Appl Biochem Biotechnol (2009) 158:200–212
DOI 10.1007/s12010-008-8317-x

Highly Thermostable Xylanase of the Thermophilic Fungus *Talaromyces thermophilus*: Purification and Characterization

Ines Maalej · Ines Belhaj · Najla Fourati Masmoudi · Hafedh Belghith

Received: 2 May 2008 / Accepted: 2 July 2008 /
Published online: 31 July 2008

Abstract A thermostable xylanase from a newly isolated thermophilic fungus *Talaromyces thermophilus* was purified and characterized. The enzyme was purified to homogeneity by ammonium sulfate precipitation, diethylaminoethyl cellulose anion exchange chromatography, P-100 gel filtration, and Mono Q chromatography with a 23-fold increase in specific activity and 17.5% recovery. The molecular weight of the xylanase was estimated to be 25kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and gel filtration. The enzyme was highly active over a wide range of pH from 4.0 to 10.0. The relative activities at pH5.0, 9.0, and 10.0 were about 80%, 85.0%, and 60% of that at pH7.5, respectively. The optimum temperature of the purified enzyme was 75°C. The enzyme showed high thermal stability at 50°C (7days) and the half-life of the xylanase at 100°C was 60min. The enzyme was free from cellulase activity. K_m and V_{max} values at 50°C of the purified enzyme for birchwood xylan were 22.51mg/ml and 1.235μmol min^{-1} mg^{-1}, respectively. The enzyme was activated by Ag^{+}, Co^{2+}, and Cu^{2+}; on the other hand, Hg^{2+}, Ba^{2+}, and Mn^{2+} inhibited the enzyme. The present study is among the first works to examine and describe a secreted, cellulase-free, and highly thermostable xylanase from the *T. thermophilus* fungus whose application as a pre-bleaching aid is of apparent importance for pulp and paper industries.

Keywords Xylanase · Fungus · *Talaromyces thermophilus* · Thermostability

Introduction

Xylan is the second most abundant biopolymer in plant cell walls after cellulose and the major hemicellulosic polysaccharide. It consists of a backbone of β-(1→4)-linked D-

I. Maalej · I. Belhaj · N. F. Masmoudi · H. Belghith (✉)
Laboratoire de Génétique Moléculaire des Eucaryotes, Centre de Biotechnologie de Sfax,
PB "K" 3038, Sfax, Tunisia
e-mail: hafeth.belghith@cbs.rnrt.tn

xylopyranosyl units substituted with *O*-acetyl, arabinose, ferulic acid, *p*-coumaric acid, and uronic acid moieties [1, 2].

The hydrolysis of the xylan backbone requires at least two enzymes, namely, an endo-β-1,4-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37). Enzymes such as α-L-arabinofuranosidase (EC 3.2.1.39), acetyl xylan esterase (EC 3.1.1.72), and ferulic or *p*-coumaric acid esterase (EC 3.2.1.73) are needed for debranching [3]. Since their discovery, xylanases have generated considerable research interest. This is partly because of their promising biotechnological applications [4, 5], the provision of a carbon source for industrial fermentations [6], the improvement of the nutritional quality and digestibility of ruminant fodder, and the facilitation of the composting process [7]. Due to their outstanding properties, xylanolytic enzymes have found use in various industrial fields, namely, in the baking industry [8] and the pulp and paper industries [9].

A wide variety of microorganisms are known to produce xylan-degrading enzymes. Among these microorganisms, thermophilic fungi have attracted growing attention because of their exceptional potential as sources of thermostable xylanases. They have been reported to be ideal candidates for industrial application in various fields. They have found application in the preparation of paper pulps, in the processing of agro-food raw materials, and in the enzymatic conversion of hemicellulose in biomass into useful chemicals. Some thermophilic fungi produce multiple forms of xylanases that differ in molecular size, stability, adsorption, or activity on insoluble substrates [10]. For commercial applications, xylanase should ideally be produced at high-level using cheap substrates derived from lignocellulosic materials [11, 12]. Although the production of xylanases by a few thermophilic or thermotolerant fungi, e.g., *Termitomyces aurantiacus* and *Talaromyces emersonii* has been reported [13, 10], these xylanases were not reported to be cellulase-free. On the other hand, *Talaromyces lanuginosus* and *Paecilomyces thermophila J18* are fungi that produce a cellulase-free xylanase [14, 15]. Xylanases produced by thermophilic fungi are usually more thermostable than those of mesophilic fungi [11, 16]. Xylanase properties have been studied in many fungal species [11, 17, 18], but only few reports are available on the properties of xylanase from thermophilic fungi strains [19, 20].

This study reports, for the first time, on the purification and characterization of a secreted, thermostable, and cellulase-free xylanase from a newly isolated thermophilic fungus *Talaromyces thermophilus* using physico-chemical techniques, namely, diethylaminoethyl (DEAE) cellulose, gel filtration, and Mono Q ion exchange chromatography.

