequently, the content of each flask was filtered through Whatman filter paper number 1. The supernatant solutions, hereafter called crude extracts, obtained from filtration procedure were stored at 4^{O}C for subsequent use as xylanase preparations.

Enzyme assays

Xylanase activity and reducing sugar were determined as reported before (Medeiros et al., 2003; Miller, 1959). Xylanase activity was expressed as μmol reducing sugar formed min^{-1} ml^{-1} enzyme solution, i.e., as IU ml^{-1}.

Pulp bleaching

The kraft industrial cellulose pulp was obtained from a combination of *Eucalyptus grandis*, *saligna* and *urophylla* with a yield of 53%. Non-delignified pulp had kappa number and viscosity of 18 and 43 cP, respectively. The pulp previously treated with oxygen had consistency, Kappa number and viscosity of 12% (w/v), 11 and 21 cP, respectively. The kappa number (Tappi T236 cm-85) was expressed as the amount (ml) of a 0.1 N $KMnO_4$ solution consumed by 1 g of moisture-free pulp. Brightness and viscosity tests were performed according to the recommendations of the Technical Association of the Pulp and Paper Industry (Atlanta, GA, USA), using protocols outlined in Tappi T452 om-92 and Tappi T230 om-94, respectively. Enzyme and water were added to pulp so that the final consistency reached 10% (10 g dry mass pulp/100 ml water). The enzyme charge was 5 IU/g moisture free pulp. Triplicates were incubated for 4 h at 50ºC and pH 7.0. A typical ECF bleaching sequence adopted was: X-D1-E-D2, where ECF is elemental chlorine free, X is enzyme, D1 and D2 are first and second stage chlorine dioxide steps and E signifies alkaline extraction (Medeiros et al. 2002). Alkaline extraction step was not used in the brightness tests. A charge of 0.5% (v/v) was applied for D1 and D2. In the control sequence the pulp underwent the same treatment with the omission of enzyme. A corresponding volume of distilled water was used instead of enzyme. After completing the bleaching sequence, the pulp was thoroughly washed three times with tap water, before further analysis. The experiments described above were carried out in triplicate. The standard deviation was less than ± 20% of the mean.

Chromophore release

The oxygen-bleached pulp was washed twice with distilled water in order to remove soluble reducing

sugars and dried for 12 h at 70ºC. The pulp was weighed to determine the humidity. Enzyme preparations and 50 mM phosphate buffer (pH 7.0) were mixed and added to the dried pulp to reach 10% consistency. The enzyme charges were 5, 10, 15, 20 and 25 IU/g moisture free pulp. Control was prepared using a denatured enzyme sample. The pulp was incubated at 50ºC for 2 h. Filtrates taken from the reaction mixture were diluted 1.5-fold to monitor reducing sugars (540 nm) or 10-fold to monitor chromophores release (237 nm, 254 nm, 280 nm, 465 nm) using a Lambda-5 UV/VIS Spectrophotometer (Perkin-Elmer). The liberation of aromatic compounds was monitored by absorbance values at 237 nm and 254 nm, while the absorbance at 280 nm indicated the presence of lignin. The remotion of hydrophobic compounds was detected by absorbance at 465 nm. Absorbances measured were subtracted from control values Reducing sugar concentrations in pulp filtrates were determined by the dinitrosalicylic acid (DNS) method (Medeiros et al. 2002) and expressed as D-xylose equivalents. The experiments described above were carried out in triplicate. The standard deviation for all calculations were below 0.5.

Scanning Electron Micrograph

The oxygen-bleached pulp showed alkaline pH, inadequate for xylanase activity, requiring pH adjustment to neutral. For this purpose, was used chloridic acid. Enzyme and water were added to pulp so that the final consistency reached 10%. The enzyme charge was 15 IU/g moisture free pulp. Control was prepared using a denatured enzyme sample. Triplicates were incubated for 4 h at 50ºC. Small samples of pulps were fixed in a freshly prepared solution of 2.5% glutaraldehyde in 0.05 M phosphate buffer pH 7.0. Samples were dehydrated through a series of increasing acetone concentrations up to 100% (v/v) and subjected to drying by the critical point method in liquid carbon dioxide and cemented to aluminium stubs and sputter-coated with gold. Specimens were examined by scanning electron microscopy (JEOL JSM 840A) operating at an accelerating voltage of 5.0 kV. The experiments described above were carried out in triplicate.

