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Biochemical characterization and low-resolution SAXS shape of a novel GH11 exo-1,4-β-xylanase identified in a microbial consortium

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Abstract

Biotechnologies that aim to produce renewable fuels, chemicals, and bioproducts from residual ligno(hemi)cellulosic biomass mostly rely on enzymatic depolymerization of plant cell walls (PCW). This process requires an arsenal of diverse enzymes, including xylanases, which synergistically act on the hemicellulose, reducing the long and complex xylan chains to oligomers and simple sugars. Thus, xylanases play a crucial role in PCW depolymerization. Until recently, the largest xylanase family, glycoside hydrolase family 11 (GH11) has been exclusively represented by endo-catalytic β-1,4- and β-1,3-xylanases. Analysis of a metatranscriptome library from a microbial lignocellulose community resulted in the identification of an unusual exo-acting GH11 β-1,4-xylanase (MetXyn11). Detailed characterization has been performed on recombinant MetXyn11 including determination of its low-resolution small angle X-ray scattering (SAXS) molecular envelope in solution. Our results reveal that MetXyn11 is a monomeric globular enzyme that liberates xylobiose from heteroxylans as the only product. MetXyn11 has an optimal activity in a pH range from 6 to 9 and an optimal temperature of 50 ºC. The enzyme maintained above 65% of its original activity in the pH range 5 to 6 after being incubated for 72 h at 50 ºC. Addition of the enzyme to a commercial enzymatic cocktail (CelicCtec3) promoted a significant increase of enzymatic hydrolysis yields of hydrothermally pretreated sugarcane bagasse (16% after 24 h of hydrolysis).

Keywords: GH11 exo-β-1,4-xylanase; Metatranscriptome; Biochemical characterization; Synergism; Small Angle X-ray Scattering.

42 **INTRODUCTION**

43 Use of residual plant biomass as a feedstock for the production of biofuel, chemicals, and
44 renewable materials, represents a feasible, sustainable and environmentally friendly alternative to fossil
45 fuel-derived products (Isikgor and Becer 2015; Sims et al. 2010; Silva et al. 2018). Conversion of plant
46 residues into these products relies on the controlled deconstruction and depolymerization of plant cell walls
47 (PCW) (Keegstra 2010). Because of the intricate ultrastructure and diversified linkage complexity of PCW,
48 efficient enzymatic processing of biomass-based feedstocks remains a challenge (Isikgor and Becer 2015;
49 Johnson 2016; Silva et al. 2018). The enzymatic depolymerization of PCW is currently one of the most
50 expensive technological steps in its valorization (Johnson 2016). Existing enzyme cocktails comprise
51 mixtures of different plant cell wall degrading enzymes (PCWDE), each one with a distinct mechanism of
52 action that synergistically reduce the PCW into simple sugars (Silva et al. 2018). Therefore, in order to
53 decrease associated costs and increase efficiency, there is a constant demand for new enzymes with
54 biochemical and biophysical properties that match industrial requirements, such as thermal and pH stability,
55 activity at a broad pH range, and different substrate specificities or new mechanisms of activity, to list a
56 few. Our current knowledge of enzymatic deconstruction of PCWs is incomplete and the discovery and
57 characterization of new enzymes with novel catalytic mechanisms will contribute to a better understanding
58 of PCW depolymerization.

59 Although PCWDE have been characterized from plants (Johansson et al. 2002; Suzuki et al. 2002)
60 and animals (Evangelista et al. 2015; Watanabe and Tokuda 2001; Pauchet et al. 2010), microorganisms
61 represent the main source for enzyme discovery. In this context, metatranscriptomic studies on complex
62 unculturable microbial communities have greatly enhanced the pace of identification of enzymes from
63 underexplored and uncultivable microorganisms (Castillo et al. 2013; Rittmann et al. 2006; Curtis et al.
64 2003; Duan and Feng 2010). For these reasons, we have conducted metatranscriptomic studies of a
65 microbial consortium, which was grown in a nutrient-limited medium enriched with sugarcane bagasse,
66 to selectively favor microorganisms capable of degrading PCW (Mello et al. 2016; Evangelista et al.
67 2018). In the course of these studies, a GH11 exo-acting β-1,4-xylanase, termed here MetXyn11, was
68 identified (GenBank accession number: ATY75129.1).

69 Xylanases cooperatively act on xylan (the major component of hemicellulose), which is composed
70 of a linear backbone chain of xylopyranose residues linked by β-1,4 glycosidic bonds and decorated with

71 β-D-galactopyranosyl, α-L-arabinofuranosyl, and α-D-glucuronic acid or its 4-*O*-methyl ether derivative
72 residues (Pollet et al. 2010; Paës et al. 2012; Kalim et al. 2015; Biely et al. 2016). Due to their essential
73 participation in biomass depolymerization, xylanases have been widely applied in several industrial sectors,
74 such as second-generation bioethanol production; prebiotic production; pulp treatment; xylitol production;
75 industrial waste treatment; and degumming of fibers for paper and textiles, to name a few (Kalim et al.
76 2015; Paës et al. 2012). Xylanases from GH10 and GH11 families are the most studied and widely used in
77 biotechnological applications. In contrast to other GH families that comprise xylanases, GH11 is known as
78 "true β-1,4-xylanase" family, because it is almost entirely composed of β-1,4-xylanases (Kalim et al. 2015;
79 Paës et al. 2012). Moreover, until very recently, all GH11 members had been characterized as endo-catalytic
80 enzymes that are highly specific for cleaving the internal linkages of the heteroxylans. However, our group
81 recently identified the first GH11 with exo-β-1,4-xylanase activity (Mello et al., 2016).

82 Considering the essential role of xylanases in PCW depolymerization and biotechnological
83 applications of these enzymes, here we report the identification and detailed characterization of a second
84 GH11 family member (MetXyn11) that displays exo-β-1,4-xylanase activity, and demonstrate its ability to
85 significantly increase sugar release when added to a commercial cellulase cocktail.

86

87 **MATERIALS AND METHODS**

88 **Cloning, heterologous expression, and purification**

89 MetXyn11was identified from a previously reported metatranscriptomic library (Mello et al. 2016;
90 Evangelista et al. 2018). Its amino acid sequence was analyzed using BLASTP (Altschul et al. 1990),
91 ExPASy (Wilkins et al. 1999), XtalPred (Slabinski et al. 2007) and SignalP 4.0 (Petersen et al. 2011)
92 software. The MetXyn11 open reading frame (ORF), devoid of the signal peptide coding sequence, was
93 cloned into the expression vector pETM11/LIC, using the ligation independent cloning (LIC) method
94 (Camilo and Polikarpov 2014). First, the DNA sequence target was amplified by PCR, in which the
95 genomic DNA extracted from the microbial consortia was used as a template. The following primers were
96 designed to amplify MetXyn11 sequence (LIC tails are shown in italic): MetXyn11_Forward 5´-
97 *CAGGGCGCCATG*GAACCCAAAATGCCACCTG-3´ and MetXyn11_Reverse 5´-
98 *GACCCGACGCGG*TTAACGGGGTGTTTCATCCC-3´. The resulting plasmid was designed to express

99 MetXyn11 fused with a 6xHis-tag at the N-terminal region, including a cleavage site for *Tobacco etch* virus
100 protease (TEV) between the two sequences (Camilo and Polikarpov 2014). This allows the proteolytic
101 6xHis-tag removal after the protein purification by Ni^{+2} affinity chromatography. The resulting plasmid
102 was propagated in *Escherichia coli* (DH5α) cells (Thermofischer, Waltham USA), and the purified plasmid
103 was used in the heat-shock transformation of *E. coli* Rosetta (DE3) cells (Novagen, Watertown USA) to
104 create the MetXyn11 expression strain.

105 MetXyn11 expression was carried out in LB medium at 37 ºC for 5 h ($O.D._{600} = 0.6$), followed by
106 an induction step at 18 ºC for 24 h, containing 1 mM IPTG. The cells were pelleted at 2.500 *x g* for 45 min
107 at 4 ºC, suspended in lyses buffer (50 mM Tris pH 8.0, 150 mM NaCl, 4 mM PMSF, 2 mM DTT, 10%
108 (v/v) glycerol and 50 $\mu g.mL^{-1}$ of lysozyme), incubated at 18 ºC for 20 min and sonicated on an ice bath
109 using a 550 Sonic Dismembrator Sonifier (Fisher Scientific, Hampton USA). Next, the cells were pelleted
110 (6,000 *x g*, 45 min at 4 ºC) and the supernatant was used for MetXyn11 protein purification.

111 Three chromatographic steps were used for MetXyn11 purification: two steps of Ni^{+2} affinity
112 chromatography, one before and one after TEV proteolysis; and a third step of size exclusion
113 chromatography. In the first step, MetXyn11 (~30 kDa) attached to 6xHis-tag was eluted using 50 mM Tris
114 pH 8.0, 150 mM NaCl and 125 mM imidazole. The sample was dialyzed to remove traces of imidazole and
115 then, incubated with 3 $mg.mL^{-1}$ of TEV protease at 4 ºC for 48 h. In the second purification step, MetXyn11
116 (~29 kDa) free of 6xHis-tag was eluted in 50 mM Tris pH 8.0 and 150 mM NaCl. The third purification
117 step was conducted on a Superdex™ 75 prep grade 16/60 (GE-Healthcare, Chicago USA) gel filtration
118 column equilibrated with 50 mM Tris pH 8.0 and 150 mM NaCl. The protein was concentrated to 1 $mg.mL^{-}$
119 ^{1}, using the 10 kDa Vivaspin Concentrator (GE-Healthcare, Chicago USA) at 1,500 *x g* and stocked at 4
120 ºC. The protein integrity and sample purity were confirmed by the 15% SDS-PAGE (sodium dodecyl
121 sulfate–polyacrylamide gel electrophoresis) analysis.