Materials and Methods

Materials

Commercial birchwood xylan, beech wood xylan, oat spelt xylan, RBB xylan, PNP xyloside, and DEAE cellulose were purchased from Sigma. DEAE cellulose was from Pharmacia. The lignocellulosic materials, namely, wheat bran and rabbit food, were obtained locally. The protein assay kit was obtained from Bio-Rad Laboratories.

Fungal Strain

Talaromyces thermophilus Stolk, a xylanase producer, was isolated from the soil samples of El Hamma thermal station in the south of Tunisia and was identified as a thermophile strain by CBS (Centraalbureau voor Schimmelcultures, Baarn, Netherlands).

Methods

Screening Medium and Method

The basal medium used for the screening of thermophilic fungi producing xylanase was a slightly modified Mandels medium [21]. The basal medium used was KH_2PO_4 1g, K_2HPO_4 2.5g, $(NH4)_2SO_4$ 1.4g, $MgSO_4{\cdot}7H_2O$ 0.3g, $CaCl_2$ 0.3g, yeast extract 1g, urea 0.7g, and Tween 80 1ml. To 1l of this medium, 1ml of an oligoelements solution ($MnSO_4$, 1.6g/l, $ZnSO_4$, 1,4g/l, $FeSO_4$, 5g/l, $CoCl_2$, 2g/l) and 18g agar–agar (Fluka) were added. After sterilization, antibiotic (ampicillin 50μg/ml and tetracycline 20μg/ml) were added. The medium was inoculated by the soil samples that were previously suspended in 10ml of sterile water. Finally, all inoculated media were incubated at 50°C for 5days.

This fungus was conserved in conidia form in glycerol 20% at −80°C.

Enzyme Production

For xylanase production, *T. thermophilus* Stolk was cultivated in a liquid medium (Mandels medium) [21] with some modifications as follows: KH_2PO_4 1g, K_2HPO_4 2.5g, $(NH_4)_2SO_4$ 1.4g, $MgSO_4{\cdot}7H_2O$ 0.3g, $CaCl_2$ 0.3g, yeast extract 1g, urea 0.7g, Tween 80 1ml, water 1l, and 2% xylan oat spelt or 2% wheat bran. The pH of the medium was 7.0. The medium was supplemented with 1ml of an oligoelements solution: $MnSO_4$ 1.6g/l, $ZnSO_4$ 1.4g/l, $FeSO_4$ 5g/l, and $CoCl_2$ 2g/l. The enzyme's production was carried out in 500-ml flasks, each containing 100ml of culture medium and was incubated for 5days at 50°C at an agitation rate of 160rpm. The initial pH was adjusted to the desired level prior to sterilization at 121° C for 20min. After sterilization, appropriate antibiotics (ampicillin 50μg/ml and tetracycline 50μg/ml) were added. The flasks were then inoculated with 10^6 spores and incubated for 6days at 50°C on a rotary shaker (160rpm). The culture broth was centrifuged at 5,000×*g* for 15min to obtain clear supernatant used as a starting material for enzyme purification.

Enzyme Purification

The purification of the xylanase was carried out in three steps. The first step involved ammonium sulfate precipitation (80% saturation) of 1,000ml cell-free supernatant. The saturated solution was left overnight at 4°C and then centrifuged, and the precipitates were dissolved in the minimum volume of 20mM phosphate buffer (pH7). The dialyzed fraction of concentrated proteins (20ml) was then loaded on to the anion exchange DEAE cellulose column (2.5 × 30cm) which was pre-equilibrated with potassium phosphate buffer. The column was operated at a flow rate of 25ml/h. A continuous NaCl gradient (0–1M) was applied and fractions (3ml each) collected. The fractions showing xylanase activity were then pooled and dialyzed at 4°C. The dialyzed enzyme preparation was loaded onto a Bio-Gel P-100 column (2.5 × 80cm) previously equilibrated with the same buffer at a flow rate of 15.0ml/h. The active fractions were pooled, dialyzed against distilled water, and then concentrated by freeze drying. In each step, the protein content and xylanase activity were determined.

Enzyme Assay

Xylanase activity was determined by measuring the release of reducing sugars from birchwood xylan (1% *w*/*v*) by dinitrosalicylic acid method [22]. One unit of xylanase was defined as the amount of enzyme required to release 1μmol of xylose from birchwood xylan

in 1min under standard assay conditions (50°C, 50mM phosphate buffer, pH7.0). The enzyme activity of β-xylosidase was determined by measuring pNP released from pNPX at 50°C [23]. The reaction mixture, consisting of 5mM pNPX in 50mM phosphate buffer (pH7.0), was incubated for 10min in a total volume of 0.25ml. The reaction was stopped by the addition of 0.75ml of 2M Na_2CO_3, and the amount pNP released was determined by measuring the absorbance at 410nm. One unit of β-xylosidase was defined as the amount of enzyme required to release 1μmol of pNP per minute under the conditions described above. The protein concentration was determined by Bradford method [24] using bovine serum albumin as a standard. Absorption at 280nm was used to monitor proteins in column effluents.

