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1 **Characterization of a purified thermostable xylanase from *Caldicoprobacter algeriensis***
2 **sp. nov. strain TH7C1^T**

3

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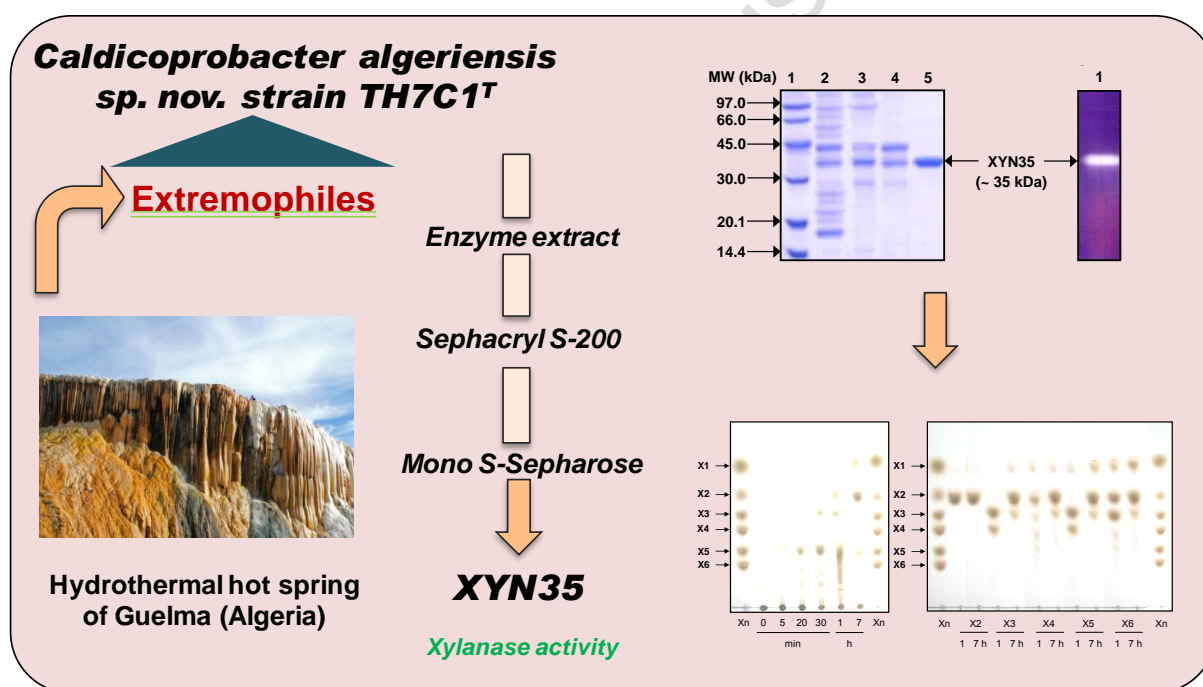
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26 **Research Highlights**

- 27 ▶ A novel *C. algeriensis* sp. nov. strain TH7C1^T was purified (XYN35) and characterized.
- 28 ▶ The optimum pH and temperature values for activity were pH 11 and 70 °C, respectively.
- 29 ▶ XYN35 was specific for xylans, followed by soluble oat-spelt and beechwood xylans.
- 30 ▶ The hydrolysis products from birchwood xylan were xylose, xylobiose, and xylotriose.
- 31 ▶ XYN35 is a potential strong candidate for application in the pulp bleaching industry.

32 **Graphical Abstract**

33

34

35 **Abstract**

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

38 nov., strain TH7C1^T, a thermophilic, anaerobic strain isolated from the hydrothermal hot
39 spring of Guelma (Algeria). The maximum xylanase activity recorded after 24 h of
40 incubation at 70 °C and in an optimized medium containing 10 g/l mix birchwood- and oats
41 spelt-xylan, was 250 U/ml. The pure protein was obtained after heat treatment (1 h at 70 °C),
42 followed by sequential column chromatographies on Sephacryl S-200 gel filtration and
43 Mono-S Sepharose anion-exchange. Matrix assisted laser desorption ionization-time of flight
44 mass spectrometry (MALDI-TOF/MS) analysis indicated that the purified enzyme is a
45 monomer with a molecular mass of 35,075.10 Da. The results from amino-acid sequence
46 analysis revealed high homology between the 21 NH₂-terminal residues of XYN35 and those
47 of bacterial xylanases. The enzyme showed optimum activity at pH 11 and 70 °C. While
48 XYN35 was activated by Ca²⁺, Mn²⁺, and Mg²⁺, it was completely inhibited by Hg²⁺ and
49 Cd²⁺. The xylanase showed higher specific activity on soluble oat-spelt xylan, followed by
50 beechwood xylan. This enzyme was also noted to obey the Michaelis-Menten kinetics, with
51 K_m and k_{cat} values on oat-spelt xylan being 1.33 mg/ml and 400 min⁻¹, respectively. Thin-
52 layer chromatography soluble oat-spelt xylan (TLC) analysis showed that the final
53 hydrolyzed products of the enzyme from birchwood xylan were xylose, xylobiose, and
54 xylotriose. Taken together, the results indicated that the XYN35 enzyme has a number of
55 attractive biochemical properties that make it a potential promising candidate for future
56 application in the pulp bleaching industry.

