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Characterization of a Thermotolerant and Alkalotolerant Xylanase from a *Bacillus* sp.

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ABSTRACT

In a recent screening for thermophilic bacteria from Azores hot springs, a Bacillus sp strain 3M, exhibiting cellulase-free extracellular xylanolitic activity, was isolated. Further enzyme characterization from liquid cultures grown on birchwood xylan revealed that the endo-1,4-β-xylanase retains 100% of activity for at least 3 d at 55°C. At 80°C, it retains 47% of its maximal activity, and the enzyme is still active at 90°C. The optimum pH of the enzyme has a broad pH range, between 6.0 and 7.5, and it is remarkably active for the alkaline region, exhibiting 89% of relative activity at pH 9.0. The enzyme was partially inactivated by different divalent metal ions. Because of its tolerance for high temperature and pH conditions, and the absence of contaminating cellulase activity, the xylanase produced by *Bacillus* sp 3M appears to be attractive for use in the pulp and paper industry. Indeed, the efficiency of the enzyme application to the kraft *Eucalyptus* pulp was studied for bleaching pretreatment, resulting in a moderate increase of pulp bleachability.

Index Entries: Thermotolerant bacteria; alkalotolerant bacteria; *Bacillus;* extracellular hydrolases; xylanase; xylan; bleachability; enzymatic pulp prebleaching.

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INTRODUCTION

Xylan, the major component of plant hemicelluloses, is constituted of 1,4-linked β-D-xylopyranose backbone units and short side chains of arabinofuranosyl and glucopyranosyl residues. Xylanases (1,4-β-D-xylanohydrolase, E.C.3.2.1.8) are hemicellulases that catalyze the hydrolysis of xylan backbone.

One of the major industrial applications of xylanases involves its use for prebleaching kraft pulps *(1)*. In a pulp manufacturing plant, wood lignin is removed at high temperature and basic pH, the so-called kraft process. This process also involves the use of chlorine or chlorine dioxide to obtain high levels of pulp brightness. An enzymatic prebleaching removes xylan, and thereby partly releases the lignin from the pulp. This strategy avoids the use of huge amounts of clorine-based chemicals commonly used in the conventional process. The major effect of the enzyme is caused by the hydrolysis of reprecipitated and readsorbed xylan and xylan–lignin complexes, which are separated during the cooking process *(2)*.

Xylanases have been widely characterized in bacteria and fungi *(3)*, but most of them reported to date have optimal pH and temperature at acidic values and below 45°C, which limits their use for pulp-bleaching processes *(4)*. Therefore, screening for novel xylanases displaying activity under high temperatures and alkaline conditions is an important feature for pulp applications.

This paper reports an alkaline and thermotolerant xylanase produced by a thermophilic *Bacillus* sp strain 3M, previously isolated from Azores hot springs.

MATERIALS AND METHODS

Strain

The organism used (*Bacillus* sp 3M) was originally isolated from terrestrial hot springs (temperature, 90°C) samples collected on São Miguel, Azores, as described elsewhere (manuscript in preparation). Stock cultures were maintained on tryptone soya agar slants at 4°C, in the dark, and transferred into fresh medium once a month.

Culture Media

The culture medium used in this work has the following composition (per liter of distilled water): 1.5 g $(NH_4)_2SO_4$; 1.7 g KH_2PO_4; 1.7 g $Na_2HPO_4 \cdot 2H_2O$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.5 mL mineral solution *(5)*; 1 g yeast extract; and 1 g peptone. The pH was adjusted to 6.9–7.0 with 1.0 *M* NaOH

or HCl, and this basal medium was sterilized by steam-autoclaving (at 121°C, 1 atm for 15 min). The medium was supplemented either with 1.0% (w/v) (0.5% in the case of fermentor cultivation) birchwood xylan (Sigma Chemical Co., St. Louis, MO), oat spelts xylan (Sigma), carboxymethylcellulose (BDH), saccharose (Merck, Darmstadt, Germany) or lactose (BDH, Poole, UK). All these C-sources were autoclaved separately.