Determination of Molecular Mass

The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate and native–native polyacrylamide gel electrophoresis and by gel filtration on a Bio-Gel P-100 column (2.5 × 80cm) using standard protein markers: 1, Dextran Blue; 2, Catalase; 3, yellow colorant; 4, red colorant; X, xylanase activity observed. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10% gel) was performed as described by Lamely [25] using low-molecular-weight (15–100kDa) electrophoresis standards. Staining of protein bands was done with Coomassie brilliant blue R-250 (Bio-Rad). After electrophoresis, xylanase activity was monitored by a zymogram gel containing 0.7% agarose, RBB xylan 0.1%, or xylan 0.2% and applied for 15min at 50°C

Determination of Temperature and pH Optima and Stability

The optimum temperature of xylanase activity was determined by carrying out the purified xylanase at selected temperatures from 30 to 100°C. In each case, the substrate was pre-incubated at the required temperature before the addition of the enzyme. In order to monitor thermal stability, buffered samples of purified enzyme were incubated for fixed time period at 40, 50, 60, 70, 80, 90, and 100°C (50mM phosphate buffer, pH7.0). Aliquots were withdrawn at timed intervals and cooled on ice before assaying to determine the residual enzyme activity using the normal assay procedure. The influence of pH on xylanase activity was determined by incubating the purified enzymes with the substrates at pH ranging between 3.0 and 10.00 using three buffers: (50mM) sodium acetate buffer at pH3–6, phosphate buffer for pH6–8, and Ampso buffer at pH8–10. In order to determine the enzyme pH stability, the purified xylanase was incubated in different buffers as mentioned above at 4°C for 24h, and then the residual activities of the treated enzymes were measured by the standard assay procedure.

Hydrolysis of Xylan by the Purified Xylanase

The hydrolysis of oat spelt xylan (10g/l) was carried out in 1ml of 50mM phosphate buffer (pH7.0) with the appropriately diluted purified xylanase enzyme at 50°C. Products of enzymatic hydrolyses were analyzed by thin-layer chromatography on silica gel G-60 using chloroform/acetic acid/water (6:7:1 by volume) as a mobile phase system. Sugars were detected with 0.2% (*w*/*v*) orcinol in sulfuric acid/methanol (10:90).

Effect of Metal Ions and Enzyme Inhibitors on Xylanase Activity

The effects of metal ions and chemical reagents on the enzyme were examined by incubating 1.0ml of enzymes solutions with each of the metal ions and chemicals reagents

at a concentration of 10mM in 50mM potassium phosphate buffer, pH7.0 with 1% oat spelt xylan as substrate at 50°C for 10min. The remaining activity of the enzyme was measured under the standard conditions described above.

Kinetic Parameters

The effect of oat spelt xylan concentration, ranging from 0.4 to 25mg/ml, on xylanase activity was evaluated under optimal assay conditions. The kinetic parameters (Michaelis–Menten constant, K_m and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots according to Lineweaver and Burk [26].

Results and Discussion

Xylanase Production by *T. thermophilus*

Xylanase properties were studied in many fungal species [2, 17]. Reports on the properties of xylanase from thermophilic fungus strains, however, are very scarce in the literature [19, 20]. The present study is a first step in throwing empirical light on this concern. It aims to purify and characterize the extracellular xylanase from a newly thermophilic fungus *T. thermophilus* isolated from a soil sample in Tunisia. The production of xylanase by *T. thermophilus* was studied in shacked flask cultures at 50°C and 160rpm using oat spelt xylan 2%, wheat bran 2%, rabbit food 2%, and xylose 5% with lactose 5% as a carbon source. Figure 1 shows the xylanase activities obtained after 5days of shacked culture. The highest titer of xylanase (5.5IU ml^{-1}) was obtained on oat spelt xylan, whereas the highest β-xylosidase activity in the culture filtrates 0.25IU ml^{-1} was obtained on wheat bran 2% (data not shown).

These data evidently show that *T. thermophilus* is producing extracellular xylanase and β-xylosidase activities. They also suggest that these latter could behave as inducible enzymes, as they were produced in great quantities in the presence of xylan or wheat bran.

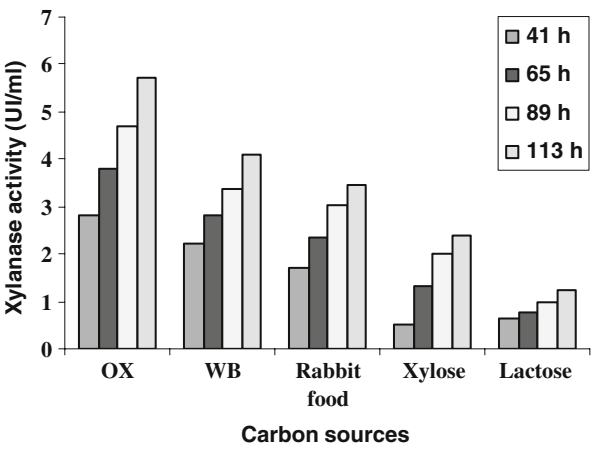

Fig. 1 Production of xylanase by *T. thermophilus* using different lignocellulosic materials as carbon sources. Oat spelt xylan (*OX*) 2%; wheat bran (*WB*) 3%; rabbit food 3%; xylose 2%; lactose 2%. Shake culture at 50 °C and 160 rpm