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

59 1. Introduction

60 Plant cell walls consist mainly of hemicellulose, cellulose (1,4- β -glucan) and lignin, with
61 hemicellulose being the second most abundant renewable polysaccharide in nature after
62 cellulose. Xylan, the major component in hemicellulose, is a heterogeneous polysaccharide
63 with a complex backbone consisting of xylopyranosyl units linked by β -1,4-glycosidic bonds
64 [1]. Xylan degradation is a complex process that involves the synergistic action of several
65 xylanolytic enzymes [2, 3]. Xylanases (EC 3.2.1.8) are among the key xylan-degrading
66 enzymes in that they cleave the long β -1,4-glycosidic bonds in the xylan backbone, resulting
67 in short xylooligosaccharides that are further hydrolyzed to D-xylose by β -1,4-xylosidases [1,
68 4, 5]. Those enzymes are major components of the xylanolytic systems produced by various
69 natural biodegradative microorganisms, including fungi and bacteria, which play crucial roles
70 in maintaining carbon flow in the carbon cycle and, hence, biomass turnover in nature.

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

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

83 xylan-degrading thermotolerant bacterial species have been purified and biochemically
84 characterized [19-26]. Thermophilic bacterial xylanases have attracted considerable attention
85 in recent research because of their biotechnological applications, particularly in the pulping
86 and bleaching bioprocesses, food and animal feed industries, and waste treatment biosystems
87 [27-30]. Most of these industrial operations involve high temperature conditions, and
88 thermostable enzymes would, therefore, offer several advantages that are not readily
89 available in other enzymes. These include (i) enhanced bioavailability compared to other options
90 (ii) increased solubility of several polymeric substrates, which leads to a decrease in viscosity
91 and chlorine utilization and, hence, the alleviation of environmental impacts, (iii) faster
92 reaction rate, and (iv) reduced risks of microbial contamination. Accordingly, thermophilic
93 organisms offer particularly attractive sources of novel thermostable enzymes [31].

94 Despite the large flow of data on the promising properties and attributes of thermostable
95 enzymes, only few studies have so far been performed on thermophilic anaerobes inhabiting
96 the various terrestrial hot springs of Algeria [27]. It was only recently that a
97 hyperthermophilic archaeon belonging to the genus *Pyrococcus* was isolated from the
98 northeast of Algeria [32]. Moreover, the authors have recently reported on the isolation of a
99 novel thermophilic anaerobic bacterium, *Caldicoprobacter algeriensis* sp. nov. strain
100 TH7C1^T, from the hydrothermal hot spring at Hammam D'Bagh (98 °C) in Guelma,
101 northeast of Algeria, which was noted to exhibit xylanolytic activity at high temperature [33,
102 34]. Overall, the findings indicated that the thermo- and alkaline-tolerant xylanase offers
103 attractive properties for the pulp and paper industry [33, 34]. Furthermore, the authors have
104 recently reported on the production and partial characterization of xylanase activity from
105 *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T [35].

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

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

112 **2. Materials and methods**

113 **2.1. Reagents**

114 Birchwood-, beechwood-, and oat spelt-xylan, as well as carboxymethyl cellulose (CMC,
115 low viscosity), avicel, *p*-nitrophenyl- β -D-xylopyranoside, *p*-nitrophenyl- β -D-glucopyranoside,
116 3,5-dinitrosalicylic acid (DNS), and bovine serum albumine (BSA) were purchased from
117 Sigma Chemical Company (St. Louis, MO, USA). Sephacryl S-200 and Mono-S Sepharose
118 were from Pharmacia (Pharmacia, Uppsala, Sweden). The protein assay kit was obtained
119 from Bio-Rad Laboratories (Inc., Hercules, CA, USA). Unless otherwise specified, all other
120 reagents and chemicals were of the analytical grade or highest level of purity available.

