Characterization of a purified thermostable xylanase from Caldicoprobacter algeriensis sp. nov. strain TH7C1T

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Research Highlights

►A novel *C. algeriensis* sp. nov. strain TH7C1^T was purified (XYN35) and characterized.

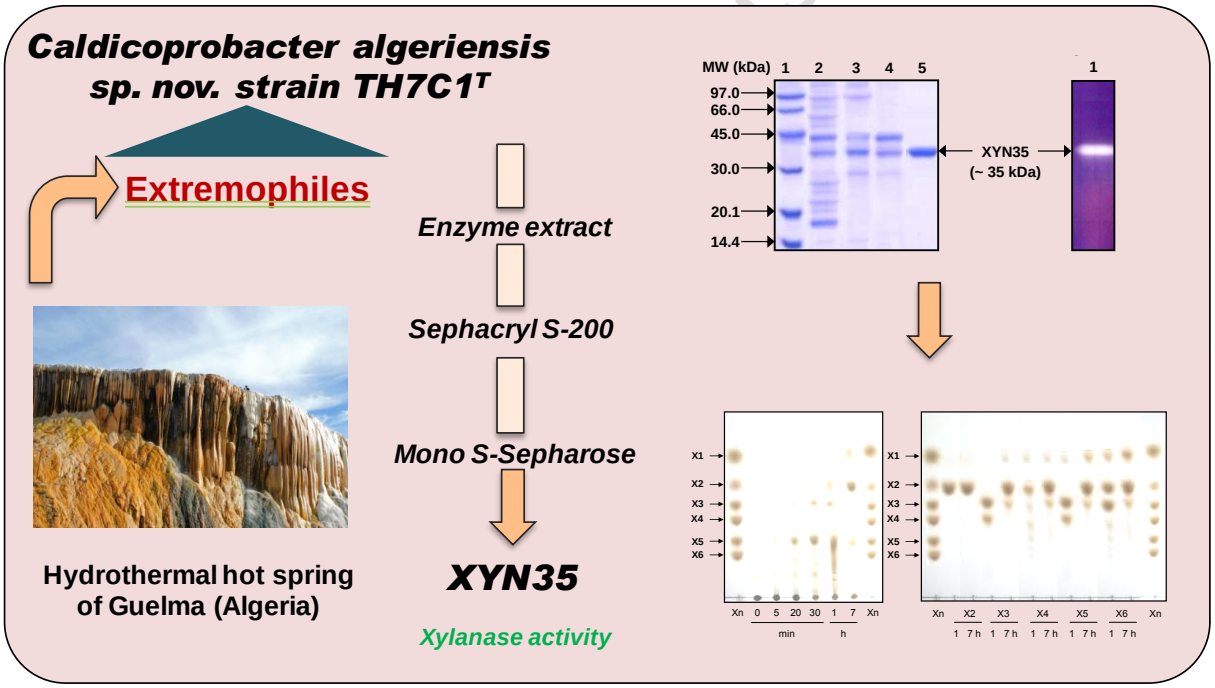
►The optimum pH and temperature values for activity were pH 11 and 70 °C, respectively.

►XYN35 was specific for xylans, followed by soluble oat-spelt and beechwood xylans.

►The hydrolysis products from birchwood xylan were xylose, xylobiose, and xylotriose.

►XYN35 is a potential strong candidate for application in the pulp bleaching industry.

Graphical Abstract



Abstract

The present study investigates the purification and biochemical characterization of an extracellular thermostable xylanase (called XYN35) from *Caldicoprobacter algeriensis* sp.

nov., strain TH7C1^T, a thermophilic, anaerobic strain isolated from the hydrothermal hot spring of Guelma (Algeria). The maximum xylanase activity recorded after 24 h of incubation at 70 °C and in an optimized medium containing 10 g/l mix birchwood- and oats spelt-xylan, was 250 U/ml. The pure protein was obtained after heat treatment (1 h at 70 °C), followed by sequential column chromatographies on Sephacryl S-200 gel filtration and Mono-S Sepharose anion-exchange. Matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) analysis indicated that the purified enzyme is a monomer with a molecular mass of 35,075.10 Da. The results from amino-acid sequence analysis revealed high homology between the 21 NH_2-terminal residues of XYN35 and those of bacterial xylanases. The enzyme showed optimum activity at pH 11 and 70 °C. While XYN35 was activated by Ca^{2+}, Mn^{2+}, and Mg^{2+}, it was completely inhibited by Hg^{2+} and Cd^{2+}. The xylanase showed higher specific activity on soluble oat-spelt xylan, followed by beechwood xylan. This enzyme was also noted to obey the Michaelis-Menten kinetics, with K_m and k_{cat} values on oat-spelt xylan being 1.33 mg/ml and 400 min^{-1}, respectively. Thin-layer chromatography soluble oat-spelt xylan (TLC) analysis showed that the final hydrolyzed products of the enzyme from birchwood xylan were xylose, xylobiose, and xylotriose. Taken together, the results indicated that the XYN35 enzyme has a number of attractive biochemical properties that make it a potential promising candidate for future application in the pulp bleaching industry.

Keywords: *Caldicoprobacter algeriensis*; Xylanase; Purification; Hydrolytic pattern; Pulp bleaching industry.

1. Introduction

Plant cell walls consist mainly of hemicellulose, cellulose (1,4-β-glucan) and lignin, with hemicellulose being the second most abundant renewable polysaccharide in nature after cellulose. Xylan, the major component in hemicellulose, is a heterogeneous polysaccharide with a complex backbone consisting of xylopyranosyl units linked by β-1,4-glycodidic bonds [1]. Xylan dgradation of is a complex process that involves the synergistic action of several xylanolytic enzymes [2, 3]. Xylanases (EC 3.2.1.8) are among the key xylan-degrading enzymes in that they cleave the long β-1,4-glycosidic bonds in the xylan backbone, resulting in short xylooligosaccharides that are further hydrolyzed to D-xylose by β-1,4-xylosidases [1, 4, 5]. Those enzymes are major components of the xylanolytic systems produced by various natural biodegradative microorganisms, including fungi and bacteria, which play crucial roles in maintaining carbon flow in the carbon cycle and, hence, biomass turnover in nature.

Xylanases have been used in a wide range of industrial applications and processes. They have been applied in the bioconversion of lignocellulosic material and agro-industrial byproducts into fermentative materials, clarification of juices, enhancement of beer consistency and enhancement of animal feed digestibility [6-8]. Due to their important activity at alkaline pH (8-11) and high temperature (60-90 °C), thermostable alkaline xylanases have attracted special attention in the pulp bio-bleaching industry [9]. Xylanase, together with other hydrolytic enzymes, have also proved useful for the generation of bio-fuels, including ethanol, from lignocellulosic biomass.

