Xylanase and β-Xylosidase from *Penicillium janczewskii*: Production, Physico-chemical Properties, and Application of the Crude Extract to Pulp Biobleaching

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Extracellular xylanase and β-xylosidase production by a *Penicillium janczewskii* strain were investigated in liquid cultures with xylan from oat spelts under different physical and chemical conditions. The selected conditions for optimized production of xylanase and β-xylosidase were 7 days, pH 6.5, at 30 °C and 8 days, pH 5.0, at 25 °C, respectively. The xylanase exhibited optimal activity in pH 5.0 at 50 °C and the β-xylosidase in pH 4.0 at 75 °C. The xylanase was more stable at pH 6.0 to 9.5, while the β-xylosidase remained stable at pH ranging from 1.6 to 5.5. The xylanase half-life (T_{50}) at 40, 50, and 60 °C was 183, 15, and 3 min, respectively. β-xylosidase half-life was 144, 8, and 4 min at 50, 65, and 75 °C, respectively. When applied to the biobleaching of *Eucalyptus* kraft pulp, xylanase dosages of 2 and 4 U/g dried pulp reduced, respectively, kappa number by 3.0 and 3.3 units after 1 h treatment, demonstrating that the use of *P. janczewskii* xylanases in this process is quite promising. The pulp viscosity was not altered, confirming the absence of cellulolytic enzymes in the fungal extract.

Keywords: Xylanolytic enzymes; Penicillium janczewskii; Enzyme production; Enzyme characterization; Pulp biobleaching

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INTRODUCTION

Degradation of the plant cell wall is a complex process involving the synergistic action of many enzymes such as cellulases, hemicellulases, pectinases, and ligninases (Aro *et al*. 2005). Xylan is the most common hemicellulose and is composed mainly of D-xylose, although other sugars may be present as substituents of the principal chain. Due to its complex structure, the complete breakdown of xylan requires several enzymes acting cooperatively, known as a xylanolytic system. Endo-β-1,4-xylanase (4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is the main enzyme responsible for xylan depolymerization, breaking the principal chain and liberating oligosaccharides, while β-xylosidase (4-β-D-xylan xylohydrolase, EC 3.2.1.37) attacks xylobiose and other xylooligasaccharides, liberating smaller xylooligasaccharides and D-xylose. Other enzymes responsible for removing xylan substituents such as α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan, feruloyl, and p-coumaroyl esterases have been referred to as auxiliary or accessory xylanolytic enzymes (Biely 1985; Bastawde 1992; Kulkarni *et al*. 1999; Polizeli *et al*. 2005).

Complete xylanolytic enzyme systems have been found to be quite widespread among fungi, actinomycetes, and bacteria, and the production of xylanolytic enzymes by *Penicillium* has been reported often (Chávez *et al*. 2006; Knob *et al*. 2010). Filamentous fungi are particularly interesting, as they secrete plant cell wall-degrading enzymes into the medium, liberating energy and nutrients from plant biopolymers (Aro *et al*. 2005). From an industrial point of view, this physiological characteristic is especially interesting because it eliminates the need for cell disruption in a bioprocess. Besides, the levels of these extracellular enzymes produced by fungi are higher than those found for yeasts and bacteria (Polizeli *et al*. 2005).

The use of xylanases as bleaching agents for wood kraft pulps has been considered the main industrial application of these enzymes (Techapun *et al*. 2003; Polizeli *et al*. 2005). Many studies have demonstrated that the pre-treatment of pulps with microbial xylanases can reduce the use of chemical agents in subsequent bleaching stages. For the use in this process, xylanases may be active at high temperature, thermo-stable, and alkalophilic (Techapun *et al*. 2003). Usually bacterial xylanases are considered more appropriate for this application, especially due to the activity in alkaline pH, although many fungal xylanases have also been evaluated, such as those produced by *Aspergillus niger* (Maximo *et al*. 1998; Raghukumar *et al*. 2004; Medeiros *et al*. 2007; Betini *et al*. 2009), *Aspergillus nidulans* (Taneja *et al*. 2002), *Acrophialophora nainiana* and *Humicola grisea* var. *thermoidea* (Medeiros *et al*. 2002; Salles *et al*. 2005), *Trichoderma longibrachiatum* (Medeiro *et al*. 2007), *Aspergillus niveus* and *Aspergillus fumigatus* (Bettini *et al*. 2009; Peixoto-Nogueira *et al*. 2009), *Aspergillus terricola* and *Aspergillus ochraceus* (Michelin *et al*. 2010), *Aspergillus sydowii* (Nair *et al*. 2010), *Thermomyces lanuginosus* (Madlala *et al*. 2001), and *Aspergillus oryzae* (Szendefy *et al*. 2006). Among *Penicillium* species, only the xylanase produced by *Penicillium corylophilum* has been evaluated for biobleaching (Medeiros *et al*. 2007). It is important to highlight that all fungal extracts were cellulase-free and the xylanases presented activity in moderate or elevated temperatures.