121 **2.2. Microbial strain, culture conditions, and identification of the microorganism**

122 The microorganisms were isolated and cultured using the anaerobic procedures described
123 by Hungate [36]. The selective medium used in the isolation process included (in g/l): mix of
124 birchwood xylan and oats spelt xylan, 10; NH₄Cl, 0.1; K₂HPO₄, 0.3; KH₂PO₄, 0.3; KCl, 0.1;
125 MgCl₂·6H₂O, 0.5; CaCl₂·2H₂O, 0.1; NaCl, 0.5; yeast extract, 2; biotrypcase, 2; cysteine-HCl,
126 0.5; together with sodium acetate (2 mM) and a Balch trace element solution (10 ml) [37].
127 Cultures were purified by the repeated use of the Hungate roll tube method, using a gelrite
128 solid medium, and transferred into a liquid medium as previously described elsewhere [38].
129 The thermophilic isolates were assayed for their abilities to produce xylanase by incubation

130 in a liquid medium containing (in g/l): mix birchwood xylan and oats spelt xylan, 10; yeast
131 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;
132 $FeSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.5; NH_4Cl , 0.2 at pH 7.2 [39]. The cultures were incubated for 72
133 h at 70 °C. One of the strains, called TH7C1^T, was noted to yield into high xylanase
134 production rates and was, therefore, selected and maintained for further experimental study.
135 The culture medium was centrifuged to remove medium debris, and the cell-free supernatant
136 was used as a crude enzyme solution, which was partially characterized by the authors [35].

137 The *Caldicoprobacter algeriensis* sp. nov. strain TH7C1^T = DSM 22661^T = JCM 16184^T
138 producing alkaline xylanase was isolated from the hydrothermal hot spring of Guelma in the
139 northeast of Algeria. It was identified on the basis of 16S rRNA gene sequencing and
140 biochemical properties as well as phenotypic, phylogenetic, and genetic characteristics [33,
141 34].

142 2.3. Enzyme assay

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

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

151 2.4. Xylanase purification procedure

152 Five hundred ml of a 24-h old culture of *C. algeriensis* sp. nov. strain TH7C1^T were
153 centrifuged for 30 min at 10,000 × g to remove microbial cells. The supernatant was
154 incubated for 1 h at 70 °C, and insoluble material was removed by centrifugation at 11,000 ×
155 g for 20 min. The clear supernatant was then recovered by centrifugation at 10,000 × g for 20
156 min and resuspended in a minimal volume of 100 mM glycine-NaOH buffer containing 5
157 mM CaCl₂ and 25 mM NaCl at pH 11 (Buffer B). The sample was subsequently dialyzed
158 overnight against repeated changes of buffer B. Insoluble material was removed by
159 centrifugation at 11,000 × g for 20 min. The supernatant was loaded on a 3 × 100 cm
160 Sephacryl S-200 column equilibrated with buffer B. The elution of xylanase was performed
161 with the same buffer at a rate of 45 ml/h. The fractions containing xylanase activity (eluted
162 between 1.8 and 2.3 void volumes) were pooled. After that, the pooled fractions were applied
163 to a Mono-S Sepharose column equilibrated in buffer B. The column (2.6 × 30 cm) was
164 rinsed with 500 ml of the same buffer. Adsorbed material was eluted with a linear NaCl
165 gradient (0-500 mM in buffer A) at a rate of 40 ml/h. Xylanase activity was eluted between
166 170 and 230 mM NaCl. The pooled fractions containing xylanolytic activity were
167 concentrated in centrifugal micro-concentrators (Amicon Inc.) with 10 kDa cut-off
168 membranes and stored at -20 °C.

