0273-2289/06/129–132/289–302/$30.00

Purification and Characterization of Two Xylanases From Alkalophilic and Thermophilic *Bacillus licheniformis* 77-2

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Abstract

The alkalophilic bacteria *Bacillus licheniformis* 77-2 produces significant quantities of thermostable cellulase-free xylanases. The crude xylanase was purified to apparent homogeneity by gel filtration (G-75) and ionic exchange chromatography (carboxymethyl sephadex, Q sepharose, and Mono Q), resulting in the isolation of two xylanases. The molecular masses of the enzymes were estimated to be 17 kDa (X-I) and 40 kDa (X-II), as determined by SDS-PAGE. The K_m and V_{max} values were 1.8 mg/mL and 7.05 U/mg protein (X-I), and 1.05 mg/mL and 9.1 U/mg protein (X-II). The xylanases demonstrated optimum activity at pH 7.0 and 8.0–10.0 for xylanase X-I and X-II, respectively, and, retained more than 75% of hydrolytic activity up to pH 11.0. The purified enzymes were most active at 70 and 75°C for X-I and X-II, respectively, and, retained more than 90% of hydrolytic activity after 1 h of heating at 50°C and 60°C for X-I and X-II, respectively. The predominant products of xylan hydrolysates indicated that these enzymes were endoxylanases.

Index Entries: Xylanase; *Bacillus licheniformis*; xylanase purification; alkalophilic bacteria; xylanase characterization.

Introduction

There is a strong interest in xylanolytic enzymes of microbial origin, owing to their numerous possibilities for industrial applications. Xylanases are typically produced as a mixture of different isoenzymes that act on xylan, degrading its backbone into small oligomers. The systems of cellulase-free xylanases have been receiving more attention, especially in systems in which the degradation of xylan without breaking cellulose fiber is required, like in the paper and pulp industries *(1)*. A new and promising

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field for xylanase application is the biorefinery. A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass (2,3). Hemicelluloses and cellulose represent more than 50% of the dry weight of agricultural residues; they can be converted into soluble sugars either by acid or enzymatic hydrolysis, so they can be used as a plentiful and cheap source of renewable energy in the world. Enzymatic hydrolysis has been preferred as it is environmentally friendly. Xylan is the main component of the hemicellulose fraction, which is degraded by xylanases, thus if complete degradation of biomass feedstocks is required, xylanase must be present.

In many microorganisms, xylanase production is accompanied by the cellulase enzymes, which disables the application of the crude enzyme in the pulp and paper industry. The most economical form of obtaining cellulase-free xylanase is the isolation of microorganisms which only produce xylanase. Genetic manipulation can also be used to obtain cellulase-free mutants through the removal of the cellulase gene from a potent xylanase-producing microorganism. Another way to obtain cellulase-free xylanase is the development of a purification process to recover the pure xylanase from the fermentation broth (4).

Many xylanolytic microorganisms produce more than one xylanase, which usually differ in their physicochemical and biochemical properties, allowing them to be separated, based on their molecular mass, electrical charge, and isoelectric point (5). Enzymatic purification is an important stage for acquiring insight into the xylanolytic system of the microorganism and to understand its mechanism of action on the substrate, with the aim of optimizing its industrial application. Working directly with the crude enzyme makes it impossible to know whether the hydrolyzation of the xylan molecule was owing to the action of a single enzyme or to an enzymatic system acting synergistically.

In a previous work, *Bacillus licheniformis* 77-2 was isolated because it is a good producer of extracellular cellulase-free xylanase. The crude enzyme presented great activity at pH 6.0 and 70°C and pH stability in the range of 5.0–10.0, with 100% stability at 40°C for 1 h (6). The crude xylanase was used in the biobleaching of eucalyptus Kraft pulp, allowing for a reduction of 33% in the ClO_2 load used for pulp delignification (κ number) or a 44% reduction to achieve the same level of brightness, in comparison with pulp that did not undergo the enzymatic treatment (1). To the best of our knowledge, only one report has been published describing the purification of the predominant form of xylanase from this microorganism and its successful application in the delignification process (7). In view of the scarcity of reports on xylanases from *B. licheniformis*, we believe that it was in the interest of certain industries to purify and characterize its isoenzymes in order to gain insight into its xylanolytic system with the aim of understanding its action during the biobleaching process.