122 **Thermofluor assays**

123 To determine the best condition for the enzyme storage and handling, the enzyme's thermal stability
124 was evaluated using in several different buffered solutions using a thermal shift fluorescence (Thermofluor)
125 assay (Table S1, Supplementary Materials). Moreover, we also assessed the MetXyn11 tertiary structure
126 stability in different pH conditions, using the 50 mM sodium acetate/ borate/ phosphate (ABF) buffer in a
127 pH range from pH 2 to 10. The experiments consisted of 20 µL reaction containing 13 µM of the enzyme

128 in 50 mM buffer with 5 µL of SYPRO Orange dye 10X (Invitrogen, Carlsbad USA). Reactions were
129 performed in triplicate in a 96-well thin-wall PCR plate (Bio-Rad, Hercules USA). The plate was sealed
130 with Optical-Quality Sealing Tape (Bio-Rad, Hercules USA), then incubated in an iCycler iQ Real-Time
131 PCR Detection System (Bio-Rad, Hercules USA). The temperature ranged from 25 to 90 ºC, increasing 1
132 °C per minute, added of a holding step of 30 seconds at each point. The extrinsic fluorescence from the
133 probe was measured at 490/530 nm of excitation/emission wavelengths. The melting temperature (T_m) was
134 calculated by Boltzmann sigmoidal function, using the GraphPad Prism 6.0 software (GraphPad Software
135 Inc., La Jolla USA).

136 **Enzymatic assays**

137 The MetXyn11 enzymatic activity was quantified by the DNS method that measures the reducing end-
138 groups of saccharides (Miller 1959). All experiments were conducted in triplicate. Enzyme specificity was
139 assessed using 15 different substrates: Avicel, carboxymethylcellulose, Sigmacell20 and
140 hydroxyethylcellulose (all from Sigma, St. Louis USA); glucuronoxylan from beechwood; arabinoxylan
141 from rye flour, arabinan, debranched arabinan, β-glucan, xyloglucan, dextrin, galactomannan, larch
142 arabinogalactan, lichenan and mannan (all from Megazyme, Bray, Republic of Ireland). Since MetXyn11
143 showed significant activity only against glucuronoxylan, this substrate was used for the subsequent
144 biochemical experiments. The reaction consisted of 35 nM of enzyme mixed with 1% (w/v) glucuronoxylan
145 and 50 mM buffer ions in 50 µL final volume, which was incubated for 10 min. The reaction was stopped
146 by the addition of 100 µl of DNS reagent and heating at 95 ºC for 10 min, followed by cooling on ice for 1
147 min for color stabilization. Product absorbance was measured at 540 nm using the MultiSkan Spectrum
148 equipment (Thermo Scientific, Waltham USA), and a standard concentration curve of D-(+)-xylose (Sigma,
149 St. Louis USA) was used to express results in reducing sugars equivalents. All the obtained data were
150 analyzed using the GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla USA).

151 The optimum pH was evaluated in pH range from 2 to 10 in ABF buffer at 50 ºC. The optimal
152 temperature was assessed in a potassium phosphate buffered solution at 50 mM concentration and pH 7.0
153 in a temperature range from 30 to 70 ºC. In both sets of experiments the reactions were performed as
154 described above. Moreover, enzyme stability assays were performed, quantifying MetXyn11 residual
155 activity after enzyme pre-incubation under long periods in different temperatures or pHs. During the
156 thermal stability assays MetXyn11 was kept at 50 ºC in potassium phosphate buffer pH 6.0 for 120 h. For

157 the pH stability assay, MetXyn11 was maintained in ABF buffer at pH range from 2 to 10 at 50 ºC for 72
158 h. The residual activity was measured under the optimal temperature and buffer conditions. Furthermore,
159 under the optimal conditions, the enzyme activity was also evaluated upon addition of 15 different chemical
160 compounds (Table S2, Supplementary Materials). To evaluate enzyme kinetics, the reactions were
161 performed under the optimal conditions enzymatic activity, varying glucuronoxylan concentration from 0.1
162 to 18 $mg.mL^{-1}$

163 **Enzymatic cleavage pattern determined by high-performance anion exchange chromatography**
164 **(HPAEC)**

165 The soluble products released by the MetXyn11 catalytic action on heteroxylans (glucuronoxylan and
166 arabinoxylan) and on xylohexaose were analyzed using HPAEC. In experiments with heteroxylans,
167 reactions containing 1% of substrate and 35 nM of MetXyn11 were conducted under optimal conditions
168 for 24 h. For comparison, we also performed experiments using a typical endo-1,4-β-xylanase
169 (rGH11Xyn11B; Ghio et al. 2017) instead of MetXyn1, to compare their heteroxylan cleavage pattern. In
170 the experiments with xylohexaose, the reactions were conducted with 55 µM of substrate and analyzed in
171 1, 5, 10 and 15 min time points. A pool containing 5 µL of each reaction conducted in triplicate was diluted
172 10-fold, then centrifuged at 13,000 *x g* for 5 min and the supernatant was analyzed by a DIONEX ICS3000
173 instrument (DIONEX, Sunnyvale USA) connected to a CarboPac PA1 4 X 250 mm column (DIONEX,
174 Sunnyvale USA). The column was equilibrated with 100 mM NaOH at 1 $mL.min^{-1}$ for 5 min; the sugars
175 were separated using a gradient from 100 mM NaOH/0 mM NaAc to 100 mM NaOH/150 mM NaAc over
176 20 minutes.

177 **Supplementation of commercial enzymatic cocktail for pretreated plant biomass saccharification**

178 Hydrothermally pretreated sugarcane bagasse was used in saccharification assays with Celic CTec3 ±
179 MetXyn11. The biomass was provided by the Raízen Group (Costa Pinto/Piracicaba, São Paulo, Brazil).
180 The raw material was rinsed with hot water (50 °C ± 5 °C) and milled using a knife mill. Next, it was dried
181 in the oven at 60 °C for 24 h and hydrothermally pretreated.

182 Hydrothermal pretreatment was performed using hot water for 30 minutes at 160 °C in a pretreatment
183 reactor AU/E-20 model (Regmed, Osasco Brazil). The pressure was kept at 7 bar and a 1:10 solid to liquid
184 ratio (grams of bagasse/mL of water) was used (Santo et al. 2018). The pretreated sugarcane bagasse used
185 in the study contained 76.8% ± 1.5% of glucan, 6.1% ± 0.1% of xylan, 17.8%± 0.5% of lignin and 1.4% ±

186 0.02% of ash content. The saccharification of pretreated sugarcane bagasse was carried out at a substrate
187 concentration of 10% (w/v). Cellic CTec3 (Novozymes, Kalundborg DKK) protein loading was 5 mg/g of
188 substrate in the control reactions. To evaluate effect of MetXyn11 supplementation 0.125 mg of the enzyme
189 per g of substrate was added to Cellic CTEc3 reaction. The reactions were conducted in a citrate buffer (50
190 mM, pH 5.0), at 50 °C up to 72 h. The soluble hydrolysate products were analyzed by high performance
191 liquid chromatography (HPLC) (Shimadzu, Kyoto Japan), equipped with refractive index detector and UV-
192 VIS spectrophotometer. Aminex HPX-87 H (Bio-Rad, Hercules USA) column was used and 5 mM H_2SO_4
193 solution at 65 °C was utilized as a mobile phase (flow rate 0.6 $mL.min^{-1}$). Glucose and xylose were used as
194 standards.

195 **Homology modelling of MetXyn11**

196 A multiple alignment was performed, using T-Coffe Server (Notredame et al. 2000), for comparative
197 analyses between MetXyn11 amino acid sequence and some of the traditional GH11 endo-β-1,4-xylanases,
198 and also Compost21_GH11 enzyme, that represents the unique previously reported GH11 exo-β-1,4-
199 xylanase (Mello et al. 2016). Moreover, a three-dimensional (3D) homology model of MetXyn11 was
200 generated using the MetXyn11 amino acid sequence and using the Compost21_GH11 crystal structure
201 (PDB id: 5VQJ) as inputs in the I-Tasser software (Yang et al. 2015). Following this, the 3D homology
202 model was superimposed with the crystal structures of the GH11 members used in our amino acid sequences
203 alignment, using Pymol program (DeLano 2002)

204 **SAXS studies**

205 Small angle X-ray scattering (SAXS) experiments were carried out at the D02A-SAXS1 beamline of
206 the Brazilian Synchrotron Light Laboratory (LNLS, Campinas, Brazil). To remove protein aggregates,
207 samples (at the concentrations of 1, 7.5 and 15 $mg.mL^{-1}$ in 50 mM potassium phosphate pH 7.0 and 150
208 mM NaCl) were centrifuged at 17,000 *x* g for 5 min at 4 °C prior to measurements, and the supernatant was
209 collected. Next, the supernatant was loaded in a 1 mm path-length capillary cell and exposed to X-rays
210 during 10 frames of 30 s, with intervals of 1 s between each frame. The data sets were collected using a
211 monochromatic X-ray beam (λ= 1.55 Å) with a Pilatus 300 area detector (Dectris, Baden Switzerland). To
212 cover a scattering vector ($q=4\pi/\lambda\sin(\theta)$, being 2θ the scattering angle) range from 0.012 to 0.400 $Å^{-1}$, the
213 distance between sample and detector was adjusted to ~1,000 mm. The scattering from buffer alone was
214 subtracted from the sample`s scattering. Comparative analysis between each frame was used to verify

215 radiation damage. Guinier analysis (Guinier and Fournet 1955; Konarev and Svergun 2015; Perry and
216 Tainer 2013) was applied to verify monodispersivity and to calculate the radius of gyration (R_g), which was
217 also estimated by an indirect Fourier transform method, using the GNOM program (Svergun 1992). The
218 distance distribution function P(r) was analyzed by GNOM and the maximum particle dimension (D_{max})
219 was determined. Ten ab initio envelope models were generated by DAMMIN (Franke et al. 2009), then
220 aligned and averaged by DAMAVER (Volkov and Svergun 2003), to build the final MetXyn11 molecular
221 envelope. The 3D-homology model of MetXyn11 structure was inputted into Crysol online software
222 (Svergun et al. 1995) to generate a theoretical SAXS profile, which was compared to the experimental
223 SAXS profile. Moreover, the 3D-homology model of MetXyn11 structure and the final model of MetXyn11
224 molecular envelope were superimposed by SUPCOMB program (Kozin and Svergun 2001).