169 **2.5. Determination of protein concentration**

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

173 **2.6. Electrophoresis and zymography**

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

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

189 **2.7. Mass spectrometry and NH_2 -terminal amino acid sequencing**

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

196 Bands of purified xylanase were separated on SDS gels and transferred to a ProBlott
197 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 CaCl₂, 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 CaCl₂. 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***

219 The effects of several metallic ions assayed at concentrations of 5 mM were also
220 investigated by adding divalent ions [Ca^{2+} (CaCl_2), Mn^{2+} (MnCl_2), Mg^{2+} (MgCl_2), Co^{2+}
221 (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),
222 and Hg^{2+} (HgCl_2)], and nonmetallic reagents, such as ethylene diamine (β -aminoethyl ether)-
223 *N,N,N',N'*-tetraacetic acid (EDTA); ethylene glycol-bis (β -aminoethyl ether)-*N,N,N',N'*-
224 tetraacetic acid (EGTA); LD-dithiothreitol (LD-DTT); 2-mercaptoethanol (2-ME); and SDS for
225 1 h at 50 °C to the reaction mixture. Activity was expressed as a percentage of activity level
226 in the absence of metallic ions or inhibitor.

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

229 **2.11. Substrate specificity and kinetic measurements of XYN35 enzyme**

230 The substrate specificity profile of the XYN35 enzyme was studied using different
231 substrates. Enzymatic reactions were carried out in buffer A containing 20 mg/ml of each
232 substrate at 70 °C for 10 min. The amount of reducing sugars produced was estimated using
233 the DNS method as described above. Activities towards *p*-nitrophenyl derivatives were
234 measured by the rate of *p*-nitrophenol formed from 1 mM of the substrates in buffer A at 70
235 °C for 10 min during hydrolysis, and detected by spectrophotometry at 410 nm. One unit of
236 xylanase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar
237 or *p*-nitrophenol per min under the above conditions. The effect of soluble oat-spelt xylan
238 concentrations, ranging from 0 to 50 mg/ml, on xylanase activity were evaluated under the
239 assay conditions.

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

243 **2.12. TLC of hydrolysis products from xylan and xylooligosacharides**

244 The hydrolyzed products from birchwood xylan and/or xylooligosacharide were
245 examined by TLC. The purified xylanase XYN35 (2 µg/ml) was incubated in buffer A
246 containing 50 mg/ml of substrate birchwood xylan or xylooligosacharides, such as xylobiose
247 (X2), xylotriose (X3), xylo-tetraose (X4), xylo-pentaose (X5), or xylo-hexaose (X6), at 50 °C
248 for an appropriate time. Enzyme reactions were stopped by boiling for 5 min. After
249 centrifugation, the supernatants were concentrated by a vacuum centrifuge and spotted onto
250 silica gel 60 F₂₅₄ TLC aluminium sheets (Merck, Whitehouse Station, NJ, USA). TLC plates
251 were developed with acetonitrile–ethyl acetate–2-propanol–water (17:5:11:10, v/v/v/v) and
252 sprayed with a mixture of methanol-sulfuric acid (95:5, v/v), followed by heating at 150°C in
253 an oven until spots appeared. Xylose and X2–X6 were used as standards.

254 **2.13. Statistical analysis**

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

262 **3. Results and discussion**

263 **3.1. TH7C1^T strain producing xylanase activity**

264 The strain designated TH7C1^T (DSM 22661^T or JCM 16184^T) and identified as
265 *Caldicoprobacter algeriensis* sp. nov. [34], was noted to display the highest rates of
266 extracellular xylanase activity [about 140 U/ml after 18 h of incubation in an initial medium
267 [35] , and 250 U/ml after 24 h of incubation in an optimized medium (this study)]. It was,
268 therefore, selected and retained for all subsequent studies.

269 **3.2. Purification of the xylanase from strain TH7C1^T**

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

280 The results of the purification procedure are summarized in Table 1. Enzyme purity was
281 estimated to be about 38.35-fold greater than that of the crude extract. Under optimum assay
282 conditions, using birchwood xylan as substrate, the purified enzyme had a specific activity of
283 603.88 U/mg, with a yield of about 27%.

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

288 from *C. algeriensis* sp. nov. strain TH7C1^T was confirmed by MALDI-TOF/MS as being
289 35075.10 Da (Fig. 1D). A xylanase having such a low-molecular mass would be highly
290 valued in pulp bleaching, since smaller enzymes can access the fiber wall structure more
291 readily and alter the pulp properties more efficiently [44]. These observations presumably
292 suggested that the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was a monomeric
293 protein comparable to those previously reported for other bacterial xylanases [20, 45-47].