Material and Methods

Microorganism and Growth Conditions

B. licheniformis 77-2 was isolated from decaying wood (6). The bacterium was grown in alkaline medium, pH 9.0 containing 1% beef extract powder, 1% peptone, 1% NaCl, 0.1% KH_2PO_4, 0.5% Na_2CO_3 (sterilized separately), and 2% Birchwood xylan. A 20 mL of the medium, in 125-mL Erlenmeyer flasks, were inoculated with 1 mL (10^7 cells) of a 16-h seed culture and incubated at 50°C with shaking at 150 rev/min. After 48 h, the bacteria were harvested by centrifugation at 10,000*g* at 4°C for 20 min. The cell-free supernatant was used as crude enzyme solution.

Enzyme and Protein Assays

Xylanase was assayed by incubating 0.1 mL of appropriately diluted enzyme, to ensure its initial rate of activity, with 0.9 mL of a suspension containing 1% of Birchwood xylan (Sigma, St. Louis, MO) in 0.1 *M* acetate buffer at pH 6.0. After incubation at 60°C for 10 min, the reducing substances released were assayed by 3,5-dinitrosalicylic acid (8). Controls were prepared with enzyme added after 10 min of boiling. One U of activity was defined as 1 µmol of xylose equivalent released/min under the above assay conditions, using a xylose standard curve. Protein content was determined according to Lowry using bovine serum albumin as the standard.

Xylanase Purification

Many steps were tried before those described here. The chromatographic steps were performed with AKTA purifier equipment (Amersham Pharmacia, Uppsala, Sweden). All steps were carried out at room temperature, except for the ethanol precipitation, which was performed at 18°C. Xylanase activity was measured after every purification step.

Ethanol Precipitation

The supernatant was cooled in an ice-bath slowly adding ethanol at –18°C (70% saturation, V/V). The mixture was incubated at 4°C overnight and the precipitate collected by centrifugation (10,000*g*, 10 min, at 4°C).

Gel Filtration Chromatography

The precipitate was suspended in Tris-HCl, 0.05 mol/L, pH 7.5, dialyzed and loaded on a Sephadex G-75 column (20×1000 mm^2) (Pharmacia) previously equilibrated with the same buffer. The flow rate was 12 mL/h. The fractions containing xylanase activity were pooled, two peaks corresponding to X-I and X-II enzymes.

Ion Exchange Chromatography

The pool containing xylanase X-I, was loaded on CM Sephadex C-50 (Pharmacia) previously equilibrated with 0.02 mol/L sodium acetate buffer

(pH 5.0). The column was washed exhaustively with equilibrating buffer to wash off unbound protein and the bound proteins were eluted with linear saline gradient (0.01–1 mol/L NaCl in elution buffer) with a flow rate of 0.06 mL/min. The levels of protein were monitored at 280 nm.

Anion Exchange Chromatography—Q-Sepharose

After the elution from G-75, the pool containing X-II xylanase was concentrated using Centriprep-10 (Millipore), dialyzed overnight against 0.05 mol/L sodium acetate buffer, pH 5.0, and loaded again on previously equilibrated Q-Sepharose, exactly as performed on Sephadex C-50. The proteins were eluted with a linear gradient of 0.1–1 mol/L NaCl in elution buffer.

Anion Exchange Chromatography—Mono Q HR

After the elution from Q-Sepharose the pool containing X-II xylanase was concentrated again using Centriprep-10 (Millipore, Beldford, MA), dialyzed overnight against Tris-HCl, 0.02 mol/L, pH 7.2, and loaded again on previously equilibrated Mono Q HR 5/5 with the same buffer, exactly as performed on Q-Sepharose. The proteins were eluted with a linear gradient of 0.1–1 mol/L NaCl in elution buffer. The active fractions were pooled, dialyzed to remove salt, concentrated by 10 kDa membrane (centricon, Millipore) and sterilized by 0.22-µm membrane filtration (Millipore).

Electrophoresis and Silver Staining

Electrophoresis was carried out on denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gel with 1.5 *M* Tris-HCl buffer (pH 8.8) as described by Laemmli *(9)*. The gels were silver stained according to Meril, 1970 *(10)*. The molecular weight markers used were albumin bovine (66 kDa), carbonic anhydrase (29 kDa), and trypsin (20 kDa).

pH and Temperature Effects

The optimal pH for pure xylanase activity was measured in a pH range 3.0–11.0 at 60°C for 10 min. The following 100 m*M* buffer systems were used: Sodium acetate buffer pH 3.0–5.6, Citrate-phosphate pH 5.5–7.0; Tris-HCl pH 6.0–8.5, and Gly-NaOH pH 8.5–11.0. Optimal pH was the pH in which the enzyme displayed its maximal activity, which was considered 100% activity. For optimum temperature activity the pure xylanase was assayed between 40°C and 90°C for 10 min, although the pH was maintained at 7.0 for X-I and 9.0 for X-II. Optimal temperature was the temperature in which the enzyme displayed its maximal activity, which was considered 100% activity. The pH stability was determined by incubating the enzyme preparation without substrate in the same buffer systems used above, for 24 h at room temperature. The remaining activity was assayed under standard conditions at 60°C. The temperature stability was checked by subjecting

the enzyme to pH 7.0 for X-I and 9.0 for X-II, without substrate at various temperatures (40–90°C) for 1 h, and then cooling in ice before measuring the residual activity under standard conditions at 60°C.

