Functional exploration of Pseudoalteromonas atlantica as a source of hemicellulose-active enzymes: Evidence for a GH8 xylanase with unusual mode of action

Sayani Ray, Jacqueline Vigouroux, Axelle Bouder, Mathilde Francin-Allami, Audrey Geairon, Mathieu Fanuel, David Ropartz, William Helbert, Marc Lahaye, Estelle Bonnin

Functional exploration of *Pseudoalteromonas atlantica* as a source of hemicellulose-active enzymes: evidence for a GH8 xylanase with unusual mode of action

Sayani Ray[1,3], Jacqueline Vigouroux[1], Axelle Bouder[1], Mathilde Francin Allami[1], Audrey Geairon[1], Mathieu Fanuel[1], David Ropartz[1], William Helbert[2], Marc Lahaye[1], Estelle Bonnin[1@]

[1] *INRA, UR 1268 Biopolymères – Interactions – Assemblages, 44 316 Nantes, France*

[2] *CERMAV-CNRS, 601 rue de la Chimie, BP53, 38041 Grenoble, France*

[3] *Present address: Department of Chemistry, The University of Burdwan, Burdwan – 713104 West Bengal, India*

[@] corresponding author: e-mail: estelle.bonnin@inra.fr; tel: +33 2 40 67 50 58

Running title: *Pseudoalteromonas atlantica* as a source of hemicellulose-degrading enzymes

Abstract

To address the need for efficient enzymes exhibiting novel activities towards cell wall polysaccharides, the bacterium *Pseudoalteromonas atlantica* was selected based on the presence of potential hemicellulases in its annotated genome. It was grown in the presence or not of hemicelluloses and the culture filtrates were screened towards 42 polysaccharides. *P. atlantica* showed appreciable diversity of enzymes active towards hemicelluloses from Monocot and Dicot origin, in agreement with its genome annotation. After growth on beechwood glucuronoxylan and fractionation of the secretome, a β-xylosidase, a α-arabinofuranosidase and an acetylesterase activities were evidenced. A GH8 enzyme obtained in the same growth conditions was further cloned and heterologously overexpressed. It was shown to be a xylanase active on heteroxylans from various sources. The detailed study of its mode of action demonstrated that the oligosaccharides produced carried a long tail of un-substituted xylose residues on the reducing end.

Keywords: CAZymes; Glycoside-hydrolases; enzyme screening; hemicellulases; GH8 xylanase

33 **1- Introduction**

34 Hemicelluloses are among the most abundant polysaccharides present in the plant cell wall
35 after cellulose. They play an important role in the regulation of cell wall mechanical
36 properties during plant growth, which impacts the various applications of plant crops quality
37 and their agro-industrial processes **[1]**. Hemicelluloses include several polysaccharides, such
38 as xyloglucans, heteroxylans, heteromannans, mix linked β-glucans **[2]**. Xyloglucans,
39 heteroxylans, heteromannans are made of (1,4) linked β-D-glucose, xylose and mannose,
40 respectively, on which several other sugars or non-sugar substituents can be linked. In
41 monocot endosperm, xylans carry (1,3)- and/or (1,2)-linked α-L-arabinofuranosyl units as the
42 main substituents to form arabinoxylans (AX) **[3]**. Heteromannans include linear mannan,
43 glucomannan (GlcM), galactomannan (GalM), and galactoglucomanan (GgM) **[4]**. Mix linked β-
44 glucans consist of a (1,4)-linked β-D-glucose backbone in which (1,3) linkages are
45 interspaced. A deeper knowledge on the fine structural details of hemicelluloses is required
46 not only to improve their degradation in biorefinery process, but also for a better
47 understanding of the impact of their versatile decorations on their functions in the cell wall.
48 Degradation with carbohydrate active enzymes, followed by chemical, chromatographic and
49 spectrometric analyses is one of the leading tools to solve the fine structure of
50 polysaccharides **[5 -8]**. However to address the structural complexity of hemicelluloses, there
51 is a need for enzymes exhibiting novel specificity. The CAZy database (http://www.cazy.org)
52 classified the Carbohydrate Active enZymes in structurally-related families **[9]**. To date the
53 database includes 156 families of glycoside hydrolases (GH), of which 43 are involved in the
54 degradation of hemicelluloses.

55 Due to the structural complexity of hemicelluloses, their enzymatic degradation requires a
56 large panel of enzymes working in synergy. For all the hemicelluloses cited above, this
57 includes the enzymes able to split linkages in the backbone and enzymes specific for the side
58 chain residues. When focusing on backbone, splitting the β-(1,4)-linkage between two glucose

59 residues in XyG requires either broadly specific endo-β-1,4-glucanases (EC 3.2.1.4), belonging
60 to 13 different GH families (5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74 and 124), or highly specific endo-
61 acting xyloglucanases (EC 3.2.1.151), classified in 6 GH families (5, 9, 12, 16, 44 and
62 74)(http://www.cazy.org). Complete degradation of heteroxylan requires the co-working of
63 endo-β-1,4-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase **[10]**.
64 Most of the microbial β-1,4-xylanases (EC 3.2.1.8) are grouped into 7 GH families: GH5, 8, 30,
65 43, 51, where a bi-functional cellulase/xylanase stands, and GH10 and 11, where the most
66 studied β-1,4-xylanases are gathered **[11–14]**. The endo-β-1,4-mannanases required for the
67 hydrolysis of heteromannan backbone are classified into the GH families 5, 26 and 113.
68 As part of a comprehensive screening of bacteria to highlight enzymatic activities towards a
69 large number of polysaccharides, *Pseudoalteromonas atlantica* was shown to produce an
70 interesting diversity of enzyme repertoire. Its genome contains several GH genes (Table 1)
71 but there is a lack of information on most of the corresponding proteins. Therefore it was
72 grown in the presence or not of different hemicelluloses to study the potential inducer effect
73 on the enzyme secretion. Its secretome was fractionated to identify the proteins involved in
74 the observed activities. One of these proteins annotated as belonging to GH8 family was
75 studied in more details.
76
77

78 2- Material and methods

79 *2-1 Culture conditions*

80 The strain *Pseudoalteromonas atlantica* T6c (ATCC, USA) was grown in its optimum growth
81 conditions (Table 1). A pre-culture was carried out by inoculating frozen cells in 10 mL of
82 specific culture medium (Table 1) in a 100 mL Erlenmeyer flask. After 36 h incubation at the
83 suitable temperature under shaking at 150 rpm in a New Brunswick incubator, 1 mL of the
84 culture was inoculated in 50 mL of fresh medium and incubated for additional 8 h under

85 similar conditions. Then, 50 mL were inoculated in 1 L of fresh medium in 5 L shaker flask.
86 After 36 h, the culture was centrifuged at 5,000 × g for 30 min at 4°C. Culture supernatant and
87 cell pellet were treated separately prior to the screening assay. The cell free culture
88 supernatant was filtered overnight on a 300 kDa membrane (PES, Millipore) in a 400 mL
89 Amicon system 8400 (Millipore). The filtrate was then diafiltered and concentrated to 20 mL
90 with 50 mM Tris–HCl buffer pH 8.5 containing a protease inhibitor cocktail (Roche,
91 Indianapolis, IN, USA), on a 10 kDa membrane (PES, Millipore) under compressed air (0.5 bar).
92 The cell pellet was washed twice with 500 mL of 50 mM Tris–HCl buffer pH 8.5 and re-
93 suspended in 10 mL of the same buffer containing anti-protease mixture. Bacterial cells were
94 disrupted by Constant cell disruption system (Constant systems Ltd, Northants, UK) and the
95 membrane fragments were removed by centrifugation at 20,000 ×g for 60 min at 4°C. The
96 clarified lysate was diluted 10 times, filtered overnight on a 300 kDa membrane and finally
97 concentrated, diafiltered with 50 mM Tris–HCl buffer pH 8.5 on a 10 kDa membrane to a final
98 volume of 20 mL. Culture supernatant and cell lysate were further used for activity screening.
99

100 *2-2 Preparation of polysaccharides for enzyme screening*

101 The forty-two polysaccharides used as substrates for the screening are shown in Table 2. All
102 the polysaccharides were dissolved in deionized water (4 mg/mL). Solutions of commercial
103 polysaccharides were diafiltered on a 10 kDa membrane (PES, Millipore, Billerica, MA, USA)
104 using a 50 mL Amicon system 8050 (Millipore). Polysaccharides purified from apple and
105 tomato and belonging to the laboratory collection were directly dissolved in deionized water.
106

107 *2-3 Screening assay*

108 The 42 polysaccharide solutions (150 μL) and 5 controls containing water were dispensed
109 into a 96-well filter microplate (10 kDa, PES, Pall) with an equal volume (150 μL) of culture
110 supernatant or cell lysate from bacterial culture **[17]**. Polysaccharide controls were done by

111 incubating 150 µL of polysaccharide solutions with 150 µL 50 mM buffer solution (Tris-HCl,
112 pH 8.5 or acetate, pH 5.2) instead of culture filtrate or cell lysate. Microplates were sealed
113 with a plastic film and incubated overnight with shaking at 34°C. The incubation medium was
114 filtered on a multiscreen HTS vacuum manifold (MSVMHTS00, Millipore) connected to a high-
115 output vacuum pressure pump (Millipore) for 3 h under about 0.5 bar pressure.
116 For the colorimetric assay, 40 µL of each incubation filtrate were transferred to a microplate
117 (Dutscher, Brumath, France). Then, 200 µL of ferricyanide solution **[18]** were added to the
118 sample and the microplate was sealed with plastic film. The plate was heated at 95°C for
119 15 min in a thermocycler (GenAmp PCR system 2700, Applied Biosystems France, Villebon-
120 sur-Yvette, France) and cooled to room temperature. The occurrence of reducing ends was
121 revealed by reading the absorbance at 420 nm of 200 µL of the samples in a microplate reader
122 (Nunc, Roskilde, Denmark) using a Wallac 1420 multilabel counter (PerkinElmer Life Sciences,
123 Boston, MA, USA). The amount of reducing ends was expressed in absorbance units. The
124 samples with absorbance higher than the corresponding bacterial filtrate or lysate alone
125 (control) minus 0.15 were considered as negative hits. The mean (X) and the standard
126 deviation (σ) of the optical density (OD) of these negative hits were calculated. When the OD
127 of the sample was lower than X-2σ, the hit was considered as positive. The polysaccharides
128 were thus classified according to four intervals based on the control X and its σ.
129

130 ### *2-4 Time course of growth of* P. atlantica

131 Two pre-cultures of *P. atlantica* were carried out by inoculating frozen cells in 10 mL of
132 sterilized bactomarine broth culture medium in a 100 mL Erlenmeyer flask with 12 h time lag.
133 After 36 h incubation at 20°C with shaking at 180 rpm in a New Brunswick incubator, 1 mL of
134 the culture was inoculated in 250 mL of fresh medium. The growth of the bacteria was
135 followed by the absorbance of 1 mL culture at 600 nm at 1 h of time intervals. Similarly the

136 time course of growth was followed using konjac GlcM or beechwood glucuronoxylan (GuX)
137 added at 1 g/L in the culture medium.
138