Purification of Xylanase from *T. thermophilus*

All steps of purification were conducted at room temperature unless otherwise specified. The culture fluid (1.5l) was centrifuged at 6,000×*g* for 15min, and 1l of the resulting clear supernatant (2,320UI) was adjusted to 80% saturation by adding solid ammonium sulfate. Precipitates were collected by centrifugation at 10,000×*g* for 25min and dialyzed against the 0.02M phosphate buffer, pH7.0. Insoluble precipitates were removed by centrifugation, and the supernatant resulted in an active starting material for further purification. This step resulted in 48% recovery and 1.15-fold purification. The enzyme was loaded on a DEAE cellulose column (1.25 × 28cm) equilibrated with 0.02M phosphate buffer, pH7.0. The elution pattern of the DEAE cellulose column is shown in Fig. 2. Two peaks were observed and eluted at 0.3M NaCl for β-xylosidase activity and at 0.6M NaCl for xylanase activity. Active fractions showing xylanase activity were pooled, concentrated with PEG, and dialyzed against 0.02M phosphate buffer, pH7.0. The xylanase fraction from step 1 was then chromatographed on Bio-Gel P100 column (1.25 × 80cm) equilibrated with 0.02M phosphate buffer, pH7.0. Elution was done at a rate of 30ml/h with the same buffer, and active fractions were pooled, concentrated, and dialyzed against 0.02M phosphate buffer, pH7.0.

The results of gel filtration and SDS-PAGE (Fig. 5a) show that this xylanase has a low molecular weight of 25kDa, which is within the MW range of xylanases belonging to the family 11 [27]. However, the molecular mass of previously reported xylanases vary, most of which are between 40 and 100kDa due to glycosylation. The apparent molecular mass of the xylanase from *T. thermophilus* is similar to the xylanase from *Paecilomyces varioti* [20] and is higher than the 20.0-kDa xylanase from *P. varioti* IMD RK 032 [20]. A xylanase of a low molecular mass can be highly useful in pulp bleaching, since smaller enzymes can access the fiber wall structure more readily and alter the pulp properties more efficiently [12].

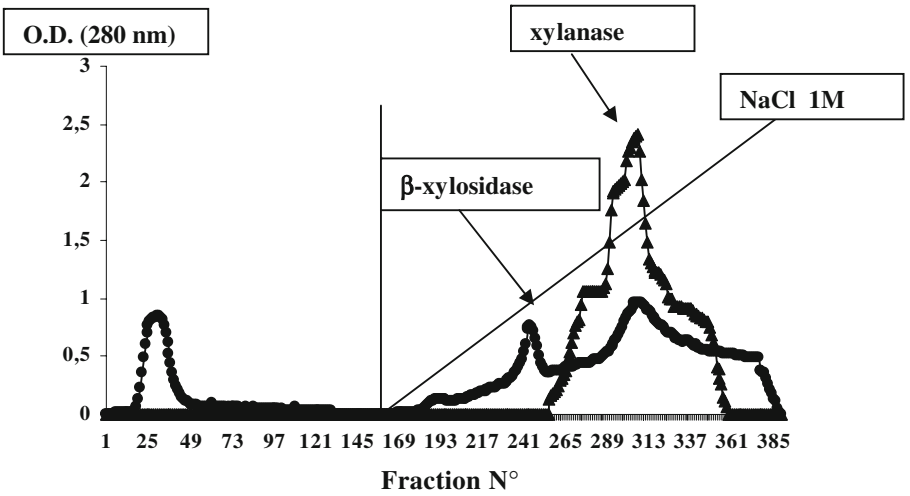

Fig. 2 Separation of proteins by DEAE cellulose chromatography. Protein pellet from ammonium sulfate precipitation was suspended in buffer, desalted, loaded on DEAE cellulose column, and then eluted with a linear gradient of NaCl in phosphate buffer. Absorbance at 280 nm was monitored continuously. Fraction (4 ml) was collected and assayed for xylanase activity (*filled triangle*); absorbance at 280 nm (*filled diamond*) and (*solid line*) sodium chloride gradient

Table 1 Purification steps of extracellular xylanase from *T. thermophilus*.

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture cupernatant	2,320	3,342	6.94	100	1
Ammonium sulfate	1,127	140	8	48	1.15
DEAE cellulose	630	16.8	37.5	27.1	5.4
Bio-Gel P-100	403.6	6.37	63.4	17.4	9.13
Q-Sepharose	400	2.5	160	17.2	23

The xylanase fraction from step 2 was then chromatographed on Mono Q-sepharose at pH8.6 using a linear gradient of 0–1M NaCl. During chromatography, a major fraction was separated. At this stage, the enzyme had a specific activity of 160U/mg on oat spelt xylan. The enzyme was then purified 23 folds and was found to contain 17.2% of the initial crude enzyme activity. The results of the purification steps are summarized in Table 1.