Xylanases are produced naturally from various biological sources, including bacteria, fungi and yeasts [6, 10-14]. Xylanolytic activity has been reported from several thermophilic bacteria, such as *Caldocellum saccharolyticum* [15], *Bacillus thermantarcticus* [16], *Bacillus aerophilus* [17], and *Streptomyces althioticus* LMZM [18]. Some xylanases from different

xylan-degrading thermotolerant bacterial species have been purified and biochemically characterized [19-26]. Thermophilic bacterial xylanases have attracted considerable attention in recent research because of their biotechnological applications, particularly in the pulping and bleaching bioprocesses, food and animal feed industries, and waste treatment biosystems [27-30]. Most of these industrial operations involve high temperature conditions, and thermostable enzymes would, therefore, offer several advantages that are not readily available in other ezymes. These include (i) enhanced bioavaibility compared to other options (ii) increased solubility of several polymeric substrates, which leads to a decrease in viscosity and chlorine utilization and, hence, the alleviation of environmental impacts, (iii) faster reaction rate, and (iv) reduced risks of microbial contamination. Accordingly, thermophilic organisms offer particularly attractive sources of novel thermostable enzymes [31].

Despite the large flow of data on the promising properties and attributes of thermostable enzymes , only few studies have so far been performed on thermophilic anaerobes inhabiting the various terrestrial hot springs of Algeria [27]. It was only recently that a hyperthermophilic archaeon belonging to the genus *Pyrococcus* was isolated from the northeast of Algeria [32]. Moreover, the authors have recently reported on the isolation of a novel thermophilic anaerobic bacterium, *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T, from the hydrothermal hot spring at Hammam D'Bagh (98 °C) in Guelma, northeast of Algeria, which was noted to exhibit xylanolytic activity at high temperature [33, 34]. Overall, the findings indicated that the thermo- and alkaline-tolerant xylanase offers attractive properties for the pulp and paper industry [33, 34]. Furthermore, the authors have recently reported on the production and partial characterization of xylanase activity from *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T [35].

In view of the scarcity of data on the thermophilic anaerobes inhabiting the hydrothermal hot springs of Algeria and considering the promising opportunities that these microorganisms

might offer for the development of novel thermostable and active enzymes, the present study was undertaken to investigate, for the first time, a purification protocol for the recovery of highly active XYN35, a novel xylanase isolated from strain TH7C1^{T}. The overall biochemical properties and hydrolytic activity of the enzyme on xylan were also studied.

2. Materials and methods

2.1. Reagents

Birchwood-, beechwood-, and oat spelt-xylan, as well as carboxymethyl cellulose (CMC, low viscosity), avicel, *p*-nitropenyl-β-D-xylopyranoside, *p*-nitropenyl-β-D-glucopyranoside, 3,5-dinitrosalycilic acid (DNS), and bovine serum albumine (BSA) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sephacryl S-200 and Mono-S Sepharose were from Pharmacia (Pharmacia, Uppsala, Sweden). The protein assay kit was obtained from Bio-Rad Laboratories (Inc., Hercules, CA, USA). Unless otherwise specified, all other reagents and chemicals were of the analytical grade or highest level of purity available.

2.2. Microbial strain, culture conditions, and identification of the microorganism

The microorganisms were isolated and cultured using the anaerobic procedures described by Hungate [36]. The selective medium used in the isolation process included (in g/l): mix of birchwood xylan and oats spelt xylan, 10; NH_4Cl, 0.1; K_2HPO_4, 0.3; KH_2PO_4, 0.3; KCl, 0.1; $MgCl_2{\cdot}6H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.1; NaCl, 0.5; yeast extract, 2; biotrypcase, 2; cysteine-HCl, 0.5; together with sodium acetate (2 mM) and a Balch trace element solution (10 ml) [37]. Cultures were purified by the repeated use of the Hungate roll tube method, using a gelrite solid medium, and transferred into a liquid medium as previously described elsewhere [38]. The thermophilic isolates were assayed for their abilities to produce xylanase by incubation

in a liquid medium containing (in g/l): mix birchwood xylan and oats spelt xylan, 10; yeast extract, 1; K_2HPO_4, 0.3; KH_2PO_4, 0.3; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.3; $MnSO_4$, 1; $FeSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.5; NH_4Cl, 0.2 at pH 7.2 [39]. The cultures were incubated for 72 h at 70 °C. One of the strains, called TH7C1^T, was noted to yield into high xylanase production rates and was, therefore, selected and maintained for further experimental study. The culture medium was centrifuged to remove medium debris, and the cell-free supernatant was used as a crude enzyme solution, which was partially characterized by the authors [35].

The *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T = DSM 22661^T = JCM 16184^T producing alkaline xylanase was isolated from the hydrothermal hot spring of Guelma in the northeast of Algeria. It was identified on the basis of 16S rRNA gene sequencing and biochemical properties as well as phenotypic, phylogenetic, and genetic characteristics [33, 34].

2.3. Enzyme assay

Xylanase activity was determined by measuring the release of reducing sugar from soluble xylan using the DNS method described by Miller [39]. In brief, 0.9 ml buffer A (20 mg/ml birchwood xylan in 100 mM glycine-NaOH buffer supplemented with 5 mM $CaCl_2$ at pH 11) were mixed with 0.1 ml enzyme solution (1 mg/ml). After incubation at 70 °C for 10 min, the reaction was terminated by adding 1.5 ml of the DNS reagent [40]. The mixture was then boiled for 5 min and cooled. Absorption was measured at 540 nm.

One unit of xylanase activity was defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to xylose per min under the assay conditions.

2.4. Xylanase purification procedure

Five hundred ml of a 24-h old culture of *C. algeriensis* sp. nov. strain TH7C1^T were centrifuged for 30 min at 10,000 × g to remove microbial cells. The supernatant was incubated for 1 h at 70 °C, and insoluble material was removed by centrifugation at 11,000 × g for 20 min. The clear supernatant was then recovered by centrifugation at 10,000 × g for 20 min and resuspended in a minimal volume of 100 mM glycine-NaOH buffer containing 5 mM $CaCl_2$ and 25 mM NaCl at pH 11 (Buffer B). The sample was subsequently dialyzed overnight against repeated changes of buffer B. Insoluble material was removed by centrifugation at 11,000 × g for 20 min. The supernatant was loaded on a 3 × 100 cm Sephacryl S-200 column equilibrated with buffer B. The elution of xylanase was performed with the same buffer at a rate of 45 ml/h. The fractions containing xylanase activity (eluted between 1.8 and 2.3 void volumes) were pooled. After that, the pooled fractions were applied to a Mono-S Sepharose column equilibrated in buffer B. The column (2.6 × 30 cm) was rinsed with 500 ml of the same buffer. Adsorbed material was eluted with a linear NaCl gradient (0-500 mM in buffer A) at a rate of 40 ml/h. Xylanase activity was eluted between 170 and 230 mM NaCl. The pooled fractions containing xylanolytic activity were concentrated in centrifugal micro-concentrators (Amicon Inc.) with 10 kDa cut-off membranes and stored at -20 °C.