The successful application of these enzymes depends on the optimization of xylanase and β-xylosidase production, which can directly result in cost reduction. Moreover, some knowledge about their physicochemical characteristics is indispensable for their further use in industrial processes. Thus, the aim of this study was to investigate the influence of culture conditions on the production of xylanase and β-xylosidase by a *Penicillium janczewskii* strain, earlier selected as a good xylanolytic enzyme producer (Terrasan *et al*. 2010), as well as to biochemically characterize the enzymes present in the crude filtrate obtained under optimized conditions. After characterization, the crude extract obtained under optimized conditions was applied to cellulose kraft pulp biobleaching.

EXPERIMENTAL

Microorganism and Growth Conditions

Penicillium janczewskii was deposited in the culture collection of the Environmental Studies Center, CEA/UNESP, Brazil. The microorganism was maintained in Vogel's solid medium (Vogel 1956) with 1.5% (w/v) wheat bran at 4 °C and cultured periodically. The cultures were inoculated in the same medium with 1.5% (w/v) glucose and incubated for 7 days at 28 °C for spore production. The spores were harvested and

suspended in sterile distilled water. The concentration was adjusted to 10^7 spores mL^{-1}, and 1 mL of this suspension was used for liquid medium inoculation.

Culture Conditions for Enzyme Production

Liquid cultures were prepared in Vogel's medium supplemented with 1.0% (w/v) xylan from oat spelts (Sigma). Erlenmeyer flasks (125 mL) containing 25 mL of the medium were inoculated with 1 mL of the spore suspension and incubated for 7 days. The pH was adjusted with 1.0 M HCl or NaOH and the temperature was maintained as required. All cultures were developed in triplicate and the results were presented as mean values.

Preparation of the Crude Extracts

The filtrate was separated by vacuum filtration with Whatman No. 541 filter paper and used for extracellular enzymatic activity assays and protein determination. The mycelium was washed with distilled water, dried on filter paper, and frozen. Cells were disrupted with sand using a mortar and pestle and suspended in McIlvaine buffer, pH 4.0. After centrifugation (9000 *g*, 20 min), the supernatant was used for intracellular protein determination.

Protein Determination

The protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as standard.

Determination of Enzyme Activities

Xylanase activity was determined according to Bailey *et al*. (1992), using 1.0% (w/v) birchwood xylan (Sigma) in a buffered reaction medium and appropriately diluted enzyme solution. Reducing sugars were quantified with DNS acid reagent (Miller 1959).

β-xylosidase and α-L-arabinofuranosidase activities were determined in a reaction mixture containing 0.25% (w/v) p-nitro-phenyl β-D-xylopiranoside and p-nitrophenyl α-L-arabinofuranoside (Sigma), respectively, in McIlvaine buffer pH 4.0 and appropriately diluted enzyme solution at 50 °C. The reaction was stopped by the addition of a saturated sodium tetraborate solution, and the absorbance was measured at 405 nm (Kersters-Hilderson *et al*. 1992).

One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of product equivalent per min in the assay conditions. Specific activities were expressed as enzyme units per milligram of protein.

Effect of Agitation, Initial pH, and Temperature on Enzyme Production

Enzyme production was assayed in standing culture for 15 days and in shaking (120 rpm) cultures for 8 days. The effect of the initial pH value of the medium on xylanase and β-xylosidase production was analyzed from pH 3.0 to 9.0 and the influence of cultivation temperature was verified in temperatures from 15 to 35 °C.

Enzyme Characterization

Electrophoresis

Mixtures containing 50 μg of protein obtained in the different culture conditions for the production of xylanase (7 days of cultivation, pH 6.5, 30 °C) and β-xylosidase (8 days of cultivation, pH 5.0, 25 °C) were applied to SDS-PAGE performed in 8 to 18%

(w/v) gradient gels, according to Laemmli, (1970). The resolved protein bands were visualized after staining with 0.1% Coomassie brilliant blue R-250 dissolved in methanol, acetic acid, and distilled water (4:1:5 v/v/v). Standard proteins (Sigma) were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14.2 kDa).

Optimum pH and pH stability

The effect of pH on enzyme activity was determined by assaying the activity in a range of pH values at 50 °C. The following buffers were used for xylanase activity: Sorensen (1.6 to 3.5), 0.05 M sodium acetate (4.0 to 5.5), 0.05 M imidazole (6.0 to 7.0), 0.05 M Tris-HCl (7.0 to 9.0), and Sorensen (9.5 and 10.0), and the following buffers for β-xylosidase activity: 0.05 M glycine-HCl (1.6 to 3.0) and McIlvaine (3.0 to 7.5). The effect of pH on enzyme stability was determined by incubating (4 °C, 24 h) the diluted (1:2 v/v) crude extract without substrate in different buffers (the same for each enzyme, as above) at pH ranging from 3.0 to 8.0. Xylanase activity was assayed in 0.05 M sodium acetate, pH 5.5, at 50 °C and β-xylosidase activity in McIlvaine buffer, pH 4.0, at 75 °C.