139 ### *2-5 Fractionation of the culture supernatant of* P. atlantica

140 Similar cultures were carried out at larger scale by inoculating 50 mL of pre-culture in 1 L of
141 fresh medium in 5 L shaker flask in the same conditions.
142 *P. atlantica* was cultivated in sterilized bactomarine broth without any added polysaccharide
143 or in the presence of konjac GlcM or beechwood GuX at 1 g/L. After 16 h, 18 h and 16 h of
144 respective growth for the three conditions, the culture was centrifuged at 5000 × g for 30 min
145 at 4°C. The cell free supernatants were recovered and complemented with a protease
146 inhibitor cocktail.
147 The culture supernatants were filtered overnight on a 300 kDa membrane (PES, Millipore)
148 using a 400 mL Amicon system 8400 (Millipore). The filtrates were diafiltered and
149 concentrated to 20 mL, with deionized water on a 10 kDa membrane (PES, Millipore) under
150 compressed air (0.5 bar) to remove small molecules. The concentrated filtrates were
151 separated into 2 x 10 mL and the pH of each half was adjusted at 8.5 or 5.2 by adding 100 mM
152 Tris–HCl or 100 mM acetate buffer, respectively. By this way, 6 culture filtrates were
153 obtained: without any added polysaccharide or with konjac GlcM or beechwood GuX, and for
154 each, at pH 8.5 and 5.2. All culture filtrates were stored at 4°C before enzyme assays.
155 The culture filtrate obtained in the presence of beechwood GuX was fractionated by ion
156 exchange chromatography on a Mini Q™ column (4.6 x 50 mm, GE Healthcare, Uppsala,
157 Sweden) installed on an AKTA purifier (GE Healthcare) and equilibrated with 50 mM Tris-HCl
158 buffer at pH 8.5. Elution was performed at a flow rate of 0.8 mL/min with 50 mM Tris-HCl
159 buffer pH 8.5 (7 column volumes) to recover the unbound fraction. Bound proteins were
160 eluted by a gradient on 10 column volumes from 35 to 75% of NaCl 1 M in 50 mM Tris-HCl
161 buffer pH 8.5. The fractions of interest were dialyzed in a 10 kDa cut off dialysis tube

162 (Nanosep 10K Omega, Pall Life Science, USA) against 50 mM Tris-HCl buffer at pH 8.5,
163 adjusted to a final volume of 3 mL and stored at 4 °C.
164

165 ### *2-6 Enzymatic activities in* P. alteromonas *culture filtrates and after anion*
166 *exchange chromatography*

167 Two hundred µL of polysaccharide solutions were incubated overnight with 200 µL of
168 bacterial culture filtrates or protein fractions eluted from ion exchange chromatography. After
169 withdrawing 100 µL aliquot, the amount of reducing ends produced in the rest of the reaction
170 medium was quantified by Nelson method **[19]** adapted to microplate **[20]**, and read on a
171 microplate reader (Multiskan Ex). The amount of reducing ends was expressed in µg/mL
172 using standard curve prepared with appropriate sugar. Controls were prepared similarly with
173 previously heat-inhibited culture filtrates and polysaccharide solutions.
174 Glycosidase activities were assayed by incubating 100 µL culture filtrates with 100 µL 4 mM
175 *para*-nitrophenyl (*p*-NP) derivatives of α-L-arabinofuranoside, α-D-galactopyranoside, β-D-
176 mannopyranoside, β-D-glucopyranoside, α-D-xylopyranoside, β-D-galactopyranoside, α-L-
177 fucopyranoside, acetate, and *ortho*-nitrophenyl (*o*-NP) β-D-xylopyranoside for 16 h at 40°C.
178 The incubation was stopped by adding 0.6 mL of 1 M sodium carbonate solution **[21]**. The
179 liberation of *p*-nitrophenol was reported as nmol/mL. Control tests were prepared by mixing
180 the culture filtrate with sodium carbonate before adding the substrate.
181

182 ### *2-7 Protein analysis in* P. alteromonas *culture filtrates*

183 The concentration of protein was determined in the culture filtrate by using the Bradford
184 reagent (Bio-Rad, Marnes-la-Coquette, France) and bovine serum albumin (Sigma-Aldrich,
185 L'Isle d'Abeau, France) as a standard (0–25 µg/mL) **[22]**.
186 <u>*Electrophoresis*</u>: polyacrylamide gel electrophoresis was performed under denaturing

187 conditions (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)). It was
188 carried out in a MiniProtean 3 apparatus (BioRad) using a continuous 10–20%
189 polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate.
190 *In-gel digestion*: Bands of interest were excised from the SDS-PAGE and subjected to in-gel
191 reduction, alkylation and trypsic digestion essentially as described [**23**].
192 *LC–MS/MS analysis*: Mass spectrometry analyses were conducted by the platform
193 "Biopolymers-Interaction-Structural Biology" located at the INRA Center of Angers-Nantes
194 (www.bibs.inra.fr). Nanoscale capillary liquid chromatography-tandem mass spectrometry
195 (LC-MS/MS) analyses of the digested proteins were performed using an Ultimate U3000 RSLC
196 system (Dionex) coupled with an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher).
197 Details of the analytical procedure are given in **[23]**.
198 *Databank search and protein identification:* The LC-MS/MS collected data files were processed
199 into mgf format using Proteome Discoverer (Thermo-Fisher). The mgf files were then
200 searched against the Uniprot databank (http://www.uniprot.org, November 2014) restricted
201 to the taxonomy *Pseudoalteromonas atlantica* T6c (Taxon ID# 342610) using the Mascot 2.2
202 program (Matrix Science). Enzymatic cleavage was declared as a trypsic digestion with one
203 possible missed cut event. The fixed modifications of Cys residues by iodoacetamide and the
204 possible oxidation of Met residues were considered. Precursor mass and fragment mass
205 tolerance were set at 5 ppm and 0.5 Da, respectively. Proteins were validated when they had
206 an e-value below 0.01, and when they were identified with a minimum of two peptides of
207 individual score above the significance threshold provided by Mascot ($p < 0.01$).
208

209 ## *2-8 Cloning and expression and purification of rPaGH8*

210 The gene *Patl_1069* (GenBank accession number ABG39595.1), coding for the glycosyl
211 hydrolase of *P. atlantica* T6c grouped in the GH8 CAZy family was amplified from genomic
212 DNA using the following primers:

213 5-GTGAGCATTGATCACTTAACGCTAACAAACAAAACGCGG-3' (forward) and
214 5'-GCGCGGCCGCTTCAGGCTCGTTTTCATTTGGCTTATAG-3' (reverse) encompassing BamH1
215 and EcoR1 restriction site respectively. The gene was cloned in pET28a which contains an N-
216 terminal fused six-histidine-tag (6His-tag) using BamH1 and EcoR1 restriction sites (pET28a-
217 GH8 plasmid) and transformed in *E. coli* strain BL21 (DE3). *E. coli* BL21 (DE3) harbouring the
218 pET28-GH8 plasmid was pre-cultured overnight in Luria-Bertani medium containing 50
219 µg/mL of kanamycin before being diluted 20 times in the same growth medium and incubated
220 under shaking at 20°C overnight.
221 Cells were harvested by centrifugation, washed with 50mM Tris-HCl NaCl 100 mM (pH 7.5),
222 and resuspended in one-tenth of the culture volume of cold Tris-HCl 50 mM pH7.5, NaCl 100
223 mM, imidazole 20 mM (pH 7.5) containing protease inhibitor (Pierce™ Protease Inhibitor
224 Mini Tablets, ThermoFisher, Illkirch, France), 5 µL DNAse (Invitrogen, ThermoFisher, Illkirch,
225 France) and 20µg/mL RNAse (Invitrogen). Cells were disrupted using a French press at 2000
226 bars twice for 1 min, centrifuged 10 min at 4°C to recover a supernatant and a pellet.
227 The supernatant was loaded on a HisTrap™ FF column (1 mL) mounted on a ÄKTA Primer
228 system (GE Healthcare) to purify the His-tag GH8 protein. The recombinant protein was
229 eluted with 20 mM Tris-HCl buffer pH 8 containing 200 mM NaCl, 300 mM imidazole. The
230 supernatant and pellet from the French press lysate, and the fractions eluted from the
231 HisTrap column were subjected to a polyacrylamide gel electrophoresis under denaturing
232 conditions (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)), together
233 with a PageRuler™ Unstained Protein Ladder (Fisher). SDS-PAGE was carried out into a
234 MiniProtean 3 apparatus (BioRad) using a continuous 10% w/v polyacrylamide gel in the
235 presence of 0.1% sodium dodecyl sulfate that was further stained with Coomassie-HCL **[24]**.
236 The fractions containing the purified rPaGH8 were pooled, dialyzed against Tris-HCl 50 mM
237 pH 8, and concentrated twenty times using a VIVASPIN 500 concentrator (PES membrane 10
238 kDa, Sartorius).

239

240 *2-9 Analysis of the oligosaccharides produced by rPaGH8*

241 The recombinant xylanase rPaGH8 was incubated overnight with xylan from *Palmaria*
242 *palmata* [**25**], beechwood GuX, water-extractable (WEAX) and water un-extractable
243 arabinoxylan (WUAX) from wheat. The results were compared with the hydrolysis by the
244 commercial GH11 xylanase M1 from *Trichoderma viride* (Megazyme, Wicklow, Ireland).
245 Reaction media aliquots were withdrawn and precipitated in 4 volumes of ethanol 96° at
246 +4°C. After centrifugation 5 min at 20,000 g and evaporation under vacuum, samples were
247 dissolved in 500 µL water and filtrated on 0.45 µm membrane. They were analysed by HPAEC
248 on a CarboPac PA 200 column (3 x 250 mm, Dionex, Sunnyvale, USA) thermostated at 25°C.
249 Samples were eluted at 0.4 mL/min with a linear gradient of sodium acetate from 0 to 170
250 mM in 100 mM NaOH **[26]**. The nomenclature for AX oligosaccharides is according to **[27]**: X:
251 D-Xyl*p* (xylose in the β-(1-4)-linked backbone); A2: α-L-Ara*f*-(1-2)-β-D-Xyl*p*; A3: α-L-Ara*f*-(1-
252 3)-β-D-Xyl*p*; A2,3: α-L-Ara*f*-(1-2)-[α-L-Ara*f*-(1-3)]-β-D-Xyl*p*. As an example, XA3X describes a
253 main chain of 3 xylose residues of which the middle one is substituted at *0*-3 by an arabinose
254 residue.

255 Unknown peaks were purified on the same Carbopac PA 200 column equipped with on-line
256 desalting by the Carbohydrate Membrane Desalter (CMD300, Dionex) to remove sodium ions.
257 The CMD unit was fed with dilute sulfuric acid (75 mM) at 3 mL/min using an AXP pump
258 (Dionex). The separated unknown oligosaccharides were collected (200 µL) and further
259 analysed by electrospray ionization mass spectrometry. ESI-MS experiments were performed
260 on a Synapt G2Si high-definition mass spectrometer (Waters Corp., Manchester, UK). Two
261 types of mass measurements were performed on the samples: firstly, a mass profile was done
262 on a mass range of 400–2000 *m/z*. Ions of interest were further fragmented by collision-
263 induced dissociation in the transfer cell of the instrument, using an appropriate collision
264 energy to obtain numerous fragments. Samples were mixed volume/volume with acetonitrile

265 and infused at 5 µL/min in the instrument. The instrument was operated in negative
266 ionization mode in the so-called 'sensitivity' mode, with an ESI capillary voltage of 2.2 kV and
267 a sampling cone voltage of 60 V. Data acquisition was carried out using MassLynx software
268 (V4.1).
269
270
271 **3- Results**
272 *3-1 Enzymatic screening after cultivation in the presence or not of polysaccharides*
273 To highlight enzymes active on various polysaccharides, a preliminary screening was
274 conducted on four bacteria from marine and terrestrial origin whose genome carried at least
275 10 sequences of hemicelluloses-degrading enzymes. As a larger number of sequences did not
276 necessarily give more positive responses for substrate degradation, *P. atlantica* was selected
277 from this screening since it offered the best performance and the greatest diversity on
278 polysaccharides. Its genome contains genes coding for GH enzymes specific for hemicellulose
279 backbone, *i.e.* β-glucanase, xyloglucanase, β-mannanase and β-xylanase (GH3, 5, 8, 10, 16 and
280 43, Table 1).
281 *P. atlantica* was first grown in the basal medium recommended (Table 1) and in the absence
282 of added polysaccharide. Forty-two terrestrial or marine polysaccharides of diverse origins
283 and structures (Table 2) were tested as substrates to screen enzymatic activities in the
284 culture supernatant and in the cell lysate (Table 3). By comparing the results, it was obvious
285 that the culture filtrate was more active and more diverse than the cell lysate. The culture
286 filtrate of *P. atlantica* was highly, moderately or slightly active on 15 substrates among the 42
287 tested, including 10 hemicellulose-like substrates, while the cell lysate was highly active on
288 only 2 substrates and slightly active on 4 substrates.
289 To enhance the enzyme production, different hemicelluloses were added to the culture
290 medium, as the addition of polysaccharides can play an important role in the induction of

