

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

Bouanane-Darenfed Amel, Boucherba Nawel, Bouacem Khelifa, Mohammed Gagaoua, Joseph Manon, Kebbouche-Gana Salima, Nateche Farida, Hacene Hocine, Ollivier Bernard, Cayol Jean-Luc, et al.

▶ To cite this version:

Bouanane-Darenfed Amel, Boucherba Nawel, Bouacem Khelifa, Mohammed Gagaoua, Joseph Manon, et al.. Characterization of a purified thermostable xylanase from Caldicoprobacter algeriensis sp. nov. strain TH7C1T. Carbohydrate Research, 2016, 419, pp.60-68. 10.1016/j.carres.2015.10.013 . hal-04156554

HAL Id: hal-04156554 https://hal.inrae.fr/hal-04156554

Submitted on 22 Sep 2023 $\,$

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

Page	1	of	33
------	---	----	----

1	Characterization of a purified thermostable xylanase from Caldicoprobacter algeriensis
2	sp. nov. strain TH7C1 ^T
3	
4	Amel Bouanane-Darenfed ^{a,b,*} , Nawel Boucherba ^c , Khelifa Bouacem ^a , Mohammed
5	Gagaoua ^d , Manon Joseph ^b , Salima Kebbouche-Gana ^e , Farida Nateche ^a , Hocine
6	Hacene ^a , Bernard Ollivier ^b , Jean-Luc Cayol ^b , Marie-Laure Fardeau ^b
7	
8	^a Laboratory of Cellular and Molecular Biology, Microbiology Team, Faculty of Biological
9	Sciences, University of Sciences and Technology of Houari Boumediene (USTHB), PO Box
10	32, El Alia, Bab Ezzouar, 16111 Algiers, Algeria
11	^b Aix Marseille University, IRD, University of Toulon, CNRS, Mediterranean Institute of
12	Oceanography (MIO), UM 110, 13288 Marseille, cedex 09, France
13	^c Laboratory of Applied Microbiology, Faculty of Nature Science and Life, University of
14	Bejaia, Targa Ouzemmour, 06000 Bejaia, Algeria
15	^d Maquav Team, Bioqual Laboratory, INATAA, Frères Mentouri Constantine 1 University,
16	Ain El-Bey Street, 25000 Constantine, Algeria
17	^e Laboratory of Biological Resources Conservation and Valuation, Faculty of Sciences,
18	M'Hamed Bougara- Boumerdes University, 06000 Boumerdes , Algeria
19	
20	
21	
22	
23	
24	* Corresponding author: Tel.: +213772546477/+2132124791; fax: +213 21247217. E-mail
25	addresses: amelbouanane@gmail.com; darenfedamel@yahoo.fr (A. Bouanane-Darenfed)

Page 2 of 33

26 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.
- 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.
- ▶ XYN35 is a potential strong candidate for application in the pulp bleaching industry.

32 Graphical Abstract



33

34

35 Abstract

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

Page 3 of 33

nov., strain TH7C1^T, a thermophilic, anaerobic strain isolated from the hydrothermal hot 38 spring of Guelma (Algeria). The maximum xylanase activity recorded after 24 h of 39 incubation at 70 °C and in an optimized medium containing 10 g/l mix birchwood- and oats 40 spelt-xylan, was 250 U/ml. The pure protein was obtained after heat treatment (1 h at 70 °C), 41 followed by sequential column chromatographies on Sephacryl S-200 gel filtration and 42 Mono-S Sepharose anion-exchange. Matrix assisted laser desorption ionization-time of flight 43 mass spectrometry (MALDI-TOF/MS) analysis indicated that the purified enzyme is a 44 monomer with a molecular mass of 35,075.10 Da. The results from amino-acid sequence 45 analysis revealed high homology between the 21 NH₂-terminal residues of XYN35 and those 46 of bacterial xylanases. The enzyme showed optimum activity at pH 11 and 70 °C. While 47 XYN35 was activated by Ca^{2+} , Mn^{2+} , and Mg^{2+} , it was completely inhibited by Hg^{2+} and 48 Cd²⁺. The xylanase showed higher specific activity on soluble oat-spelt xylan, followed by 49 beechwood xylan. This enzyme was also noted to obey the Michaelis-Menten kinetics, with 50 $K_{\rm m}$ and $k_{\rm cat}$ values on oat-spelt xylan being 1.33 mg/ml and 400 min⁻¹, respectively. Thin-51 layer chromatography soluble oat-spelt xylan (TLC) analysis showed that the final 52 hydrolyzed products of the enzyme from birchwood xylan were xylose, xylobiose, and 53 xylotriose. Taken together, the results indicated that the XYN35 enzyme has a number of 54 55 attractive biochemical properties that make it a potential promising candidate for future application in the pulp bleaching industry. 56