Optimum temperature and thermostability

The optimum temperature was determined by assaying xylanase activity in 0.05 M sodium acetate buffer, pH 5.5, and at temperatures ranging from 20 to 70 °C and β-xylosidase activity in McIlvaine buffer, pH 4.0, at temperatures ranging from 30 to 90 °C. Thermostability was determined by assaying residual activities after incubation of the crude extract without substrate from zero to 210 min at 40, 50, and 60 °C for xylanase assays, and from zero to 150 min at 50, 65, and 75 °C for β-xylosidase assays. Xylanase activity was assayed in 0.05 M sodium acetate buffer, pH 5.5, at 50 °C and β-xylosidase activity in McIlvaine buffer, pH 4.0, at 75 °C.

Effect of ions and other substances

$CuCl_2$, $ZnSO_4$, $MnSO_4$, $BaCl_2$, $CaCl_2$, NH_4Cl, NaCl, $MgSO_4$, $CoCl_2$, $HgCl_2$, $Pb(CH_3COO)_2$, sodium citrate, EDTA, SDS, PMSF, DTT, and β-mercaptoethanol were added to the enzymatic reactions at final concentrations of 2 and 10 mM. The relative activities were expressed as a percentage against the control (without any substance). Xylanase activity was assayed in 0.05 M sodium acetate buffer, pH 5.5, at 50 °C and β-xylosidase activity in McIlvaine buffer, pH 4.0, at 75 °C.

Biobleaching

The *Eucalyptus* spp. kraft pulp was produced at the Laboratory of Chemistry, Pulp and Energy at Forest Science Department, ESALQ/USP, SP State, Brazil. The oxygen pre-bleached pulp (8.5 initial kappa number) was treated with the *P. janczewskii* crude extract obtained under optimized conditions for xylanase production. Enzymatic treatments were based on the xylanase activity corresponding to charges of 2, 4, 8, 18, and 32 U/g oven-dried pulp. The experiments were carried out in polyethylene plastic bags at 10% pulp consistency, pH 5.5, incubation at 50 °C, for 1 and 2 h. After the treatment, the pulp was filtered using a Büchner funnel, rinsed with 200 mL distilled water and used for determination of kappa number and viscosity parameters. Control samples were prepared with distilled water instead of enzyme. The experiments were carried out in triplicate and the results were presented as mean values.

Kappa number and viscosity

Kappa number and pulp viscosity were determined according to the recommendations of the Technical Association of the Pulp and Paper Industry (Atlanta, GA, USA), using protocols outlined in TAPPI test methods T-236cm-85 and T 230 om-08, respectively.

RESULTS AND DISCUSSION

Optimization of Culture Conditions

Earlier, the production of xylanase and β-xylosidase by *P. janczewskii* was studied using different carbon sources, and the highest xylanase and β-xylosidase production was verified with xylan from oat spelts (Terrasan *et al*. 2010). Thus, a step-by-step optimization was carried out to determine the best culture conditions for the production of xylanase and β-xylosidase by this fungal strain in a liquid culture medium with this carbon source. Time-courses of *P. janczewskii* growth and xylanase and β-xylosidase production were followed both in standing and agitated conditions in a liquid medium for 15 and 7.5 days, respectively. As observed in Fig. 1a, xylanase production in the standing conditions increased substantially until the 7th day of culture (29.2 U mL^{-1}), slightly decreasing in the subsequent days. The highest levels of specific xylanase activity were observed from the 4th to 10th day of cultivation.