Cultivation Conditions

The microorganism was grown at 55°C in 500-mL Erlenmeyer flasks containing 150 mL of the basal medium previously described, shaken at 150 rpm on an orbital shaker (New Brunswick Scientific Co., Edison, NJ), without pH control. Batch cultures were also carried out in a 5-L Braun fermentor with a 3.5-L working volume. The aeration rate was set at 1 vvm, and the stirring at 500 rpm, at uncontrolled pH value. The bacterial growth was followed by measuring the optical density at 580 nm.

Culture Sampling

The bacterial cells were removed from the culture broth by centrifugation (4000*g* for 15 min at 4°C), and the cell-free supernatant dialyzed (cutoff = 6–8 kDa) against distilled water overnight at 4°C. These dialysates were further used for the enzymatic assays.

Enzymatic Assays

Endo-1,4-β-xylanase (EC 3.2.1.8) was assayed using 1% (w/v) birchwood 4-O-methyl-glucuronoxylan (Sigma) as substrate, at 55°C and pH 5.3, according to Bailey et al. *(6)*. One U of endo-1,4-β-xylanase activity was defined as that amount of enzyme that catalyzes the release of 1 μmol of xylose equivalents/min under the assay conditions. Filter-paper activity (FPase), which describes the overall cellulolytic activity, was assayed using the International Union of Pure and Applied Chemistry (IUPAC) method *(7)*, using Whatman No. 1 filter paper (about 50 mg) as substrate, at 55°C and pH 4.8. One U of enzyme activity was reported as μmol/min of glucose equivalents released/min under standard conditions. β-Xylosidase (EC 3.2.1.37) activity was determined, at 55°C and pH 4.8, using the synthetic substrate *p*-nitrophenyl-β-D-xylopyranoside (Sigma), as described by Li et al. *(8)*. One U of activity was defined as that amount of enzyme that catalyzes the release of 1 μmol/min of *p*-nitrophenol/min under the assay conditions.

Optimal Temperature and pH

For determination of the enzyme optimal temperature, the enzymatic assay was performed for the temperature range of 30–95°C, at pH 5.3. For the optimum pH determination, 50 m*M* buffer solutions, ranging from

pH 4.0 to 10.6, were used to assay the xylanase activity at 55°C. Sodium citrate buffer was used for pH 4.0–6.0, potassium phosphate buffer for pH 6.0–8.0, Tris-HCl buffer for pH 8.0–9.0, and glycine-NaOH buffer for pH 9.0–10.6.

Localization of Xylanases

For extracellular activity, the cell-free medium was used for xylanase activity assay. For cell-wall-bound enzyme activity, bacterial cells were resuspended in 50 m*M* citrate buffer (pH 5.3) and subjected to low-speed centrifugation for contaminant removal. The intact cells were resuspended in 0.2 *M* Tris-HCl buffer (pH 8.0) with 0.5 *M* sucrose and 0.5 *M* ethylenediaminetetraacetic acid (EDTA), and incubated in the presence of lysozyme during 15 min at 30°C, to obtain spheroplasts. After centrifugation, the supernatant was used for determination of cell-wall-bound activity. For soluble enzymatic activity, the spheroplasts were sonicated in 0.2 *M* Tris-HCl buffer (pH 7.5) for 10 min on ice, and the supernatant obtained by ultracentrifugation was used in the xylanase assay.

Thermostability

To study xylanase thermal stability, enzyme preparations in 50 m*M* citrate buffer (pH 5.3) were incubated at temperatures ranging from 55°C to 90°C for 3 d, and assayed daily for xylanase activity.

Influence of Metal Ions

The effect of different divalent metal ions on xylanase activity was determined by the addition of the corresponding ion at a final concentration of 10 m*M* to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of magnesium, calcium, manganese, zinc, and cobalt chlorides, and copper and cadmium sulphates.