RESULTS AND DISCUSSION

The bleaching ability of crude xylanase preparations from *T. longibrachiatum*, *P. corylophilum* and *A. niger* was tested on non-delignified and oxygen-bleached kraft pulps. Release of chromophore is a better indicator of xylanase effect on the cellulose pulp than the liberation of reducing sugar, whereas the latter will continue being produced as result of xylanase attack on xylooligomers released by the initial hydrolysis of the xylan coating the fiber surface (Garg et al. 1998). Release of material absorbing at 237, 254 and 280 nm by xylanase preparations increased with enzyme dosage, suggesting that it was released as a result of xylanase activity (Baraznenok et al. 1999). UV absorbance spectra of materials released from eucalyptus oxygen-bleached kraft pulp gave the highest bleaching effect at 237 nm after 2 h of reaction time (Figs. 1-3). Compared with the *P. corylophilum* and *T. longibrachiatum* xylanase activities, xylanase from *A. niger* released more chromophores. According to Elegir et al. (1995), the liberation of material absorbing at 237 nm correlates with color release and enhances pulp brightness following bleaching. At enzyme dosages of 10-20 IU/g dry weight pulp, the amount of reducing sugars liberated by enzyme preparation from *P. corylophilum* was greater than that for xylanases from *T. longibrachiatum* and *A. niger* (Table 1). On the other hand, for an enzyme dosage of 25 IU/g dry weight pulp, the best performance was from xylanase preparation of *T. longibrachiatum*. The liberation of products absorbing at 465 nm by xylanases from *P. corylophilum* and *T. longibrachiatum* did not correlated with the release of chromophoric material (Figs. 1 and 2). However, the pulp treatment with xylanase from *A. niger* showed a small increased release in absorption at 465 nm (Fig. 3). The results obtained above suggested that the xylanases from *P. corylophilum* and *T. longibrachiatum* and not some other factor were responsible for the release of chromophoric material and there was a significant decrease in the aromaticity of residual lignin without a concomitant decrease in the color. The same result was described for xylanase preparations from *Streptomyces thermoviolaceus* (Garg et al. 1996).

The chemical properties of non-delignified and oxygen-bleached kraft pulps treated with 5 IU/g moisture free pulp of xylanase preparations from

T. longibrachiatum, *P. corylophilum* and *A. niger* are summarized in Table 2. The kappa number of non-delignified pulp was reduced to 4.9, 4.8 and 4.3 with xylanase preparations from *P. corylophilum*, *A. niger* and *T. longibrachiatum*, respectively.

Table 1 - Release of reducing sugars from *Eucalyptus* kraft pulp by xylanases from *A. niger*, *P. corylophilum* and *T. longibrachiatum.*

Xylanase source	Reducing sugars (mg/ml)					
	Dosage of enzyme (IU/ g dry weight pulp)					
	5	10	15	20	25	Control
A. niger	0.146	0.199	0.278	0.299	0.300	0.130
P. corylophilum	0.161	0.279	0.332	0.369	0.343	0.134
T. longibrachiatum	0.163	0.246	0.307	0.316	0.393	0.132

Table 2 - Effect of xylanase enzyme treatment on *Eucalyptus* kraft pulp.

Xylanase source	Brightness (% ISO)	Viscosity (cP)	Kappa number
Control1	66.7	25.1	5.4
Control2	83.3	21.5	1.5
A. nigera	70.8	31.6	4.8
A. nigerb	81.6	20.0	2.3
P. corylophiluma	73.0	33.4	4.9
P. corylophilumb	82.7	23.7	0.6
T. longibrachiatuma	71.6	33.0	4.3
T. longibrachiatumb	82.3	23.0	0.9

[1] Non-delignified pulp without enzyme pretreatment.
[2] Oxygen-bleached pulp without enzyme pretreatment.
[a] Non-delignified pulp.
[b] Oxygen-bleached pulp.