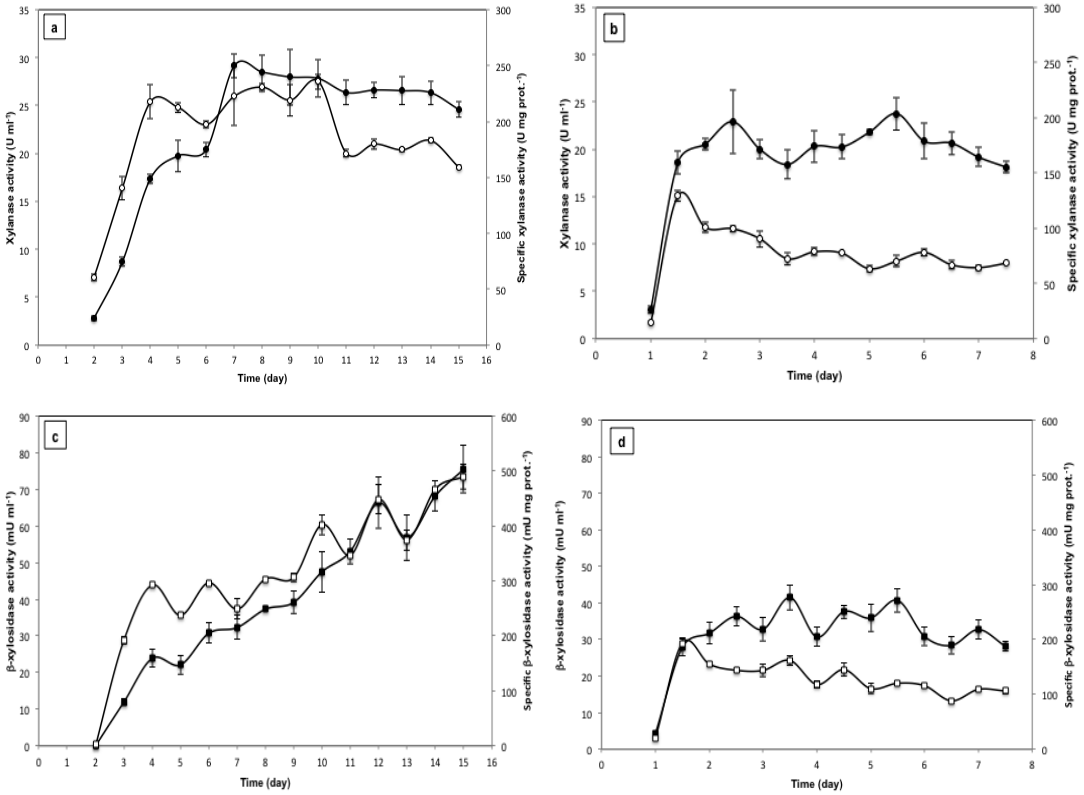

Fig. 1. Time-courses of xylanase (a and b) and β-xylosidase (c and d) production by *P. janczewskii* in stationary (a and c) and shaking (b and d) conditions. Culture conditions: Vogel medium with 1.0 % (w/v) xylan from oat spelts at pH 6.5, 28 ºC. (●) Xylanase activity (U ml^{-1}), (○) specific xylanase activity (U mg prot^{-1}), (■) β-xylosidase activity (mU ml^{-1}), (□) specific β-xylosidase activity (mU mg prot^{-1}). All cultures were developed in triplicate and the results were presented as mean values.

In shaking conditions (Fig. 1b), two peaks (2.5 and 5.5 days) with elevated xylanase activity were observed. The activity remained stable from the 3rd to the 5th day, and from the 6th day the activity decreased. The highest specific activity was observed in 1.5 day-old cultures (129.2 U mg $prot^{-1}$), decreasing from this period on. Maximum fungal growth (end of exponential phase) was observed at 7 (1.4 mg prot) and 2.5 days (1.7 mg prot) of cultivation in standing and shaking conditions, respectively, which were coincident with the peaks of maximal enzyme production. According to Kulkarni *et al*. (1999), the highest xylanase levels are usually observed at the end of the exponential phase and in the beginning of the stationary phase. Besides, better fungal growth was observed in shaking conditions. However, this better growth did not result in higher enzyme production.

In stationary conditions, β-xylosidase production (Fig. 1c) increased until the 15th day of culture, in which it was maximal (75.5 mU mL^{-1} and 489.7 mU mg $prot^{-1}$). Under shaking conditions (Fig. 1d), three peaks with high activity were observed at 2.5, 3.5, and 5.5 days of culture. These results are in accordance with the literature in relation to enzyme production levels and cultivation periods (Mishra *et al*. 1985; Lenartovicz *et al*. 2003; Medeiros *et al*. 2003; Li *et al*. 2007). However, higher enzyme production by *P. janczewskii* was verified in standing conditions, rather than under shaking conditions. Stationary cultures were also reported ideal for xylanase and β-xylosidase production by *Penicillium sclerotiorum* (Knob and Carmona 2009a; Knob and Carmona 2009b). Although it is important to maintain medium homogeneity, excessive agitation can disrupt the fungal hyphae (Palma *et al*. 1996), and, in this case, can have a marked negative influence on xylanase and β-xylosidase production.

Stationary conditions were selected for the subsequent experiments; the culture period for xylanase production by *P. janczewskii* was 7 days, which was the peak of xylanase production. For β-xylosidase production, 8 days of culture was chosen. The period corresponded, in the culture filtrate, to elevated enzyme activity that was secreted by the microorganism, but not to an additional amount present in the filtrate due to cell lysis. The microorganism still was in stationary phase, as verified by the intracellular protein content (data not shown).