2.5. Determination of protein concentration

Protein concentration was determined by the method of Bradford [41] using a Dc protein assay kit purchased from Bio-Rad Laboratories (Hercules, CA, USA), with BSA as a reference.

2.6. Electrophoresis and zymography

The analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed following the method of Laemmli [42]. The protein bands were visualized with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) staining. After electrophoresis, the protein bands were stained with Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The low molecular weight markers LMW from Amersham Biosciences were used as protein markers standards.

Zymogram analysis was monitored as reported by Kubata et al. [43]. Briefly, after proteins (50 µg) were separated by SDS-PAGE, the gel was incubated in a renaturation buffer (Buffer A) for 30 min at 70 °C with gentle shaking. The treated gel was then overlaid on a pre-cast 5 mg/ml agar gel containing 10 mg/ml birchwood xylan in buffer A. The agar plates were then incubated for 15 min at room temperature. After incubation the polyacrylamide gel was removed, and the agar gel was immersed in 30 mg/ml Congo red dye for 30 min and washed with 1.5 M NaCl solution to visualize zones of clearance corresponding to xylanase activity.

2.7. Mass spectrometry and NH_2-terminal amino acid sequencing

The molecular mass of the purified xylanase was analyzed, in the linear mode by the method of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) using a Voyager DE-RP instrument (Applied Biosystems/PerSeptive Biosystems, Inc., Framingham, MA, USA). The data were collected by a Tektronix TDS 520 numeric oscillograph and analyzed using the GRAMS/386 software (Galactic Industries Corporation, Salem, NH, USA).

Bands of purified xylanase were separated on SDS gels and transferred to a ProBlott membrane (Applied Biosystems, Foster City, CA, USA). N-terminal sequence analysis was

198 performed by automated Edman's degradation using an Applied Biosystem Model 473A gas-
199 phase sequencer.

200 ***2.8. Effects of pH on activity and stability of XYN35 enzyme***

201 Xylanase activity was assayed for 10 min over a range of pH 2-13 at 70 °C using
202 birchwood xylan as a substrate. pH stability was determined by pre-incubating the purified
203 xylanase in buffers of different pH values in the range of 6-12 for 12 h at 50 °C. Aliquots
204 were then withdrawn, and residual enzymatic activity was determined as described in the
205 xylanase activity assay.

206 The following buffer systems, supplemented with 5 mM $CaCl_2$, were used at 100 mM:
207 glycine-HCl for pH 2-5; 2-(*N*-morpholino) ethanesulfonic acid (MES) for pH 5-6; 4-(2-
208 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for pH 6-8; Tris-HCl for pH 8-9;
209 and glycine-NaOH for pH 9-13.

210 ***2.9. Effects of temperature on xylanase activity and stability of XYN35 enzyme***

211 The effect of temperature on xylanase activity was examined at 40-100 °C for 10 min at
212 pH 11. In order to monitor thermostability, buffered samples of the purified enzyme were
213 incubated for 12 h at 70, 80, 90, and 100 °C (100 mM glycine-NaOH buffer, pH 11) in the
214 presence and absence of 5 mM $CaCl_2$. Aliquots were withdrawn at timed intervals and cooled
215 on ice before being assayed to determine residual enzymatic activity under standard
216 conditions. The non-heated enzyme, which was cooled on ice, was considered as a control
217 (100%).

218 ***2.10. Effects of metallic ions and inhibitors on enzyme activity***

The effects of several metallic ions assayed at concentrations of 5 mM were also investigated by adding divalent ions [Ca^{2+} ($CaCl_2$), Mn^{2+} ($MnCl_2$), Mg^{2+} ($MgCl_2$), Co^{2+} ($CoCl_2$), Cu^{2+} ($CuCl_2$), Zn^{2+} ($ZnCl_2$), Ba^{2+} ($BaCl_2$), Fe^{2+} ($FeCl_2$), Ag^{2+} ($AgNO_3$), Cd^{2+} ($CdCl_2$), and Hg^{2+} ($HgCl_2$)], and nonmetallic reagents, such as ethylene diamine (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacitic acid (EDTA); ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacitic acid (EGTA); LD-dithiothreitol (LD-DTT); 2-mercaptoethanol (2-ME); and SDS for 1 h at 50 °C to the reaction mixture. Activity was expressed as a percentage of activity level in the absence of metallic ions or inhibitor.

Xylanase activities, measured with birchwood xylan as a substrate in the absence of any metallic ions or reagent, were taken as control (100%).

2.11. Substrate specificity and kinetic measurements of XYN35 enzyme

The substrate specificity profile of the XYN35 enzyme was studied using different substrates. Enzymatic reactions were carried out in buffer A containing 20 mg/ml of each substrate at 70 °C for 10 min. The amount of reducing sugars produced was estimated using the DNS method as described above. Activities towards *p*-nitrophenyl derivatives were measured by the rate of *p*-nitrophenol formed from 1 mM of the substrates in buffer A at 70 °C for 10 min during hydrolysis, and detected by spectrophotometry at 410 nm. One unit of xylanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar or *p*-nitrophenol per min under the above conditions. The effect of soluble oat-spelt xylan concentrations, ranging from 0 to 50 mg/ml, on xylanase activity were evaluated under the assay conditions.

The kinetic parameters were estimated by Lineweaver–Burk plots. The kinetic constants, Michaelis–Menten constant (K_m) and maximal reaction velocity (V_{max}) values were calculated using the Hyper32 software package.