Other Methods

Soluble protein was estimated spectrophotometrically by the method of Lowry et al. *(9)*, using bovine serum albumin (BSA) as standard protein. Reducing sugar in the assay reaction mixture was estimated by the dinitrosalicylic acid (DNS) method *(10)*.

Enzymatic Pulp Prebleaching

Concentrated crude enzyme preparation was obtained (approx 12-fold) by tangential ultrafiltration of the extracellular culture broth in a Minitan system (Millipore, Bedford, MA) equipped with a 10-kDa molec-

ular cutoff membrane. The concentrated enzyme preparation displayed an endo-1,4-β-xylanase activity of 2.7 U/mL. A 4.5-g sample of unbleached pulp from *Eucalyptus globulus*, obtained from the pulp mill of Portucel, (Kappa number, 14.0; dry wt content, 91.6% w/w) was suspended in 50 m*M* citrate buffer, pH 5.3, for a final consistency of 5%, and incubated with the crude enzyme preparation at a concentration ranging from 5–50 U xylanase/g oven-dried pulp, at 55°C for 1–24 h, in a sealed polyethylene bag. After dewatering the samples by vacuum filtration on a Buchner apparatus, pulps were extracted under alkaline conditions, in order to reveal the effect of the enzymatic pretreatment. This alkaline extraction consisted of pulp-treating with 1% NaOH at 1% (w/v) consistency for 1 h at 60°C, followed by washing with distilled water until pH decreased to 7.0 *(11)*. A reference pulp sample was assayed identically to enzyme-treated pulp, being the enzyme solution replaced with an equivalent volume of buffer. The final pulp Kappa number was determined according to TAPPI standard method T236 cm-85.

Total reducing sugars present in the filtrates obtained after each of the two stages were determined by the DNS method as xylose equivalents. These filtrates were also analyzed by high-pressure liquid chromatography (HPLC), using a Aminex HPX-42A (Bio-Rad, Richmond, CA) column, using water as eluent at a flow rate of 0.5 mL/min and 85°C. Xylan, xylose, glucose, and xylo- and gluco-oligosaccharide standards were used for identification of the eluted carbohydrates.

RESULTS AND DISCUSSION

Bacterial Growth Profile

Figure 1 reports the time-course profile of *Bacillus* sp 3M grown in basal medium supplemented with 1.0% birchwood xylan, as described in the Materials and Methods section. The exponential growth phase was very fast, and lasted only 2 h with a doubling time of 96 min.

Endo-β-1,4-Xylanase Activities

The production of extracellular endo-1,4-β-xylanase activity during growth of *Bacillus* sp 3M, on media supplemented either with birchwood or oat spelts xylan, is shown in Fig. 1. Both enzyme activity profiles are similar, but much higher activities were reached using oat spelts xylan as substrate. There was no obvious correlation between growth and extracellular xylanase activity. Activity was detectable in the culture supernatant only in the end of the exponential growth phase (after 4 h of growth). Bacterial xylanase excretion rate was fairly constant up to 9 h of cultivation time, and the highest volumetric activities (46 and 169 mU/mL on birch-