The purification procedure steps provided a homogeneous preparation of xylanase as determined by SDS-PAGE. The enzyme migrated as a single 25-kDa band on SDS-PAGE-stained with Coomassi blue (Fig. 3). After electrophoresis, xylanase activity was revealed by a zymogram gel containing 0.7% agarose, RBB xylan 0.1%, or xylan 0.2% (Fig. 4).

Most previously studied xylanases are reported to show both xylanase and low cellulase activities [4]. For more detailed information on, for example, xylanase from *P. varioti* Bainier and *T. lanuginosus* strains, see [17, 20]. Interestingly, our purified xylanase hydrolyzed only xylan and was free from all other examined enzyme activities including those of carboxymethyl cellulase, β-xylosidase, and β-glucosidase (Table 2). This result can indicate that the xylanase of *T. thermophilus* Stolk belongs to the glycosylic family G11; the xylanases of this family have no activity on cellulose and are the low-molecular-weight enzymes. This property of xylanases is very interesting for the industrial applications such as the bleaching of paper.

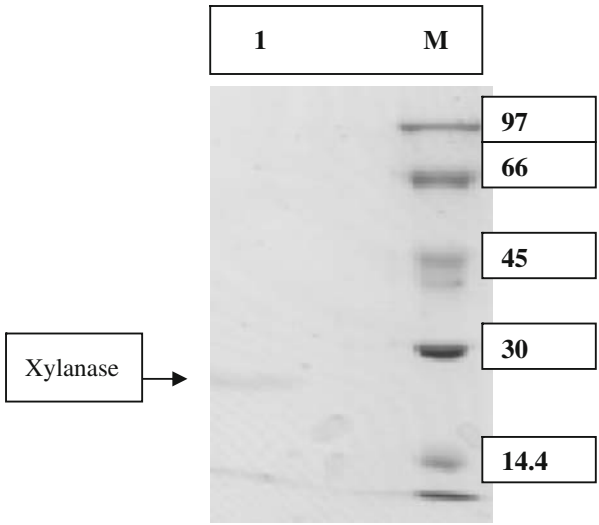

Fig. 3 SDS-polyacrylamide gel of Mono Q-purified xylanase from *T. thermophilus*. *Lane M* low-molecular-weight calibration kit; *lane 1* purified xylanase

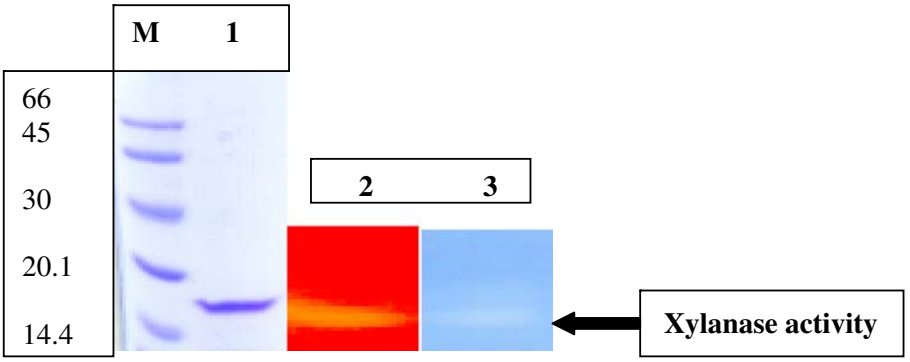

Fig. 4 Zymogram of the purified xylanase from *T. thermophilus*. *Lane M* low-molecular-weight calibration kit; *lane 1* purified xylanase; *2* substrate RBBX; *3* xylane colored with Congo red

Effect of pH on Xylanase Activity and Stability

Most of the so far reported xylanases had an optimum pH between 5.0 and 7.0 [9]. The purified xylanase from *T. thermophilus* had an optimal pH around 7.0–8.0 (Fig. 5). Interestingly, according to the pH stability data, the enzyme retained more than 80% of its activity after being incubated for 24h at different pH values ranging between 4.8 and 9.2 (Fig. 6). The residual activity was almost 40% at pH3 and pH11. These data are in line with the previously reported xylanases isolated from some thermophilic fungi, such as *T. lanuginosus* and *T. emersonii* strains which were stable over a wide pH range [10, 17].

Effect of Temperature on Enzyme Activity and Thermostability

The optimum temperature of the purified enzyme was found to be 75–80°C (Fig. 7) compared to the 50°C observed with *P. varioti IMD RK 032* [19]. The optimal temperature of the purified xylanase was similar to those of the xylanases from *P. thermophila J18* and *T. emersonii* [10, 18]. These xylanases are usually more thermostable than those of mesophilic fungi [11, 16]. The xylanase in the present investigation retained 100% of its activity after incubation at 50°C for 7days and 50% of its activity after 1h incubation at 100°C (Fig. 8). The thermal stability of this xylanase was higher than that of the *T. aurantiacus* xylanase [28]. Our enzyme, therefore, exhibits a number of highly appealing and promising features that make it a strong candidate for future industrial applications.