2.12. TLC of hydrolysis products from xylan and xylooligosacharides

The hydrolyzed products from birchwood xylan and/or xylooligosaccharide were examined by TLC. The purified xylanase XYN35 (2 µg/ml) was incubated in buffer A containing 50 mg/ml of substrate birchwood xylan or xylooligosacharides, such as xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), or xylohexaose (X6), at 50 °C for an appropriate time. Enzyme reactions were stopped by boiling for 5 min. After centrifugation, the supernatants were concentrated by a vacuum centrifuge and spotted onto silica gel 60 F_{254} TLC aluminium sheets (Merck, Whitehouse Station, NJ, USA). TLC plates were developed with acetonitrile–ethyl acetate–2-propanol–water (17:5:11:10, v/v/v/v) and sprayed with a mixture of methanol-sulfuric acid (95:5, v/v), followed by heating at 150°C in an oven until spots appeared. Xylose and X2–X6 were used as standards.

2.13. Statistical analysis

All determinations were performed in three independent replicates, and the control experiment without xylanase was carried out under the same conditions. The experimental results were expressed as the mean of the replicate determinations and standard deviation (mean ± SD). Statistical significance was evaluated using t-tests for two-sample comparison and one-way analysis of variance (ANOVA) followed by t-test. The results were considered statistically significant for P values of less than or equal to 0.05. Statistical analysis was performed using the R package Version 3.1.1 (Vanderbilt University, USA).

3. Results and discussion

3.1. TH7C1^T strain producing xylanase activity

The strain designated TH7C1^T (DSM 22661^T or JCM 16184^T) and identified as *Caldicoprobacter algeriensis* sp. nov. [34], was noted to display the highest rates of extracellular xylanase activity [about 140 U/ml after 18 h of incubation in an initial medium [35] , and 250 U/ml after 24 h of incubation in an optimized medium (this study)]. It was, therefore, selected and retained for all subsequent studies.

3.2. Purification of the xylanase from strain TH7C1^T

The supernatant was obtained by the centrifugation of a 24-h old culture of the TH7C1^T strain using broth (500 ml) as a crude enzyme solution. The enzymatic preparation was heat-treated for 1 h at 70 °C. The precipitate formed was collected by centrifugation, dissolved in a minimum amount of buffer B, and then dialyzed overnight against repeated changes of the same buffer. Fractions corresponding to xylanase activity were pooled, and then loaded on a Sephacryl S-200 column equilibrated with buffer B. Purification to homogeneity was achieved using Mono-S Sepharose anion-exchange chromatography. Bound proteins were eluted with a linear gradient of NaCl from 0 to 500 mM in buffer C at a rate of 40 ml/h. The protein elution profile obtained at the final purification step indicated that the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was eluted at 170-230 mM NaCl (Fig. 1A).

The results of the purification procedure are summarized in Table 1. Enzyme purity was estimated to be about 38.35-fold greater than that of the crude extract. Under optimum assay conditions, using birchwood xylan as substrate, the purified enzyme had a specific activity of 603.88 U/mg, with a yield of about 27%.

The SDS-PAGE analysis of the pooled fractions showed one band corresponding to an apparent molecular mass of approximately 35 kDa (Fig. 1B). Zymogram activity staining revealed one zone of xynolytic activity for the purified sample co-migrating with proteins of a molecular mass of 35 kDa (Fig. 1C). The exact molecular mass of the purified xylanase

from *C. algeriensis* sp. nov. strain TH7C1^T was confirmed by MALDI-TOF/MS as being 35075.10 Da (Fig. 1D). A xylanase having such a low-molecular mass would be highly valued in pulp bleaching, since smaller enzymes can access the fiber wall structure more readily and alter the pulp properties more efficiently [44]. These observations presumably suggested that the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was a monomeric protein comparable to those previously reported for other bacterial xylanases [20, 45-47].

3.3. NH_2-terminal amino acid sequence

The twenty one NH_2-terminal amino acid residues were determined to be AQTITTDQTGYDGMYYTPWIF. This sequence was compared to other protein sequences available at Swiss-Prot database using the BLASTP search programs (Table 2). The results revealed high sequence homology with endo-1, 4-β-xylanases belonging to GH-11 family, particularly those from *Actinomadura* sp. Cpt20 (84% of identity), *Streptomyces roseiscleroticus* (79% of identity), and *Streptomyces* sp. SWU10 (74% of identity). The sequence was absent in all known GH-families and could, therefore, be considered as a signature for the GH-11 family. It could, therefore, be possible to deduce that the XYN35 xylanase from *C. algeriensis* sp. nov. strain TH7C1^T belongs to the GH-11 family [48, 49].

3.4. The effect of metallic ions and inhibitors on the purified xylanase

Several metallic ions and inhibitors were assayed for their effects on XYN35 activity (Table 3). The results indicated that xylanase activity was strongly inhibited by Cd^{2+}, Hg^{2+}, and Ba^{2+} and moderately inhibited by Fe^{2+}, Mg^{2+}, Ag^{2+}, and SDS. The enzyme was activated by Ca^{2+}, Mn^{2+}, Co^{2+}, and Cu^{2+} as compared to the control. Xylanase activity was not inhibited by chelating reagents such as EDTA and EGTA and slightly inhibited by disulfide-reducing agents 2-ME and DTT, which suggested that disulfide bonds were not essential to maintain

the active conformation of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T. This could lead to the conclusion that, like the crude enzyme [35], the purified xylanase XYN35 is not a metalloenzyme.

3.5. Effects of pH on xylanase activity and stability of XYN35 enzyme

The results presented in Fig. 2A show that the XYN35 enzyme was active over a wide range of pH between 6 and 12, with optimal activity being recorded at pH 11. The purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T showed enzyme stability over a broad pH range of 6-12 at 70 °C, and retained more than 75% of its activity (Fig. 2A). This xylanase was, therefore, an alkaline enzyme. Most of the bacterial xylanases so far reported had an optimum pH between 5 and 8 [10, 20-22]. Interestingly, the findings pertaining to pH stability showed that the enzyme retained more than 75% of its activity after being incubated for 12 h at different pH values ranging between 6 and 12 (Fig. 2B). Its residual activity was 81 and 93% at pH 6 and pH 7, respectively.

The pH profiles showed that the behaviors displayed by the purified xylanase XYN35 were similar to the crude enzyme previously reported by the authors [35]. These results are in agreement with those previously reported for xylanases isolated from several thermophilic actinomyces, such as *Actinomadura* sp. Cpt20, *Actinomadura* sp. S14, and *Actinomadura* sp. FC7 strains, which were stable over a wide pH range [10, 20-22].

Therefore, the tolerance to a broad pH range, especially to alkaline and high temperature conditions, exhibited by the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T is promising with regard to future industrial applications.