Determination of the Kinetic Parameters

The enzymatic reaction for the kinetic study of the xylanases of *B. licheniformis* 77-2 was carried out at pH 6.0 and 60°C for 10 min, using the specific activity of 18 and 18.3 U/mg protein of XI and XII, respectively, with different Birchwood xylan concentrations (0.2–20 mg/mL). The kinetic constants (K_m and V_{max}) were determined from the Lineweaver–Burk plot.

Analysis of Hydrolytic Products

A 5 U of the purified enzyme was incubated at 60°C for 2 h with 0.9 mL of 2% Birchwood xylan (Sigma) in 50 m*M*, pH 6.0 Tris-HCl buffer, with a final volume of 1 mL. After the incubation period, the enzyme was inactivated by 10 min of boiling. The identification of the hydrolysis products was carried out by the method of descending paper chromatography (Watman N. 1), using as run solvent ethyl acetate, isopropanol and water, in the proportion 6:3:1, respectively. The revelation solvent was acetone and saturated silver nitrate, washed with alcoholic silver hydroxide to visualize the stains. The standards used were xylotriose, xylobiose, and xylose.

Results and Discussion

Summary of Purification

Diagram 1
Stages of Purification

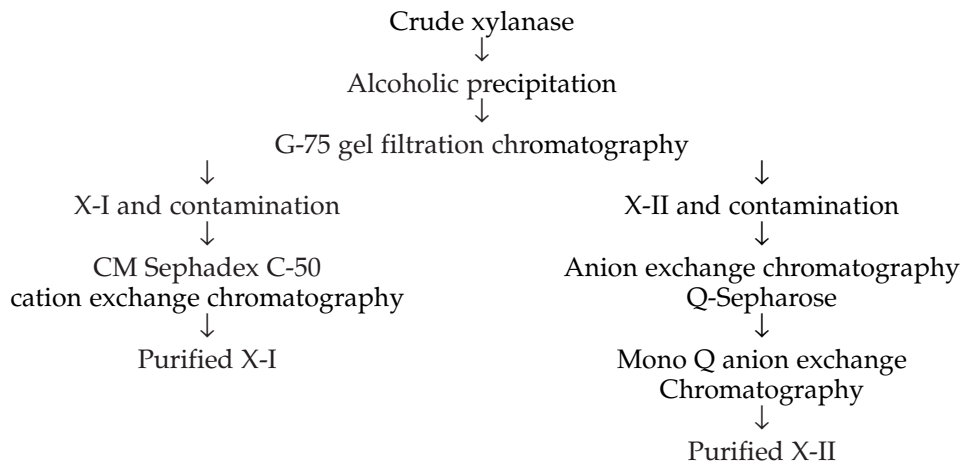

Table 1
Summary of the Purification of the Xylanases Produced by *B. licheniformis* 77-2

Step		Volume (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Supernatant		480	931	8640	9.3	100	1
Precipitation and dialysis		20	370	3700	10	42.8	1.1
Gel filtration (G-75)	X-I	118	12	1286	107	14.9	11.5
	X-II	136	22	2285	104	26.5	11.2
CM sephadex	X-I	78	0.78	562	720	6.5	77.4
Q-Sepharose	X-II	54	5.4	1350	250	15.6	27
Concentration centriprep	X-II	3.8	3.9	904	232	10.5	25
Mono Q HR5/5	X-II	4.5	0.27	99	367	1.2	40

Diagram 1 summarizes the purification stages realized. Starting from the crude supernatant of xylanase from *B. licheniformis* 77-2, two xylanases named X-I and X-II were separated.