294 3.3. *NH₂-terminal amino acid sequence*

295 The twenty one NH₂-terminal amino acid residues were determined to be
296 AQTITTDQGTGYDGMYYTPWIF. This sequence was compared to other protein sequences
297 available at Swiss-Prot database using the BLASTP search programs (Table 2). The results
298 revealed high sequence homology with endo-1, 4-β-xylanases belonging to GH-11 family,
299 particularly those from *Actinomadura* sp. Cpt20 (84% of identity), *Streptomyces*
300 *roseiscleroticus* (79% of identity), and *Streptomyces* sp. SWU10 (74% of identity). The
301 sequence was absent in all known GH-families and could, therefore, be considered as a
302 signature for the GH-11 family. It could, therefore, be possible to deduce that the XYN35
303 xylanase from *C. algeriensis* sp. nov. strain TH7C1^T belongs to the GH-11 family [48, 49].

304 3.4. *The effect of metallic ions and inhibitors on the purified xylanase*

305 Several metallic ions and inhibitors were assayed for their effects on XYN35 activity
306 (Table 3). The results indicated that xylanase activity was strongly inhibited by Cd²⁺, Hg²⁺,
307 and Ba²⁺ and moderately inhibited by Fe²⁺, Mg²⁺, Ag²⁺, and SDS. The enzyme was activated
308 by Ca²⁺, Mn²⁺, Co²⁺, and Cu²⁺ as compared to the control. Xylanase activity was not inhibited
309 by chelating reagents such as EDTA and EGTA and slightly inhibited by disulfide-reducing
310 agents 2-ME and DTT, which suggested that disulfide bonds were not essential to maintain

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

314 **3.5. Effects of pH on xylanase activity and stability of XYN35 enzyme**

315 The results presented in Fig. 2A show that the XYN35 enzyme was active over a wide
316 range of pH between 6 and 12, with optimal activity being recorded at pH 11. The purified
317 xylanase from *C. algeriensis* sp. nov. strain TH7C1^T showed enzyme stability over a broad
318 pH range of 6-12 at 70 °C, and retained more than 75% of its activity (Fig. 2A). This
319 xylanase was, therefore, an alkaline enzyme. Most of the bacterial xylanases so far reported
320 had an optimum pH between 5 and 8 [10, 20-22]. Interestingly, the findings pertaining to pH
321 stability showed that the enzyme retained more than 75% of its activity after being incubated
322 for 12 h at different pH values ranging between 6 and 12 (Fig. 2B). Its residual activity was
323 81 and 93% at pH 6 and pH 7, respectively.

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

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

332 **3.6. Effects of temperature on xylanase activity and stability of XYN35 enzyme**

333 Using birchwood xylan as substrate, the optimum temperature for xylanase activity at pH
334 11 was 70 °C in the absence of CaCl₂ and 70 °C in the presence of 5 mM Ca²⁺ (Fig. 2C). The
335 optimal temperature of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was similar
336 to those of other previously reported for the crude enzyme previously described by the
337 authors [35], and the other purified bacterial xylanases [10, 20, 44]. Recently, the purified
338 xylanase from *Scytalidium thermophilum* ATCC No. 16454, have an optimal temperature of
339 65 °C [50].

340 The half-lives times of the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T in the
341 absence of any of the additive used were 8, 4, 2, and 0.7 h at 70, 80, 90, and 100 °C,
342 respectively. The addition of different concentrations of CaCl₂ (1-10 mM) was observed to
343 enhance the thermostability of the enzyme. The maximal thermostability was achieved with 5
344 mM Ca²⁺. As shown in Fig. 2D, in the presence 5 mM CaCl₂, the half-lives times of the
345 purified xylanase at 70, 80, 90, and 100 °C increased to 9, 6, 3, and 1.3 h, respectively. The
346 thermostability exhibited by the xylanase from *C. algeriensis* sp. nov. strain TH7C1^T was
347 higher than those of the GH-11 family xylanases from *Actinomadura* sp. Cpt20 [10],
348 *Actinomadura* sp. S14 [20], *Streptomyces olivaceoviridis* A1 [46], and *Sporotrichum*
349 thermophile (StXyn1) [51], but it was lower than that of *Anoxybacillus* sp. Ip-C. Enzyme
350 activity and stability were previously reported to be enhanced by Ca²⁺ [10, 52, 53]. The
351 improvement of enzyme thermostability against thermal inactivation in the presence of CaCl₂
352 could be attributed to the strengthening of interactions inside the protein molecules and to the
353 binding of Ca²⁺ to the autolysis site [6]. This result suggests also that the calcium ion
354 protected the enzyme against thermal denaturation and played a vital role in maintaining the
355 active conformation of the enzyme at higher temperatures, as there are probably two
356 carbohydrate binding modules (CBMs) with calcium [48, 49]. Similar effects were also