291 enzymatic activity **[28 -31]**. The polysaccharides tested were chosen with respect to the GH
292 enzymes annotated in the genome. *P. atlantica* genome possesses one sequence in GH5, one in
293 GH8, one in GH10, and three sequences in GH43 (Table 1) potentially active on β-linked-1,4-
294 mannan, glucan and xylan. For this reason, *P. atlantica* culture broth was complemented with
295 either konjac GlcM or beechwood GuX or a mixture of CMC, tara GalM and beechwood GuX
296 (abbreviated as '3P', Table 3). Beechwood GuX added alone or in admixture with CMC and
297 tara GalM (3P) in the culture broth reinforced the activities against itself, and against rye and
298 wheat AX, while the activities towards apple and tomato XyG disappeared.
299 The improvement of the secretion of hemicellulolytic enzymes by adding polysaccharide to *P.*
300 *atlantica* culture broth was further investigated.
301

302 *3-2 Effect of polysaccharides on growth kinetics and enzyme production*

303 Growth of *P. atlantica* without any added polysaccharide was followed over 48 h (Figure 1).
304 Growth was slow for the first 5 h and increased rapidly to reach a plateau after 20 h culture.
305 Adding konjac GlcM did not lead to a significant change in the bacterial growth in comparison
306 to culture in the absence of added polysaccharide. Conversely beechwood GuX positively
307 affected the growth rate of *P. atlantica* as it induced faster growth from 10 to 20 h.
308 The culture filtrates were recovered from each culture and tested for their enzymatic activity
309 on 12 different hemicelluloses and 8 nitrophenyl-derivatives at pH 5.2 and 8.5 to identify the
310 secreted activities (Figure 2). The culture filtrates obtained without added polysaccharide and
311 in the presence of konjac GlcM exhibited roughly similar activity profiles on hemicelluloses
312 (Figures 2A and 2C, respectively). Activities on xylan-like polysaccharides were favoured at
313 pH 8.5. Also the activities towards nitrophenyl-derivatives were similar in the two culture
314 filtrates (Figures 2B and 2D). The activities towards *o*-NP β-D-xylopyranoside, *p*-NP α-L-
315 arabinofuranoside and *p*-NP acetate were higher at pH 5.2 than at pH 8.5. Comparison of
316 Figures 2A and 2B and Figures 2C and 2D demonstrated that adding konjac GlcM in the

317 culture medium did not improve the production of hemicellulases or glycosidases. This result
318 agreed with the growth kinetics study, which showed that konjac GlcM did not significantly
319 enhance the bacterial growth.
320 In the culture filtrate produced in the presence of beechwood GuX (Figures 2E and 2F), the
321 activities on heteroxylans were reinforced, mostly at pH 8.5. Conversely to what was observed
322 in the other culture conditions, the activity on *o*-NP xylopyranoside was much higher at pH
323 8.5.
324

325 *3-3 Fractionation of culture filtrate obtained on beechwood glucuronoxylan*

326 The culture filtrate obtained in the presence of beechwood GuX was fractionated by ion
327 exchange chromatography. The activities were evaluated in the collected fractions on konjac
328 GlcM, CMC, beechwood GuX and wheat AX, and also on *o*-NP β-D-xylopyranoside and *p*-NP α-
329 L-arabinofuranoside (Figure 3). The results suggested that enzymes active on konjac GlcM
330 and CMC (Figure 3A) eluted mainly with xylosidase activity (Figure 3B), whereas enzymes
331 active on beechwood GuX and wheat AX eluted later in the gradient (Figure 3A).
332 The gel electrophoresis of the fractions eluted between 7 and 13 mL elution volume of the ion
333 exchange chromatography (Fractions B2 to B12, Figure 4A) showed that the major band in
334 fractions B4 to B6 stand around 45 kDa and could correspond to the GH5 β-glucanase
335 Patl_1404. These fractions also contained a slight band around 55 kDa and a band at 72 kDa
336 possibly corresponding to the GH43 arabinofuranosidase Patl_0842 and the GH3-like protein
337 Patl_4162, respectively. In the fractions B7 to B9, the major band was around 42 kDa and
338 could correspond to the GH10 β-xylanase Patl_2657. Thus the electrophoresis demonstrated
339 the proteins associated with the enzyme activities recovered from the chromatography and is
340 an additional element to suggest the β-glucanase activity of GH5 Patl_1404, the
341 arabinofuranosidases activity of GH43 Patl_0842 and the β-xylanase activity of GH10
342 Patl_2657.

343 In the fractions B9 to B12 a lighter band around 96 kDa possibly corresponded to the GH8
344 protein Patl_1069. The band was excised and submitted to trypsic digestion. The LC-MS/MS
345 analysis of the digests confirmed that it corresponded to Patl_1069 (Supplementary data 1).
346 As the activity of this GH8 protein was not yet described, it was cloned and expressed in order
347 to analyse further its activity and specificity.
348

349 *3-4 Production and activity of the recombinant enzyme rPaGH8.*

350 The GH8 protein Patl_1069 was expressed in the heterologous system *E. coli* with a poly-His
351 tag fused to its N-terminus. The recombinant protein rPaGH8 was purified on a His trap
352 affinity column. Three fractions eluting in the imidazole gradient and containing a band at 96
353 kDa (Fractions 4 to 6 on Figure 4B) were pooled and further tested for their enzymatic
354 activity. The pooled fraction exhibited 289 nkat/mL on soluble mix-linked xylan from
355 *Palmaria palmata* confirming that rPaGH8 is a xylanase. Conversely, rPaGH8 did not degrade
356 o-NP xyloside, and thus has no β-xylosidase activity.
357

358 *3-5 Specificity and mode of action of rPaGH8*

359 The hydrolysis products released by rPaGH8 from heteroxylans from various sources were
360 analysed by HPAEC and compared to the hydrolysis products released by the commercial
361 GH11 xylanase M1 (Figure 5A). The first substrate tested was the soluble mix-linked xylan
362 from *Palmaria palmata*, which contains mainly (1,4) linkages but also about 20% (1,3)
363 linkages **[25]**. The GH11 xylanase M1 produced monomer (X1), β(1,4) dimer (X2), β(1,4)
364 trimer (X3), traces of tetramer (X4), and two peaks eluting at 13.2 min and 14.1 min,
365 potentially containing (1,3) linkages. On the contrary, rPaGH8 did not release X1 but released
366 significant amount of X3 and β(1,4) hexamer (X6) eluting at 12.4 min. It produced two
367 additional unknown peaks eluted at 13.4 min and 14.5 min, the later being minor (Table 4).

368 When beechwood glucuronoxylan was hydrolysed by xylanase M1 or rPaGH8, the later
369 released three unidentified products absent with the former, at 17.4 min, 18.3 min and 19.0
370 min (Figure 5B and Table 4). When water-extractable or water-unextractable arabinoxylans
371 from wheat (WEAX and WUAX, respectively) were submitted to xylanase M1 (Figure 5C and
372 D), many different products appeared including X1 and X2 and many ramified
373 oligosaccharides, of which the majority has been previously identified **[32]**. rPaGH8 produced
374 X1 to X4 and many ramified oligosaccharides. Some of them were similar to those produced
375 by xylanase M1, but in different amounts. Remarkably rPaGH8 released lower amounts of
376 oligosaccharides with 1 or 2 un-substituted xylose at the reducing end (such as XA3X and
377 XA3XX) and higher amount of oligosaccharides with 3 un-substituted xylose at the reducing
378 end (such as XA2,3XXX). Three additional peaks appeared at 19.0 min, 19.7 min, 23.7 min. The
379 same unidentified peaks were produced from both WEAX and WUAX, and the peak at 19.0
380 min was also produced from beechwood GuX (Table 4).
381 From all these substrates, rPaGH8 produced linear xylooligosaccharides from degree of
382 polymerisation 1 (as traces) to 4, suggesting that the enzyme is unable to hydrolyse X4. X5
383 was absent (from WEAX, WUAX) or present in very minor amounts (from beechwood GuX),
384 suggesting that it was hydrolysed in X3 + X2. X6 accumulated from *Palmaria* xylan, suggesting
385 that rPaGH8 was not able to degrade it. As the polysaccharide contained (1,3) as well as (1,4)
386 linkages, it is likely that this accumulating oligosaccharide contained one (1,3) linkage that
387 prevented its degradation by rPaGH8.
388 The unknown peak eluting at 19.0 min (marked with a star on Figure 5) was chosen to
389 tentatively elucidate its structure because it was released from wheat arabinoxylans and
390 beechwood glucuronoxylan. To this aim, the peak was isolated, desalted and analysed by mass
391 spectrometry. The MS analysis showed a m/z of 1073.35, corresponding to an
392 oligosaccharide with a degree of polymerisation of 8 (Figure 6A). The MS/MS analysis did not
393 allow a complete characterization of this structure. Since all hydroxyl functions could not be

394 isolated via intracyclic fragments, several structures were possible. However, the elution time
395 of the linear X8 may be estimated around 14 min taking into account the elution time of the
396 available linear oligosaccharides (X1 to X6). Thus, it is unlikely that the unknown peak
397 corresponds to the linear oligosaccharide X8. Consequently, it could correspond to a branched
398 oligosaccharide with 1 arabinose and 7 xylose residues (A1X7) or 2 arabinose and 6 xylose
399 residues (A2X6) (Figure 6C). In this last case, the two arabinose residues could be located
400 either on the *O*3 of the two non-reducing xylose residues or on *0*2 and *0*3 of the last non-
401 reducing xylose residue (see the orange-marked hydroxyl positions on Figure 6C). The
402 structure presented on Figure 6C was the most probable as the non-reducing xylose residue
403 was free of any substituent. Indeed during catalysis, this xylose was bound to the sub-site +1
404 of the enzyme, which is not supposed to accept a substituted xylose. From the MS/MS
405 spectrum (Figure 6B) it is sure that this oligosaccharide contained at least 4 un-substituted
406 xylose residues at its reducing end.
407

408 ## 4- Discussion

409 Hemicellulose-degrading enzymes contribute to the saccharification process of biomass for
410 the production of platform molecules or second-generation bioethanol **[34 -39]**. They also
411 have a great impact in animal feeding **[40]**. At an analytical level, polysaccharide-active
412 enzymes are important tools to solve the fine structure of their substrates. They are produced
413 by many microorganisms and bioinformatic tools are essential to select those potentially
414 producing enzymes of interest for their numerous applications.
415 *P. atlantica* is a marine bacterium known to produce enzymes active on seaweeds
416 polysaccharides such as agarases [**41**], alginate lyase [**42**] and porphyranase [**43**]. The
417 genome annotation suggested 14 genes coding for putative hemicellulases (Table 1) [**44**].
418 Patl_1404 gene is the only gene from *P. atlantica* T6c coding a GH5 protein. Patl_2657 gene
419 was annotated as belonging to GH10, which suggested it as a xylanase. These predicted endo-