Hydrolysis of Xylane by the Purified Xylanase

The hydrolysis of birchwood xylan catalyzed by the purified xylanase was followed by an analysis of the reaction products using thin-layer chromatography (TLC; Fig. 9). The

Table 2 Effect of potential inhibitors and activators on *T. thermophilus* xylanase activity.

Substrates (2%)	Crude enzyme (UI/ml)	Purified enzyme (UI/ml)
Cellulose	0	0
CMC	0	0
Xylan oat spelt	6.8	5.4
PNP-xylopyranose	0.5	0

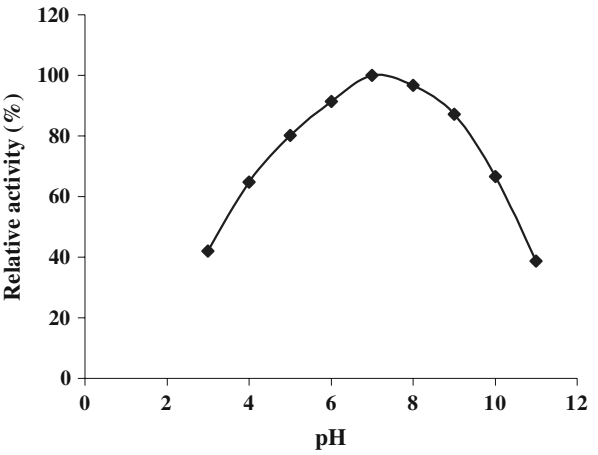

Fig. 5 Effect of pH on the purified *T. thermophilus* xylanase; values were triplicates at each pH

products contained only xylo-oligosaccharides, and no xylose was detected. This pattern of hydrolysis classified the *T. thermophilus* xylanase as an endoenzyme (β-1, 4-xylan xylanohydrolase).

Kinetic Study and Additives Effect

The activity of *T. thermophilus* xylanase was determined. The Michaelis constant K_m for xylan at 50°C was 22.51mg/ml of xylan, and the maximal velocity V_{max} was 1.235μmol min^{-1} mg^{-1}. The effect of potential inhibitors or activators on the purified xylanase is shown in Table 3. Xylanase activity of *T. thermophilus* was affected by $HgCl_2$ (Hg^{2+}), which is a strong inhibitor of most xylanases including those from *T.*

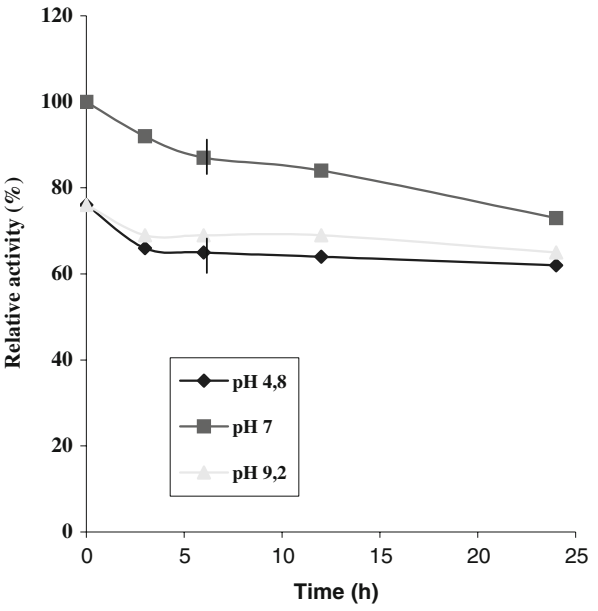

Fig. 6 pH stability of the xylanase. Purified enzyme was kept in buffer at 4 °C for 24 h, and the residual activity was measured under standard assay conditions

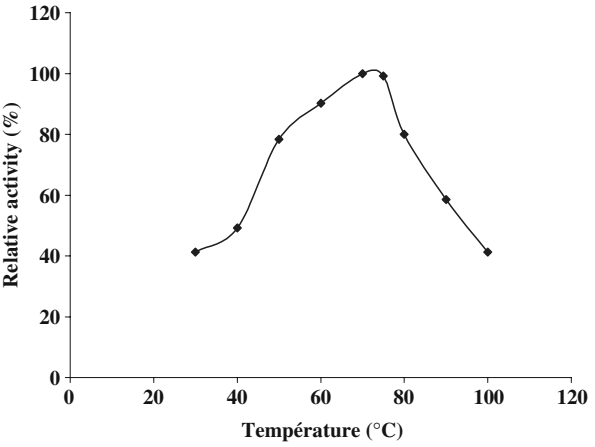

Fig. 7 Optimum temperature of *T. thermophilus* xylanase activity

lanuginosus [29, 30]. While Hg^{2+}, Ba^{2+}, and Mn^{2+} ions at a final concentration of 10mM had a strong inhibitory effect on the enzyme activity, the ions Zn^{2+}, Fe^{2+}, and Mg^{2+} had a minor negative effect (Table 3). The ions Cu^{2+}, Co^{2+}, and Ag^{+} also stimulated xylan hydrolysis (Table 3).