The first purification stage was precipitation with alcohol. In this stage, the specific activity of the crude xylanase increased about 10 times and the total activity of xylanase was decreased from 8640 U to 3700 U, corresponding to a yield of 42%, as shown in Table 1. In the gel filtration process five protein peaks were observed and the xylanase activity appeared in two peaks that were designated X-I and II. X-I was separated from X-II with a yield of 15% and 26%, respectively. The purification of X-I by CM Sephadex C-50 raised the specific activity from 107 U/mg of protein to 720 U/mg of protein, resulting in an 80-fold purification factor and a yield of 6.5. The low yield of the X-I fraction may be explained on the basis of its low stability at pH 5.0 (Fig. 1A) used in the elution buffer and the fact that negative resins may have formed a chelate and eliminated certain metal ions important for enzyme activity *(11)*.

X-II did not bind to CM Sephadex C-50, thus it was applied on Q-Sepharose resin. The purification stage of xylanase II on the Q-Sepharose resin increased the purification factor from 11 to 28. Therefore, this stage was important in the purification of the enzyme, but it did not present sufficient resolution to separate it from all the contaminants.

With the application of X-II on the Mono Q HR 5/5 resin, the xylanase was completely separated. X-II was eluted with about 0.6 *M* of NaCl. In this stage, a low yield was obtained, but it was important for complete purification, as viewed in the electrophoresis stage (Fig. 2). The low yield of the X-II fraction may be also explained on the basis of its low stability in

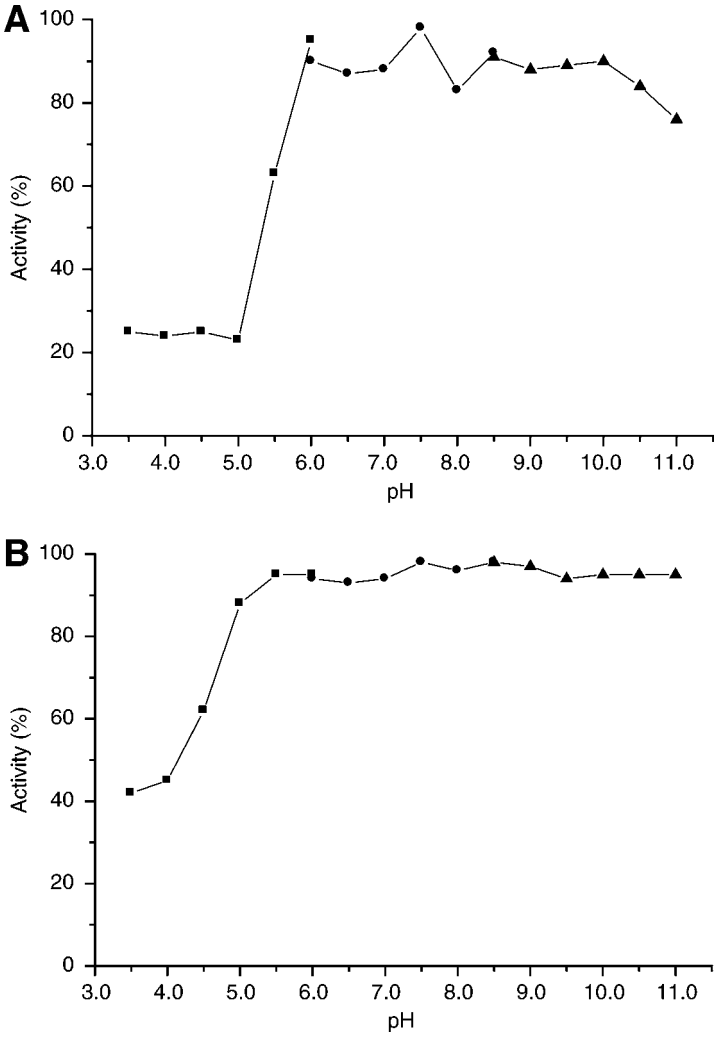

Fig. 1. Effects of pH on the stability of Xylanases from *B. licheniformis* **(A)** X-I and **(B)** X-II. The enzymes were incubated for 24 h at room temperature in the following 0.1 *M* buffer system: sodium acetate (pH 3.0–5.5); citrate-sodium phosphate (pH 5.5–7.0); Tris-HCl (pH 7.0–8.0) and Gly-NaOH (pH 8.5–10.0). The remaining activity was assayed at 60°C for 10 min at pH 6.0. A 100% activity was 2.1 U/mL for X-I and 6.6 U/mL for X-II.

the pH 5.0 buffer used for enzyme elution (Fig. 1B) and owing to the number steps used for complete purification of the enzyme. Generally, the recovery of proteins is inversely proportional to the number steps used to accomplish the purification procedure. Another reason for the low yields could be owing to fact that X-II showed evidence of glycosylation in results obtained on zymogram stained with Schiff reagent and in another separate experiment in which the xylanase bound to concanavalin A resin