420 glucanase and endo-xylanase could explain the presence of activities on glucan, GlcM and
421 xylan in *P. atlantica* culture filtrate. Three sequences belong to GH43 and 4 to GH16. The
422 substrate specificity of these proteins, together with that belonging to GH8 (Patl_1069), is less
423 straightforwardly predictable, as these families are multispecific (5, 14, and 9 EC numbers in
424 families GH8, GH16 and GH43, respectively). Moreover, the choice of substrates is crucial to
425 succeed in evidencing enzymatic activities. As an example, the activities of *P. atlantica* culture
426 filtrates were much lower on maize AX in comparison to wheat AX and beechwood xylan. In
427 maize AX the xylan backbone can be decorated not only with arabinose but also with xylose
428 and galactose, and the arabinose moiety can be further decorated with xylose, galactose,
429 ferulic acid or one or more arabinose residues [**45-46**]. This gives it a more complex
430 structure that may make it more resistant to enzymatic hydrolysis than other AX.
431 Consequently, the hydrolysis of maize AX required a wider set of enzymes acting
432 synergistically, some of which may be lacking in *P. atlantica* secretome.
433 A β-xylosidase activity was shown in *P. atlantica* culture filtrates. The genome contains two
434 GH3-like sequences (Patl_3730, Patl_4162) whose family includes β-xylosidases, α-
435 arabinofuranosidases and β-glucosidases. It also contains 3 putative α-arabinofuranosidases
436 belonging to GH43 family (Patl_0810, Patl_0842 and Patl_3728) of which it is known that
437 many characterized enzymes showed a dual-activity α-arabinofuranosidase/ β-xylosidase
438 [**47-50**]. Thereby, there is two possible explanations for this β-xylosidase activity, either a
439 GH3 β-xylosidase or a GH43 dual-enzyme.
440 The xylanase activity shown after fractionation of *P. atlantica* secretome could be ensured by
441 two enzymes, the GH10 Patl_2657 and the GH8 Patl_1069. GH10 xylanases are characterized
442 by their high versatility since they hydrolyse many types of heteroxylans in which they can act
443 near a substituted xylose residue [**51 – 53**]. GH8 family contains xylanases for which little
444 data is available to date, and reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156),
445 which hydrolyses β-(1→4)-D-xylose residues from the reducing end of xylooligosaccharides

446 (www.cazy.org/GH8) and can be active on branched oligosaccharides [**54**]. To date, no
447 xylanase was described in *Pseudoalteromonas atlantica*. As we demonstrated that rPaGH8
448 produced oligosaccharides from wheat arabinoxylans, this ensures that it is an endoxylanase
449 (EC 3.2.1.8) and not a reducing-end-xylose releasing exo-oligoxylanase. In addition, the mode
450 of action of this xylanase on arabinoxylan was unusual since it favoured oligosaccharides with
451 side arabinose residues grouped on the non-reducing end and with an un-substituted tail on
452 the reducing end. Interestingly, two other GH8 enzymes were demonstrated in other
453 *Pseudoalteromonas* strains, the psychrotolerant *P. arctica* [**55**] and the psychrophilic *P.*
454 *haloplanktis* [**56**]. GH8 xylanase from *P. haloplanktis* has an inverting mechanism and a
455 $(\alpha/\alpha)_6$-fold. The increase in arabinose substitution on the substrate hindered *P. haloplanktis*
456 xylanase suggesting that the tolerance of GH8 xylanase towards the arabinose may be lower
457 than that of GH10 ones [**57**] and higher than those of GH11 which can only hydrolyse
458 xylosidic bonds between two un-branched xylose residues [**51**]. The crystal structure analysis
459 showed a long substrate-binding cleft from +4 to -3 subsites [**58**], which can be consistent
460 with the structure shown in the present study for the hydrolysis products. Moreover, *P.*
461 *haloplanktis* xylanase highlights a secondary binding site at the surface of the protein, which
462 favoured significantly the activity towards insoluble substrates [**59**]. However, its molar mass
463 was 48.4 kDa, two times less than PaGH8 xylanase, suggesting that the latter may be multi-
464 modular. Using the genome browser on biocyc.org allowed showing that the corresponding
465 gene may belong to a short operon with a gene whose product was annotated as a
466 hypothetical protein (Supplementary data 2). This gene did not belong therefore to an operon
467 with another GH, or to a polysaccharide-utilizing locus that could have helped the concerted
468 work of polysaccharides-active enzymes.
469 PaGH8 was active on various xylan-like substrates, originating from the seaweed *Palmaria*
470 *palmata* or from terrestrial plants, although *P. atlantica* originates from marine environment.
471 Although it remains unclear why marine bacteria secrete enzymes active towards plant

472 polysaccharides, this observation is consistent with the previous evidence that marine
473 bacteria secrete pectin-degrading enzymes in the presence of pectin. This was demonstrated
474 for bacteria close to *P. atlantica* such as *P. haloplanktis* [**60**] and *Alteromonas macleodii* [**61**].
475 The polysaccharides tested as substrates in the present study have rather known structures.
476 The structure of wheat AX was extensively studied and shown to vary largely in its level of
477 arabinose di-substitution [**62**]. NMR spectroscopy revealed that beechwood GuX contained
478 approximately one 4-O-methylglucuronic acid every 15 xylose residues [**63**]. From these
479 polysaccharides PaGH8 produced oligosaccharides in which the arabinose substitutions were
480 gathered at the non-reducing side of the molecule, showing that the initial polysaccharides
481 contained such stretches of xylose residues without any substitution. These new
482 oligosaccharides pave the way to elucidate further the chemical structure of the
483 polysaccharides thanks to PaGH8 particular behaviour.
484 Altogether the present results highlighted *P. atlantica* as a source of hemicellulose-degrading
485 enzymes. Our data showed that the use of various polysaccharides generated a wide variety of
486 enzymes. Only those produced in the presence of glucuronoxylan were studied, which particularly
487 revealed the new GH8 xylanase. Results obtained with one or more other polysaccharide substrates
488 lead to the production of a wide range of enzymes that remain to be valued in the near future.
489

Acknowledgments

We thank Maud Fer (CERMAV, Grenoble) for her help in the enzymatic screening, Marija Pavlovic and Hélène Rogniaux (INRA, UR 1286 BIA, Nantes, BIBS platform) for the proteomics analyses, and Anne-Laure Chateigner-Boutin (INRA, UR 1286 BIA, Nantes) for her help in using the web tool biocyc.org. SR was supported by a scholarship from the Pays de la Loire and Brittany regions within the GlycoOuest research network.

Authors' contributions

EB, ML, WH designed the research. SR, JV, AB, AG, MF performed the research experiments. SR, MFA, MF, DR, WH, ML and EB analyzed the data. SR and EB drafted the manuscript. All authors read and approved the final manuscript.

Conflict of interest

None declared

505 **References**

506 [1] P. Albersheim, A.G. Darvill, K. Roberts, R. Sederoff, A. Staehelin, Biochemistry of the cell wall
507 molecules, Plant Cell Walls, Garland Science, Taylor & Francis Group, LLC, New York, 2011, pp.
508 67-118.

509 [2] H.V. Scheller, P. Ulvskov, Hemicelluloses, Ann. Rev. Plant Biol 61 (2010) 263-289.

510 [3] M.S. Izydorczyk, C.G. Biliaderis, Cereal arabinoxylans : advances in structure and
511 physicochemical properties, Carbohydr. Polym 28 (1995) 33-48.

512 [4] C.L.O. Petkowicz, F. Reicher, H. Chanzy, F.R. Taravel, R. Vuong, Linear mannan in the
513 endosperm of *Schizolobium amazonicum*. Carbohydr. Polym 44 (2001) 107-112.

514 [5] J.J. Ordaz-Ortiz, M.-F. Devaux, L. Saulnier, Classification of wheat varieties based on structural
515 features of arabinoxylans as revealed by endoxylanase treatment of flour and grain, J. Agric. Food
516 Chem 53 (2005) 8349-8356.

517 [6] E. Nguema-Ona, J.P. Moore, A. Fagerstrom, J.U. Fangel, W.G.T. Willats, A. Hugo, M.A. Vivier,
518 Profiling the main cell wall polysaccharides of tobacco leaves using high-throughput and
519 fractionation techniques, Carbohydr. Polym 88(3) (2012) 939-949.

520 [7] L. Coulier, Y. Zha, R. Bas, P.J. Punt, Analysis of oligosaccharides in lignocellulosic biomass
521 hydrolysates by high-performance anion-exchange chromatography coupled with mass spectrometry
522 (HPAEC–MS), Biores. Technol 133 (2013) 221-231.

523 [8] S. Ray, J. Vigouroux, B. Quémener, E. Bonnin, M. Lahaye, Novel and diverse fine structures in
524 LiCl-DMSO extracted apple hemicelluloses, Carbohydr. Polym 108 (2014) 46-57.

525 [9] V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-
526 active enzymes database (CAZy) in 2013, Nucleic Acids Res 42 (2014) D490–D495.

527 [10] D. Shallom, Y. Shoham, Microbial hemicellulases, Curr. Op. Microbiol 6 (2003) 219-228.

528 [11] K. Kolenova, M. Vrsanska, P. Biely, Mode of action of endo-β-1,4-xylanases of families 10 and
529 11 on acidic xylooligosaccharides, J. Biotechnol 121(3) (2006) 338-345.

530 [12] J.-G. Berrin, N. Juge, Factors affecting xylanase functionality in the degradation of

531 arabinoxylans, Biotechnol. Lett 30(7) (2008) 1139-1150.
532 [13] J. Zhang, M. Siika-aho, T. Puranen, M. Tang, M. Tenkanen, L. Viikari, Thermostable
533 recombinant xylanases from *Nonomuraea flexuosa* and *Thermoascus aurantiacus* show distinct
534 properties in the hydrolysis of xylans and pretreated wheat straw, Biotechnol. Biofuels 4 (2011) 12-
535 24.
536 [14] G. Paës, J.G. Berrin, J. Beaugrand, GH11 xylanases: Structure/function/properties relationships
537 and applications, Biotechnol. Adv 30 (2012) 564-592.
538 [15] G. Gauthier, M. Gauthier, R. Christen, Phylogenetic analysis of the genera *Alteromonas*,
539 *Shewanella*, and *Moritella* using genes coding for small-subunit rRNA sequences and division of the
540 genus *Alteromonas* into two genera, *Alteromonas* (Emended) and *Pseudoalteromonas* gen. nov., and
541 proposal of twelve new species combinations, Intern. J. System. Bacteriol 45(4) (1995) 755-761.
542 [16] C. Assor, B. Quemener, J. Vigouroux, M. Lahaye, Fractionation and structural characterization
543 of LiCl–DMSO soluble hemicelluloses from tomato, Carbohydr. Polym 94 (2013) 46-55.
544 [17] M. Fer, A. Préchoux, A. Leroy, J.-F. Sassi, M. Lahaye, C. Boisset, P. Nyvall-Collén, W.
545 Helbert, Medium-throughput profiling method for screening polysaccharide-degrading enzymes in
546 complex bacterial extracts, J. Microbiol. Meth 89(3) (2012) 222-229.
547 [18] D.K. Kidby, D.J. Davidson, A convenient ferricyanide estimation of reducing sugars in the
548 nanomole range, Anal. Biochem 55 (1973) 321-325.
549 [19] N. Nelson, A photometric adaptation of the Somogyi method for determination of glucose, J.
550 Biol. Chem 153 (1944) 375-380.
551 [20] R.J. Sturgeon, Monosaccharides, in: P.M. Dey, J.B. Harbone (Eds.), Methods in Plant
552 Biochemistry, Academic Press, London (UK), 1990, pp. 1-38.
553 [21] X. Rouau, E. Odier, Purification and properties of two enzymes from *Dichomitus squalens*
554 which exhibit both cellobiohydrolase and xylanase activity, Carbohydr. Res 145 (1986) 279-292.
555 [22] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of
556 protein utilizing the principle of protein-dye binding, Anal. Biochem 72 (1976) 248-255.