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

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

362 **3.7. Substrate specificity and determination of kinetic parameters of the xylanase**

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

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

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

385 **3.8. Hydrolysis of birchwood xylan and xylooligosaccharides with the purified XYN35**

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

389 The enzyme liberated xylooligosaccharides from birchwood xylan in 5 min as shown in
390 Fig. 3A. In fact, the xynalase XYN35 initially released xylooligosaccharides with high
391 molecular masses [degree of polymerization (DP) of > 5], and the molecular masses became
392 smaller (DP, 1–6) as the enzyme reaction progressed. Finally, the products converged on X1,
393 X2, and X5. The xylobiose was the final major product and xylobiose–xylotetraose was
394 increased with extended incubation time. In 7 h, a trace amount of xylose was detected, but
395 this can be a product from xylotriose together with xylobiose. Thus, the enzyme exhibited β -
396 1,4-endoxylanase activity and suggesting its good potential in xylooligosaccharides
397 production. The xylooligosaccharides are sugar oligomers produced during the hydrolysis of
398 xylan which is the major component of plant hemicellulose, a heteropolysaccharide with
399 homopolymeric backbone of xylose units [1].

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

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

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

416 **4. Conclusion**

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

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

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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 column (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 CaCl₂. The activity of the enzyme at 70 °C in the absence of CaCl₂ 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 CaCl₂ 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 CaCl₂ 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, xylo-tetraose; X5, xylo-pentaose; X6, xylo-hexaose; 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 NH₂-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 <i>Caldicoprobacter algeriensis</i> sp. nov. strain TH7C1 ^T [N-terminal; 21 amino acids] (this work)	AQTITTDQTGYDGMYYTPWIF	100
Endo-1, 4- β -xylanase from <i>Actinomadura</i> sp. Cpt20 [N-terminal; 19 amino acids]	A TITT QTGY GMYYPW	84
Endo-1, 4- β -xylanase from <i>Streptomyces roseiscleroticus</i> [N-terminal; 31 amino acids; (A57001)]	A TITT QTGYDGMYY W	79
Endo-1, 4- β -xylanase precursor from <i>Streptomyces</i> sp. SWU10 [241 amino acids; (BAK53483)]	A TITT QTG DGMYY W	74
Endo-1, 4- β -xylanase precursor from <i>Actinosynnema mirum</i> ATCC 29888 [233 amino acids; (CP001630)]	AQTITT QTG YY	71
Endo-1, 4- β -xylanase precursor from <i>Thermobispora bispora</i> 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
<i>p</i> NP-β-D-xylopyranoside	0	0
<i>p</i> NP-β-D-glucoxyranoside	0	0