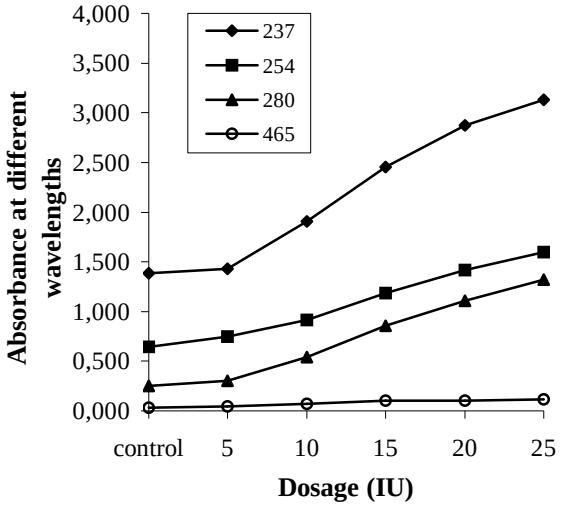

Figure 1 - Release of chromophoric material from oxygen-bleached eucalyptus kraft pulp by xylanase from *P. corylophilum.*

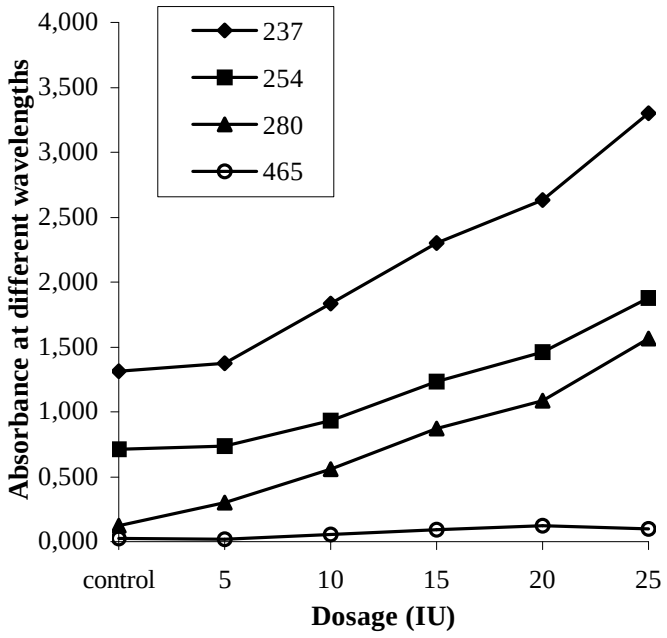

Figure 2 - Release of chromophoric material from oxygen-bleached eucalyptus kraft pulp by xylanase from *T. longibrachiatum*.

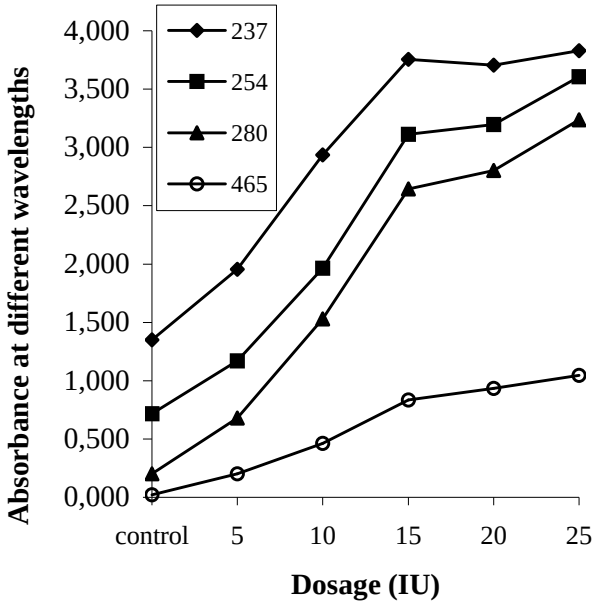

Figure 3 - Release of chromophoric material from oxygen-bleached eucalyptus kraft pulp by xylanase from *A. niger*.