557 [23] M. Suliman, A.-L. Chateigner-Boutin, M. Francin-Allami, A. Partier, B. Bouchet, J. Salse, C.
558 Pont, J. Marion, H. Rogniaux, D. Tessier, F. Guillon, C. Larré, Identification of glycosyltransferases
559 involved in cell wall synthesis of wheat endosperm, J. Proteomics 78 (2013) 508-521.
560 [24] A. Lawrence, H. Besir, Staining of proteins in gels with Coomassie G-250 without organic
561 solvent and acetic acid, J. Vis. Exp. (2009) 30, doi: 10.3791/1350.
562 [25] M. Lahaye, J. Vigouroux, Liquefaction of dulse (*Palmaria palmata* L. Kuntze) by a commercial
563 enzyme preparation and a purified endo-β-1,4-xylanase, J. Appl. Phycol 4 (1992) 329-337.
564 [26] B. Quémener, D. Bertrand, I. Marty, M. Causse, M. Lahaye, Fast data preprocessing for
565 chromatographic fingerprints of tomato cell wall polysaccharides using chemometric methods, J.
566 Chromatogr A 1141(1) (2007) 41-49.
567 [27] R. Fauré, C.M. Courtin, J.A. Delcour, C. Dumon, C.B. Faulds, G.B. Fincher, S. Fort, S.C. Fry,
568 S. Halila, M.A. Kabel, L. Pouvreau, B. Quémener, A. Rivet, L. Saulnier, H.A. Schols, H. Driguez,
569 M.J. O'Donohue, A brief and informationally rich naming system for oligosaccharide motifs of
570 heteroxylans found in plant cell walls, Austr. J. Chem 62(533-537) (2009).
571 [28] H.M. Ferreira, E.X.F. Filho, Purification and characterization of a β-mannanase from
572 *Trichoderma harzianum* strain T4. Carbohydr. Polym (2004)57, 23-29.
573 [29] J. Gomes, K. Terler, R. Kratzer, E. Kainz, W. Steiner, Production of thermostable β-
574 mannosidase by a strain of Thermoascus aurantiacus: Isolation, partial purification and
575 characterization of the enzyme, Enzyme Microb. Technol 40 (2007) 969-975.
576 [30] S.R. Ab, I. Darah, I.C. Omar, Utilization of palm kernel cake for the production of mannanase
577 by an indigenous filamentous fungus, *Aspergillus niger* USM F4 under solid substrate fermentation,
578 The Internet J. Microbiol 9(1) (2009).
579 [31] D.Y. Kim, S.-J. Ham, H.J. Lee, Y.-J. Kim, D.-H. Shin, Y.H. Rhee, K.-H. Son, H.-Y. Park, A
580 highly active endo-β-1,4-mannanase produced by *Cellulosimicrobium* sp. strain HY-13, a
581 hemicellulolytic bacterium in the gut of *Eisenia fetida*, Enzyme Microb. Technol 48 (2011) 365-370.
582 [32] L. Saulnier, B. Quémener, Enzymatic mapping of arabinoxylan structure, in: P.R. Shewry, J.L.

583 Ward (Eds.), Analysis of Bioactive Components in small grain cereals, AACC International, St Paul,
584 Minnesota 55121, USA, 2009, pp. 191-202.
585 [33] B. Domon, C.E. Costello, Structure elucidation of glycosphingolipids and gangliosides using
586 high performance tandem mass spectrometry, Biochem 27(5) (1988) 1534-1543.
587 [34] F.M. Girio, C. Fonseca, F. Carvalheiro, L.C. Duarte, S. Marques, R. Bogel-Lukasik,
588 Hemicelluloses for fuel ethanol: A review, Bioresource Technology Special Issue on Lignocellulosic
589 Bioethanol: Current Status and Perspectives 101(13) (2010) 4775-4800.
590 [35] R.E.H. Sims, W. Mabee, J.N. Saddler, M. Taylor, An overview of second generation biofuel
591 technologies, Biores. Technol 101(6) (2010) 1570-1580.
592 [36] F. Talebnia, D. Karakashev, I. Angelidaki, Production of bioethanol from wheat straw: An
593 overview on pretreatment, hydrolysis and fermentation, Biores. Technol 101(13) (2010) 4744-4753.
594 [37] T. Hasunuma, F. Okazaki, N. Okai, K.Y. Hara, J. Ishii, A. Kondo, A review of enzymes and
595 microbes for lignocellulosic biorefinery and the possibility of their application to consolidated
596 bioprocessing technology, Biores. Technol 135(0) (2013) 513-522.
597 [38] R. Goldbeck, A.R.L. Damasio, T.A. Gonçalves, C.B. Machado, D.A.A. Paixao, L.D. Wolf, F.
598 Mandelli, G.J.M. Rocha, R. Ruller, F.M. Squina, Development of hemicellulolytic enzyme mixtures
599 for plant biomass deconstruction on target biotechnological applications, Appl Microb Biotechnol 98
600 (2014) 8513-8525.
601 [39] S. Brethauer, M.H. Studer, Biochemical conversion processes of lignocellulosic biomass to
602 fuels and chemicals - A review, Chimia 69(10) (2015) 572-581.
603 [40] E. Kiarie, L.F. Romero, C.M. Nyachoti, The role of added feed enzymes in promoting gut
604 health in swine and poultry, Nutr Res Rev 26 (2013) 71-88.
605 [41] D. Groleau, W. Yaphe, Enzymatic hydrolysis of agar: purification and characterization of
606 beta-neoagarotetraose hydrolase from *Pseudomonas atlantica*, Can. J. Microbiol 23 (1977) 672-679.
607 [42] R. Matsushima, H. Danno, M. Uchida, K. Ishihara, T. Suzuki, M. Kaneniwa, Y. Ohtsubo, Y.
608 Nagata, M. Tsuda, Analysis of extracellular alginate lyase and its gene from a marine bacterial strain,

609 *Pseudoalteromonas atlantica* AR06, Appl. Microbiol. Biotechnol 86(2) (2010) 567-576.
610 [43] C. Przybylski, G. Correc, M. Fer, F. Gonnet, W. Helbert, R. Daniel, MALDI-TOF MS and ESI-
611 LTQ-Orbitrap tandem mass spectrometry reveal specific porphyranase activity from a
612 *Pseudoalteromonas atlantica* bacterial extract, RSC Advances 5(98) (2015) 80793-80803.
613 [44] A. Copeland, S. Lucas, A. Lapidus, K. Barry, J.C. Detter, T. Glavina del Rio, N. Hammon, S.
614 Israni, E. Dalin, H. Tice, S. Pitluck, E. Saunders, T. Brettin, D. Bruce, C. Han, R. Tapia, P. Gilna, J.
615 Schmutz, F. Larimer, M. Land, L. Hauser, N. Kyrpides, E. Kim, A.C. Karls, D. Bartlett, B.P.
616 Higgins, P. Richardson, Complete sequence of *Pseudoalteromonas atlantica* T6c. (2006) Submitted
617 (15-JUN-2006) US DOE Joint Genome Institute, 2800 Mitchell Drive B100, Walnut Creek, CA
618 94598-1698, USA.
619 [45] L. Saulnier, C. Marot, E. Chanliaud, J.-F. Thibault, Cell wall polysaccharide interactions in
620 maize bran, Carbohydr. Polym 26 (1995) 279-287.
621 [46] L. Saulnier, J. Vigouroux, J.-F. Thibault, Isolation and partial characterization of feruloylated
622 oligosaccharides from maize bran, Carbohydr. Res 272 (1995) 241-253.
623 [47] K. Wagschal, C. Heng, C.C., Lee, D.W. Wong, Biochemical characterization of a novel dual-
624 function arabinofuranosidase/xylosidase isolated from a compost starter mixture, Appl. Microbiol.
625 Biotechnol 81 (2009) 855–863.
626 [48] M. Ravanal, E. Callegari, J. Eyzaguirre, Novel bifunctional α-L-
627 Arabinofuranosidase/Xylobiohydrolase (ABF3) from *Penicillium purpurogenum*, Appl. Environ.
628 Microbiol 76 (2010) 5247-5253.
629 [49] J. Zhou, L. Bao, L. Chang, Y. Zhou, H. Lu, Biochemical and kinetic characterization of GH43
630 β-D-xylosidase/ α-L-arabinofuranosidase and GH30 α-L-arabinofuranosidase/β-D-xylosidase from
631 rumen metagenome, J. Indus. Microbiol. Biotechnol 39 (2012) 143–152.
632 [50] A.H. Viborg, K.I. Sorensen, O. Gilad, D.B. Steen-Jensen, A. Dilokpimol, S. Jacobsen, B.
633 Svensson, Biochemical and kinetic characterisation of a novel xylooligosaccharide-upregulated
634 GH43 β-D-xylosidase/α-L-arabinofuranosidase (BXA43) from the probiotic *Bifidobacterium*

635 *animalis* subsp. lactis BB-12, AMB Express 3 (2013) 56.
636 [51] P. Biely, M. Vrsanska, M. Tenkanen, D. Kluepfel, Endo-β-1,4-xylanase families: differences in
637 catalytic properties, J. Biotechnol 57 (1997) 151-166.
638 [52] J. Beaugrand, G. Chambat, V.W.K. Wong, F. Goubet, C. Rémond, G. Paës, S. Benamrouche, P.
639 Debeire, M.J. O'Donohue, B. Chabbert, Impact and efficiency of GH10 and GH11 thermostable
640 endoxylanases on wheat bran and alkali-extractable arabinoxylans, Carbohydr. Res 339 (2004) 2529-
641 2540.
642 [53] M. Lafond, A. Tauzin, V. Desseaux, E. Bonnin, A. El Hassan, T. Giardina, GH10 xylanase D
643 from *Penicillium funiculosum*: biochemical studies and xylooligosaccharides production, Microb.
644 Cell Fact 10(20) (2011) 1-8.
645 [54] S.V. Valenzuela, S. Lopez, P. Biely, J. Sanz-Aparicio, F.I.J. Pastor, The Glycoside Hydrolase
646 family 8 reducing-end xylose-releasing exo-oligoxylanase Rex8A from *Paenibacillus barcinonensis*
647 BP-23 is active on branched xylooligosaccharides, Appl. Environ. Microbiol 82(17) (2016) 5116-
648 5124.
649 [55] S. Elleuche, H. Piascheck, G. Antranikian, Fusion of the OsmC domain from esterase EstO
650 confers thermolability to the cold-active xylanase Xyn8 from *Pseudoalteromonas arctica*,
651 Extremophiles 15 (2011) 311-317.
652 [56] T. Collins, M.-A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A novel family 8
653 xylanase, functional and physicochemical characterization, J. Biol. Chem 277(38) (2002) 35133-
654 35139.
655 [57] A. Pollet, J. Schoepe, E. Dornez, S.V. Strelkov, J.A. Delcour, C.M. Courtin, Functional analysis
656 of glycoside hydrolase family 8 xylanases shows narrow but distinct substrate specificities
657 and biotechnological potential, Appl. Microbiol. Biotechnol 87 (2010) 2125-2135.
658 [58] F. Van Petegem, T. Collins, M.-A. Meuwis, C. Gerday, G. Feller, J. Van Beeumen, The
659 structure of a cold-adapted family 8 xylanase at 1.3 Å resolution, J. Biol. Chem 278 (2003) 7531-
660 7539.