225

226 **RESULTS**

227 **Bioinformatic analysis**

228 Bioinformatic analysis revealed that MetXyn11 has 268 amino acid residues (including a putative 21-
229 residue signal peptide) and a molecular mass of ~28 kDa. The enzyme has a calculated isoelectric point of
230 6.22. MetXyn11 has the highest amino acid sequence similarity (id > 80%) with Compost21_GH11 (PDB
231 id: 5VQJ) and another GH11 exo-β-1,4-xylanase (GenBank: ATY75130.1), identified in the same
232 metatranscriptomic library. The organism(s) that carries (carry) these exo-β-1,4-xylanase genes is(are) still
233 unknown, however, MetXyn11 also shows high similarity to other known GH11 endo-β-1,4-xylanase from
234 bacterial sources, particularly with the enzymes from the *Cellvibrio* genus including *Cellvibrio* sp.
235 PSBB006, *Cellvibrio mixtus*, *Cellvibrio* sp. PSBB023 and *Cellvibrio* sp. pealriver (all with id = 78%).

236 Comparative analyses between the amino acid sequences and 3D structures of MetXyn11,
237 Compost21_GH11 and some typical GH11 endo-β-1,4-xylanases revealed some notable differences
238 between these enzymes (Fig. 1). The first noticeable difference between these enzymes is the extended
239 amino acid sequence at the N-terminus of MetXyn11, which was predicted as being an extra α-helix. The
240 second difference between these enzymes, is the two extra loops shared by MetXyn11 and
241 Compost21_GH11, which are absent in typical GH11 endo-β-1,4-xylanases.

242 **Cloning, heterologous expression and purification**

243 MetXyn11 ORF was cloned into the pETM11/LIC expression vector, and used to transform *E. coli*
244 Rosetta cells. The enzyme was successfully overexpressed as a soluble protein fused to the 6xHis-tag (Fig.
245 2). The enzyme was purified in three purification steps and eluted as a single peak after size exclusion
246 chromatography, confirming sample purity. The MetXyn11 molecular mass estimated by SDS-PAGE (~28
247 kDa) is in agreement with the theoretical molecular mass predicted from its amino acid sequence (Fig. 2).

248 **Substrate specificity and hydrolytic products**

249 MetXyn11 specific activity was tested against 15 different plant polysaccharides. Among all the
250 tested potential substrates, the enzyme showed detectable enzymatic activity only against glucuronoxylan
251 and arabinoxylan. MetXyn11 has a much higher activity for glucuronoxylan than to arabinoxylan. Indeed,
252 as evaluated by the DNS method, MetXyn11 exhibited a 50 times lower specific activity for arabinoxylan
253 when compared to glucuronoxylan. To evaluate the products released by MetXyn11 on these substrates,
254 we analyzed them using HPAEC-PAD (Fig. 3). For both substrates, the enzyme released xylobiose as the
255 unique product, indicating an exo catalytic pattern. For comparison both substrates were also hydrolysed
256 by *Paenibacillus* sp. A59 GH11 endoxylanase (rGH11XynB; Ghio et al., 2018) kindly donated by Prof.
257 Eleonora Campos (INTA, Argentina), revealing strikingly different hydrolytic patterns. Liberation of
258 xylobiose by MetXyn11 was also confirmed by the experiments using xylohexaose as a substrate (Fig. 4).
259 In these experiments, the profile of xylo-oligosaccharides evaluated over the 15 min of reaction revealed
260 the conversion of xylohexaose into xylotetraose plus xylobiose, then, the conversion of xylotetraose to
261 xylobiose. Since the xylohexaose used here had a small amount of xylopentaose contamination (Fig. S1,
262 Supplementary Materials), it was also possible to observe the conversion of the xylopentaose in xylotriose
263 plus xylobiose.

264 **Optimal conditions for MetXyn11 stability**

265 Optimal conditions for MetXyn11 stability were determined by assessing its tertiary structural
266 integrity in several different buffer solutions at different temperatures by Thermofluor analysis (Ericsson
267 et al. 2006) (Table S1, Supplementary Materials). The results showed a good fit with the Boltzmann
268 sigmoidal equation, which is usually applied for non-linear fitting of thermal denaturation data, revealing
269 that MetXyn11 tertiary structure has a T_m value of 55 ºC in its best buffer conditions (Fig. 5). MetXyn11 is
270 most stable between pHs 5.5 and 7.0, in sodium phosphate pH 5.5, MES pH 5.8-6.5, Bis-Tris pH 6.0-7.0
271 and HEPES pH 7.0 (T_m = 55 ºC) buffers, followed by several other buffers with pHs between 4.7 and 8.5

272 (T_m = 51-54 ºC). The enzyme was least stable at the extreme pHs: HCl pH 2.0 (T_m = 26 ºC), citric acid pH
273 3.0 (T_m = 46 ºC) and sodium carbonate pH 9.5-10.0 (T_m = 46 ºC).

274 **Effects of metal ions and chemicals on enzyme activity**

275 MetXyn11 activity was evaluated in the presence of different metal ions and chemicals (Table S2,
276 Supplementary Materials). The major detrimental effects were observed for SDS (sodium dodecyl sulfate)
277 and Fe^{+3} that completely inactivated the enzyme, followed by Mn^{+2}, Fe^{+2}, Cu^{+2}, Co^{+2} and Ca^{+2} which
278 imparted a loss of approximately 78%, 46%, 46%, 21% and 20% of its catalytic activity, respectively. In
279 contrast, Li^{+2}, Ni^{+2}, K^{+} and Mg^{+2} enhanced MetXyn11 activity by approximately 6%, 10%, 12% and 23%,
280 respectively. Neutral surfactants, Tween-20 and Triton-100X respectively provoked a loss of 16.5 % and a
281 gain of 8% on MetXyn11 activity, respectively. The reducing agents DTT and β-mercaptoethanol caused
282 decrease of the enzyme activity equal to 15% and 5%, respectively.

283 **MetXyl11 optimal activity and the enzyme kinetics**

284 MetXyn11 activity was highest around 50 ºC; however, the enzyme maintained over 80% of its
285 optimal activity at both 40 ºC and 60 ºC (Fig. 6A). We also determined the MetXyn11 activity profile
286 following variations of pH. The enzyme showed the best performance at pH 6-7, retaining above 60% of
287 the maximum activity at pH 5, approximately 90% at pH 8-9 and 80% at pH 10. No significant activity was
288 detected at pHs below 5 (Fig. 6B). According to the BRENDA database (Schomburg et al. 2017), the
289 optimal temperature of GH11 xylanases (derived from several distinct microorganisms) varies from 22 to
290 90 ºC and the optimal pH from 2 to 11. However, most of these enzymes have an optimal activity between
291 40 and 65 ºC and pH 4-7. Therefore, the MetXyn11 activity profile is consistent with those of most of
292 GH11 xylanases. Kinetics assays were performed under the enzyme optimal conditions, using
293 glucuronoxylan as a substrate (Fig. 6C). Interestingly, the reactions revealed a "first-order reaction" profile
294 even at 18 mg/L of substrate. Nevertheless, based on the data obtained predicted values of V_{max} =50.30
295 $\mu M.s^{-1}$, K_M of 121 $mg.mL^{-1}$, k_{cat} of 1437 s^{-1} and a catalytic efficiency (k_{cat}/K_M) of 11.88 $mL.s^{-1}.mg^{-1}$,
296 respectively, were obtained using GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla USA).
297 The obtained K_M is consistent with a very poor binding of the enzyme to insoluble substrate
298 (glucuronoxylan). A high K_M value offsets an elevated turnover number, resulting in a low catalytic
299 efficiency of the enzyme on glucuronoxylan.

300 **Thermal and pH stability of MetXyn11**

301 MetXyn11 stability was evaluated both as a capacity to maintain the enzyme fold and a capacity
302 to maintain its catalytic activity. For the former, we used Thermofluor analyses, in which MetXyn11 was
303 exposed to a wide pH range during a linear increment on temperature until its thermal denaturation. The
304 results show a signal from the fluorescence probe detected at the beginning of the experiment at pH 2,
305 indicating enzyme denaturation (Fig. 5B). At pH 3, the MetXyn11 structure exhibited a low T_m value of 34
306 ^oC, increasing to 44 ^oC at pH 4 until the maximum value of 52 ^oC at pH 6, and then discretely decreasing
307 until 43 ^oC at pH 10 (Fig. 5B). Furthermore, residual activity of MetXyn11 was measured during 120 h
308 under the optimal conditions for its enzymatic activity (Fig. 5C). MetXyn11 maintained almost 80% of its
309 original activity after being incubated for 24 h, followed by 67%, 65%, 45% and 20%, respectively, after
310 48 h, 72 h, 96 h and 120 h. MetXyn11 residual activity was also measured after 72 h at optimal temperature,
311 but varying the pH from 2 to 10 (Fig. 5D). MetXyn11 presented no significant residual activity for pHs 2
312 and 3. At pH 4, MetXyn11 retained 14% of its initial activity, about 70% at pH 5-6, 40% at pH 7, followed
313 by a gradual decrease to 18% at pH 10.