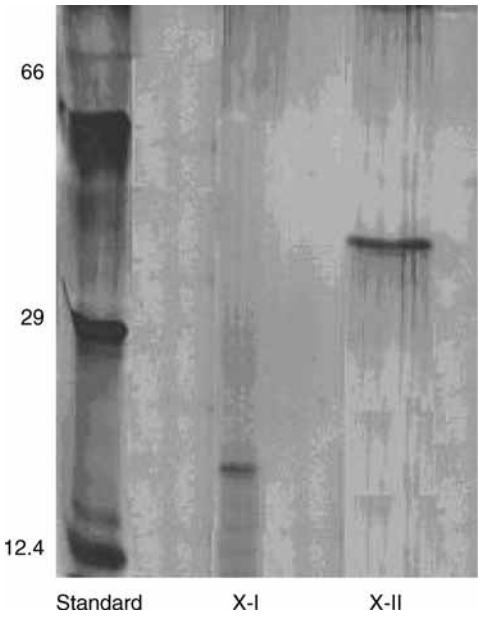

Fig. 2. SDS-PAGE of purified xylanases from *B. licheniformis*: The standards were β-amylase bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa).

(data not shown). Glycoproteins display interactions with many chromatography gels *(12)*, which may result in lower recuperation. In this work, many modifications of the mobile phase were attempted, such as the use of high pH, decreased polarity and denatured condition, and so on, to reduce these interactions. In both xylanase purification procedures, no problems like protein precipitation were observed.

Determination of the Molecular Mass

The determination of the molecular mass in gel filtration is not an efficient methodology for many xylanases of the *Bacillus* spp., owing to their interaction with the resin. In such cases, SDS-PAGE is more suitable *(12)*. X-I and X-II fractions and standard proteins (bovine serum albumin, carbonic anhydrase, and cytochrome C) were applied to the 10% SDS-PAGE and proteins were stained with silver. Then, gel was dried between two sheets of cellophane. The molecular masses of the xylanases were determined (not digitized) by plotting the relative mobility (R_f value) of each protein against the log of its molecular weight using the standard protein (Fig. 2). X-I presented a molecular mass of 17 kDa and X-II presented a molecular mass of 40 kDa.

The xylanase from *B. amyloliquefaciens* displayed a molecular mass of 18 kDa *(12)*, which is very close to the value of X-I, and *Bacillus* TAR-1 showed a 40 kDa xylanase *(13)*, which was the same value presented by X-II. The molecular mass of xylanase from *B. licheniformis* A99 was 45 kDa.

Purified xylanases have been reported in the range of 5.5–85 kDa *(7)*. Xylanases with low molecular mass are appropriate for the pulp and paper industry, because the size of xylanases is directly related to their accessibility to the fiber pore and the consequent removal of lignin *(14)*.

Determination of the Kinetic Parameters

The kinetic study was carried out at 60°C for 10 min with different xylan concentrations (Birchwood, Sigma, St. Louis, MO). The results were analyzed according to the graphical procedure proposed by Lineweaver and Burk (data not shown). V_{max} was 7.05 and 9.1 U/mg of protein and K_m was 1.8 and 1.05 mg/mL, for the enzymes X-I and X-II, respectively. These values of K_m are in agreement with the reported range of K_m for xylanases *(15)*, such as that of *Bacillus stearothermophilus* T-6 which produced a xylanase with a K_m of 1.63 mg/mL *(16)*. These values are smaller than the K_m of 4.5 and 3.3 mg/mL of the xylanases from *B. amyloliquefaciens (15)* and *B. licheniformis (7)*, respectively, and this difference is even greater when in comparison with the K_m of 10.14 mg/mL of the xylanase from the mushroom *Trichoderma longibrachiatum (17)*.

Effect of pH on Activity and Stability of Xylanases

X-I presented maximum activity at pH 7.0, retaining about 80% of its activity at pH 8.0 and about 50% of its activity at pH 9.0 (Fig. 3A). Nath and Rao *(18)*, and Archana and Satyanarayana *(7)* describe the enzymatic activity of Xyl II from *Bacillus* spp. (NCIM 59) and xylanase from *B. licheniformis* A99, respectively, with maximum activity in the pH range of 6.0–7.0. The high pH optimum of xylanase is a good property, as this is required for industrial pulping processes. The enzyme X-II presented an unusual pH-dependence behavior in the determination of its optimum pH (Fig. 3B). The bell-shaped curve was not observed, though a large range of optimum pH was observed, from pH above 7.0 until pH 11.0; the upper limit used in this paper. Both xylanases were very stable for 24 h over a large range of pH. X-I presented an average of about 90% stability from pH 6.0 to 10.0, and at pH 11.0 it still retained about 75% of its enzymatic activity. The enzyme X-II was similarly stable in a range from pH 5.5 to 11.0 (Fig. 1A,B). These results are also interesting for the application of these enzymes in the paper industry.