3.6. Effects of temperature on xylanase activity and stability of XYN35 enzyme

Using birchwood xylan as substrate, the optimum temperature for xylanase activity at pH 11 was 70 °C in the absence of $CaCl_2$ and 70 °C in the presence of 5 mM Ca^{2+} (Fig. 2C). The optimal temperature of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was similar to those of other previously reported for the crude enzyme previously described by the authors [35], and the other purified bacterial xylanases [10, 20, 44]. Recently, the purified xylanase from *Scytalidium thermophilum* ATCC No. 16454, have an optimal temperature of 65 °C [50].

The half-lives times of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T in the absence of any of the additive used were 8, 4, 2, and 0.7 h at 70, 80, 90, and 100 °C, respectively. The addition of different concentrations of $CaCl_2$ (1-10 mM) was observed to enhance the thermostability of the enzyme. The maximal thermostability was achieved with 5 mM Ca^{2+}. As shown in Fig. 2D, in the presence 5 mM $CaCl_2$, the half-lives times of the purified xylanase at 70, 80, 90, and 100 °C increased to 9, 6, 3, and 1.3 h, respectively. The thermostability exhibited by the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was higher than those of the GH-11 family xylanases from *Actinomadura* sp. Cpt20 [10], *Actinomadura* sp. S14 [20], *Streptomyces olivaceoviridis* A1 [46], and *Sporotrichum* thermophile (StXyn1) [51], but it was lower than that of *Anoxybacillus* sp. Ip-C. Enzyme activity and stability were previously reported to be enhanced by Ca^{2+} [10, 52, 53] . The improvement of enzyme thermostability against thermal inactivation in the presence of $CaCl_2$ could be attributed to the strengthening of interactions inside the protein molecules and to the binding of Ca^{2+} to the autolysis site [6]. This result suggests also that the calcium ion protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at higher temperatures, as there are probably two carbohydrate binding modules (CBMs) with calcium [48, 49]. Similar effects were also

observed with the crude enzyme reported by Bouacem el al. [35] and the other bacterial xylanases described in the literature [20, 45-47].

These findings further indicate that the XYN35 xylanase presented here exhibits a number of attractive and promising features that make it a strong candidate for future applications in the pulp bleaching industry.

3.7. Substrate specificity and determination of kinetic parameters of the xylanase

The substrate specificity of xylanase is often attributed to the glycosidic preceding the bond they hydrolyze. The hydrolysis activity of the XYN35 enzyme with regard to a variety of substrates was also studied (Table 4). The results revealed that the enzyme showed high specificity towards several of the xylans tested, with the highest activity being observed with soluble oat-spelt xylan, followed by beechwood xylan. However, no activity towards filter paper, CMC, avicel, *p*NP-β-D-xylopyranoside, and *p*NP-β-D-glucopyranoside were observed. Furthermore, the results indicated that XYN35 enzyme displayed the classical Michaelis-Menten kinetics for the soluble oat-spelt xylan used as substrate (Fig. 2E). The kinetic parameters K_m and V_{max} values of XYN35 were 1.33 mg/ml and 595 µmol min^{-1} mg^{-1}, respectively, using oat-spelt xylan as the substrate. Its deduced turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) were found to be 400 min^{-1} and 300.75 min^{-1} mM^{-1}, respectively.

Above all, the results indicated that XYN35 enzyme from *C. algeriensis* sp. nov. strain TH7C1^T was a "true xylanase". In fact, most of the xylanases previously investigated in the literature were reported to display both xylanase and low cellulase activities [54]. Interestingly, XYN35 was noted to hydrolyze only xylan and to be free from all the activities of the other enzymes investigated, including those of carboxymethyl cellulase, β-xylosidase, and β-glucosidase (Table 4).

Based on this result, the XYN35 xylanase of *C. algeriensis* sp. nov. strain TH7C1^T can be inferred to belong to the GH-11 family; the xylanases of this family have no activity on cellulose and are low-molecular mass enzymes [10, 55]. This property of xylanase is highly valued by several industrial applications and processes, particularly those pertaining to the biobleaching of paper.

3.8. Hydrolysis of birchwood xylan and xylooligosaccharides with the purified XYN35

In order to analyze reaction products, birchwood xylan and various xylooligosaccharides (X2–X6) were hydrolyzed with purified XYN35, and the reaction products were analyzed by TLC using a silica gel plate and the suitable solvent system (Fig. 3).

The enzyme liberated xylooligosaccharides from birchwood xylan in 5 min as shown in Fig. 3A. In fact, the xynalase XYN35 initially released xylooligosaccharides with high molecular masses [degree of polymerization (DP) of > 5], and the molecular masses became smaller (DP, 1–6) as the enzyme reaction progressed. Finally, the products converged on X1, X2, and X5. The xylobiose was the final major product and xylobiose–xylotetraose was increased with extended incubation time. In 7 h, a trace amount of xylose was detected, but this can be a product from xylotriose together with xylobiose. Thus, the enzyme exhibited β-1,4-endoxylanase activity and suggesting its good potential in xylooligosaccharides production. The xylooligosaccharides are sugar oligomers produced during the hydrolysis of xylan which is the major component of plant hemicellulose, a heteropolysaccharide with homopolymeric backbone of xylose units [1].

In hydrolysis tests of xylooligosaccharides, the enzyme hydrolyzed X3–X6 and produced X1 and X2 as the final hydrolysates after 7 h incubation, but no reaction products were observed when X2 was used as a substrate (Fig. 3B). The enzyme did not hydrolyze xylobiose into xylose even after 7 h incubation. The small amount of xylose found in a

reaction mixture of xylan seems to be derived from xylotriose. The degradation rates were: xylohexose > xylopentose > xylotetraose > xylotriose. These results suggested that the xynalase XYN35 recognizes xylooligosaccharides more avidly than xylotriose for hydrolysis activity. Purified XYN35 hydrolyzed xylan to xylooligosaccharides, in which xylobiose was a final product of all xylans. As deduced from degradation patterns of xylooligosaccharides (xylobiose–xylohexaose), the enzyme must recognize xylooligosaccharides with a DP greater than five.

Ideally, for industrial application, xylanases should have alkaline pH optimum and good thermal stability [48, 49]. Alkaline and thermostable xylanases have gained importance due to their application in the development of eco-friendly technologies used in the paper biobleaching industry as these enzymes are able to hydrolyse xylan, which is soluble in alkaline solutions, indicating the potential industrial use of the XYN35 enzyme.