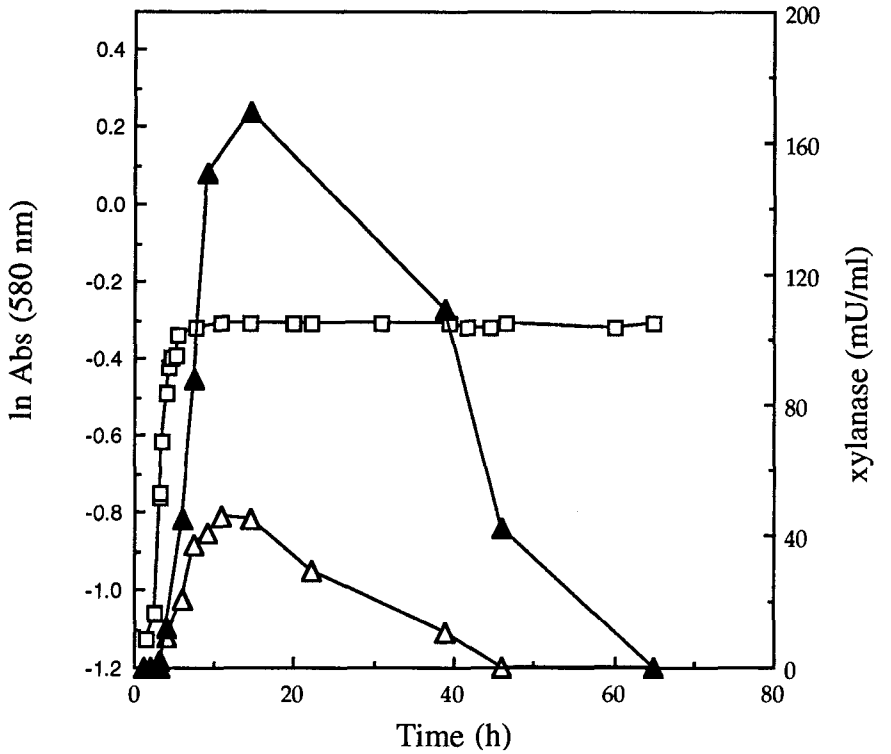

Fig. 1. Time-course of endo-β-1,4-xylanase production by *Bacillus* sp 3M at 55°C on 1% birchwood xylan (△) and 1% oat spelts xylan (▲). The growth profile shown (□) was obtained on birchwood xylan.

wood and oat spelts xylan, respectively) were obtained at about 15 h of cultivation time (early stage of stationary-growth phase), followed by a slower decrease in the enzyme activity, until approx 48 h of cultivation time.

Production of extracellular xylanase was also analyzed for growth on media supplemented with saccharose and lactose, and on the basal medium with no supplement. After 15 h of cultivation (time corresponding to the maximal activity on both birchwood and oat spelts xylan), the endo-1,4-β-xylanase activities obtained were residual: 12, 24, and 19 mU/mL on saccharose, lactose, and basal media, respectively. The authors conclude that xylanase is synthesized constitutively to some extent by *Bacillus* sp 3M, but levels increased from two- to 15-fold by growth on xylan. This result was strengthened by the presence of enzyme activity, even when growth was carried out in the basal medium supplemented with saccharose, but without any supplementation of yeast extract or peptone, to ensure the absence of any possible C source inducer of xylanase activity. In these conditions, a volumetric activity of 21 mU/mL was observed after 21 h.

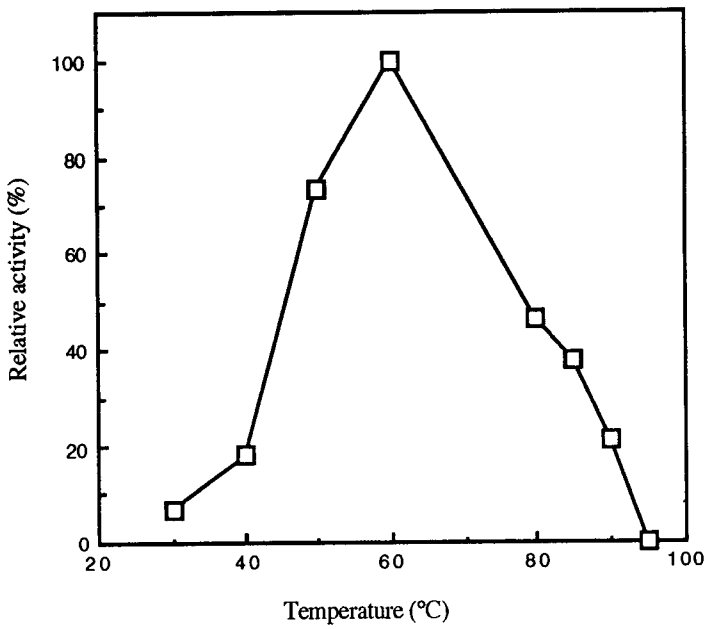

Fig. 2. Effect of temperature on activity (pH 5.3) of the xylanase produced by *Bacillus* sp 3M grown at 55°C for 10.8 h on 1% birchwood xylan. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 62 mU/mL).