Effect of Temperature on Activity and Stability of Xylanases

The two enzymes presented optimal activities at 70–75°C. X-I presented 50% activity at 90°C (Fig. 4A), and X-II presented 80% activity at the same temperature (Fig. 4B). The xylanases of *Bacillus* spp. and *B. licheniformis* A99 showed a maximum activity at 55°C and 60°C respectively. *B. amiloliquefaciens* produced a xylanase with an optimum temperature of 80°C and retained 90% of its activity at 90°C *(11)*. High temperatures of action for xylanases are also quite exciting from the point of view of their industrial application.

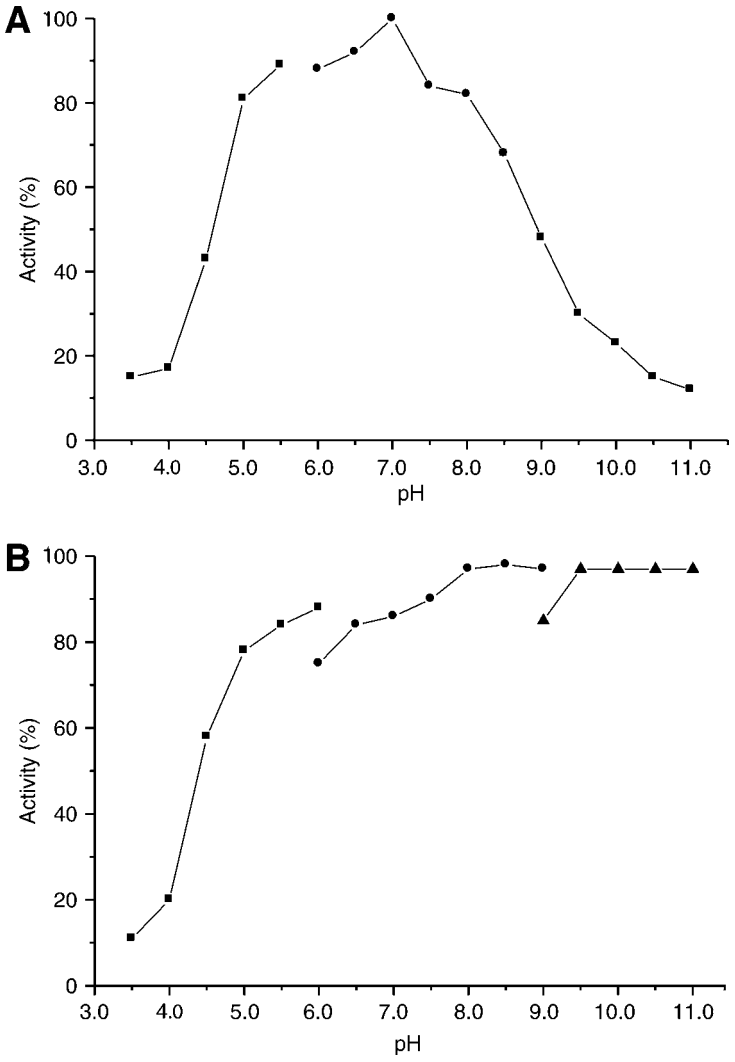

Fig. 3. Effects of pH on xylanases from *B. licheniformis* **(A)** X-I and **(B)** X-II. The effect of pH on xylanases was measured at 60°C for 10 min using Birchwood xylan as substrate in the following 0.1 *M* buffer system: sodium acetate (pH 3.0–5.5); citrate-sodium phosphate (pH 5.5–7.0); Tris-HCl (pH 7.0–8.5) and Gly-NaOH (pH 8.5–10.0). The 100% activity was 7 U/mL for X-I and 3.6 U/mL for X-II.

Both xylanases were 100% stable after one hour at 40°C. At 55°C X-I retained about 40% of its activity after 1 h of treatment, and that percentile remained up to 90°C (Fig. 5A). The enzyme X-II retained about 85% of its activity for temperatures up to 65°C. Approx 30% of the original activity was retained between 75 and 90°C (Fig. 5B).