Temperature and pH are important factors that affect microbial growth and the production and secretion of enzymes. When cultivated in a different pH medium, intracellular protein contents above 1.2 mg of protein were verified, indicating fungal growth in all pH ranges. However, better growth was observed within an acidic pH range, with the maximum observed at pH 4.0 (above 2.0 mg prot). Higher xylanase production was also observed in the acidic pH range (Fig. 2a**)**, especially at pH 5.0 and 6.5, with the highest production verified at pH 6.5 (29.2 U ml^{-1} and 179.1 U mg $prot^{-1}$). The highest β-xylosidase production **(**Fig. 2c**)** was verified at pH 5.0 (65.1 mU ml^{-1}) and the highest β-xylosidase specific activity at pH 6.5 (171.8 mU mg $prot^{-1}$). In general, the production of enzymes gradually decreased from these pH values.

After the selection of culture pH, the effect of the temperature of cultivation on xylanase and β-xylosidase production was investigated. As observed in Fig. 2b, higher xylanase production was obtained at 25 and 30 °C, with the maximum activity observed at 30 °C (24.4 U mL^{-1}). The specific activity directly increased with an increase in temperature, with the maximum observed at 35 °C (90.1 U mg $prot^{-1}$). β-xylosidase production **(**Fig. 2d**)** revealed itself to be more susceptible to temperature variation, since maximum production was verified at 25 °C (67.8 U mL^{-1} and 184.5 U mg $prot^{-1}$), with a

decrease at higher temperatures. Additionally, better fungal growth was observed at 20 to 30 °C, confirming the mesophilic character of the microorganism.

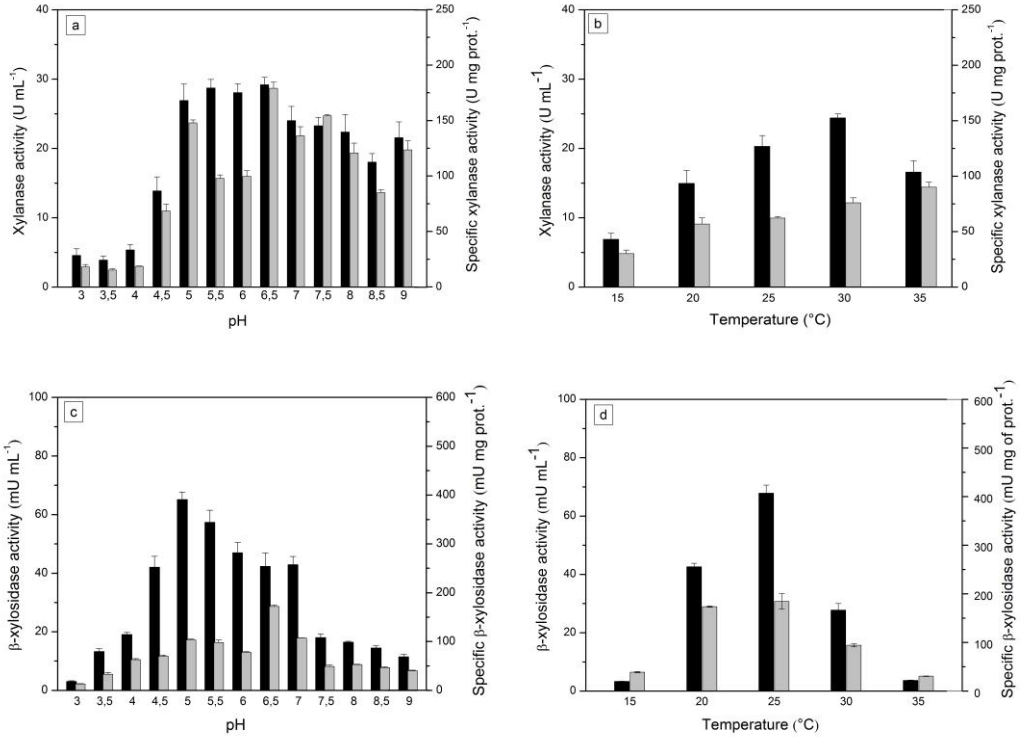

Fig. 2. Effect of pH and temperature on xylanase (a and b) and β-xylosidase (c and d) production by *P. janczewskii*. Culture conditions: Vogel medium with 1.0% (w/v) xylan from oat spelts, 28 ºC (left), and pH 6.5 for xylanase or 5.0 for β-xylosidase production (right). (■) Enzyme activity (U ml^{-1} or mU ml^{-1}), (■) Specific enzyme activity (U mg $prot^{-1}$ or mU mg $prot^{-1}$). All cultures were developed in triplicate, and the results were presented as mean values.