Oxygen affects xylanase production in this *Bacillus* sp 3M. The production of xylanase increased remarkably when *Bacillus* sp 3M was grown in batch-aerated fermentor which allows a nonlimiting oxygen growth. The xylanase yield, after 10 h of cultivation, was about fourfold higher in the bioreactor (177 and 296 mU/mL on 0.5% birchwood and oat spelts xylan, respectively) than that obtained in the parallel shake-flask cultures, which, by definition, is always oxygen-limiting cultivation.

Other Enzymatic Activities

The xylanolytic system of the *Bacillus* sp 3M also contains β-xylosidase activity, albeit in lower levels than endo-1,4-β-xylanase level (the maximal activity detected was 0.8 mU/mL after 7.5 h on birchwood xylan at 55°C). β-xylosidase seems to be the rate-limiting enzyme in xylan hydrolysis by the *Bacillus* sp 3M xylanolytic system. No filter-paper hydrolase (FPase) activity was detected in the extracellular broth from xylan-grown bacterial cultivation.

Temperature Effect

Xylanase from *Bacillus* sp 3M exhibited a temperature profile (Fig. 2) with a sharp peak of maximal activity at 60°C and showing activity between 30 and 90°C.

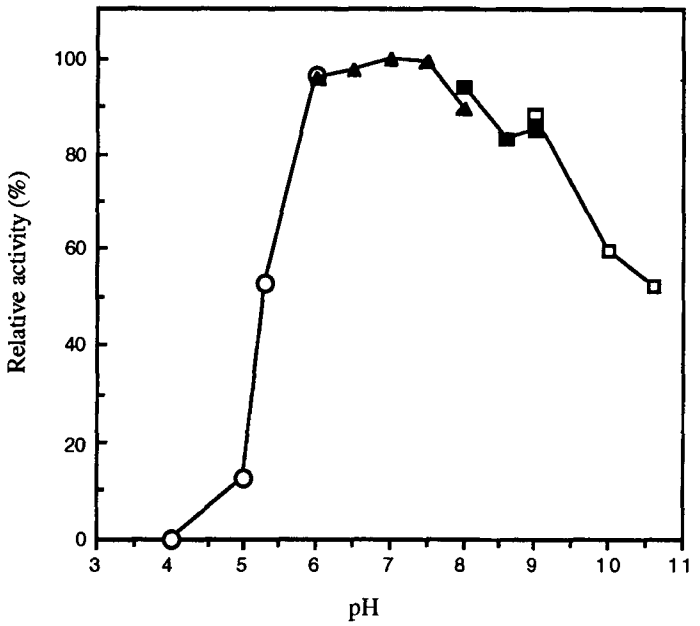

Fig. 3. Effect of pH on activity (55°C) of the xylanase produced by *Bacillus* sp 3M grown at 55°C for 7.5 h on 1% birchwood xylan. The ionic strength for all buffers was 50 m*M*: (○), sodium citrate; (△) potassium phosphate; (■), Tris-HCl; (□) Glycine-NaOH. Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 37 mU/mL).

pH Effect

A pH range between 4.0 and 10.6 was used to study the effect of pH on xylanase activity (Fig. 3). Optimum pH was found to be 7.0, but a broad pH plateau between 6.0 and 7.5, exhibiting a relative activity near the optimal value, was observed. For pH values between 7.0 and 9.0, there was only a slight activity decrease (89% of maximal activity remained at pH 9.0), and 52.0% of xylanase relative activity was still measured at pH 10.6, which means that the enzyme is strongly alkalotolerant.