When xylanases from *A. niger*, *T. longibrachiatum* and *P. corylophilum* were used in the bleaching of oxygen-bleached kraft pulps, the kappa number decreased to 2.3, 0.9 and 0.6 after 2 h incubation, respectively. In comparison to control, treatment of both unbleached and oxygen-bleached pulps with xylanase from *P. corylophilum* viscosity increased to 33.06% and 10.23%, respectively. According to Ragauskas et al. (1994), this improvement in the pulp viscosity could be caused by an accumulate of high molecular polysaccharides, which occurred when xylan was selectively removed. However, a small loss of viscosity was observed when oxygen-bleached pulp was treated with xylanase preparation from *A. niger*. This effect could be due to some other reason than cellulase activity (Medeiros et al., 2002). Medeiros et al. (2003) reported that all xylanases preparations were not active against filter paper and carboxymethyl cellulose as substrates. For xylanase preparations from *Acrophialophora nainiana*, *Humícola grisea* var. *thermoidea* and *Trichoderma harzianum*, the viscosity of kraft pulp produced in the sequence X-D1-E-D2 was higher than the control sequence (Medeiros et al., 2002).

All tested xylanase preparations were effective in increasing the brightness of the pulps. In this case, the enzyme preparation from *P. corylophilum* was more efficient with an increase of 82.7% in the degree of brightness on oxygen-bleached pulps.

The results of scanning electron microscopy studies of control and xylanase-treated pulps are presented in Fig. 4.