Xylanase and β-xylosidase Characterization

The SDS-PAGE presented in Fig. 3 compares the protein profiles from the mixtures obtained in the different culture conditions for optimized production of xylanase and β-xylosidase by *P. janczewskii*. As can be observed, similar protein profiles were obtained since quite similar culture conditions were used for the production of each enzyme. It is noteworthy the presence of a very intense band of a protein with molecular weight (MW) between 30 and 43 kDa in both profiles, which is in the MW-range commonly observed for fungal xylanases (Polizeli *et al*. 2005), suggesting it is the main xylanase produced by the fungus. Besides, in the profile of optimized β-xylosidase production there were observed protein bands with higher intensity of MW from 67 to 94 kDa and, on the top of the gel, a very intense band of a higher MW protein, suggesting it can be the β-xylosidase produced by the fungus, since microbial xylosidases are usually high MW proteins (Knob *et al*. 2010).

Table 1 summarizes the main properties of the crude extracellular xylanase and β-xylosidase produced by *P. janczewskii* in stationary cultures under optimized conditions.

High xylanase activity was verified in the pH range from 2.0 to 7.0, presenting peaks at pH 5.0 and 3.5, and another at pH 6.5 with expressive activity. These peaks of

high activity are probably the result of the presence of isozymes optimally active at different pH.

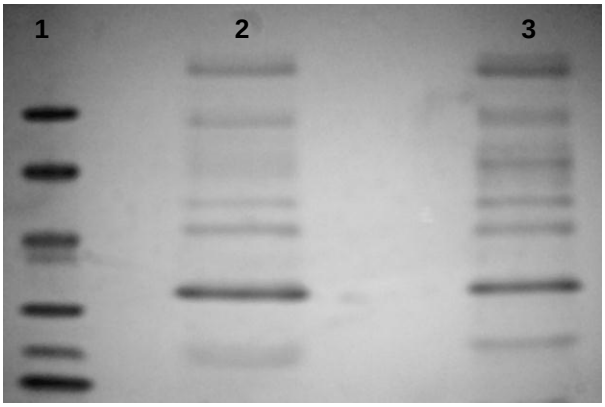

Fig. 3. SDS-PAGE (8-18%) of the crude extracellular extract from *P. janczewskii*. Lane 1: standard proteins, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14,4 kDa); Lane 2: protein mixture (50 µg) obtained under optimal conditions for xylanase production; Lane 3: protein mixture (50 µg) obtained under optimal conditions for β-xylosidase production.

The production of xylanases with different physico-chemical properties has been verified and it is related to the substrate heterogeneity and its accessibility, favoring the development of the microorganism in different conditions (Wong *et al*. 1988). β-xylosidase was optimally active at pH 4.0, maintaining more than 60% of the maximum activity at pH values ranging from 3.0 to 5.0, decreasing in more acidic pH and also in neutral and alkaline pH. This optimum pH is similar to those observed for most *Penicillium* xylanases and β-xylosidases (Chávez *et al*. 2006; Knob *et al*. 2010). It is interesting to note that both the xylanase and the β-xylosidase from *P. janczewskii* presented different optimum pH values. In contrast, both xylanase and β-xylosidase from *Penicillium funiculosum* presented optimal activity at pH 4.0 (Mishra *et al*. 1985).

Table 1. Crude extracellular Xylanase and β-xylosidase Properties from *P. janczewskii*

Characteristic	Enzyme	
	Xylanase	β-xylosidase
Optimum pH	5.0	4.0
Optimum temperature (°C)	50	75
pH stability	5.0-9.5	1.6-5.5
Half-life (min)	183 (40 °C) 15 (50 °C) 3 (60 °C)	144 (50 °C) 8 (65 °C) 4 (75 °C)

Temperature has a profound influence on enzyme activity and, in many cases, it is desirable that enzyme preparations suitable for industrial applications must not only present high activity but also stability at elevated temperatures. In this study, high

xylanase activity (more than 60% from the maximum) was observed at temperatures from 40 to 60 °C, with the peak of optimum activity at 50 °C. High β-xylosidase activity (more than 80% of the maximum), was observed up to 85 °C, with the peak of maximum activity at 75 °C. The optimum temperature of *P. janczewskii* xylanase is similar to xylanases from other *Penicillium* strains (Chávez *et al*. 2006); however, the optimum temperature of the β-xylosidase was elevated, considering that a mesophilic micro-organism produced it. Usually, the optimum temperatures verified for mesophilic fungal β-xylosidases remain between 30 and 55 °C (Knob *et al*. 2010). Similar results were observed only for the β-xylosidases from thermotolerant fungal strains, which presented optimum temperatures between 70 and 75 °C (Rizzatti *et al*. 2001; Lenartovicz *et al*. 2003; Pedersen *et al*. 2007).

The *P. janczewskii* xylanase was stable over a broad pH range, retaining more than 80% of its activity in the range from 5.0 to 9.5; however, at pH 10.0 a loss of 80% of its activity was verified. In pH ranging from 1.6 to 4.5, xylanase retained more than 40% of its activity. The stability in this wide pH range indicates potential application in different industrial processes that require this characteristic. Substantial alkali tolerance may also be important for application in the biobleaching processes. Conversely, β-xylosidase was more stable in acidic pH, retaining almost 100% of the activity at pH 1.6 to 5.5, and more than 60% of the activity was observed above this pH range. The stability presented by the *P. janczewskii* β-xylosidase was very high; usually in the acid pH range lower stabilities are observed (Pedersen *et al*. 2007; Knob and Carmona 2009a; 2011).