Localization of Xylanolytic Enzymes

The localization of xylanolytic activity was investigated in cultures of *Bacillus* sp 3M grown for 7.5 h on birchwood xylan. The activity was determined in culture supernatants, in crude extracts prepared by ultrasonic disruption of cells, and in lyzosyme-treated cells (Fig. 4). These data show that almost all (93.5%) of the total xylanase activity in xylan-grown cultures is extracellular. The intracellular, as well as the cell-wall-bound, activities were rather low (1.4% and 5.1%, respectively). For this reason,

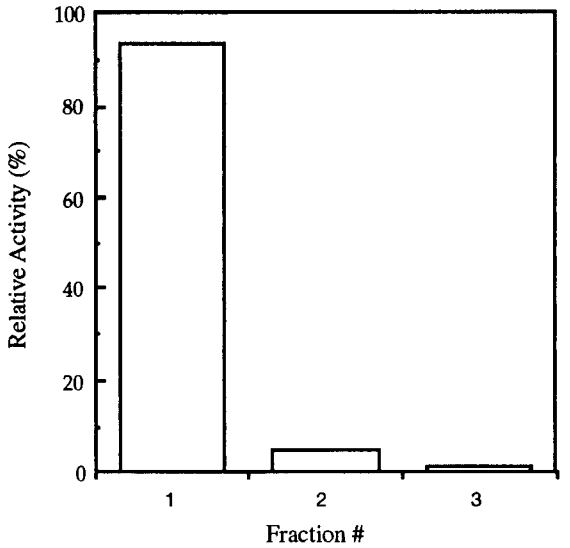

Fig. 4. Distribution of xylanase activity in *Bacillus* sp 3M grown on 1% birchwood xylan for 7.5 h at 55°C. 1, extracellular; 2, cell-wall-bound; 3, intracellular. Relative activity is expressed as a percentage of the total activity (100% of enzyme activity = 49.4 $mU/mg_{protein}$).

further assays of enzyme activity in this work were only carried out using the extracellular enzymatic fraction.

Thermostability

Because the authors intend to study the potential technological importance of this xylanase, apart from its pH and temperature optima, its thermal stability is a very important property. This study showed that the enzyme was highly stable at 55°C, retaining 100% of its original activity after a 3-d preincubation at this temperature and pH 5.3. It tolerated a temperature of 60°C at pH 5.3, but showed a large degree of inactivation after 1 d (retaining only 7, 5, and 4%, respectively, after 1, 2, and 3 d of incubation). Incubation at higher temperatures (70, 80, and 90°C) completely inactivated the enzyme after 1 d.

Influence of Metal Ions

As can be observed in Table 1, the endo-1,4-β-xylanase did not require any specific ion for its activity. Because metal ions could be generated from equipment corrosion, specially when subject to acid hydrolysis, the authors tested the presence of some metal ions at the concentration of 10 m*M*. All of them decreased the enzyme activity, although to different