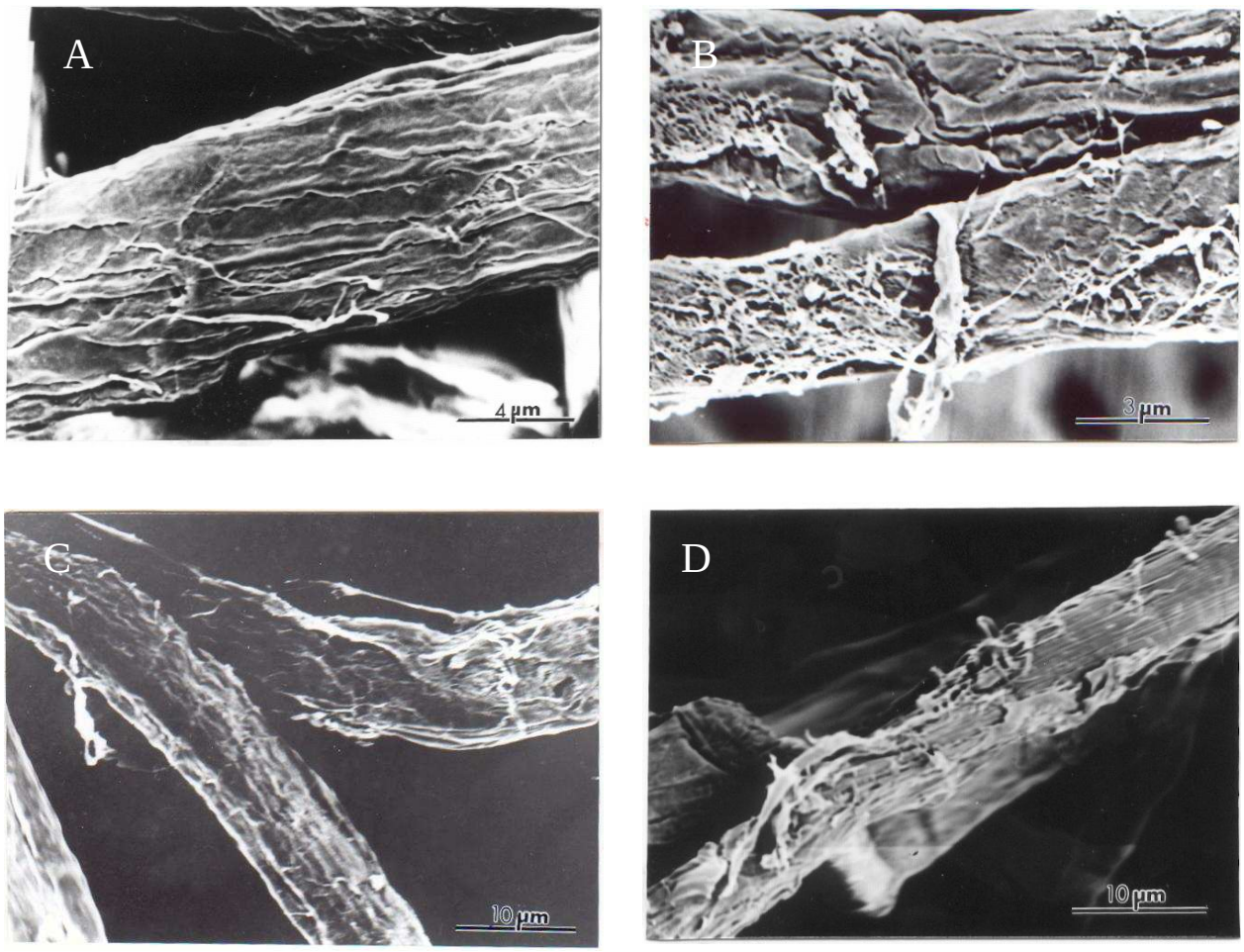

Figure 4 - Scanning electron micrograph of eucalyptus oxygen-bleached kraft pulp: control (A); treated with xylanase from *A. niger* (B); treated with xylanase from *P. corylophilum* (C) and treated with xylanase from *T. longibrachiatum* (D).

Xylanases were responsible for noticeable changes in the surface architecture of the oxygen-bleached pulp compared to the surface of untreated pulp. Some reports in the literature have focused on scanning electron microscopy of xylanase treated pulps and shown that enzymatic prebleaching opens up the pulp structure (Salles et al. 2005). Morphological changes, including holes, cracks, filaments and peeling of the fiber surfaces were evident after the pulp treatment with xylanase preparations of *A. niger*, *P. corylophilum* and *T. longibrachiatum*. The same result was described for cellulase-free xylanases from *Acrophialophora nainiana* and *Humicola grisea* var. *thermoidea* (Salles et al., 2005). Garg et al. (1998) reported that the treatment of celullose fibers with xylanase from *S. thermoviolaceus* showed extensive separation of microfibrils. Scanning electron microscopy of eucalyptus pulp after treatment with *Streptomyces* sp. QG-11-3 xylanase resulted in better porosity, swelling up and separation of pulp microfibrils and pulp fibers (Beg et al. 2000). In other reports, the use of an enzyme preparation, containing cellulase and xylanase activities from *H. insolens* on Douglas-fir kraft pulps did not present cleavages or pit enlargments of the fiber surfaces and changes to internal morphology of the fibers (Mansfield et al. 1997). However, it did show changes in the outermost fiber surface of the pulp.

In conclusion, results showed that crude xylanase preparations from *T. longibrachiatum*, *P. corylophilum* and *A. niger* were effective for improving some properties of cellulose kraft pulps. The results of the scanning electron microscopy studies revealed that a defibrillation of the microfibrils was detected for the pulp enzymatically treated when compared with the control. Further experiments should be focused on the use of different enzyme mixtures and their effects on treatment of kraft pulps.

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RESUMO

Amostras de xilanases de extratos brutos de *Penicillium corylophilum, Aspergillus niger e Trichoderma longibrachiatum* foram utilizadas no branqueamento de polpa kraft de eucalipto antes das seqüências alcalina e dióxido de cloro. O pré-tratamento enzimático melhorou a alvura e o processo de deslignificação de amostras de polpa kraft de eucalipto não-tratada e tratada com oxigênio. Amostras de xilanases de *T. longibrachiatum* e *P. corylophilum* foram mais efetivas na redução do número kappa da polpa. A polpa tratada com oxigênio sofreu uma pequena redução na sua viscosidade quando incubada com amostra de xilanase de *A. niger*. Para todas as amostras de xilanases, a maior liberação de cromóforos da polpa foi a 237 nm. A amostra de xilanase de *P. corylophilum* liberou maior quantidade de açúcar redutor da polpa, utilizando dosagem de 10-20 UI/g de peso seco da polpa. Estudos de microscopia eletrônica de varredura revelaram várias alterações morfológicas da polpa tratada com oxigênio tais como a formação de buracos, rachaduras e filamentos.

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