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

3

Page 4 of 33

59 **1. Introduction**

60 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 61 cellulose. Xylan, the major component in hemicellulose, is a heterogeneous polysaccharide 62 63 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 64 xylanolytic enzymes [2, 3]. Xylanases (EC 3.2.1.8) are among the key xylan-degrading 65 enzymes in that they cleave the long β -1,4-glycosidic bonds in the xylan backbone, resulting 66 in short xylooligosaccharides that are further hydrolyzed to D-xylose by β -1,4-xylosidases [1, 67 4, 5]. Those enzymes are major components of the xylanolytic systems produced by various 68 natural biodegradative microorganisms, including fungi and bacteria, which play crucial roles 69 in maintaining carbon flow in the carbon cycle and, hence, biomass turnover in nature. 70

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

Page 5 of 33

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

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

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

5

Page 6 of 33

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.

112 **2. Materials and methods**

113 **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.

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

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

Page 7 of 33

in a liquid medium containing (in g/l): mix birchwood xylan and oats spelt xylan, 10; yeast extract, 1; K₂HPO₄, 0.3; KH₂PO₄, 0.3; NaCl, 0.5; MgSO₄·7H₂O, 0.3; MnSO₄, 1; FeSO₄·7H₂O, 0.1; CaCl₂, 0.5; NH₄Cl, 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].

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 CaCl₂ 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.

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.

151 2.4. Xylanase purification procedure

Page 8 of 33

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

169 **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.

173 2.6. Electrophoresis and zymography

Page 9 of 33

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 181 proteins (50 µg) were separated by SDS-PAGE, the gel was incubated in a renaturation buffer 182 (Buffer A) for 30 min at 70 °C with gentle shaking. The treated gel was then overlaid on a 183 184 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 185 was removed, and the agar gel was immersed in 30 mg/ml Congo red dye for 30 min and 186 washed with 1.5 M NaCl solution to visualize zones of clearance corresponding to xylanase 187 188 activity.

189 2.7. Mass spectrometry and NH₂-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

Page 10 of 33

- 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

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

The following buffer systems, supplemented with 5 mM CaCl₂, were used at 100 mM: glycine-HCl for pH 2-5; 2-(*N*-morpholino) ethanesulfonic acid (MES) for pH 5-6; 4-(2hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) for pH 6-8; Tris-HCl for pH 8-9; and glycine-NaOH for pH 9-13.

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

The effect of temperature on xylanase activity was examined at 40-100 °C for 10 min at pH 11. In order to monitor thermostability, buffered samples of the purified enzyme were incubated for 12 h at 70, 80, 90, and 100 °C (100 mM glycine-NaOH buffer, pH 11) in the presence and absence of 5 mM CaCl₂. Aliquots were withdrawn at timed intervals and cooled on ice before being assayed to determine residual enzymatic activity under standard conditions. The non-heated enzyme, which was cooled on ice, was considered as a control (100%).