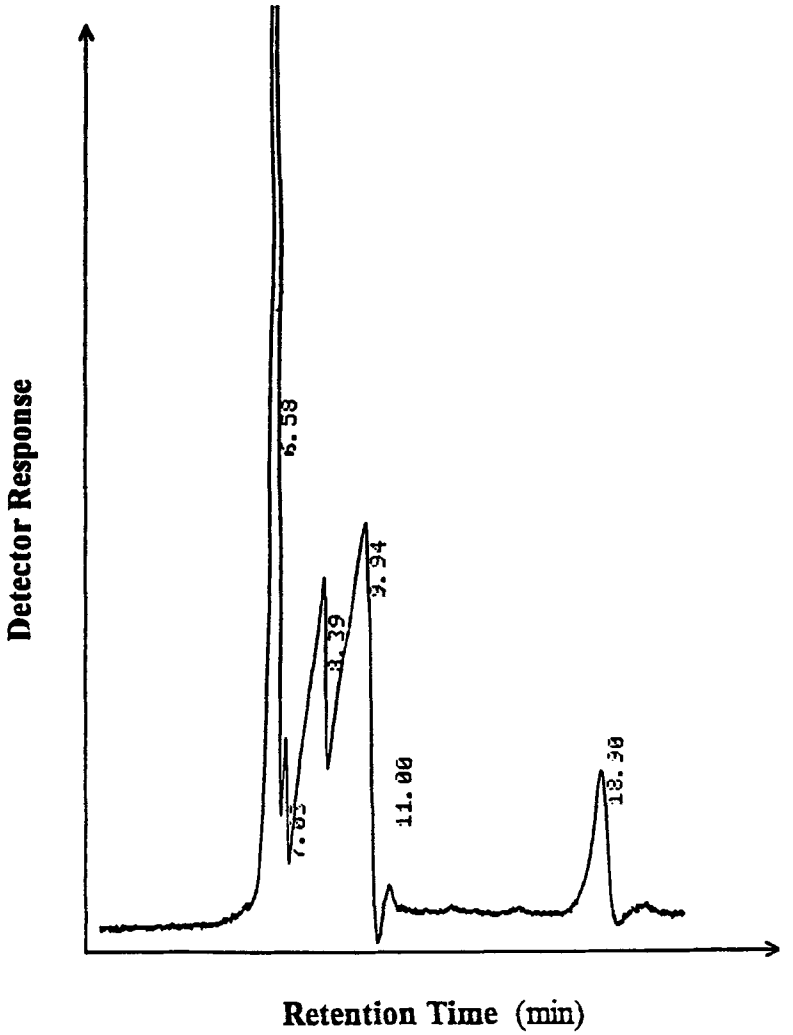

Fig. 5. HPLC chromatogram for the filtrate obtained after treatment (55°C, pH 5.3, 24 h) of Eucalyptus kraft pulp (final consistency, 5%) with the *Bacillus* sp 3M xylanase (50 $U/g_{oven\text{-}dried\ pulp}$). Retention times (min): xylose, 18.9; >DP 7, 9.94; >DP 10, 8.39.

degrees. A slight inhibition was produced in the activity by Mg^{2+}, Ca^{2+}, and Mn^{2+}, and a stronger inhibitory effect was observed in the presence of Zn^{2+}, Cu^{2+}, Cd^{2+}, and Co^{2+}.

Bleaching Study

To evaluate the effects of the xylanase enzyme on hardwood kraft pulp bleachability, i.e., the extent of lignin removal, an enzymatic pretreatment of pulp, combined with an alkaline extraction step, was carried out.

Table 1
Effect of Different Ions on Xylanolytic Activity Produced by *Bacillus* sp. 3M Grown at 55°C During 10.8 h on 1% Birchwood Xylan

Ion[a]	Relative activity[b] (%)
Control (no addition)	100
Mg^{2+}	89
Ca^{2+}	83
Mn^{2+}	79
Zn^{2+}	22
Cu^{2+}	20
Cd^{2+}	16
Co^{2+}	15

[a] The final concentration in the reaction mixture was 10 m*M*.
[b] Relative activity is expressed as a percentage of the maximum (100% of enzyme activity = 46 mU/mL)

Table 2
Results Obtained with Pine Kraft Pulp (5% consistency) Treated (55°C; pH 5.3; 24 h) with *Bacillus* sp. 3M Xylanase (50 $U/g_{oven\text{-}dried\ pulp}$) Prior to Bleaching Sequence

	Reducing sugars liberated[a] (mmol/L)		Kappa number[b]	
	Enzyme		Enzyme	
Stage	+	−	+	−
After enzymatic pretreatment	8.2	0	—	—
After alkaline extraction	0	0	9.1	9.7

[a] Reducing sugars determined as xylose equivalents.
[b] Original Kappa number = 14.0.