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

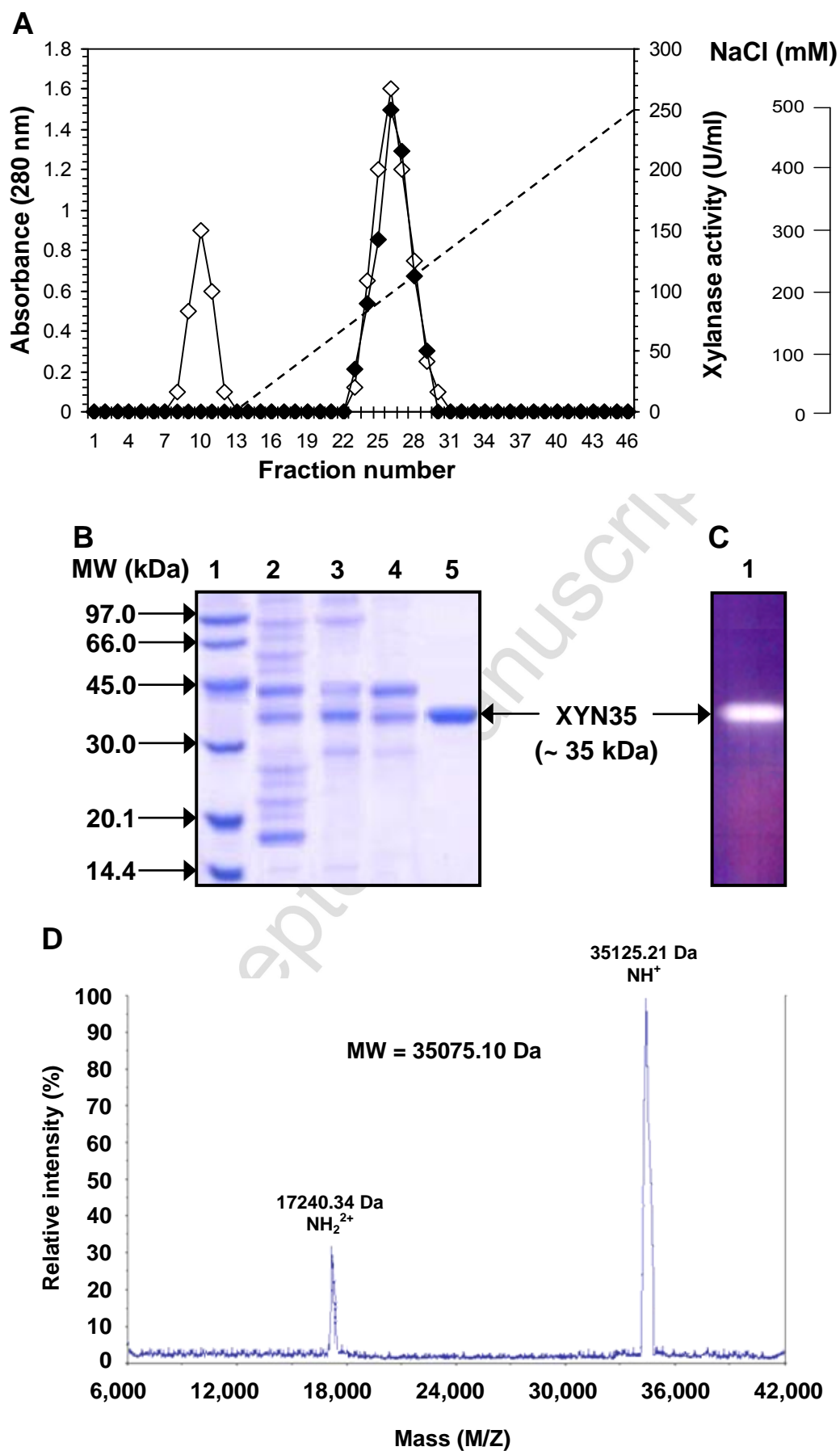


Figure 1