T. thermophilus xylanase was resistant to 10mM SDS and retained almost all its activity. However, ethylenediaminetetraacetic acid (EDTA), a chelating reagent, and dithiothreitol (DTT), a disulfide-reducing agent, were found to inhibit the enzyme activity, suggesting that disulfide bonds are essential to maintain the active conformation of the enzyme.

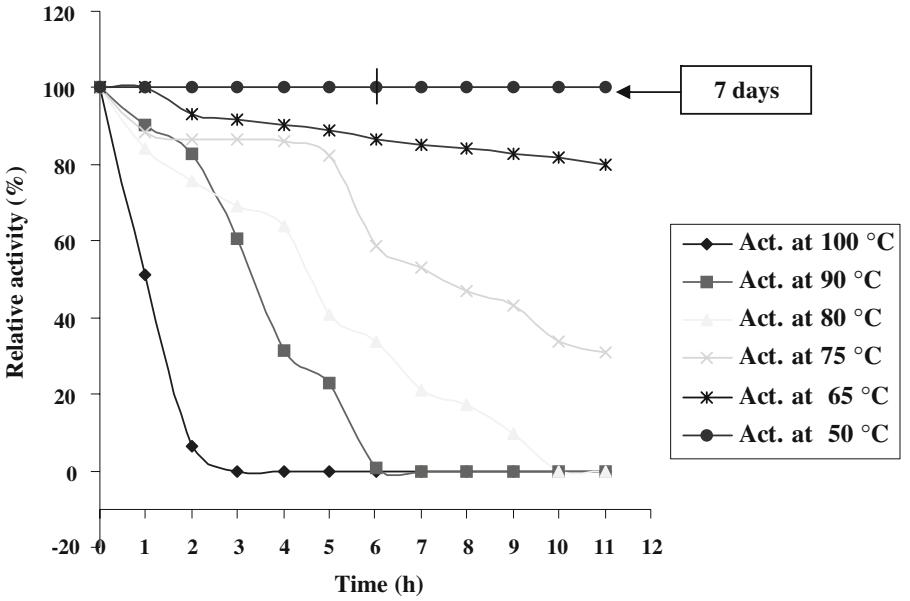

Fig. 8 Thermal stability. Effect of temperature on the stability of the purified xylanase: Pure samples diluted in 50 mM phosphate buffer (pH 7) were heated at the relevant temperature for different period of time, placed on ice for 30 min, and residual xylanase activity determined using the standard assay procedure at 50 °C

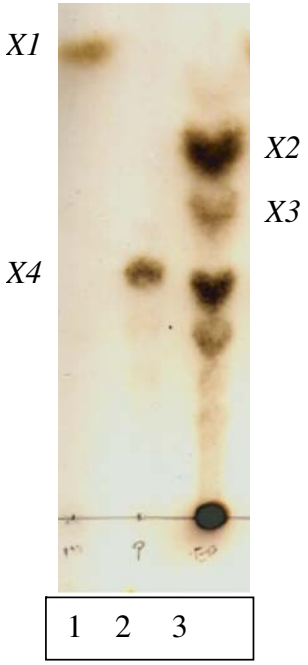

Fig. 9 TLC analysis of xylan hydrolysis by the purified xylanase (*1* xylose; *2* xylotetraose; *3* birchwood xylan)

Conclusion

The purification and characterization of a xylane-degrading enzyme from *T. thermophilus* Stolk fungi indicate that this enzyme displays a number of biochemical properties that make it a potentially strong candidate for industrial and commercial application in pulp bleaching. Of particular interest is the fact that it is a cellulase-free xylanase. It is also an enzyme which has a thermal stability and a low molecular weight when compared to the currently used commercial fungal xylanases. These features actually provide a strong stimulation for

Table 3 Effect of potential inhibitors or activators on the purified xylanase.

Substances at 10 mM	Relative xylanase activity (%)
Non	100
$AgNO_3$	125
$HgCl_2$	65
$BaCl_2$	65
$CoCl_2$	112
$CuSO_4$	105
$MgSO_4$	80
$FeSO_4$	80
$ZnSO_4$	80
$MnSO_4$	65
EDTA	90
DTT	85
SDS	100

further research on the structure of the purified xylanase so as to establish a method of xylanase immobilization to produce xylo-oligosaccharides.

Acknowledgments This work has been supported by grants from the Tunisian government's "Contrat-Programme" through the "Ministère de la Recherche Scientifique, de la Technologie et du Développement des Compétences" of Tunisia. The authors wish to express their sincere gratitude to Prof. Ali Gargouri (Head of the Laboratory of Molecular Genetics of Eukaryotes, CBS Sfax Tunisia) for his critical reading and valuable suggestions during the experimental work. They also wish to extend their sincere thanks to Mr. Anouar Smaoui from FSS for his constructive editing of the current paper and careful proofreading of its Englishness.