314 **Enhancement of biomass hydrolysis by MetXyn11 supplementation to commercial enzymatic**
315 **cocktail**

316 To test the capacity of MetXyn11 to enhance biomass hydrolysis by a commercial enzymatic
317 cocktail, we hydrolysed pretreated sugar cane bagasse using Cellic CTex3 alone and also supplemented by
318 MetXyn3. Although MetXyn11 action alone did not lead to any detectable levels of released xylose or
319 glucose, the enzyme addition to Cellic CTec3 led to a significantly enhanced hydrolytic activity of the
320 cocktail on sugarcane bagasse as compared with the Cellic CTec3 alone. The levels of cellulose hydrolysis
321 achieved in 24 h, 48 h, and 72 h, were 46.9%, 59,4%, and 63.3% respectively compared to Cellic CTec3
322 alone, while they reached 54.5%, 67%, and 68.7% when Cellic CTec3 was combined with MetXyn11 (Fig.
323 7A). Corroborating with the literature (Väljamäe et al. 1999; Boisset et al. 2001; Pellegrini et al. 2018), the
324 relative increase in the hydrolysis yields was higher at the beginning of the reaction (at 24 h an increase in
325 cellulose conversion was 16.6%) whereas at 48 h and 72 h the observed gains were 13.3%, and 8.6%. (Fig.
326 7B). Levels of xylan hydrolysis, which reached 51.5%, 59.5% and 64% in 24 h, 48 h and 72 h using Cellic
327 CTec3 alone, were increased to 53.9%, 62.2% and 66.7% when Cellic CTec3 was supplemented by

328 MetXyn11 (Fig. 7C&D). Thus, MetXyn11 addition provoked a significant increase of enzymatic hydrolysis
329 of pretreated sugarcane bagasse when used in combination with Celic CTec3.

330

331 **Low resolution shape of MetXyn11 in solution**

332 SAXS studies were performed to determine the MetXyn11 low-resolution molecular envelope in
333 solution. Structural parameters, data plot curves and the molecular envelope model obtained from SAXS
334 experiments are summarized in Table S3 (Supplementary Materials), Figs. 8 and 9. Analysis of the initial
335 *q*-region of the scattering curves using Guinier approximation (*lnI(q) versus* q^2) exhibits the linear
336 correlation expected for monodisperse samples, indicating homogeneity of the particles in solution (Fig.
337 8A). In addition, the calculated R_g (18.52 Å) from each scattered frame remained constant (within the
338 experimental errors) along the different protein concentrations, indicating: first, an absence of radiation
339 damage and, second, an absence of attractive or repulsive interactions between the particles.

340 The distance-distribution p(r) plot has an almost perfect bell-shape, which is characteristic of well-
341 defined spherical particles, but the curve also shows an extended tail with a small second peak at the highest
342 *q*-region (Fig. 8B), indicating that MetXyn11 might have a small module that protrudes from the overall
343 globular shape (Perry and Tainer 2013). Moreover, D_{max} and R_g of MetXyn11 are equal to, respectively, 58
344 Å and 17.67 Å, which is similar to the data obtained from Guinier analysis. The Porod-Debye plot (q^4 *x I(q)*
345 *versus* q^4) displays a clear plateau, which is consistent with the observation that MetXyn11 is a globular-
346 like protein that lacks disordered regions (Fig. 8C) (Perry and Tainer 2013; De Oliveira et al. 2015; Rambo
347 and Tainer 2011). The dimensionless Kratky curve (q^2 *x I(q) versus q*), which provides a notion about the
348 degree of the particle compactness, has a well-defined maximum very close to 1.1 at $qR_g = 1.6$. That is,
349 again, coherent with a nearly globular protein in solution (Fig. 8D) (Rambo and Tainer 2011; Perry and
350 Tainer, 2013). Furthermore, at $qR_g > 6$ there is a subtle elevated baseline, suggesting that MetXyn11 may
351 exhibit some degree of flexibility (Perry and Tainer 2013; De Oliveira et al. 2015). All the experimental
352 SAXS data fitted well to the theoretical SAXS plots generated from the 3D-homology model of MetXyn11
353 structure. Finally, the low-resolution envelope snugly fits the 3D-homology model of the MetXyn11,
354 revealing a monomeric globular-like protein with a protuberance at its N-terminus (Fig. 9).

355

356 **DISCUSSION**

357 **A novel GH11 xylosidase**

358 Substrate specificity assays using different plant polysaccharides revealed that MetXyn11
359 exhibited activity only on glucuronoxylan and arabinoxylan. These results are in line with the previous
360 observations since all known GH11 members are specific towards heteroxylans. Indeed, GH11 xylanases
361 are considered to be "true" β-1,4-xylanases as compared to the other GH families (Pollet 2010; Paës et al.
362 2012), which are frequently able to act on different polysaccharides in addition to heteroxylans. For
363 example, there are known bifunctional xylanases from GH16, GH43 and GH62 that have two distinct
364 catalytic domains, one with xylanase activity, while the other having glucanase activity (for GH16) or
365 arabinase activity (for both GH43 and GH62) (Paës et al. 2012). Moreover, GH5, GH8, GH10, and GH30
366 xylanases, which have a unique catalytic domain, show more versatility than members of the GH11 family.
367 Some GH5 enzymes are specific for heteroxylans, however the GH5 family also includes cellulases,
368 glucanases and mannanases (Pollet et al. 2010; Cantarel et al. 2009; Lombard et al. 2013). The same applies
369 for the GH8 family, with a substitution of glucanases and mannanases for chitosanases and licheninases
370 (Pollet et al. 2010; Cantarel et al. 2009; Lombard et al. 2013). The GH30 family contains both glucanases
371 and galactanases (Cantarel et al. 2009; Lombard et al. 2013). Finally, the GH10 family mostly comprises
372 endo-β-1,4-xylanases, with a few examples of endo-β-1,3-xylanases and β-1,4-xylosidases. Furthermore,
373 GH10 enzymes can hydrolyze some glucose-derived substrates such as aryl-cello-oligosaccharides (Pollet
374 et al. 2010).

375 The much lower activity of MetXyn11 towards arabinoxylan as compared to glucuronoxylan could
376 be explained by a difference in the substrate´s decorations. Glucuronoxylan used in our experiments is only
377 ~13% decorated by glucuronic acid, while arabinoxylan is ~40% decorated with arabinose residues. It is
378 well known that GH11 xylanases have a narrow catalytic cleft unable to accommodate and cleave branched
379 substrates (Pollet et al. 2010; Paës et al. 2012), which could explain MetXyn11 preference for
380 glucuronoxylan as compared to arabinoxylan.

381 Quite remarkably though, despite having a profile of substrate specificity considered common
382 among all GH11 endo-β-1,4-xylanases, MetXyn11 revealed an uncommon pattern of hydrolytic products
383 (Fig. 3 and Fig. 4). Typical GH11 endo-β-1,4-xylanases release both xylobiose and xylotriose as main
384 undecorated products from heteroxylans (Biely et al. 2016; Pollet et al. 2010). In addition, GH11 family

385 members also generate longer xylooligosaccharides (such as xylotetraose) linked to aldopentauronic acid
386 or by L-arabinofuranosyl residue at the penultimate xylopyranosyl residue from the non-reducing end as
387 their major decorated products released from glucuronoxylan and arabinoxylan, respectively (Biely et al.
388 2016). In contrast, our HPAEC analyses identified xylobiose as the unique product released by MetXyn11
389 both from glucuronoxylan and arabinoxylan, which is characteristic with its exo-catalytic activity (Fig. 3).
390 Furthermore, our HPAEC results clearly show a conversion of xylohexaose into xylotetraose plus
391 xylobiose, xylotetraose in xylobioses, and xylopentaose in xylotriose plus xylobiose. These results indicate
392 that MetXyn11 cleaves off terminal xylobiose molecules from xylooligosacharides and, also heteroxylans.

393 Our results are perfectly in line with the only other reported GH11 exo-β-1,4-xylanase
394 (Compost21_GH11) (Mello et al. 2017). The Compost21_GH11 hydrolysis of glucuronoxylan and
395 xylooligosaccharides also revealed xylobiose as a unique product liberated by the enzyme. The crystal
396 structure of Compost21_GH11 (PDB: 5VQJ) has the typical β-jelly-roll fold shared by all the other 32
397 GH11 xylanase structures deposited in PDB. However, Compost21_GH11 has two extra loops not present
398 in the other family members (Fig. 1B). These two extra loops are very close to the cleft and one of them
399 clearly blocks part of the catalytic groove, evidencing its contribution for the uncommon exo-catalytic
400 mechanism. Amino acid sequences alignment between MetXyn11 and Compost21_GH11 (Fig. 1A)
401 revealed that MetXyn11 also presents the two extra loops, which was also confirmed by the 3D
402 superposition between the crystal structure of Compost21_GH11 and the 3D-homology model of the
403 MetXyn11 structure (Fig. 1C). Therefore, a MetXyn11 exo-catalytic mechanism could be mediated by the
404 presence of these two extra loops.

405 Finally, our enzymatic kinetics results are consistent with the structural determinants of MetXyn11
406 (Fig. 6). A typical narrow cleft of all GH11 family enzymes decorated with two uncommon extra loops
407 (that might mediate MetXyn11 action as an exo-xylanase) significantly restrict MetXyn11 binding and
408 recognition of heteroxylans. This could explain why MetXyn11 kinetics display a "first-order reaction"
409 profile (as expected for very low concentrations of substrate binding-sites) even at high concentrations of
410 glucuronoxylan.