Half-lives (T_{50}) of 183, 15, and 3 min were observed for xylanase at 40, 50, and 60 °C, respectively, and 144, 8, and 4 min for the β-xylosidase at 50, 65, and 75 °C, respectively. Comparing thermostability at 50 °C, β-xylosidase was less susceptible to heat denaturation than xylanase, with a T_{50} approximately 10-fold higher. The xylanase was less thermostable than those from some other *Penicillium* strains (Lenartovicz *et al*. 2003; Sinitsyna *et al*. 2003), but more stable than that from *P. sclerotiorum*, which presented T_{50} lower than 4 min at 50 °C (Knob and Carmona 2009b). However, the β-xylosidase from *P. janczewskii* was less thermostable than that from *P. sclerotiorum* that presented T_{50} of 240 min at 50 °C (Knob and Carmona 2009a).

Among the ions evaluated (data not shown), activation was observed only for β-xylosidase by Ca^{2+}. The increase (6 and 17%) was directly related to the concentration of the ion, suggesting that it may be required for the enzyme as cofactor. Nevertheless, EDTA inhibited, on different levels, both enzymes. This suggests that some other divalent metallic ion may be required as cofactor. The ion Hg^{2+} completely inhibited xylanase but only moderately inhibited β-xylosidase. The inhibition by Hg^{2+} seems to be a general property of xylanases, suggesting the presence of thiol groups of cysteine next to or in the active site of the enzyme (Bastawde 1992), but occurring only for some β-xylosidases (Rizzatti *et al*. 2001; Guerfali *et al*. 2008) and not for others (Deleyn and Claeyssens 1977; Knob and Carmona 2009b). Cu^{2+} and Pb^{2+} were moderate to strong inhibitors of both activities, while Zn^{2+}, Mn^{2+}, Ba^{2+}, NH_4^+, Na^+, and citrate inhibited only the β-xylosidase.

In addition, β-mercaptoethanol and DTT-stimulated xylanase activity can be explained by the prevention of oxidation of the -SH groups in the presence of these agents, or by a reduction of S-S bridges, restoring the native conformation of the enzyme or some specific region of the catalytic site. The inhibition of both enzymes verified with SDS indicates the importance of hydrophobic interactions for the three-dimensional structures of these enzymes.

Biobleaching

The results of enzyme treatment with *P. janczewskii* culture filtrate on the delignification of a pre-treated *Eucalyptus* kraft pulp are shown in Fig. 4. For practical reasons, the treatment was carried out based on quantification of xylanolytic activity. However, other enzymes, such as β-xylosidases (43.7 mU mL^{-1}) and α-L-arabinofuranosidases (37.4 mU mL^{-1}) are also present in this mixture and may be acting cooperatively with the xylanases in the process. Treatments of pulp with 2 and 4 U/g pulp for 1 h promoted kappa number reductions of 3 and 3.3 units, respectively. With a higher enzyme concentration, only a small reduction in kappa number was observed. According to Suurnäkki *et al*. (1997), although the hydrolysis degree increases with the enzyme content, above a certain amount, only small additional benefits are verified. Other studies also verified that, beyond a threshold, greater amounts of enzyme and longer incubation did not improve xylanase treatments (Maximo *et al*. 1998; Taneja *et al*. 2002). The same reduction pattern was observed in 2 h treatments, and kappa number values were only slightly lower than those observed in 1 h treatments. These results indicated that after more extended treatment periods no additional benefits in terms of kappa number reduction are achieved.

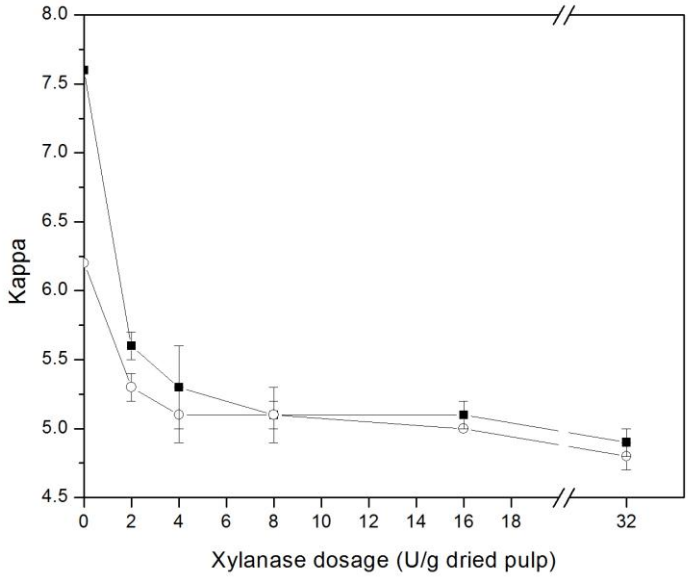

Fig. 4. Effect of time and enzymatic dosage on kappa number of *Eucalyptus* kraft pulp treated with crude *P. janczewskii* xylanase. (■) 1 h treatment; (○) 2 h treatment. The experiments were carried out in triplicate and the results were presented as mean values.