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

Page 11 of 33

219 The effects of several metallic ions assayed at concentrations of 5 mM were also investigated by adding divalent ions [Ca²⁺ (CaCl₂), Mn²⁺ (MnCl₂), Mg²⁺ (MgCl₂), Co²⁺ 220 (CoCl₂), Cu²⁺ (CuCl₂), Zn²⁺ (ZnCl₂), Ba²⁺ (BaCl₂), Fe²⁺ (FeCl₂), Ag²⁺ (AgNO₃), Cd²⁺ (CdCl₂), 221 and Hg^{2+} (HgCl₂)], and nonmetallic reagents, such as ethylene diamine (β -aminoethyl ether)-222 N,N,N',N'-tetraacitic acid (EDTA); ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-223 tetraacitic acid (EGTA); LD-dithiothreitol (LD-DTT); 2-mercaptoethanol (2-ME); and SDS for 224 1 h at 50 °C to the reaction mixture. Activity was expressed as a percentage of activity level 225 in the absence of metallic ions or inhibitor. 226

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

The substrate specificity profile of the XYN35 enzyme was studied using different 230 substrates. Enzymatic reactions were carried out in buffer A containing 20 mg/ml of each 231 232 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 233 measured by the rate of *p*-nitrophenol formed from 1 mM of the substrates in buffer A at 70 234 °C for 10 min during hydrolysis, and detected by spectrophotometry at 410 nm. One unit of 235 xylanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar 236 237 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 238 assay conditions. 239

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.

Page 12 of 33

243 2.12. TLC of hydrolysis products from xylan and xylooligosacharides

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

254 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 \pm 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).

262 **3. Results and discussion**

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

Page 13 of 33

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.

269 **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 270 strain using broth (500 ml) as a crude enzyme solution. The enzymatic preparation was heat-271 treated for 1 h at 70 °C. The precipitate formed was collected by centrifugation, dissolved in 272 273 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 274 Sephacryl S-200 column equilibrated with buffer B. Purification to homogeneity was 275 achieved using Mono-S Sepharose anion-exchange chromatography. Bound proteins were 276 eluted with a linear gradient of NaCl from 0 to 500 mM in buffer C at a rate of 40 ml/h. The 277 protein elution profile obtained at the final purification step indicated that the xylanase from 278 C. algeriensis sp. nov. strain $TH7C1^{T}$ was eluted at 170-230 mM NaCl (Fig. 1A). 279

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

Page 14 of 33

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 NH₂-terminal amino acid residues were determined to be 295 AQTITTDQTGYDGMYYTPWIF. This sequence was compared to other protein sequences 296 297 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, 298 particularly those from Actinomadura sp. Cpt20 (84% of identity), Streptomyces 299 roseiscleroticus (79% of identity), and Streptomyces sp. SWU10 (74% of identity). The 300 301 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 302 xylanase from *C. algeriensis* sp. nov. strain TH7C1^T belongs to the GH-11 family [48, 49]. 303

304 **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

Page 15 of 33

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.

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 range of pH between 6 and 12, with optimal activity being recorded at pH 11. The purified 316 xylanase from C. algeriensis sp. nov. strain $TH7C1^{T}$ showed enzyme stability over a broad 317 318 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 319 320 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 for 12 h at different pH values ranging between 6 and 12 (Fig. 2B). Its residual activity was 322 323 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.

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

Page 16 of 33

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

Page 17 of 33

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.

362

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 363 364 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 365 366 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 paper, CMC, avicel, $pNP-\beta$ -D-xylopyranoside, and $pNP-\beta$ -D-glucopyranoside were observed. 368 Furthermore, the results indicated that XYN35 enzyme displayed the classical Michaelis-369 370 Menten kinetics for the soluble oat-spelt xylan used as substrate (Fig. 2E). The kinetic parameters $K_{\rm m}$ and $V_{\rm max}$ values of XYN35 were 1.33 mg/ml and 595 μ mol min⁻¹ mg⁻¹, 371 respectively, using oat-spelt xylan as the substrate. Its deduced turnover number (k_{cat}) and 372 catalytic efficiency (k_{cat}/K_m) were found to be 400 min⁻¹ and 300.75 min⁻¹ mM⁻¹, respectively. 373 Above all, the results indicated that XYN35 enzyme from C. algeriensis sp. nov. strain 374 TH7C1^T was a "true xylanase". In fact, most of the xylanases previously investigated in the 375 literature were reported to display both xylanase and low cellulase activities [54]. 376 Interestingly, XYN35 was noted to hydrolyze only xylan and to be free from all the activities 377 378 of the other enzymes investigated, including those of carboxymethyl cellulase, β -xylosidase, and β -glucosidase (Table 4). 379

Page 18 of 33

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.