661 [59] S. Cuyvers, E. Dornez, J.A. Delcour, C.M. Courtin, The secondary substrate binding site of the
662 *Pseudoalteromonas haloplanktis* GH8 xylanase is relevant for activity on insoluble but not soluble
663 substrates, Appl. Microbiol. Biotechnol 92 (2011) 539-549.
664 [60] J.-H. Hehemann, L.V. Truong, F. Unfried, N. Welsch, J. Junker, S.E. Heiden, S. Junker, D.
665 Becher, A. Thürmer, R. Daniel, R. Amann, T. Schweder, Aquatic adaptation of a laterally acquired
666 pectin degradation pathway in marine gammaproteobacteria, Environ. Microbiol 19(6) (2017) 2320-
667 2333.
668 [61] H. Koch, A. Dürwald, T. Schweder, B. Noriega-Ortega, S. Vidal-Melgosa, J.-H. Hehemann, T.
669 Dittmar, H.M. Freese, D. Becher, M. Simon, M. Wietz, Biphasic cellular adaptations and ecological
670 implications of *Alteromonas macleodii* degrading a mixture of algal polysaccharides, The ISME J 13
671 (2019) 92-103.
672 [62] G. Dervilly, L. Saulnier, P. Roger, J.-F. Thibault, Isolation of homogeneous fractions from
673 wheat water-soluble arabinoxylans. Influence of the structure on their macromolecular
674 characteristics, J. Agric. Food Chem 48 (2000) 270-278.
675 [63] A. Teleman, M. Tenkanen, A. Jacobs, O. Dahlmana, O. Characterization of *O*-acetyl-(4-*O*-
676 methylglucurono)xylan isolated from birch and beech, Carbohydr. Res 337 373–377.
677

678 **Figure legends**

679

680 **Figure 1:** *P. atlantica* growth in the absence of added polysaccharide (black line), and in the
681 presence of konjac glucomannan, GlcM (green line) and beechwood glucuronoxylan, GuX (red
682 line).

683

684 **Figure 2:** Enzymatic activities measured in the culture supernatants of *P. atlantica* on
685 hemicelluloses (**A, C, E**, respectively) and *p*-NP-sugar derivatives (**B, D, F**, respectively). Three
686 culture conditions were applied, without any added polysaccharide (**A, B**), in the presence of
687 konjac glucomanann, GlcM (**C, D**) and in the presence of beechwood glucuronoxylan, GuX (**E,**
688 **F**). Each activity was measured at pH 5.2 (black bars) and 8.5 (grey bars).

689

690 **Figure 3:** Enzyme profile after fractionation by anion exchange chromatography of the
691 culture filtrate of *P. atlantica* grown in the presence of beechwood glucuronoxylan (GuX).
692 (**A**) Activities measured on carboxymethyl cellulose, CMC (black full line), konjac
693 glucomannan, GlcM (black dotted line), beechwood glucuronoxylan GuX (grey full line) and
694 wheat arabinoxylan AX (grey dotted line).
695 (**B**) Activities measured on *p*-NP α-L-arabinofuranoside, *p*-NP α-L-Ara (full line), and *o*-NP β-
696 D-xylopyranoside, *o*-NP β-D-Xyl (dotted line).

697

698 **Figure 4:** SDS-PAGE
699 (A) The labels B2 to B12 referred to the fractions recovered between 7 and 13 mL elution
700 volume of the anion exchange chromatography of the *P. atlantica* culture filtrate obtained on
701 beechwood glucuronoxylan (see Figure 3).

702 (B) The labels 1 to 8 referred to the fractions eluted from the His trap affinity column for the
703 purification of the recombinant rPaGH8. The fractions 4 to 6 were pooled to get the
704 recombinant rPaGH8.
705 M: molecular weight markers.
706
707 **Figure 5:** HPAEC analysis of the reaction products released by rPaGH8 (black line) and the
708 commercial xylanase M1 (green line) from different xylan substrates. The labels refer to the
709 structure of the oligosaccharides, using the nomenclature of Fauré et al., 2009 [**27**].
710 (**A**): Xylan from *Palmaria palmata*
711 (**B**): Beechwood glucuronoxylan
712 (**C**): Water extractable arabinoxylan from wheat endosperm
713 (**D**): Water unextractable arabinoxylan from wheat endosperm
714 *: oligosaccharide whose structure has been subsequently identified by mass spectrometry
715
716 **Figure 6**: Mass spectrometry analysis of the HPAEC fraction collected at the retention time of
717 19.0 min (see Figure 5).
718 (**A**) Zoom of the MS spectrum with the peak corresponding to an AX DP8 (*m/z* 1073.35).
719 (**B**) MS/MS spectrum of the 1073.35 *m/z* species after further fragmentation.
720 (**C**) One possible structure of observed fragment depicted according to the nomenclature of
721 Domon and Costello, 1998 [**33**]. The two pentoses in orange can be placed in any orange-
722 marked position. Blue annotations: intercyclic fragments; Red annotations: intracyclic
723 fragments; Stars: fragments with the loss of one water molecule.
724
725

726 **Supplementary data**

727 **Supplementary data 1:** Amino-acid sequence of the GH8 protein Patl_1069 excised from the
728 PAGE gel (see Figure 4). The matched peptides were identified by LC-MS/MS and are
729 highlighted in red. They lead to sequence coverage of 8%.

730

731 **Supplementary data 2:** *P. atlantica* genome visualized using the genome browser on
732 biocyc.org (position 1,214,005 to 1,348,215 bp). The gene PATL_RS05460 (in brown and
733 framed in bold in the centre of the figure) corresponds to the protein UniProt Q15WZ3
734 (Patl_1069) and its locus is located between position 1,279,741 and 1,282,479 bp. The colours
735 refer to putative operons. The gene PATL_RS05460 forms a putative operon with the gene
736 PATL_RS05455 located just upstream on the genome. The product of this gene is annotated as
737 a hypothetical protein.

738

739 **Table 1**: Presentation of *Pseudoalteromonas atlantica* T6c strain [**15**]: origin, culture
740 conditions and occurrence of the hemicellulose-modifying GH families.

General information	Strain	Origin
	ATCC BAA-1087	Marine
Culture conditions		
Medium	Bactomarine broth (DIFCO 2216)	
pH	7.4	
T (°C)	20	
Occurrence of hemicellulose-related GH families		
GH family	Number of sequences	Gene entry
GH3	4	Patl_0825
		Patl_1716
		Patl_4162
		Patl_3730
GH5	1	Patl_1404
GH8	1	Patl_1069
GH10	1	Patl_2657
GH16	4	Patl_0805
		Patl_0824
		Patl_0843
		Patl_0880
GH43	3	Patl_0810
		Patl_0842
		Patl_3728

741
742

743 **Table 2**: List of polysaccharides used as substrates for the screening of polysaccharides-
744 degrading enzymes.

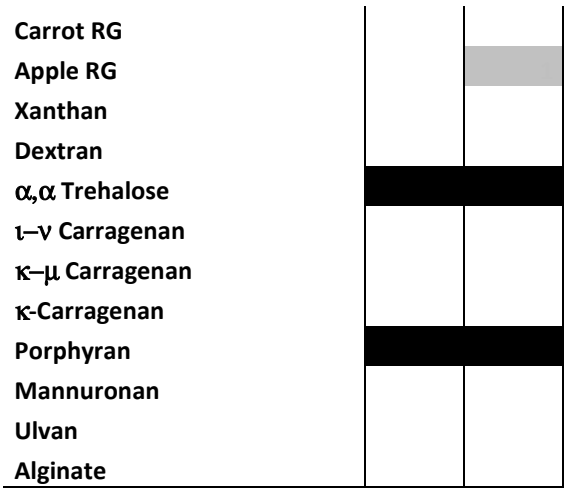
	Polysaccharide	Biological origin	Source
1	**Arabinoxylan (AX)**	Maize	Lab collection
2	**Arabinoxylan (AX)**	Rye	Megazyme
3	**Arabinoxylan (AX)**	Wheat	Megazyme
4	**Glucuronoxylan (GuX)**	Beechwood	Sigma
5	**Glucuronoxylan (GuX)**	Tomato	[**16**]
6	**Glucuronoarabinoxylan (GAX)**	Apple	[**8**]
7	**Glucomannan (GlcM)**	*Amorphophallus konjac* (Konjac powder)	Dextra Lab
8	**Galactomannan (GalM)**	*Caesalpina spinosa* (Tara gum)	Dextra Lab
9	**Galactomannan (GalM)**	*Cyamopsis tetragonolobus* (Guar gum)	Dextra Lab
10	**Galactomannan (GalM)**	*Ceretonia siliqua* (Locust bean gum)	Dextra Lab
11	**β (1,4) Mannan**	Carob	Megazyme
12	**Galactoglucomannan (GgM, E3)**	Apple	[**8**]
13	**Galactoglucomannan (GgM, A1)**	Apple	[**8**]
14	**Galactoglucomannan (GgM)**	Tomato	Lab collection
15	**CarboxyMethyl Cellulose (CMC)**		Sigma
16	**Avicel**		Sigma
17	**Xyloglucan (XyG)**	*Tamarindus indica* (Tamarind gum)	Dextra Lab
18	**Acetylated xyloglycan (AcXyG)**	Apple	[**8**]
19	**Xyloglucan (XyG)**	Apple	[**8**]
20	**Xyloglucan (XyG)**	Tomato	[**16**]
21	**Partially depectinated AIR* (pDAIR)**	Apple	[**8**]
22	**Arabinan**	Sugar beet	Megazyme
23	**Welan gum**	*Alcaligenes* sp.	Dextra Lab
24	**Tragacanth gum**	*Astragalus*	Dextra Lab
25	**Arabic gum**	*Acacia* sp.	Dextra Lab
26	**Amylopectin**	Waxy corn	TCI
27	**Amylose**	Potato	Lab collection
28	**Pullulan**	*Scerotium rolfsii*	Dextra Lab
29	**High methylated Pectin (HMPectin)**	Apple	[**8**]
30	**Low methylated Pectin (LMPectin)**	Citrus	Cargill
31	**Rhamnogalacturonan (RG)**	Carrot	Lab collection
32	**Rhamnogalacturonan (RG)**	Apple	[**8**]
33	**Xanthan**	*Xanthomonas campestris*	Dextra Lab
34	**Dextran**	*Leuconostoc mesenteroides*	Dextra Lab
35	**α,α Trehalose**		Carbosynth Ltd
36	**ι–ν Carragenan**	*Kappaphycus alvarezzi*	CP-Kelco
37	**κ–μ Carragenan**	*Eucheuma denticulatum*	CP-Kelco
38	**κ-Carragenan**	*Kappaphycus alvarezzi*	CP-Kelco
39	**Porphyran**	*Porphyra umbilicalis*	Lab collection
40	**Mannuronan**		Lab collection
41	**Ulvan**	*Ulva rotundata*	Lab collection
42	**Alginate**	*Ascophyllum* sp.	Lab collection

* AIR = Alcohol Insoluble Residue

745
746

747 **Table 3**: Colorimetric profiling of the hemicellulose-degrading enzymes on 42
748 polysaccharides in the extracellular (E) and intracellular (I) culture filtrates of
749 *Pseudoalteromonas atlantica* T6c grown without or with added polysaccharides: mixture of
750 carboxymethyl cellulose + tara galactomannan + beechwood xylan (abbreviated as '3P'),
751 konjac glucomannan (GlcM) and beechwood xylan. Positive hits of polysaccharide
752 degradation were classed according to 4 intervals based on the control mean (x) and standard
753 deviation (σ) of negative hit polysaccharides. The extent of degradation was indicated
754 according to grey intensity, : x −2σ< absorbance < x−4σ ;
755 : x−4σ< absorbance < x−6σ; : x−6σ < absorbance < x−8σ ; : x−8σ< absorbance.

Added Polysaccharides	No		3P	GlcM	Xylan
Culture filtrate*	E	I	E	E	E
Protein concentration (µg/mL)	1265	1470	821	606	660
Screening substrate					
Maize AX					
Rye AX					
Wheat AX					
Beechwood xylan					
Tomato GuX					
Apple GAX					
Konjac GlcM					
Tara GalM					
Guar GalM					
Locust GalM					
β (1,4) Mannan					
Apple GgM					
Apple GgM					
Tomato GgM					
CMC					
Avicel					
Tamarind XyG					
Apple AcXyG					
Apple XyG					
Tomato XyG					
pDAIR					
Arabinan					
Welan gum					
Tragacanth gum					
Arabic gum					
Amylopectin					
Amylose					
Pullulan					
Apple HM Pectin					
Citrus LM Pectin					



* Two type of culture filtrates: E = extracellular, I = intracellular

758 **Table 4**: Hydrolysis products released by rPaGH8 from various hemicelluloses. Crosses (+) in
759 bold indicate oligosaccharides produced exclusively by rPaGH8 and not by xylanase M1. The
760 number of + is used as an indication of the peak size.