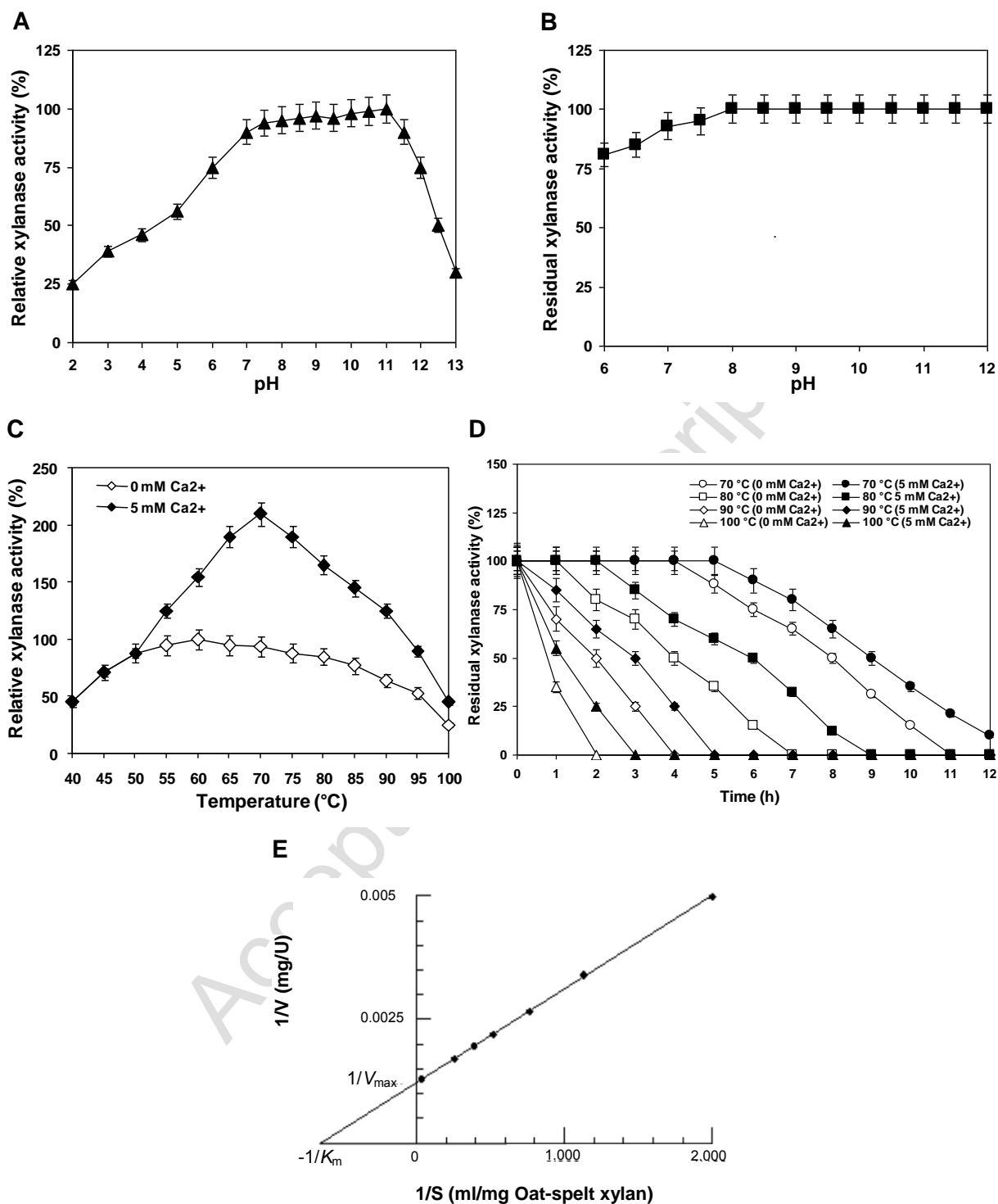
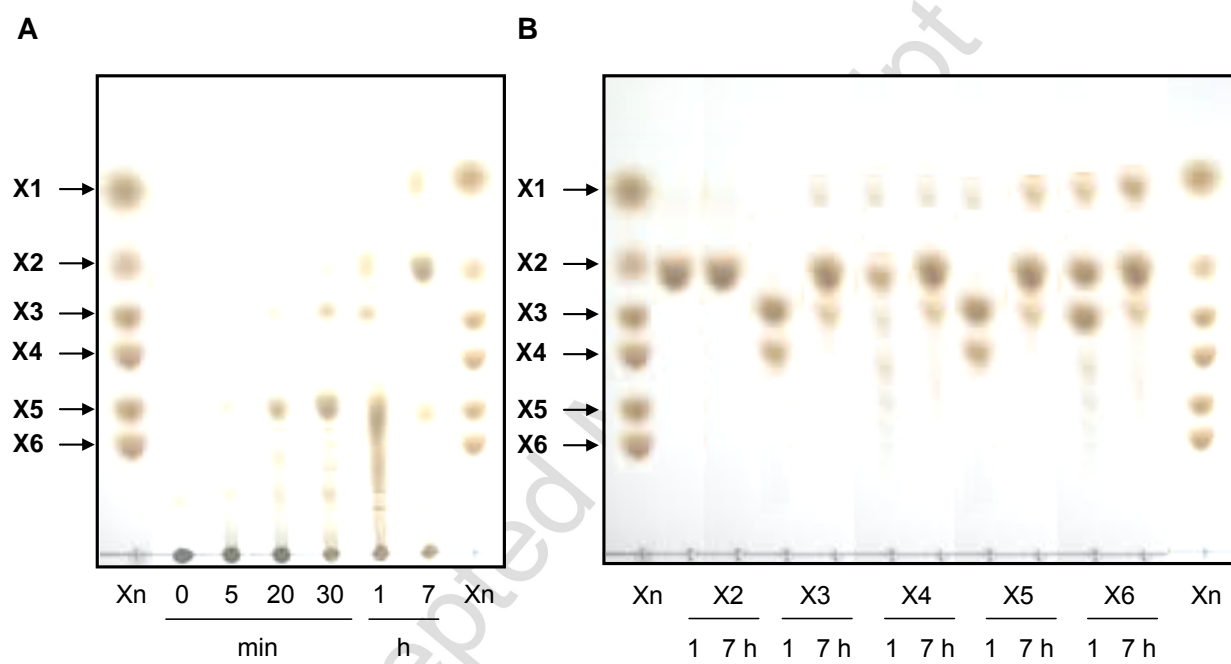


Figure 2

**Figure 3**