The kappa number is indicative of the residual lignin in the pulp, and its enzymatic reduction after treatment indicates a reduction in the amount of active chlorine to be used in later bleaching stages. A comparison among the results of this study and those using other xylanases is difficult, due to differences in the origin and characteristics of the pulp, differences in pulp pretreatment, different processing conditions, and also due to enzyme characteristics. The reduction obtained in the treatments with *P. janczewskii* xylanases was higher than to those obtained with some other fungal xylanases, which normally present reductions between 0.9 and 4.6 units in kappa number

(Betini *et al*. 2009; Madlala *et al*. 2001; Maximo *et al*. 1998; Medeiros *et al*. 2007; Michelin *et al*. 2010; Nair *et al*. 2010; Peixoto-Nogueira *et al*. 2009; Taneja *et al*. 2002). Only one study reported greater reduction, *i.e*., 10.0, treating a cellulose pulp with *A. niger* xylanases (Raghukumar *et al*. 2004).

In addition, it was observed that the treatment with *P*. *janczewskii* xylanase did not affect the pulp viscosity (Fig. 5). This fact indicates the maintenance of the pulp integrity, confirming the absence of cellulolytic enzymes in the extract, as previoulsy verified (Terrasan *et al*. 2010). This is crucial for a further application of this enzymatic extract, since the presence of cellulases could damage the fibers, resulting in loss of strength and performance. As verified in this work (especially in 2 h treatments) and in other studies (Medeiros *et al.* 2002; Betini *et al.* 2009), maintenance of pulp viscosity or even its increase can be attributed to the selective removal of the pulp hemicellulose, which could be interfering with the overall pulp viscosity (Suurnäkki *et al*. 1997).

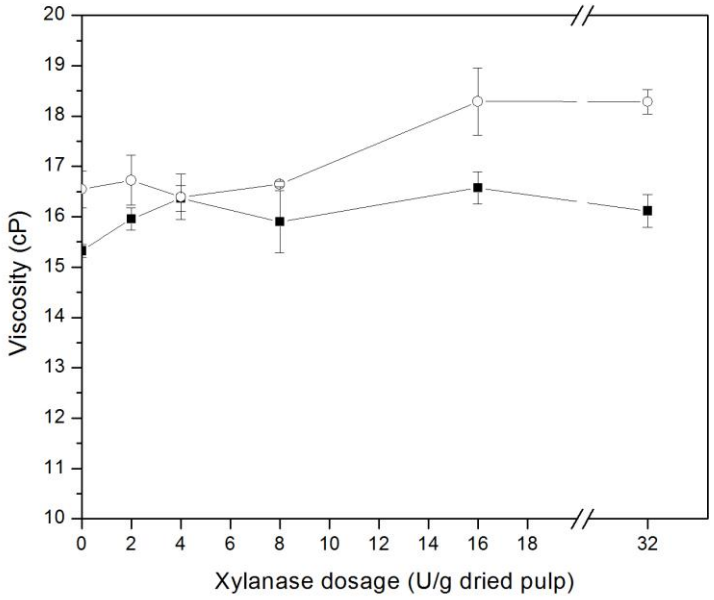

Fig. 5. Effect of time and enzymatic dosage on viscosity of *Eucalyptus* kraft pulp treated with crude *P*. *janczewskii* xylanase. (■) 1 h treatment; (○) 2 h treatment. The experiments were carried out in triplicate and the results were presented as mean values.

CONCLUSIONS

1. The optimized conditions for xylanase production were the following: 7 days of cultivation at pH 6.5 at 30 °C. For β-xylosidase production, optimized conditions were the following: 8 days at pH 5.0 at 25 °C, both in standing conditions.
2. The activity and the stability of the enzymes in moderate and elevated temperatures, as well the cellulase absence, allows their application prior to bleaching stages of a cellulose kraft pulp, since kappa number reduction of 3.0 units was observed after the treatment.

3. More prolonged treatments are not necessary since only small differences in terms of kappa number reduction were observed after 1 and 2 h treatments. Different pulp pre-treatments may be assayed in order to obtain greater reduction of chlorine compounds utilized in bleaching stages.
4. The activity of the enzymes at acidic pH also allows their application in other processes, as in wine making and juice processing, as well as for the production of feed with improved nutritional quality.

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