4. Conclusion

GH-11 family xylanase without cellulase activity from *C. algeriensis* sp. nov. strain TH7C1^T was purified and characterized in this investigation. The time course for xylanase accumulation by *C. algeriensis* sp. nov. strain TH7C1^T in submerged anaerobic fermentation showed that the highest xylanase activity reached 250 U/ml in an optimized medium with mix of birchwood and oats spelt xylan used as a substrate after 24 h of cultivation. The extracellular xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was purified (called XYN35) and biochemically characterized. The results revealed that the enzyme was highly stable and active at high temperature and alkaline pH. Properties of XYN35 such as high specific activity, wide range of pH optimum and stability, and thermostability at elevated temperature, are appropriate for industrial application. Additionally, the lack of detectable cellulase activity might be useful for application using cellulose fibers to produce high-

quality pulp (xylan can be removed without damaging the cellulose) and to the production of pure xylooligosaccharides from agricultural wastes that generally include cellulose and xylan, because the products do not include glucooligosaccharides. Interestingly, XYN35 presented high xynolytic activity, and was very effective in the pulp bleaching industry, thus offering a potential promising candidate for application in biotechnological bioprocesses. Accordingly, further studies, some of which are currently underway, are needed to investigate the XYN35 encoding gene, perform site-directed mutagenesis, and determine its structure-function relationships.

Acknowledgments

This work was funded by the Algerian Ministry of Higher Education and Scientific research. The authors wish to express their gratitude to Dr. H. Laribi-Habchi (Laboratory of Chemical Engineering, Department of Process Engineering, Faculty of Technology, University of Blida 1, Blida, Algeria) for its valuable help during the preparation of this work. Thanks are also due to Mr. A. Smaoui and Mrs H. Ben Salem from the English Language Unit at the Faculty of Science of Sfax, Tunisia for their valuable proofreading and language polishing services. The authors would also like to extend their thanks to the anonymous reviewers for their valuable comments and efforts during the revision of the present manuscript.

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Figure Legends

Fig. 1. Purification and identification of the enzyme. Chromatography of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T on Mono-S Sepharose. The colomn (2.6 × 30 cm) was equilibrated with buffer B. Adsorbed material was eluted with a linear NaCl gradient (0-500 mM in buffer B) at a flow rate of 40 ml/h, and assayed for protein content at 280 nm (◊) and xylanase activity (♦) as described in Section 2 (**A**). SDS-PAGE (12%) of the purified xylanase. Lane 1, protein markers, using low molecular weight markers (Amersham Biosciences); lane 2, crude extract; lane 3, heat-treatment; lane 4, a sample of XYN35 obtained after Sephacryl S-200 chromatography; and lane 5: purified xylanase XYN35 from *C. algeriensis* sp. nov. strain TH7C1^T (50 µg) obtained after Mono-S Sepharose chromatography (fractions 23-29) (**B**). The gel was stained with Coomassie brilliant blue R-250 to reveal proteins. Zymogram of the purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T. Lane 1, 50 U of purified xylanase (**C**). MALDI-TOF spectrum of 30 pmol purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T. The mass spectrum shows a series of multiply protonated molecular ions. The molecular mass of the enzyme was found to be 35075.10 Da (**D**).

Fig. 2. Physico-chemical and kinetic proprieties of XYN35 enzyme. Effects of pH on the activity **(A)** and stability **(B)** of the purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T. Xylanase activity was evaluated in the pH range of 2-13 at 70 °C using buffers of different pH values with birchwood xylan. The pH stability of the enzyme was determined by incubating the xylanase in different buffers from 6-12 for 12 h at 50 °C, and the residual activity was measured under the standard assay procedure. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2. Effects of temperature on the activity **(C)** and stability **(D)** of the

purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T. The temperature profiles were determined by assaying xylanase activity at temperatures between 40 and 100 °C in the absence or presence of 5 mM $CaCl_2$. The activity of the enzyme at 70 °C in the absence of $CaCl_2$ was taken as 100%. The thermostability of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was examined by incubating the enzyme in the absence or presence of $CaCl_2$ at various temperatures ranging from 70 to 100 °C. Residual xylanase activity was determined from 0 to 12 h at 1 h intervals. The activity of the non-heated enzyme was taken as 100%. Each point represents the mean (n = 3) ± standard deviation. Double reciprocal plot of the purified xylanase XYN35 from *C. algeriensis* sp. nov. strain TH7C1^T. Xylanase activity was measured in 100 mM glycine-NaOH buffer (pH 11) supplemented with 5 mM $CaCl_2$ at 100 °C for 10 min using oat-spelt xylan as the substrate **(E)**.

Fig. 3. TLC analysis of hydrolysates with the purified xylanase XYN35. After hydrolysis of birchwood xylan (**A**) and xylooligosaccharides (**B**) with XYN35 for various reaction times, hydrolysates were developed on a silica gel plates. Abbreviations used: X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; X6, xylohexaose; and Xn, standards mixture of X1–X6.

Table 1

Flow sheet purification of xylanase XYN35 from *C. algeriensis* sp. nov. strain TH7C1^{T}.

Purification step[a]	Total activity (units)[a,b]	Total protein (mg)[a,c]	Specific activity (U/mg of protein)[a]	Activity recovery rate (%)	Purification factor (fold)
Crude extract	125,000 ± 7,500	7,951 ± 98	15.72 ± 3	100	1
Heat treatment (1 h at 70 °C)	97,500 ± 4,755	967 ± 41	100.82 ± 10	78	6.41
Sephacryl S-200	38,025 ± 1,211	98 ± 4	388.01 ± 23	39	24.68
Mono-S Sepharose	10,266 ± 0,350	17 ± 2	603.88 ± 37	27	38.35

[a] The experiments were conducted three times and ± standard errors are reported.

[b] One unit of xylanase activity is defined as the amount of enzyme that released 1 µmol of reducing sugar equivalent to xylose per min under the experimental conditions used. Specific activity was then expressed as U/mg of protein.

[c] Amounts of protein were estimated by Bradford method [41].

Table 2

Alignment of the NH_2-terminal amino acid sequence of the purified xylanase from *C. algeriensis* sp. nov. strain TH7C1^T with the sequences of other bacterial xylanases.

Enzyme	NH_2-terminal amino acid[a]	Identity (%)[b]
Endo-1, 4-β-xylanase from *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T [N-terminal; 21 amino acids] (this work)	AQTITTDQTGYDGMYYTPWIF	100
Endo-1, 4-β-xylanase from *Actinomadura* sp. Cpt20 [N-terminal; 19 amino acids]	A TITT QTGY GMYYTPW	84
Endo-1, 4-β-xylanase from *Streptomyces roseiscleroticus* [N-terminal; 31 amino acids; (A57001)]	A TITT QTGYDGMYY W	79
Endo-1, 4-β-xylanase precursor from *Streptomyces* sp. SWU10 [241 amino acids; (BAK53483)]	A TITT QTG DGMYY W	74
Endo-1, 4-β-xylanase precursor from *Actinosynnema mirum* ATCC 29888 [233 amino acids; (CP001630)]	AQTITT QTG YY	71
Endo-1, 4-β-xylanase precursor from *Thermobispora bispora* DSM 43833 [343 amino acids; (YP_003651058)]	AQTI QTGY Y	65

[a] Amino acid sequences for comparison were obtained using the BLASTP (NCBI, NIH, USA) program database. The GenBank accession number is in parentheses.