411 **Molecular shape of MetXyn11**

412 The analyses of amino acid sequence, SDS-PAGE, 3D-homology model and SAXS data provided
413 important information about the size, shape, compactness, and flexibility of MetXyn11. These data show

414 that MetXyn11 is a monomeric globular-like enzyme of ~28 kDa, with a β-jelly-roll fold and a high degree
415 of compactness, R_g between 17.6 and 18.5 Å and D_{max} close to 58 Å. These results are consistent with other
416 GH11 xylanases from a wide diversity of microorganisms, as reviewed by Paës and collaborators on the
417 basis of comparison of the biochemical and biophysical properties of 164 GH11 enzymes (Paës et al. 2012).
418 This paper shows that xylanases uniquely formed by the catalytic module are dense globular proteins with
419 molecular masses between 18 and 31 kDa, which have the same β-jelly-roll fold. Moreover, SAXS studies
420 of a 21-kDa enzyme from *Trichoderma longibrachiatum* showed R_g and D_{max} close to 17 Å and 50 Å,
421 respectively (Kozak 2006). As suggested by several authors, a relative small size and compactness of GH11
422 xylanases facilitate their penetration into the inner part of PCW, consequently, indicating that these
423 enzymes might initiate the PCW deconstruction, thus favoring consecutive action of larger enzymes (i.e
424 multi-domains enzymes) (Paës et al. 2012; Beaugrand et al. 2005).

425 Despite having the β-jelly-roll fold (two twisted antiparallel β-sheets and a single α-helix,
426 resembling the shape of a partially closed right hand) that is traditional for all GH11 enzymes, our 3D-
427 homology model and SAXS data revealed an unusual extra α-helix at the MetXyn11 N-terminus, which is
428 not present in all the 33 GH11 xylanases deposited in PDB, including the Compost21_GH11. This α-helix
429 has a considerable degree of flexibility, as suggested by the Kratky plot, and promotes a clear protuberance
430 at the N-terminal region of the MetXyn11 molecular envelope. The role of the additional α-helix is not yet
431 clear, thus requiring further experimental studies.

432 **Possible biotechnological applications for MetXyn11**

433 As aluded to above, xylanases, especially those from GH10 and GH11 families, have a wide range
434 of industrial applications (Polizeli et al. 2005; Kalim et al. 2015; Biely et al. 2016). The ability of
435 MetXyn11 to liberate xylobiose as the only soluble product might be advantageous for pre- and pro-
436 biotic formulations. Xylo-oligosaccharides find applications in the food sector due to their health benefits
437 and some biochemical characteristics which are considered advantageous when compared to other oligo-
438 saccharides. For example, xylo-oligosaccharides are stable over a wide pH range (2.5-8.0) and also at
439 temperatures up to 100 °C, in contrast to others non-digestible oligosaccharides such as fructo-
440 oligosaccharides, for example, that are unstable at the human gastric acid pH (Vazquez et al. 2000; Kumar
441 et al. 2012). Furthermore, xylobiose has a higher antifreeze activity than glucose, sucrose and maltose, and
442 also has a water activity similar to that shown by glucose (Vazquez et al. 2000; Kumar et al. 2012). Besides,

443 xylobiose is not carcinogenic, has acceptable odor and low calories, which favors its use in diet products
444 formulation (Vazquez et al. 2000; Kumar et al. 2012).

445 In addition, MetXyn11 has the highest specific activity around 50 ºC, and also presents good
446 stability at this temperature for 72 h or longer. These are important enzymatic properties for industrial
447 applications, since temperatures close or above 50 ºC are usually required in the processing of complex
448 polysaccharides to reduce its high viscosity and also help to prevent the undesirable growth of mesophilic
449 contaminants (Kozak 2006). Moreover, MetXyn11 has a wide pH range of activity (pH 5-10),
450 demonstrating high stability (70%) between pHs 5 and 6 after 72 h at 50 ºC. These results suggest that
451 MetXyn11 could be used both in the processes that require alkaline pHs (i.e pulp and paper biobleaching)
452 (Walia et al. 2017) and also in the processes which need acid pHs (i.e fruit juice and wine preparation)
453 (Beaugrand et al. 2005).

454 Finally, MetXyn11 could be used as a complement of commercial enzymatic cocktails aiming for
455 better yields of plant biomass enzymatic hydrolysis, as demonstrated by our Cellic CTec3 complementation
456 experiments. MetXyn11 supplementation not only enhances levels of xylan hydrolysis, but also
457 considerably increases levels of cellulose hydrolysis by Cellic CTec3 cellulases (Fig. 7). It is known that
458 that Cellic CTec3 has endo-β-1,4-xylanase and β-xylosidase acitivities (Sun et al 2015; Hu et al 2016). It
459 may be expected that xylobiose generated by the exo-xylanase is hydrolyzed into xylan as well as that
460 primary sugarcane bagasse xylan hydrolysis products generated by the xylanase activity of Cellic CTec3
461 are subsequently shortened by exo-xylanase activity of MetXyn11, resulting in the enhance of
462 hemicellulose hydrolysis. It was shown that the xylan polymer can bind tightly to the hydrophilic surfaces
463 of the cellulose crystallites 2_1-fold helical screw conformation within the plant cell wall, thus significantly
464 interfering with the cellulose enzymatic hydrolysis (Busse-Wicher et al 2014). Furthermore, pretreatment
465 solubilized hemicellulose oligomers partly aggregate on the cellulose surfaces and physically block access
466 of cellulases on the cellulose fibres (Kabel et al. 2007; Kumar et al. 2018). Moreover, solubilized fragments
467 of the xylan backbone (xylooligosaccharides, XOS) and mixed-linkage β-glucans are strong inhibitors of
468 cellulases (Kont et al 2013). Therefore, it was shown that β-xylanase supplementation increases cellulose
469 hydrolysis in xylan-containing lignocellulosic materials (Zhang et al. 2011; Kont et al 2013; Sun et al 2015;
470 Kumar et al 2018). Here we demonstrated that MetXyn11 supplementation of Cellic CTec3 leads to a
471 significant increase in rates and yields of cellulose and xylan conversion from pretreated lignocellulosic
472 biomass.

473 **Final considerations: MetXyn11 is an unusual GH11 β-1,4-xylosidase**

474 Our current work supports an importance of metatranscriptomic approaches for discovering novel
475 and unusual enzymes that are not accessible by the traditional approaches of microbial cultivation in pure
476 cultures, as so far, MetXyn11 is the second reported GH11 enzyme with a exo-β-1,4-catalytic mechanism.
477 Homology modeling and amino acid sequence comparison indicate that the same two extra loops already
478 described for the first reported GH11 exo-β-1,4-xylanase (Compost21_GH11) are also present in
479 MetXyn11. In addition, MetXyn11 has an extra N-terminus α-helix which does not exist in other GH11
480 enzymes deposited in PDB, including the Compost21_GH11. Our results also reveal that MetXyn11 is a
481 monomeric globular-like enzyme with biochemical properties that could be attractive for biotechnological
482 applications, such as: high substrate specificity; wide pH range of activity (pH 5-10) and high pH stability
483 at pH 5-6 (at optimum temperature); high thermal stability at 50 ºC (at optimum pH) for long periods of
484 time; and a unique hydrolytic product release pattern. Indeed, the enzyme exclusively produces xylobiose,
485 which is considered an added-value molecule for various applications. Furthermore, the enzyme addition
486 provoked considerable increase in Cellic CTec3 hydrolysis yields of pretreated sugar cane bagasse, which
487 could be of interest for second generation bioethanol production.

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494 **AUTHORS CONTRIBUTIONS**

495 I.P and D.E.E designed the experiments and wrote the manuscript. D.E.E and V.O.A.P performed
496 MetXyn11 biochemical and biophysical characterization. D.E.E performed the SAXS experiments. M.E.S
497 provided pretreated bagasse samples. I.P., S.M.M., N.C.B., D.E.E. and V.O.A.P. contributed to discussion
498 of the results and editing of the manuscript. All the authors approved the final version.

499 **CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.

COMPLIANCE WITH ETHICAL STANDARDS AND ETHICAL APPROVAL

This article does not contain any studies with human or animal participants.