385

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).

389 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 390 molecular masses [degree of polymerization (DP) of > 5], and the molecular masses became 391 smaller (DP, 1–6) as the enzyme reaction progressed. Finally, the products converged on X1, 392 393 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 394 this can be a product from xylotriose together with xylobiose. Thus, the enzyme exhibited β -395 1,4-endoxylanase activity and suggesting its good potential in xylooligosaccharides 396 production. The xylooligosaccharides are sugar oligomers produced during the hydrolysis of 397 398 xylan which is the major component of plant hemicellulose, a heteropolysaccharide with 399 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

Page 19 of 33

404 reaction mixture of xylan seems to be derived from xylotriose. The degradation rates were: 405 xylohexose > xylopentose > xylotetraose > xylotriose. These results suggested that the 406 xynalase XYN35 recognizes xylooligosaccharides more avidly than xylotriose 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.

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.

416 **4. Conclusion**

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

Page 20 of 33

428 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, 429 because the products do not include glucooligosaccharides. Interestingly, XYN35 presented 430 high xynolytic activity, and was very effective in the pulp bleaching industry, thus offering a 431 potential promising candidate for application in biotechnological bioprocesses. Accordingly, 432 further studies, some of which are currently underway, are needed to investigate the XYN35 433 encoding gene, perform site-directed mutagenesis, and determine its structure-function 434 relationships. 435

436 Acknowledgments

This work was funded by the Algerian Ministry of Higher Education and Scientific 437 research. The authors wish to express their gratitude to Dr. H. Laribi-Habchi (Laboratory of 438 Chemical Engineering, Department of Process Engineering, Faculty of Technology, 439 University of Blida 1, Blida, Algeria) for its valuable help during the preparation of this 440 work. Thanks are also due to Mr. A. Smaoui and Mrs H. Ben Salem from the English 441 Language Unit at the Faculty of Science of Sfax, Tunisia for their valuable proofreading and 442 language polishing services. The authors would also like to extend their thanks to the 443 anonymous reviewers for their valuable comments and efforts during the revision of the 444 present manuscript. 445

Page 21 of 33

446 **References**

- 447 [1] T. Collins, C. Gerday, G. Feller, FEMS Microbiol Rev, 29 (2005) 3-23.
- 448 [2] H.L. Trajano, S. Pattathil, B.A. Tomkins, T.J. Tschaplinski, M.G. Hahn, G.J. Van
- 449 Berkel, C.E. Wyman, Bioresour Technol, 179C (2014) 202-210.
- 450 [3] J. Zhang, L. Viikari, Appl Biochem Biotechnol, 174 (2014) 1393-1402.
- 451 [4] C.R. Terrasan, B. Temer, M.C. Duarte, E.C. Carmona, Bioresour Technol, 101 (2010)
 452 4139-4143.
- 453 [5] Z. Xie, W. Lin, J. Luo, J Biotechnol, 214 (2015) 57-58.
- 454 [6] F. Elgharbi, H.B. Hlima, A. Farhat-Khemakhem, D. Ayadi-Zouari, S. Bejar, A. Hmida455 Sayari, Int J Biol Macromol, 74 (2015) 263-270.
- 456 [7] F. Elgharbi, A. Hmida-Sayari, Y. Zaafouri, S. Bejar, Int J Biol Macromol, 79 (2015)
 457 103-109.
- 458 [8] A.K. Badhan, B.S. Chadha, J. Kaur, H.S. Saini, M.K. Bhat, Bioresour Technol, 98
 459 (2007) 504-510.
- 460 [9] C. Techapun, N. Poosaran, M. Watanabe, K. Sasaki, J Biosci Bioeng, 95 (2003) 298461 301.
- 462 [10] Z. Taibi, B. Saoudi, M. Boudelaa, H. Trigui, H. Belghith, A. Gargouri, A. Ladjama,
 463 Appl Biochem Biotechnol, 166 (2012) 663-679.
- 464 [11] A. Sunna, G. Antranikian, Crit Rev Biotechnol, 17 (1997) 39-67.
- 465 [12] P. Zhou, H. Zhu, Q. Yan, P. Katrolia, Z. Jiang, Appl Biochem Biotechnol, 164 (2011)
 466 944-956.
- 467 [13] S.F. Yuan, T.H. Wu, H.L. Lee, H.Y. Hsieh, W.L. Lin, B. Yang, C.K. Chang, Q. Li, J.
- Gao, C.H. Huang, M.C. Ho, R.T. Guo, P.H. Liang, J Biol Chem, 290 (2015) 57395748.