References

1. Bastawde, K. B. (1992). *World Journal of Microbiology & Biotechnology*, *8*, 353–368. doi:10.1007/BF01198746.
2. Joseleau, J. P., Comtat, J., & Rue, l. K. (1992). *Progress in Biotechnology*, *7*, 1–15.
3. Biely, P. (1985). *Trends in Biotechnology*, *3*, 286–290. doi:10.1016/0167-7799(85)90004-6.
4. Kulkarni, N., Shendye, A., & Rao, M. (1999). *FEMS Microbiology Reviews*, *23*, 411–456. doi:10.1111/j.1574-6976.1999.tb00407.x.
5. Beg, Q. K., Kapoor, M., Mahajan, L., & Hoondal, G. S. (2001). *Applied Microbiology and Biotechnology*, *56*, 326–338. doi:10.1007/s002530100704.
6. Zaldivar, J., Nielsen, J., & Olsson, L. (2001). *Applied Microbiology and Biotechnology*, *56*, 17–34. doi:10.1007/s002530100624.
7. Yang, X., Chen, H., Gao, H., & Li, Z. (2001). *Bioresource Technology*, *78*, 277–280. doi:10.1016/S0960-8524(01)00024-4.
8. Maat, J., Roza, M., Verbakel, J., Stam, H., Santos de Silva, M. J., & Bosse, M. (1992). Amsterdam, The Netherlands: Elsevier, pp. 349–360.
9. Madlala, A. M., Bissoon, S., Singh, S., & Christov, L. (2001). *Biotechnology Letters*, *23*, 345–351. doi:10.1023/A:1005693205016.
10. Tuohy, M. G., & Coughlan, M. P. (1992). *Bioresource Technology*, *39*, 131–137. doi:10.1016/0960-8524(92)90131-G.
11. Haltrich, D., Nidetzky, B., Kulbe, K. D., Steiner, W., & Zupancic, S. (1996). *Bioresource Technology*, *58*, 137–161.
12. Kuhad, R. C., & Singh, A. (1993). *Critical Reviews in Biotechnology*, *13*, 151–172. doi:10.3109/07388559309040630.
13. Yu, E. K. C., Tan, L. U. L., Chan, M. K., & Saddler, J. N. (1993). *Enzyme and Microbial Technology*, *9*, 16–24. doi:10.1016/0141-0229(87)90044-5.
14. Coughlan, M. P., Tuohy, M. G., Filho, E. X. F., Puls, J., Clayessens, M., Vrsanská, M., et al. (1993). In M. P. Coughlan & G. P.Hazlewood (Eds.) (pp. 53–84). London: Portland.
15. Gomes, J., Purkarthofer, H., Hany, M., Kapplmüller, J., Sinner, M., & Steiner, W. (1993). *Applied Microbiology and Biotechnology*, *39*, 700–707. doi:10.1007/BF00164453.
16. Maheshwari, R., Bharadwaj, G., & Bhat, M. K. (2000). *Microbiology and Molecular Biology Reviews*, *64*, 461–488. doi:10.1128/MMBR.64.3.461-488.2000.
17. Singh, S., Madlala, A. M., & Prior, B. A. (2003). *FEMS Microbiology Reviews*, *27*, 3–16. doi:10.1016/S0168-6445(03)00018-4.
18. Lite, Li., Hongmei, T., Yongqiang, C., Zhengqiangand, J., & Shaoqing, Y. (2005). *Enzyme and Microbial Technology*, *38*, 780–787.
19. Kelly, C. T., O'Mahony, M. R., & Fogarty, W. M. (1989). *Biotechnology Letters*, *11*, 885–890. doi:10.1007/BF01026846.
20. Singh, S., Pillay, B., & Prior, B. A. T. (2000). *Enzyme and Microbial Technology*, *20*, 502–508.
21. Mandels, M., & Weber, J. (1969). *Advances in Chemistry Series*, *95*, 391–413.
22. Miller, G. L. (1959). *Analytical Chemistry*, *31*, 426–428. doi::10.1021/ac60147a030.
23. Lacke, A. H. (1988). *Methods in Enzymology*, *160*, 679–684.
24. Bradford, M. (1976). *Analytical Biochemistry*, *72*, 248–254. doi:10.1016/0003-2697(76)90527-3.
25. Laemmli, U. K. (1970). *Nature*, *227*, 680–685. doi:10.1038/227680a0.

26. Lineweaver, H., & Burk, D. (1934). *Journal of the American Chemical Society*, *56*, 658–666. doi:10.1021/ja01318a036.
27. Wong, K. K. Y., Tan, L. U. L., & Saddler, J. N. (1988). *Microbiological Reviews*, *52*, 305–317.
28. Yu, E. K. C., Tan, L. U. L., Chan, M. K.-H., Deschatelets, L., & John Saddler, N. (1987). *Enzyme and Microbial Technology*, *9*, 16–24. doi:10.1016/0141-0229(87)90044-5.
29. Damaso Monica, C. T., Andrade, C. M. M. C., Nei, P. E. R. E. I., & Ra, J. R. (2002). *Brazilian Journal of Microbiology*, *33*, 333–338. doi:10.1590/S1517-83822002000400011.
30. Cesar, T., & Mrsa, V. (1996). *Enzyme and Microbial Technology*, *19*, 289–296. doi:10.1016/0141-0229(95)00248-0.