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671 **Figure Captions**

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673 acid sequences from MetXyn11, Compost21_GH11 (PDB id: 5VQJ), and several typical GH11 endo-1,4-
674 β-xylanases (PDB ids: 1XNK, 1H1A, 1XYP, 2JIC and 4XQD). The yellow box shows the extended amino
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676 existent only in MetXyn11 and Compost21_GH11 exo-1,4-β-xylanases. (B) Multiple superposition of the
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712 **Fig. 6: Optimal activity conditions and kinetics of MetXyn11.** The optimum pH was evaluated in pH
713 range from 2 to 10 in ABF buffer at 50 ºC, while the optimal temperature was assessed in a potassium
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721 xylose under optimal CTec3 conditions of pH and temperature, using hydrothermal pretreated sugarcane
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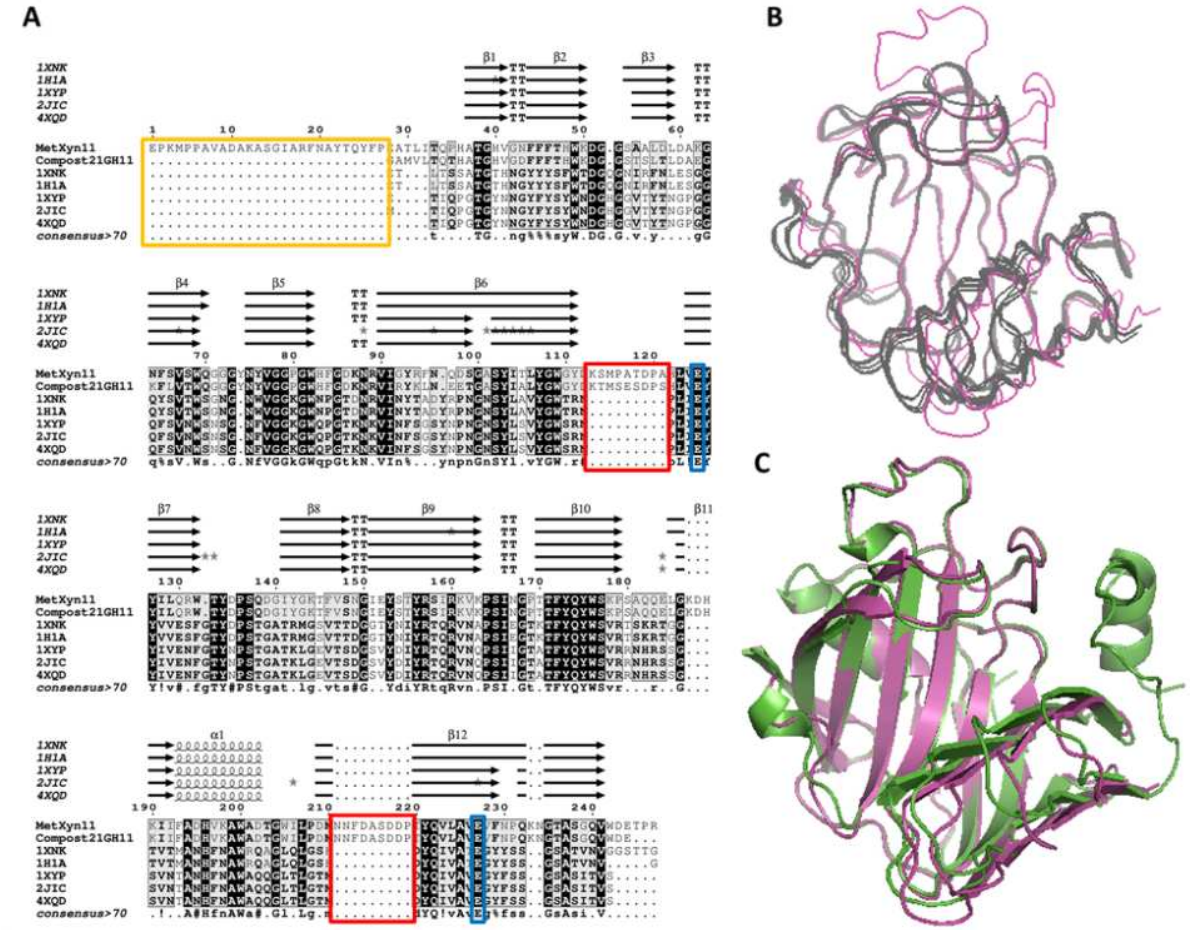

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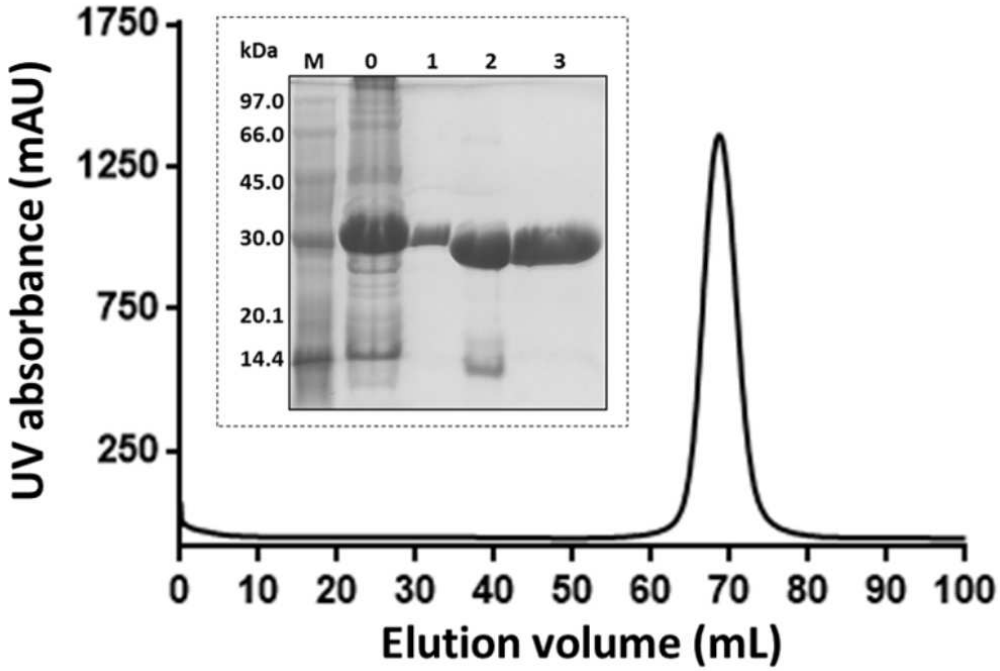

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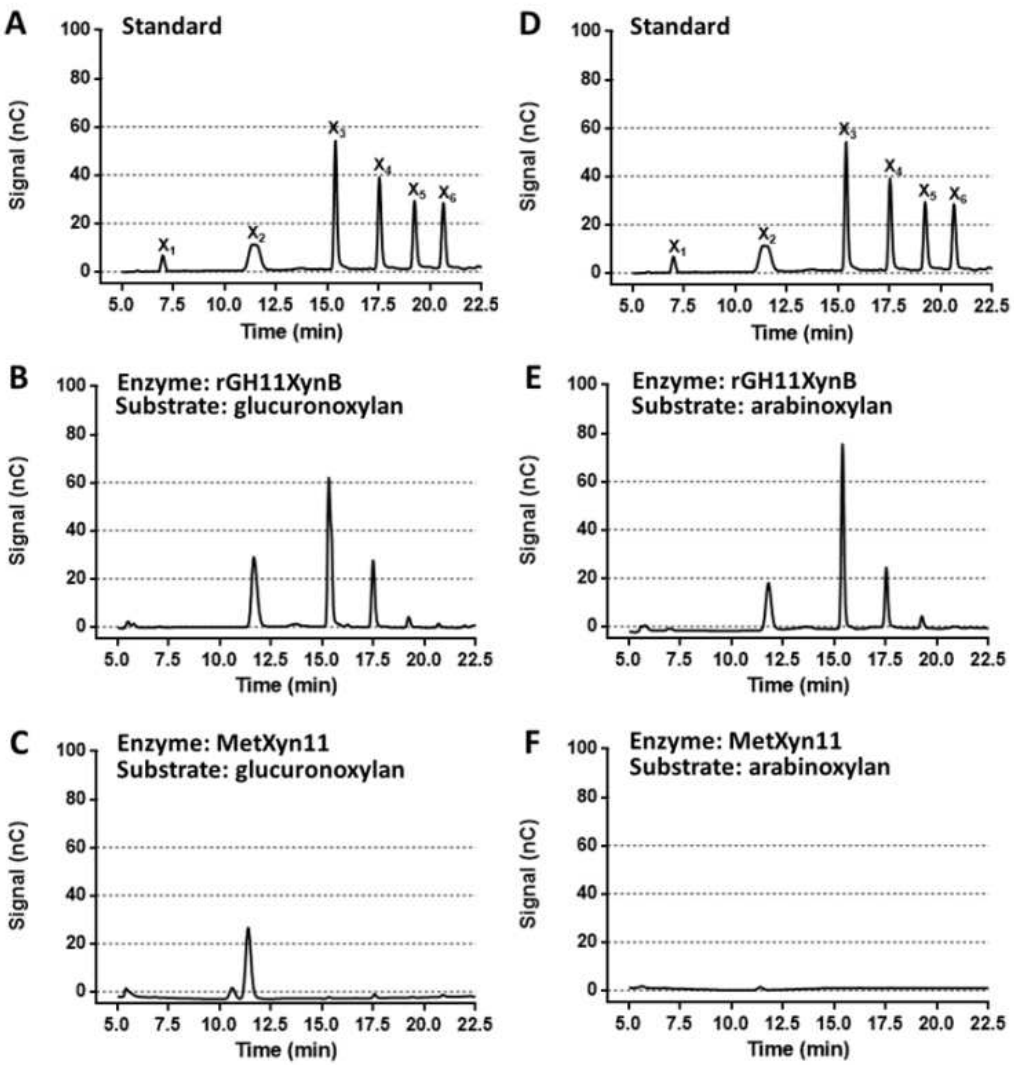

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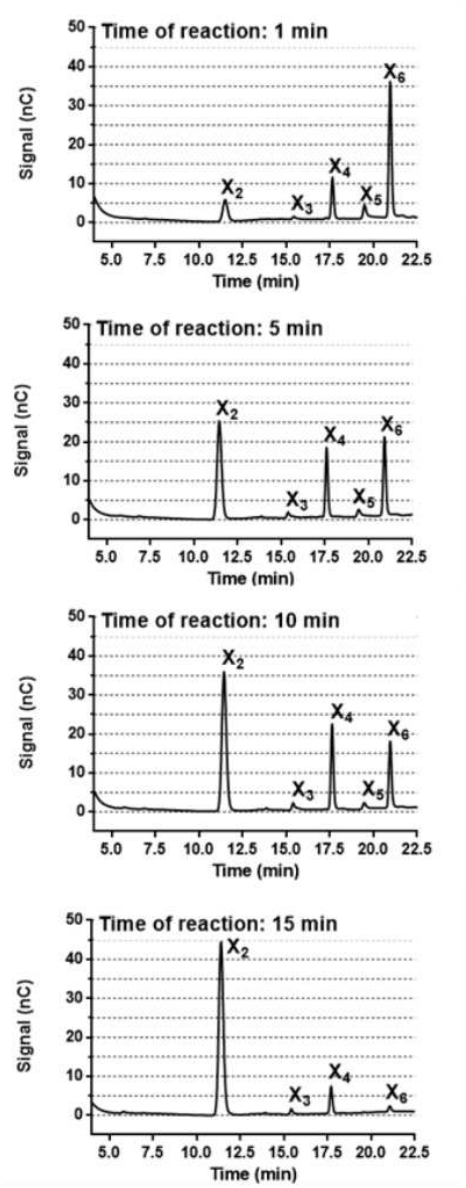