In the traditional chemical process to produce kraft pulps, the extent of lignin leached during alkaline extraction can be used to predict the effectiveness of enzyme treatment in pulp bleaching *(12)*. Thus, this study abstained from the application of the complete bleaching sequence to the enzymatic-treated pulp. The main variables affecting the enzymatic treatment efficiency are temperature, pH, pulp consistency, enzyme dosage, and residence time. The pH used was 5.3, since most of the measurements for enzyme characterization in this work were carried out at this pH value.

For the assay temperature, the optimal value (60°C) was not chosen, because, at this temperature, the enzyme showed a large degree of inactivation (93%) after a 24-h incubation. Treatment was carried out at 55°C, at which the xylanase retained 100% of its original activity after 3 d of incubation. Because consistency in the range of 1–9% has shown to yield very little effect on the efficiency *(13)*, 5% consistency pulps were always used during the enzyme stage. Enzyme dosages ranging from 5–50 $U_{xylanase}/g_{oven\text{-}dried\ pulp}$, and residence times from 1–24 h, were assayed. The best results were achieved at an enzyme dosage level of 50 U/g for a 24-h pretreatment (Table 2). From these data, the authors observe that reducing sugars were only liberated when xylanase preparation was added. The enzyme pretreatment resulted in reduction of final lignin content (as evaluated by the κ number) in pulp. This means that incubation with *Bacillus* sp 3M xylanase increased the bleachability of pulp, and that the enzymatic pretreatment should improve the efficiency of the bleaching sequence.

However, these results show that, even with a very high enzyme loading and prolonged incubation period, the increase in alkali extractability of lignin was limited (6% decrease in final κ number observed, relative to that of control enzyme-untreated pulp). Enzyme accessibility to the substrate might limit the solubilization of the xylan. It is probable that a process similar to the sequential bleaching/extraction steps used in the pulp industry will be required for optimum treatment of pulps by enzymes *(14)*. After 24 h of pulp incubation in the presence of xylanolytic extract, intact xylan, xylo-oligosaccharides exhibiting a degree of polimerization (DP) higher than 7, and xylose were detected by HPLC analysis (Fig. 5). This suggests, in addition to endo-β-1,4-xylanase activity, the presence of β-xylosidase activity, which fully hydrolyzes lower oligosaccharides to xylose as final product. The absence of any glucose or gluco-oligosaccharides indicates that the crude xylanase from *Bacillus* sp 3M was only active toward xylan, and does not attack the cellulosic fraction of the pulp.

CONCLUSIONS

In comparison with the fungal enzymes, the xylanase production time by bacteria is much shorter (as demonstrated with the strain in this study), which is a crucial beneficial factor for large-scale production *(11)*. The bacterial genus *Bacillus* has an important role in this production, since it is considered safe for human uses *(4)*. The *Bacillus* strain studied here also has the advantage that nearly all the total xylanase activity in xylan-grown cultures is extracellular, therefore avoiding the need for the operation unit of enzyme extraction from bacterial cells.

Moreover, our results demonstrated that, of the maximal xylanase activity produced by *Bacillus* sp 3M, 47% was present at 80°C and 89% at pH 9.0 (conditions generally used in the kraft pulp process *[1]*). The enzyme was also stable at 55°C for 3 d, which could be useful for industrial processes.

The xylanolytic system produced by *Bacillus* sp 3M lacks cellulase as a contaminating enzyme activity. This absence of cellulase activity contrasts with that of filamentous fungi, in which cellulolytic and xylanolytic activities often occur together *(15)*, and is a crucial feature, because the cellulase attack on the cellulose would result in fiber damage and reduced pulp strength *(1)*. The absence of cellulase makes possible the use of *Bacillus* sp 3M crude supernatant for treating kraft pulps without further protein purification.

In conclusion, all these features indicate that the *Bacillus* sp 3M xylanase may find applications in the pulp and paper industry, but additional pilot studies, using the complete bleaching sequence, should be carried out to judge the importance of this thermotolerant and alkalotolerant bacterial strain as an industrial enzyme producer.

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