21

Page 22 of 33

- 470 [14] N. Boucherba, M. Gagaoua, E. Copinet, A. Bettache, F. Duchiron, S. Benallaoua, Appl
- 471 Biochem Biotechnol, 172 (2014) 2694-2705.
- 472 [15] E. Luthi, N.B. Jasmat, P.L. Bergquist, Appl Environ Microbiol, 56 (1990) 2677-2683.
- 473 [16] L. Lama, V. Calandrelli, A. Gambacorta, B. Nicolaus, Res Microbiol, 155 (2004) 283474 289.
- 475 [17] D. Gowdhaman, V.S. Manaswini, V. Jayanthi, M. Dhanasri, G. Jeyalakshmi, V.
 476 Gunasekar, K.R. Sugumaran, V. Ponnusami, Int J Biol Macromol, 64 (2014) 90-98.
- 477 [18] L. Luo, J. Cai, C. Wang, J. Lin, X. Du, A. Zhou, M. Xiang, J Chem Technol
 478 Biotechnol, (2015) In press. DOI: 10.1002/jctb.4690.
- 479 [19] M. Irfan, M. Nadeem, Q. Syed, Protein Pept Lett, 20 (2013) 1225-1231.
- [20] T. Sriyapai, P. Somyoonsap, K. Matsui, F. Kawai, K. Chansiri, J Biosci Bioeng, 111
 (2011) 528-553.
- 482 [21] C. Holtz, H. Kaspari, J.H. Klemme, Antonie Van Leeuwenhoek, 59 (1991) 1-7.
- 483 [22] J.F. Ethier, S. Harpin, C. Girard, C. Beaulieu, C.V. Déry, R. Brzezinski, Can J
 484 Microbiol, 40 (1994) 362-368.
- 485 [23] S.P. George, A. Ahmad, M.B. Rao, Bioresour Technol, 78 (2001) 221-224.
- 486 [24] J. Blanco, J.J. Coque, J. Velasco, J.F. Martin, Appl Microbiol Biotechnol, 48 (1997)
 487 208-217.
- 488 [25] W. Sukhumsirichart, W. Deesukon, T. Kawakami, S. Matsumoto, W. Seesom, T.
 489 Sakamoto, Appl Biochem Biotechnol, 172 (2014) 436-446.
- 490 [26] W. Deesukon, Y. Nishimura, T. Sakamoto, W. Sukhumsirichart, Mol Biotechnol, 54
 491 (2013) 37-46.
- 492 [27] K. Bouacem, A. Bouanane-Darenfed, H. Laribi-Habchi, M.B. Elhoul, A. Hmida-Sayari,
- 493 H. Hacene, B. Ollivier, M.-L. Fardeau, B. Jaouadi, S. Bejar, Int J Biol Macromol, 81
- 494 (2015) 299-307.