		Xylan *Palmaria*	Beechwood GuX	WEAX	WUAX
Ara	3.3				+
X1	3.7		+	+	+
X2	5.4	+	+++	+	+
X3	8.1	++	+++	+	+
X4	10	+	+++	+	+
X5	11.2		+		
X6	12.4	**++**			
X7 ?	13.2	+			
Unknown	13.4	**+**			
XA3X	13.8			++	++
Unknown	14.2				
XA3XX	14.5			++	++
Unknown#	14.5	**+**			
XXA3XX	15.3		+	+	+
Unknown	16.3		++		
Unknown	17.4		**++**		
XA2,3XXX	18			+	+
Unknown	18.3		**++**		
Unknown*	19.0		**++**	**+**	**+**
Unknown	19.7		**+**	**+**	**+**
Unknown	20.1				
Unknown	21.4			+	+
XA3A3XX	21.7			+	+
XA3XA3XX	22.2			+	+
Unknown	23.7			**+**	**+**
XA3A2,3XX	24.2			+	+

761 #this peak eluted at the same time as XA3XX but corresponds to another oligosaccharide
762 as the xylan from *Palmaria* does not contain any arabinose.
763 * this peak was further identified by mass spectrometry
764

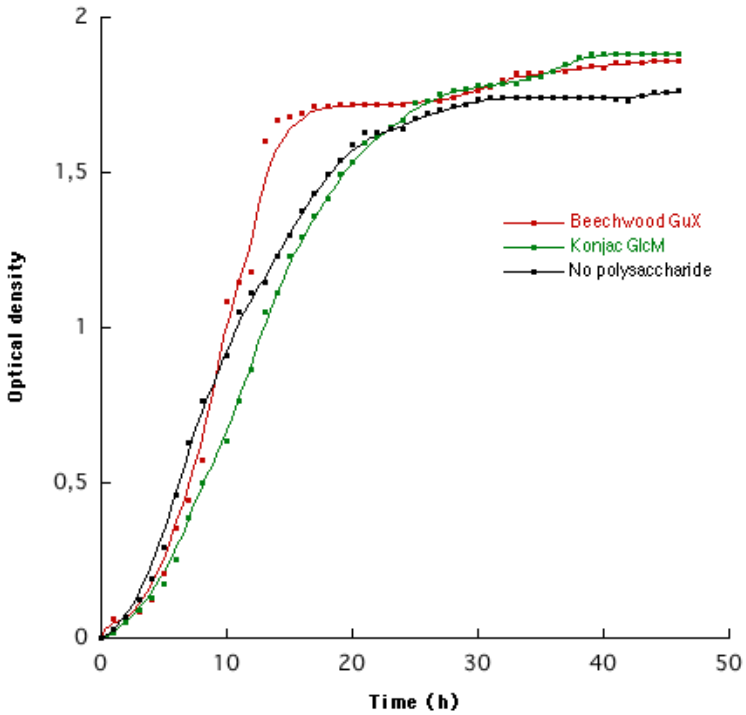


Figure 1: *P. atlantica* growth in the absence of added polysaccharide (black line), and in the presence of konjac glucomannan, GlcM (green line) and beechwood glucuronoxylan, GuX (red line).

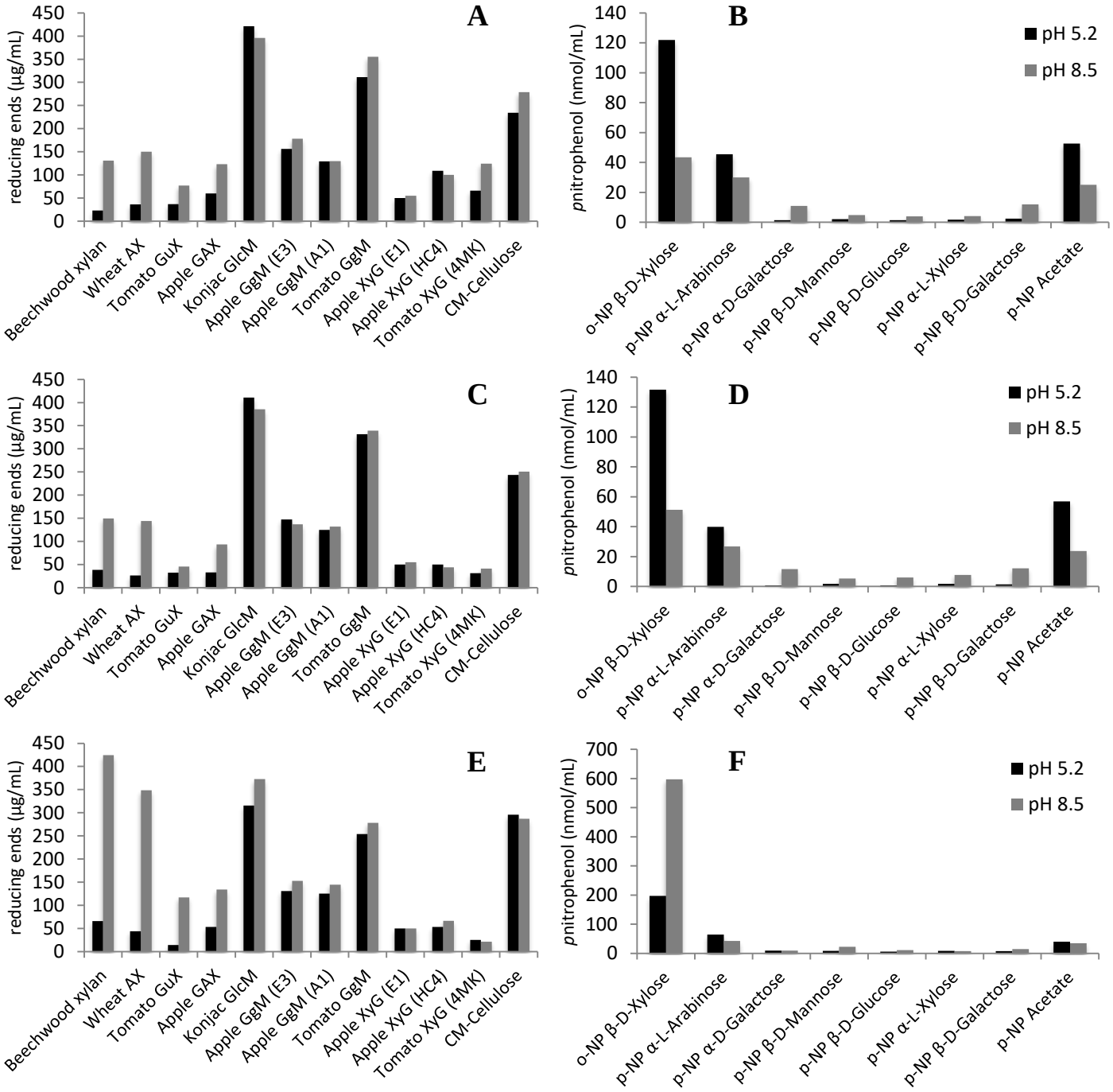


Figure 2: Enzymatic activities measured in the culture supernatants of *P. atlantica* on hemicelluloses (**A, C, E**, respectively) and *p*-NP-sugar derivatives (**B, D, F**, respectively). Three culture conditions were applied, without any added polysaccharide (**A, B**), in the presence of konjac glucomanann, GlcM (**C, D**) and in the presence of beechwood glucuronoxylan, GuX (**E, F**). Each activity was measured at pH 5.2 (black bars) and 8.5 (grey bars).

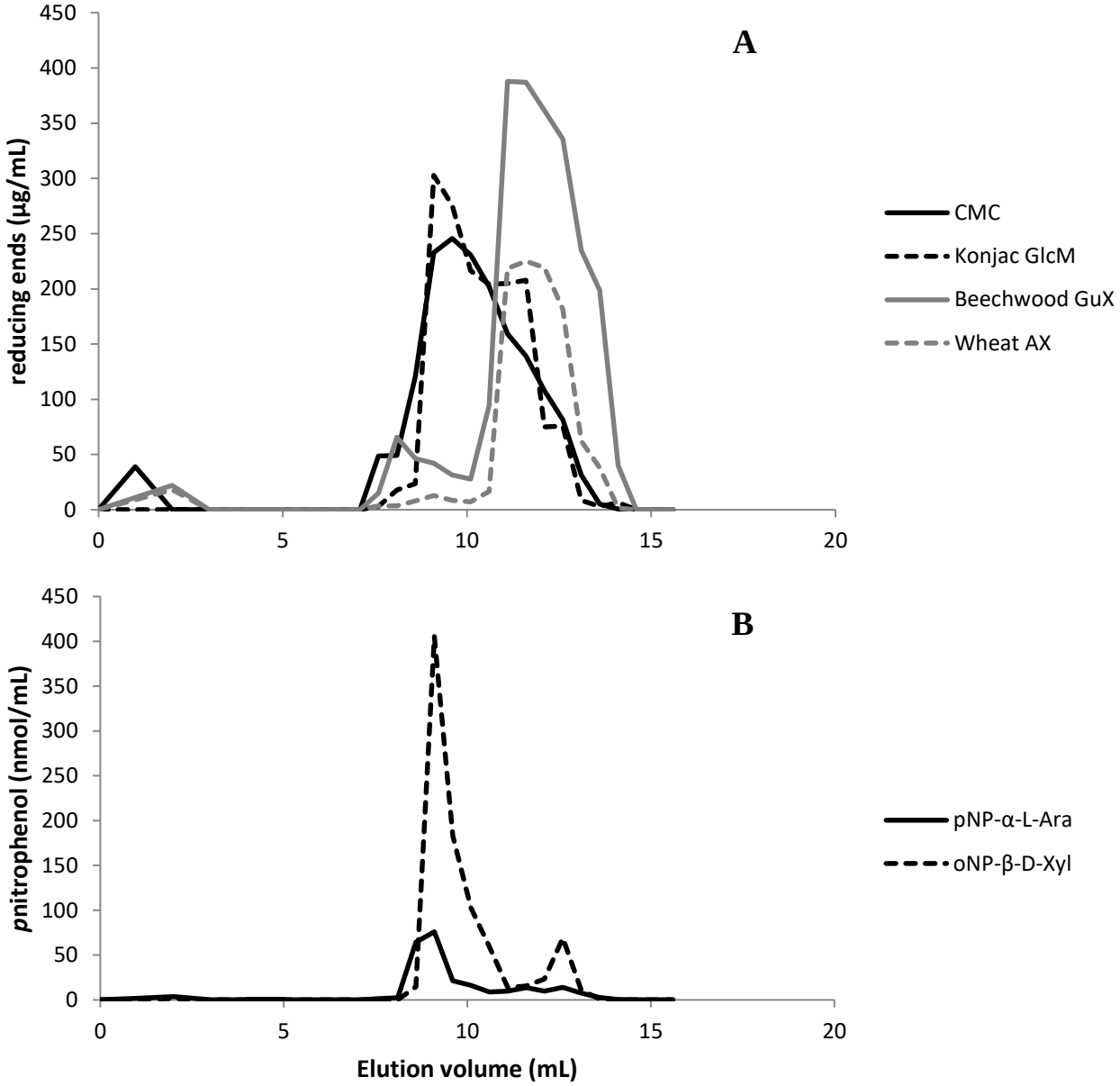


Figure 3: Enzyme profile after fractionation by anion exchange chromatography of the culture filtrate of *P. atlantica* grown in the presence of beechwood glucuronoxylan (GuX).
(**A**) Activities measured on carboxymethyl cellulose, CMC (black full line), konjac glucomannan, GlcM (black dotted line), beechwood glucuronoxylan GuX (grey full line) and wheat arabinoxylan AX (grey dotted line).
(**B**) Activities measured on *p*-NP α-L-arabinofuranoside, *p*-NP α-L-Ara (full line), and *o*-NP β-D-xylopyranoside, *o*-NP β-D-Xyl (dotted line).