Identification of the Hydrolysis Products

The hydrolysis products released from Birchwood (Sigma) by *B. licheniformis* X-I and X-II were analyzed by paper chromatography (Fig. 6).

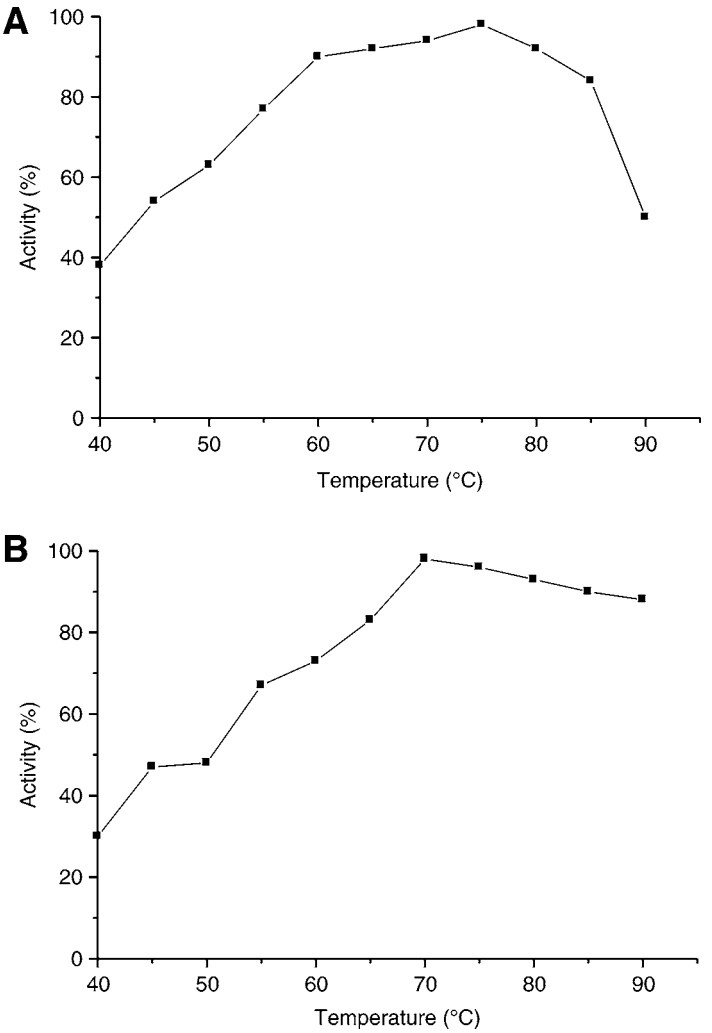

Fig. 4. Effects of temperature on xylanases from *B. licheniformis* **(A)** X-I and **(B)** X-II. Activity was measured at various temperatures for 10 min using Birchwood xylan as substrate in 0.1 *M* sodium acetate, pH 5.0.

X-I released mainly tetraose and larger xylooligosaccharides. In contrast, X-II liberated mainly xylotetraose, xylotriose, and xylobiose, whereas xylose was observed in smaller amounts. According to Biely et al. *(3)*, enzymes with larger substrate binding sites hydrolyze a small extension of the xylan generating larger products. The results obtained with X-I xylanase are in accordance with this description *(19)*.

Summary of the Characterization

Table 2 displays the characterization of the xylanases produced by *B. licheniformis* 77-2. X-I presented a molecular mass and hydrolysis products quite different from X-II, besides presenting smaller range of temperature