Page 23 of 33

- 495 [28] Q.K. Beg, B. Bhushan, M. Kapoor, G.S. Hoondal, Enzyme Microb Technol, 27 (2000)
 496 459-466.
- 497 [29] O.V. Belova, A.V. Lisov, N.G. Vinokurova, A.A. Kostenevich, L.I. Sapunova, A.G.
 498 Lobanok, A.A. Leont'evskii, Prikl Biokhim Mikrobiol, 50 (2014) 171-176.
- 499 [30] M.N. de Almeida, V.M. Guimaraes, D.L. Falkoski, G.B. Paes, J.I. Ribeiro, Jr., E.M.
- 500 Visser, R.F. Alfenas, O.L. Pereira, S.T. de Rezende, Appl Biochem Biotechnol, 172
 501 (2014) 1332-1346.
- 502 [31] P. Turner, G. Mamo, E. Karlsson, Microbial Cell Factories, 6 (2007) 9.
- 503 [32] M. Kecha, S. Benallaoua, J.P. Touzel, R. Bonaly, F. Duchiron, Extremophiles, 11 504 (2007) 65-73.
- 505 [33] A. Bouanane-Darenfed, W. Ben Hania, H. Hacene, J.L. Cayol, B. Ollivier, M.L.
 506 Fardeau, Int J Syst Evol Microbiol, 63 (2013) 2049-2053.
- 507 [34] A. Bouanane-Darenfed, M.L. Fardeau, P. Gregoire, M. Joseph, S. Kebbouche-Gana, T.
 508 Benayad, H. Hacene, J.L. Cayol, B. Ollivier, Curr Microbiol, 62 (2011) 826-832.
- 509 [35] K. Bouacem, A. Bouanane-Darenfed, N. Boucherba, M. Joseph, M. Gagaoua, W. Ben
- Hania, M. Kecha, S. Benallaoua, H. Hacene, B. Ollivier, M.L. Fardeau, Appl Biochem
 Biotechnol, 174 (2014) 1969-1981.
- 512 [36] R.E. Hungate, in, A roll tube method for the cultivation of strict anaerobes. In: Norris
- JR, Ribbons DW (eds) Methods in microbiology, Vol 3B. Academic Press, New York,
 USA, (1969) pp. 117-132.
- 515 [37] W.E. Balch, G.E. Fox, L.J. Magrum, C.R. Woese, R.S. Wolfe, Microbiol Rev, 43
 516 (1979) 260-296.
- 517 [38] M.L. Fardeau, B. Ollivier, J.L. Cayol, in, Genus III. Caldanaerobacter. In: De Vos P,
- 518 Garrity GM, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman B
- 519 (eds) The Firmicutes, Vol 3, 2nd edn. Springer, Dordrecht, (2009) pp. 1241-1244.

Page 24 of 33

- 520 [39] G.L. Miller, Anal Chem, 31 (1959) 426-428.
- 521 [40] I. Maalej, I. Belhaj, N.F. Masmoudi, H. Belghith, Appl Biochem Biotechnol, 158
 522 (2009) 200-212.
- 523 [41] M.M. Bradford, Anal. Biochem., 72 (1976) 248-254.
- 524 [42] U.K. Laemmli, Nature, 227 (1970) 680-685.
- 525 [43] B.K. Kubata, T. Suzuki, H. Horitsu, K. Kawai, K. Takamizawa, Appl Environ
 526 Microbiol, 60 (1994) 531-535.
- 527 [44] N.A. Camacho, O.G. Aguilar, Appl Biochem Biotechnol, 104 (2003) 159-172.
- 528 [45] H.-Y. Fang, S.-M. Chang, C.-H. Lan, T.J. Fang, Process Biochem, 43 (2008) 49-55.
- 529 [46] J. Georis, F. Giannotta, J. Lamotte-Brasseur, B. Devreese, J. Van Beeumen, B. Granier,
- 530 J.M. Frere, Gene, 237 (1999) 123-133.
- [47] D. Zouari Ayadi, A. Hmida Sayari, H. Ben Hlima, S. Ben Mabrouk, M. Mezghani, S.
 Bejar, Int J Biol Macromol, 72 (2015) 163-170.
- 533 [48] W. Qiao, S. Tang, S. Mi, X. Jia, X. Peng, Y. Han, J Mol Catal B-Enzym, 107 (2014) 8534 16.
- 535 [49] G. Paës, J.-G. Berrin, J. Beaugrand, Biotechnol Adv, 30 (2012) 564-592.
- 536 [50] D. Sutay Kocabaş, S. Güder, N. Özben, J Mol Catal B-Enzym, 115 (2015) 66-75.
- 537 [51] C. Vafiadi, P. Christakopoulos, E. Topakas, Process Biochem, 45 (2010) 419-424.
- 538 [52] B. Jaouadi, B. Abdelmalek, D. Fodil, F.Z. Ferradji, H. Rekik, N. Zarai, S. Bejar,
 539 Bioresour Technol, 101 (2010) 8361-8369.
- [53] N. Boucherba, S. Benallaoua, C. Estelle, H. Hakim, F. Duchiron, Process Biochem, 46
 (2011) 519-525.
- 542 [54] N. Kulkarni, M. Lakshmikumaran, M. Rao, Biochem Biophys Res Commun, 263543 (1999) 640-645.
- 544 [55] B. Henrissat, Biochem J, 280 (Pt 2) (1991) 309-316.