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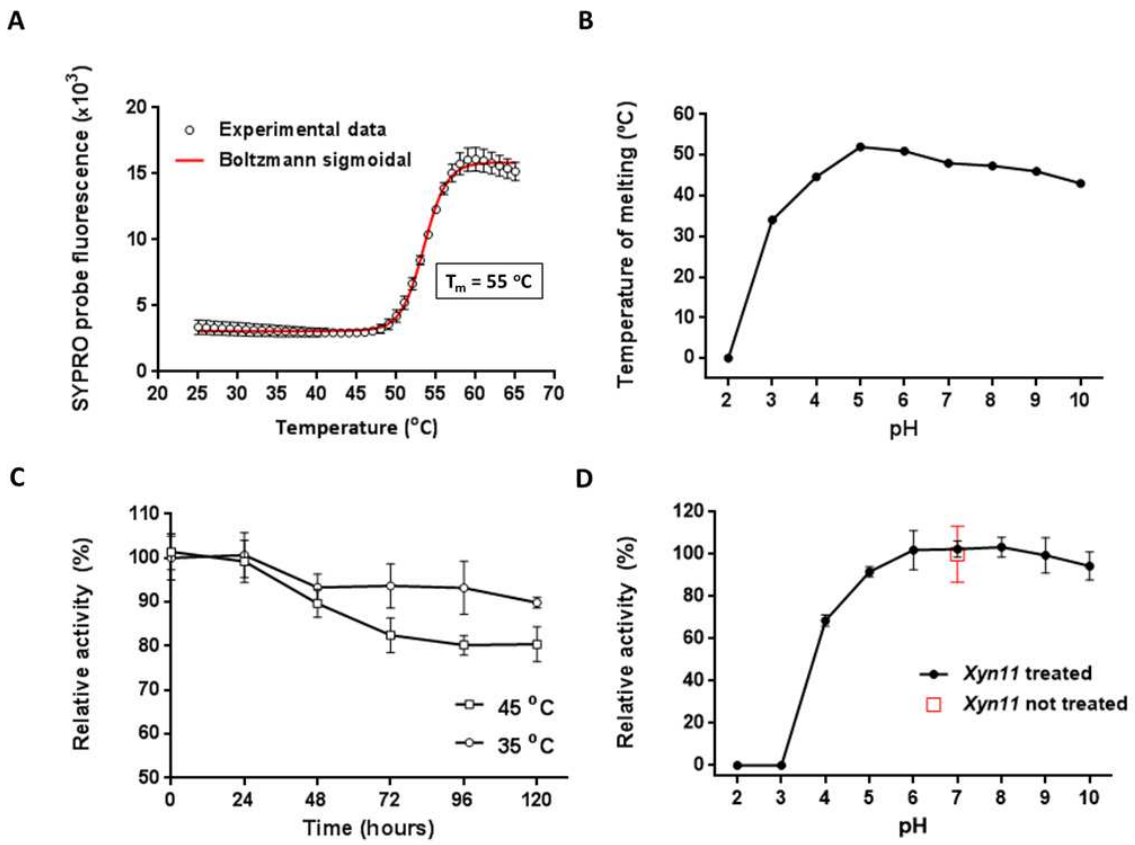

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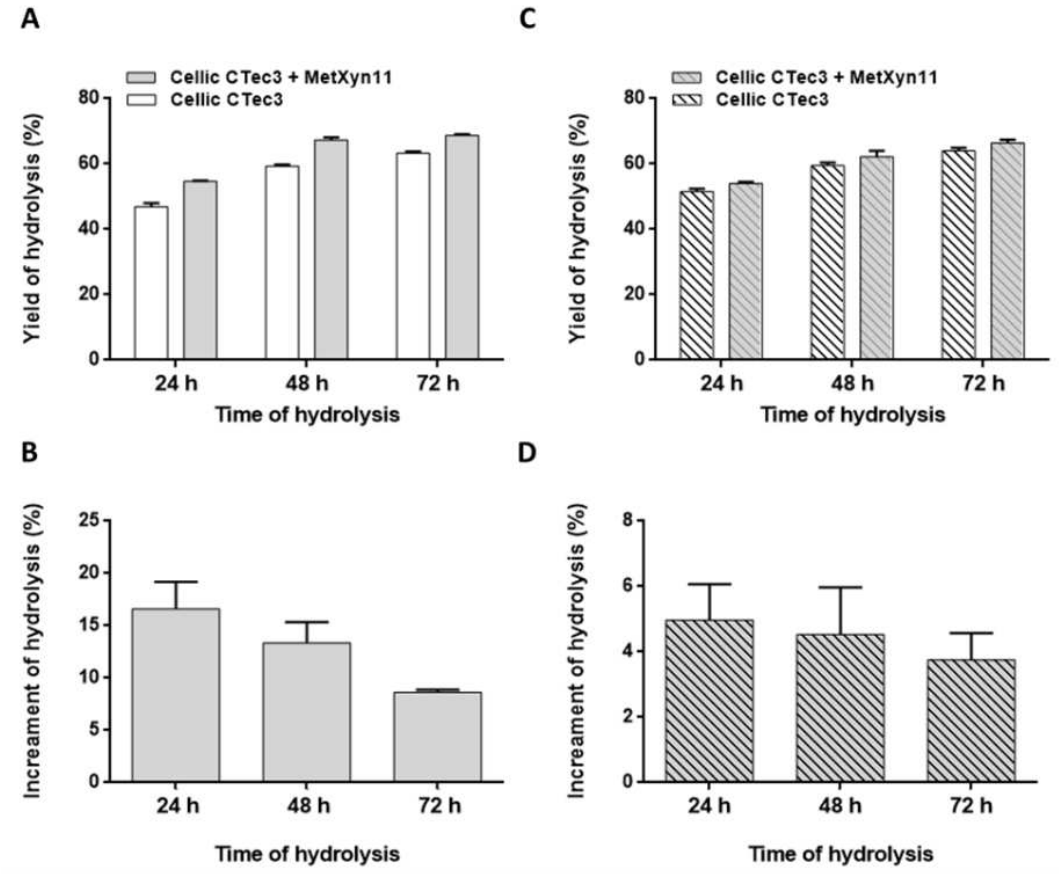

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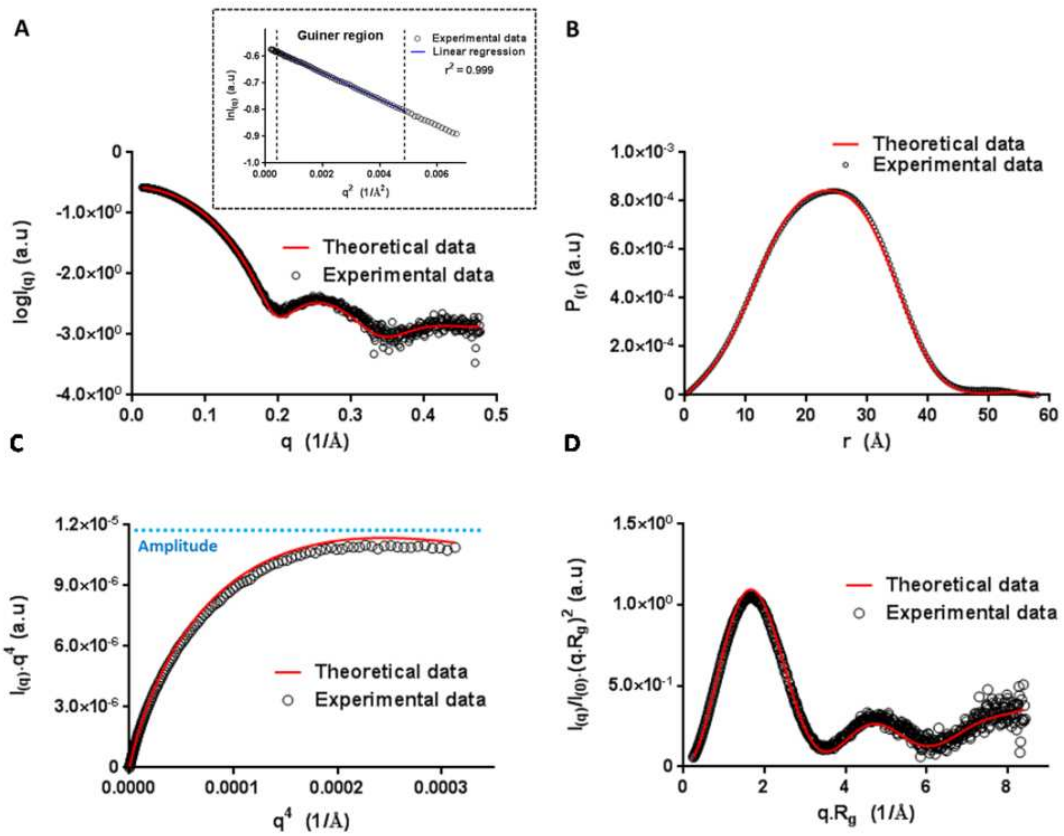