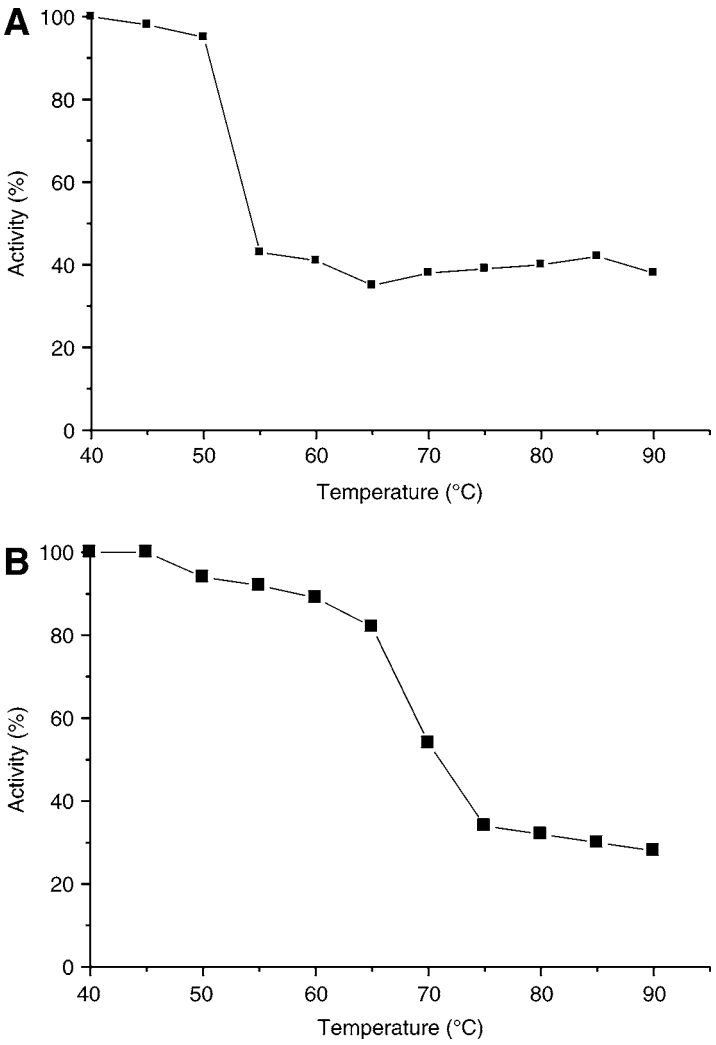

Fig. 5. Thermal stability of xylanases from *B. licheniformis*. **(A)** X-I and **(B)** X-II. The enzyme was maintained for 1 h at temperatures from 20°C to 90°C. The remaining activity was measured under standard assay conditions.

stability. *B. licheniformis* 77-2 studied here produces two different xylanase isoforms. The occurrence of more than one xylanase in *Bacillus* spp is quite common *(7)*. These enzymes can be the products of distinct genes *(20)* or they can be secreted by the same gene, but undergo different posttranslational modifications, such as limited proteolysis and/or glycosylation *(7,21)*.

X-I presented a low molecular mass in comparison with X-II, which can be an indication that the latter might have suffered differentiated glycosylation. The glycosylation of the isoenzyme X-II is reinforced by its greater stability at higher temperatures, when in comparison with X-I. Glycosylation has been reported as having an important role in stabilizing protein structure *(22–29)*. The behavior of the xylanases concerning pH

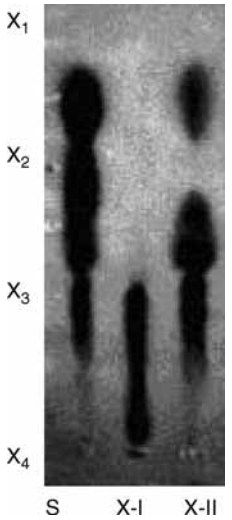

Fig. 6. Hydrolysis products of Birchwood by xylanases from *B. licheniformis* Standard (s): X_1 = xylose; X_2 = xylobiose; X_3 = xylotriose; X_4 = xylotetraose.

Table 2
Summary of the Characterization of Xylanases produced by *B. licheniformis* 77-2

	X-I	X-II
Molecular weight (kDa)	17	40
K_m (mg/mL)	1.8	1.05
V_{max} (U/mg)	7.05	9.1
Optimum pH	7.0	5.5–11.0
pH of stability	6.0–11.0	5.5–11.0
Optimum temperature (°C)	70–75	70–75
Temperature of stability (°C)	50	65
Products of hydrolyzation	$>X_4$ e X_4	X_4, X_3, X_2 e X_1

was also quite different, presenting a typical pH curve (bell-shaped), with optimum activity at pH 7.0. The atypical results found when analyzing the optimum pH of X-II may also be explained by glycosylation, because in the same way that groups of sugars linked to the protein contribute to the increased thermostability of the enzyme, they can also contribute to stabilizing the enzyme regarding changes in pH, which would explain its activity in the large range of alkaline pH. This is in agreement with Hamada et al. *(27)*, who reported the stabilizing effect of glycosylation on the pH stability of cellulase (Ex-2). Furthermore, Wang et al. *(25)* demonstrated that, in addition to the apparent stabilizing effect, glycosylation may protect the enzyme against aggregation during heat treatment under acidic condition. Both xylanases acted as endoxylanases, but the hydrolysis products

of xylan for X-I were quite different from that produced by X-II, indicating that these enzymes act differently on the xylan molecule.

Acknowledgment

The authors are grateful to the FAPESP and CNPq for their financial support.

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