[b] Residues not identical with the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T are indicated in black box.

Table 3

Effect of various reagents on the xylanase XYN35 from *C. algeriensis* sp. nov. strain TH7C1^{T}.

Reagents (at 5 mM)	Specific activity (U/mg of protein)	Relative activity (%)[a]
None	603 ± 37	100
Ca^{2+} ($CaCl_2$)	995 ± 43	165
Mn^{2+} ($MnSO_4$)	886 ± 41	147
Mg^{2+} ($MgCl_2$)	428 ± 22	71
Cu^{2+} ($CuCl_2$)	693 ± 39	115
Zn^{2+} ($ZnCl_2$)	603 ± 37	100
Co^{2+} ($CoCl_2$)	784 ± 40	130
Ba^{2+} ($BaCl_2$)	92 ± 10	15
Fe^{2+} ($FeSO_4$)	410 ± 17	68
Ag^{2+} ($AgNO_3$)	452 ± 24	75
Cd^{2+} ($CdCl_2$)	0	0
Hg^{2+} ($HgCl_2$)	0	0
2-ME	440 ± 25	73
DTT	470 ± 27	78
SDS	482 ± 31	81
EDTA	585 ± 37	97
EGTA	518 ± 30	86

[a] The values represent the mean of three independent replicates and standard errors are reported.

Table 4

Substrate specificity of the xylanase XYN35 from *C. algeriensis* sp. nov. strain TH7C1^{T}.

Substrate	Specific activity (U/mg of protein)	Relative activity (%)[a]
Birchwood xylan	603 ± 37	100
Beechwood xylan	796 ± 40	132
Oat-spelt xylan	711 ± 39	118
Soluble oat-spelt xylan	874 ± 41	147
Insoluble oat-spelt xylan	645 ± 38	107
Wheat bran xylan	452 ± 24	75
Filter paper	0	0
CMC	0	0
Avicel	0	0
*p*NP-β-D-xylopyranoside	0	0
*p*NP-β-D-glucopyranoside	0	0

[a] The activity for birchwood xylan was defined as 100%. The results are expressed in mean ± standard errors from three separate experiments.