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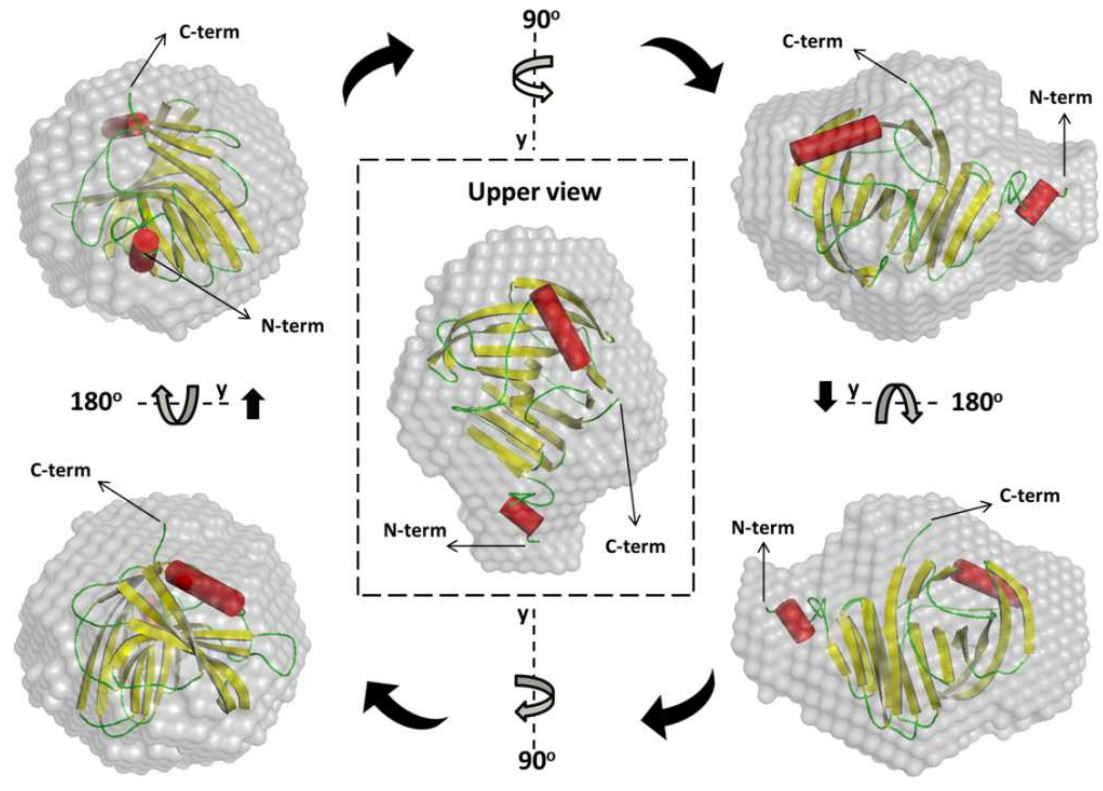

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Applied Microbiology and Biotechnology

Supplementary Materials

Biochemical characterization and low-resolution SAXS shape of a novel GH11 exo-1,4-β-xylanase identified in a microbial consortium

Danilo Elton Evangelista[1a], Vanessa de Oliveira Arnoldi Pellegrini[1a], Melissa Espirito Santo[1], Simon McQueen-Mason[2], Neil C. Bruce[2] and Igor Polikarpov[1*].

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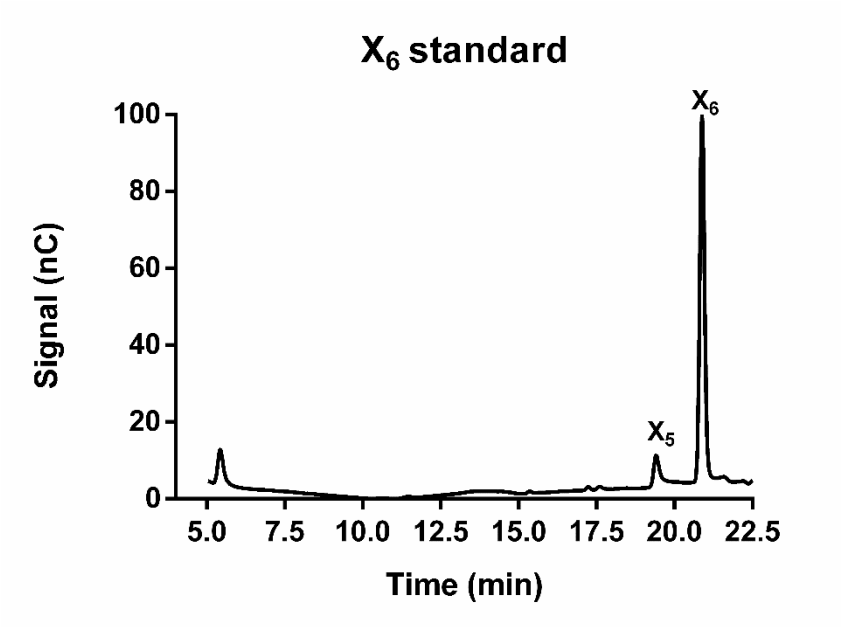

Figure S1. HPAEC-PAD analysis of xylohexaose (X6) standard. Small contamination with xylopentaose (X5) can be clearly visualized.

Table S1. Thermofluor analysis. Effect of different buffer solutions on tertiary structure stability of MetXyn11. Asterisk indicates the highest T_m values.

pH	Buffer (50 mM)	T_m (ºC)
7.0	Control (water)	55*
1.2	HCl	26
2.0	HCl	45
3.0	Citric acid	26
4.0	Sodium citrate	46
4.5	Sodium acetate	48
4.7	Sodium citrate	51
5.0	Sodium acetate	52
5.0	Potassium phosphate	54
5.5	Sodium citrate	54
5.5	Sodium phosphate	55*
5.8	MES	55
6.0	Potassium phosphate	54
6.0	Bis-Tris	55*
6.2	MES	55*
6.5	Sodium phosphate	53
6.5	Sodium cacodylate	54
6.5	Bis-Tris	55*
6.5	MES	55*

6.7	Bis-Tris	55*
7.0	Potassium phosphate	53
7.0	HEPES	55*
7.0	Bis-Tris	55*
7.3	Amonium acetate	54
7.5	Sodium phosphate	50
7.5	Tris	54
8.0	Imidazole	52
8.0	HEPES	52
8.0	Tris	52
8.0	Bicine	52
8.5	Tris	51
9.0	Bicine	49
9.5	Sodium carbonate	46
10.0	Sodium carbonate	45

Table S2. Metal ions and chemicals influence. The effects of the metal ions, reducing agents and surfactants on MetXyn11 enzymatic performance were assessed and compared with other GH11 xylanases. N/A = Not available.

Enzyme name	MetXyn11	Xyn11A	Xyn11B	Xyn11B119
Organism	Unknown	*P. oxalicum*	*P. oxalicum*	*Streptomyces sp.*
Reference	Present study	Liao et al. 2015	Liao et al. 2015	Zhou et al., 2011
Metal ions		**Residual activity (%)**		
	(10 mM)	(10 mM)	(10 mM)	(10 mM)
Control	100.0 ± 1.8	100.0 ± 3.7	100.0 ± 0.6	100.0 ± 2.6
Ca^{+2}	81.3 ± 4.2	118.9 ± 1.7	100.1± 3.4	98.3 ± 5.1
Co^{+2}	79.0 ± 6.8	124.9 ± 2.5	81.7 ± 1.6	97.3 ± 2.8
Cu^{+2}	54.3 ± 1.7	2.3 ± 0.16	41.6 ± 2.6	N/A
Fe^{+2}	53.6 ± 3.5	115.5 ± 3.1	101.3 ± 2.5	101.0 ± 1.6
Fe^{+3}	0.50 ± 0.7	22.8 ± 1.0	33.0 ± 0.5	N/A
Mn^{+2}	21.9 ± 10.3	108.8 ± 2.8	100.1 ± 2.7	65.6 ± 0.9
Mg^{+2}	123.1 ± 6.9	127.6 ± 2.0	101.8 ± 2.7	99.0 ± 0.8
Ni^{+2}	110.2 ± 1.6	116.1 ± 2.5	79.1 ± 3.1	122.3 ± 2.8
K^{+}	112.3 ± 10.3	N/A	N/A	101.4 ± 2.6
Li^{+2}	106.1 ± 6.35	119.4 ± 2.3	89.6 ± 3.9	101.3 ± 3.6
Reducing agents		**Residual activity (%)**		
	(1 mM)	(1 mM)	(1 mM)	(10 mM)
β-Mercaptoethanol	84.8 ± 0.9	41.7 ± 0.1	101.0 ± 5.3	126.5 ± 2.0
DTT	95.1 ± 1.7	100.2 ± 1.2	98.3 ± 2.6	N/A
Surfactants		**Residual activity (%)**		
	(0.1%)	(0.1%)	(0.1%)	(0.3%)
Tween-20	83.5 ± 0.3	100.8 ± 1.2	104.6 ± 7.6	N/A
Triton-100X	107.9 ± 0.4	100.0 ± 1.1	88.8 ± 2.9	N/A
SDS	0.0 ± 0.0	23.9 ± 1.8	3.5 ± 0.3	110.8 ± 7.2

Table S3. SAXS data collection and processing.

Data collection	
Beamline	LNLS-SAXS1
Wavelength (Å)	1.55
q range ($Å^{-1}$)	0.0138 – 0.47699
Exposure time per frame (s)	30
Concentration range ($mg.mL^{-1}$)	1.0, 3.5 and 15.0
Temperature (°C)	20
Data Analysis	
$I_{(0)}$	0.26 ± 0.00
Guinier q-region ($Å^{-1}$)	0.0138 – 0.06983
R_g (Å) from Guinier (± SE)	18.52 ± 0.04
R_g (Å) from GNOM (± SE)	17.67 ± 0.06
D_{max} (Å)	58
Resolution ($2\pi.q_{max}^{-1}$) (Å)	13.17
Oligomeric state	Monomer
Ab initio modeling	
Number of models	10
NSD	0.572 ± 0.008
Software employed	
Primary data reduction	Fit2D
Data processing	Primus
Theoretical data fitting	Crysol
Envelope modeling	Dammin
3D-Homology modeling	I-Tasser