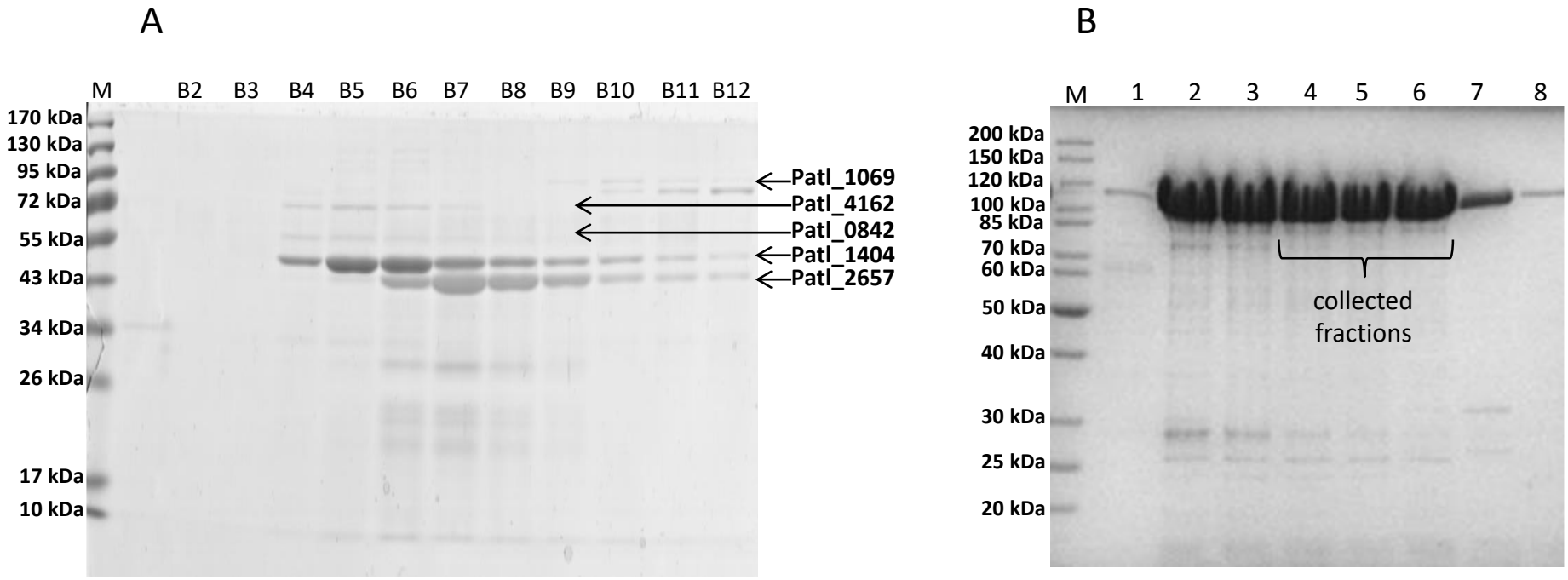


Figure 4: SDS-PAGE
(**A**) The labels B2 to B12 referred to the fractions recovered between 7 and 13 mL elution volume of the anion exchange chromatography of the *P. atlantica* culture filtrate obtained on beechwood glucuronoxylan (see Figure 3).
(**B**) The labels 1 to 8 referred to the fractions eluted from the His trap affinity column for the purification of the recombinant rPaGH8. The fractions 4 to 6 were pooled to get the recombinant rPaGH8.
M: molecular weight markers.

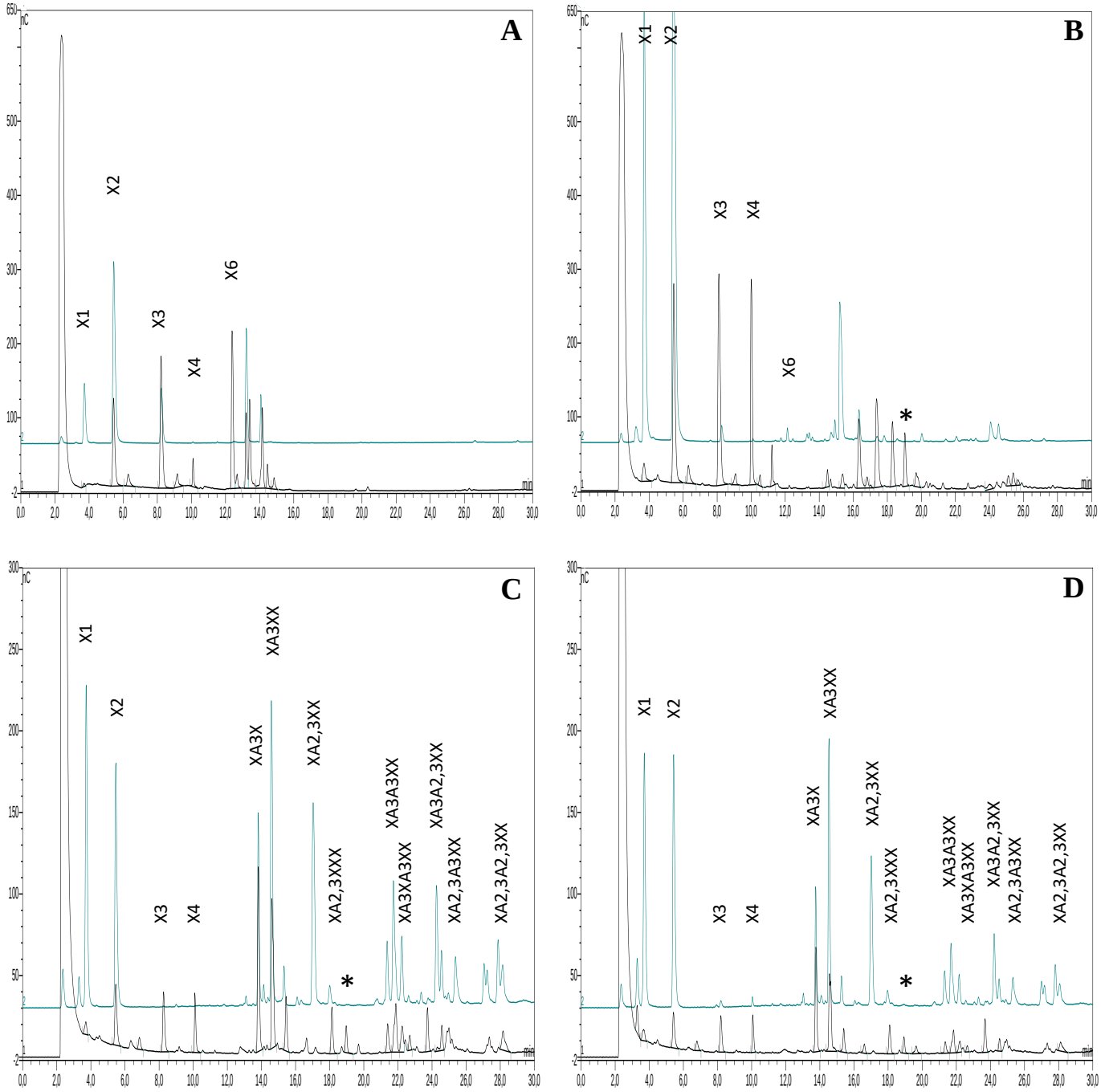


Figure 5: HPAEC analysis of the reaction products released by rPaGH8 (black line) and the commercial xylanase M1 (green line) from different xylan substrates. The labels refer to the structure of the oligosaccharides, using the nomenclature of Fauré et al., 2009 [**27**].
(**A**): Xylan from *Palmaria palmata*
(**B**): Beechwood glucuronoxylan
(**C**): Water extractable arabinoxylan from wheat endosperm
(**D**): Water unextractable arabinoxylan from wheat endosperm
*: oligosaccharide whose structure has been subsequently identified by mass spectrometry

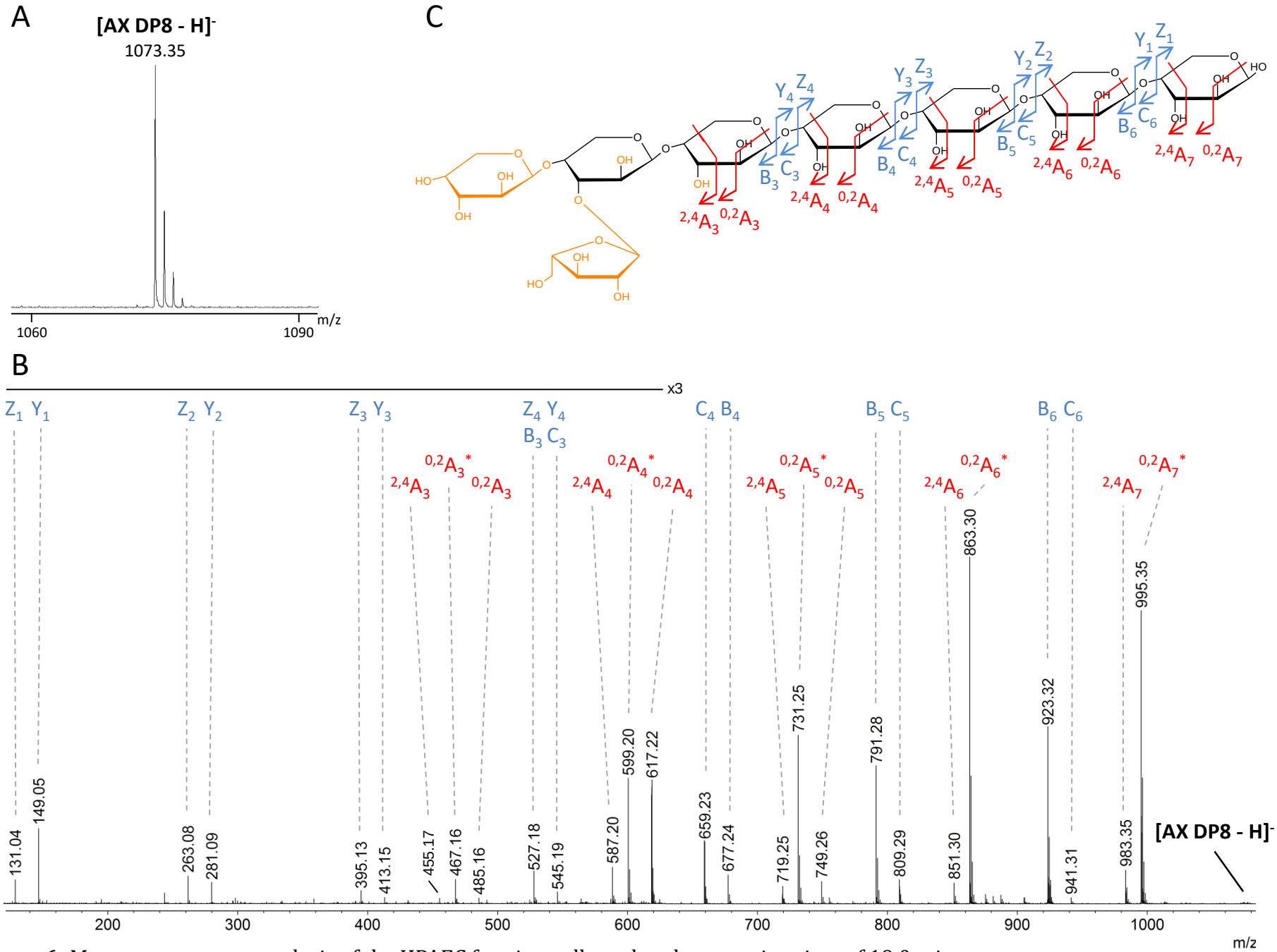


Figure 6: Mass spectrometry analysis of the HPAEC fraction collected at the retention time of 19.0 min.