Page 25 of 33

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 (\diamond) and xylanase activity (\bullet) 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 (**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

Page 26 of 33

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, xylotetraose; X5, xylopentaose; X6, xylohexaose; and Xn, standards mixture of X1–X6.

Page 27 of 33

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	$125,000 \pm 7,500$	$7,951 \pm 98$	15.72 ± 3	100	1
Heat treatment (1 h at 70 °C)	$97{,}500\pm4{,}755$	967 ± 41	100.82 ± 10	78	6.41
Sephacryl S-200	$38,025 \pm 1,211$	98 ± 4	388.01 ± 23	39	24.68
Mono-S Sepharose	$10,266 \pm 0,350$	17 ± 2	603.88 ± 37	27	38.35

^a The experiments were conducted three times and \pm standard errors are reported.

^b One unit of xylanase activity is defined as the amount of enzyme that released 1 μ mol 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].

ACCOR

Page 28 of 33

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.

Identity (%) Enzyme NH₂-terminal amino acid^a Endo-1, 4-β-xylanase from *Caldicoprobacter algeriensis* sp. AQTITTDQTGYDGMYYTPWIF 100 nov. strain TH7C1^T [N-terminal; 21 amino acids] (this work) A TITT QTGY GMYYTPW Endo-1, 4-β-xylanase from Actinomadura sp. Cpt20 [N-84 terminal; 19 amino acids] A TITT QTGYDGMYY W Endo-1, 4-β-xylanase from Streptomyces roseiscleroticus [N-79 terminal; 31 amino acids; (A57001)] Endo-1, 4-β-xylanase precursor from *Streptomyces* sp. A TITT QTG DGMYY W 74 SWU10 [241 amino acids; (BAK53483)] Endo-1, 4-β-xylanase precursor from Actinosynnema mirum AQTITT QTG YY 71 ATCC 29888 [233 amino acids; (CP001630)] Endo-1, 4-β-xylanase precursor from Thermobispora bispora AQTI QTGY Y 65 DSM 43833 [343 amino acids; (YP 003651058)]

^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.

Page 29 of 33

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
$\operatorname{Zn}^{2+}(\operatorname{ZnCl}_2)$	603 ± 37	100
$\mathrm{Co}^{2+}(\mathrm{Co}\mathrm{Cl}_2)$	784 ± 40	130
$Ba^{2+}(BaCl_2)$	92 ± 10	15
Fe^{2+} (FeSO ₄)	410 ± 17	68
$Ag^{2+}(AgNO_3)$	452 ± 24	75
$\mathrm{Cd}^{2+}(\mathrm{CdCl}_2)$	0	0
$\mathrm{Hg}^{2+}(\mathrm{HgCl}_2)$	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

PCOX

reported.

Page 30 of 33

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
СМС	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 \pm standard errors from three separate experiments.

PC.eQ

Page 31 of 33



Figure 1

Page 32 of 33



Figure 2

Page 33 of 33



Figure 3