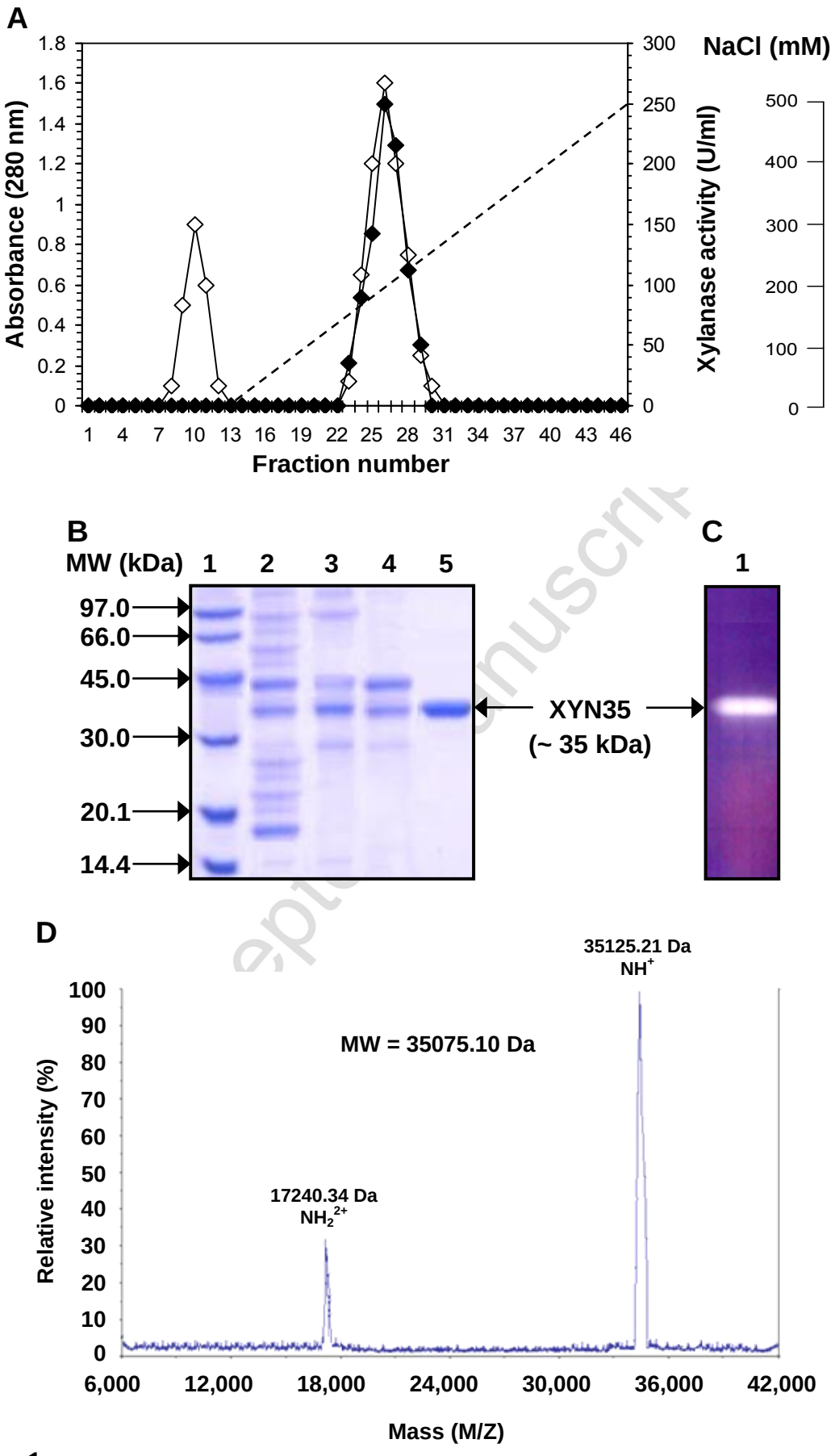

Figure 1

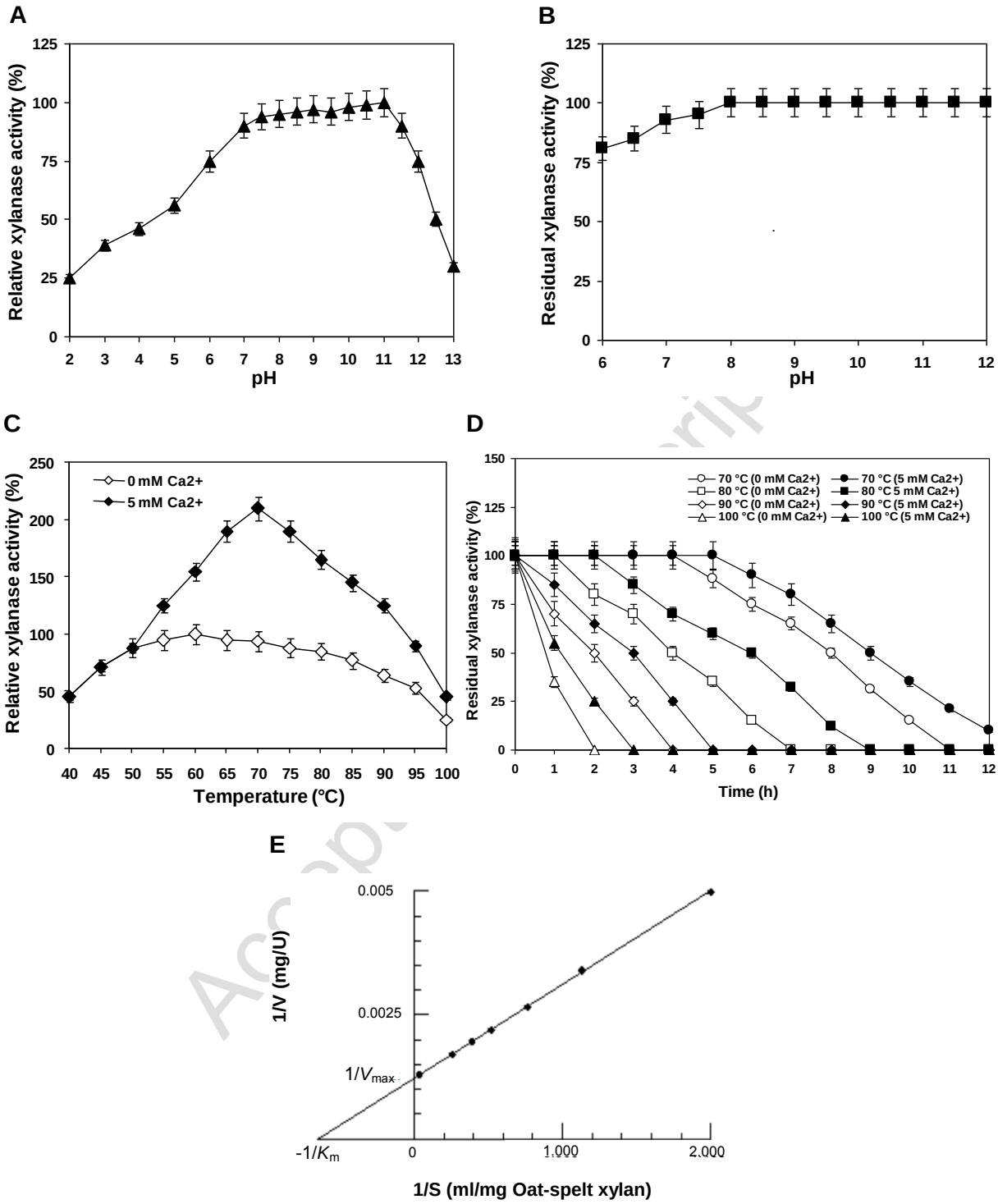

Figure 2

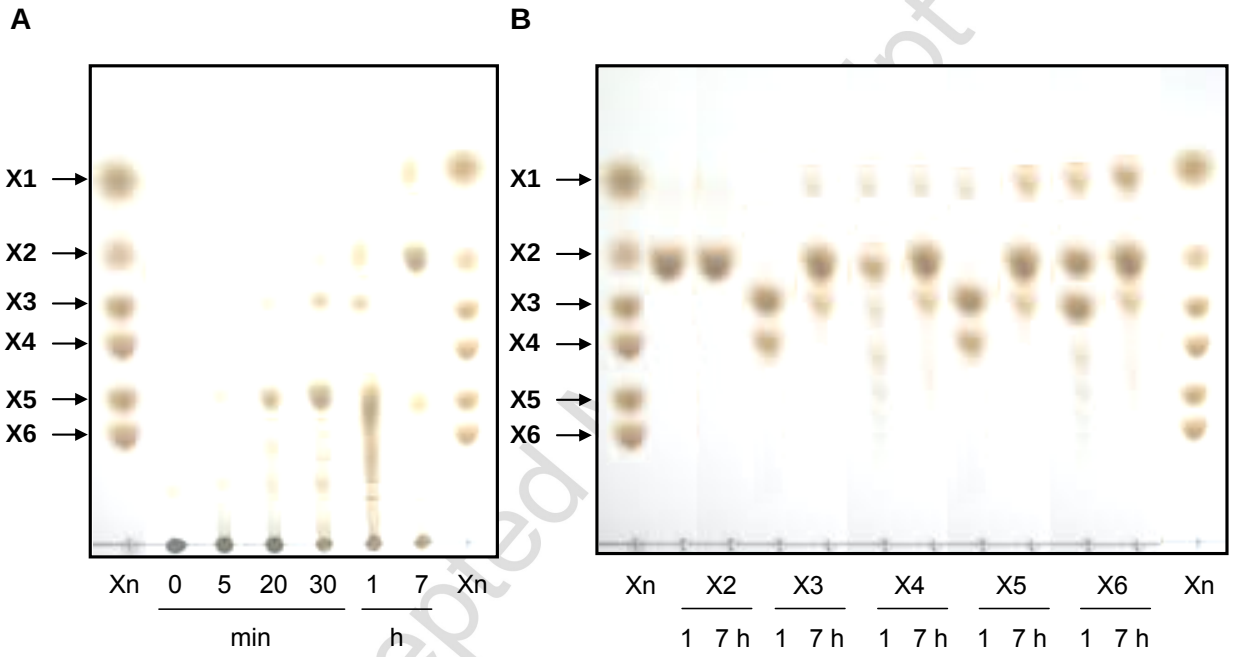

Figure 3