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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

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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

1- Introduction

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

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

residues in XyG requires either broadly specific endo-β-1,4-glucanases (EC 3.2.1.4), belonging to 13 different GH families (5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 74 and 124), or highly specific endo-acting xyloglucanases (EC 3.2.1.151), classified in 6 GH families (5, 9, 12, 16, 44 and 74)(http://www.cazy.org). Complete degradation of heteroxylan requires the co-working of endo-β-1,4-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase and esterase **[10]**. Most of the microbial β-1,4-xylanases (EC 3.2.1.8) are grouped into 7 GH families: GH5, 8, 30, 43, 51, where a bi-functional cellulase/xylanase stands, and GH10 and 11, where the most studied β-1,4-xylanases are gathered **[11–14]**. The endo-β-1,4-mannanases required for the hydrolysis of heteromannan backbone are classified into the GH families 5, 26 and 113.

As part of a comprehensive screening of bacteria to highlight enzymatic activities towards a large number of polysaccharides, *Pseudoalteromonas atlantica* was shown to produce an interesting diversity of enzyme repertoire. Its genome contains several GH genes (Table 1) but there is a lack of information on most of the corresponding proteins. Therefore it was grown in the presence or not of different hemicelluloses to study the potential inducer effect on the enzyme secretion. Its secretome was fractionated to identify the proteins involved in the observed activities. One of these proteins annotated as belonging to GH8 family was studied in more details.

2- Material and methods

2-1 Culture conditions

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

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

2-2 Preparation of polysaccharides for enzyme screening

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

2-3 Screening assay

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

incubating 150 µL of polysaccharide solutions with 150 µL 50 mM buffer solution (Tris-HCl, pH 8.5 or acetate, pH 5.2) instead of culture filtrate or cell lysate. Microplates were sealed with a plastic film and incubated overnight with shaking at 34°C. The incubation medium was filtered on a multiscreen HTS vacuum manifold (MSVMHTS00, Millipore) connected to a high-output vacuum pressure pump (Millipore) for 3 h under about 0.5 bar pressure.

For the colorimetric assay, 40 µL of each incubation filtrate were transferred to a microplate (Dutscher, Brumath, France). Then, 200 µL of ferricyanide solution **[18]** were added to the sample and the microplate was sealed with plastic film. The plate was heated at 95°C for 15 min in a thermocycler (GenAmp PCR system 2700, Applied Biosystems France, Villebon-sur-Yvette, France) and cooled to room temperature. The occurrence of reducing ends was revealed by reading the absorbance at 420 nm of 200 µL of the samples in a microplate reader (Nunc, Roskilde, Denmark) using a Wallac 1420 multilabel counter (PerkinElmer Life Sciences, Boston, MA, USA). The amount of reducing ends was expressed in absorbance units. The samples with absorbance higher than the corresponding bacterial filtrate or lysate alone (control) minus 0.15 were considered as negative hits. The mean (X) and the standard deviation (σ) of the optical density (OD) of these negative hits were calculated. When the OD of the sample was lower than X-2σ, the hit was considered as positive. The polysaccharides were thus classified according to four intervals based on the control X and its σ.

2-4 Time course of growth of P. atlantica

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

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

2-5 Fractionation of the culture supernatant of P. atlantica

Similar cultures were carried out at larger scale by inoculating 50 mL of pre-culture in 1 L of fresh medium in 5 L shaker flask in the same conditions.

P. atlantica was cultivated in sterilized bactomarine broth without any added polysaccharide or in the presence of konjac GlcM or beechwood GuX at 1 g/L. After 16 h, 18 h and 16 h of respective growth for the three conditions, the culture was centrifuged at 5000 × g for 30 min at 4°C. The cell free supernatants were recovered and complemented with a protease inhibitor cocktail.

The culture supernatants were filtered overnight on a 300 kDa membrane (PES, Millipore) using a 400 mL Amicon system 8400 (Millipore). The filtrates were diafiltered and concentrated to 20 mL, with deionized water on a 10 kDa membrane (PES, Millipore) under compressed air (0.5 bar) to remove small molecules. The concentrated filtrates were separated into 2 x 10 mL and the pH of each half was adjusted at 8.5 or 5.2 by adding 100 mM Tris–HCl or 100 mM acetate buffer, respectively. By this way, 6 culture filtrates were obtained: without any added polysaccharide or with konjac GlcM or beechwood GuX, and for each, at pH 8.5 and 5.2. All culture filtrates were stored at 4°C before enzyme assays.

The culture filtrate obtained in the presence of beechwood GuX was fractionated by ion exchange chromatography on a Mini Q™ column (4.6 x 50 mm, GE Healthcare, Uppsala, Sweden) installed on an AKTA purifier (GE Healthcare) and equilibrated with 50 mM Tris-HCl buffer at pH 8.5. Elution was performed at a flow rate of 0.8 mL/min with 50 mM Tris-HCl buffer pH 8.5 (7 column volumes) to recover the unbound fraction. Bound proteins were eluted by a gradient on 10 column volumes from 35 to 75% of NaCl 1 M in 50 mM Tris-HCl buffer pH 8.5. The fractions of interest were dialyzed in a 10 kDa cut off dialysis tube

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

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

Two hundred µL of polysaccharide solutions were incubated overnight with 200 µL of bacterial culture filtrates or protein fractions eluted from ion exchange chromatography. After withdrawing 100 µL aliquot, the amount of reducing ends produced in the rest of the reaction medium was quantified by Nelson method **[19]** adapted to microplate **[20]**, and read on a microplate reader (Multiskan Ex). The amount of reducing ends was expressed in µg/mL using standard curve prepared with appropriate sugar. Controls were prepared similarly with previously heat-inhibited culture filtrates and polysaccharide solutions.

Glycosidase activities were assayed by incubating 100 µL culture filtrates with 100 µL 4 mM *para*-nitrophenyl (*p*-NP) derivatives of α-L-arabinofuranoside, α-D-galactopyranoside, β-D-mannopyranoside, β-D-glucopyranoside, α-D-xylopyranoside, β-D-galactopyranoside, α-L-fucopyranoside, acetate, and *ortho*-nitrophenyl (*o*-NP) β-D-xylopyranoside for 16 h at 40°C. The incubation was stopped by adding 0.6 mL of 1 M sodium carbonate solution **[21]**. The liberation of *p*-nitrophenol was reported as nmol/mL. Control tests were prepared by mixing the culture filtrate with sodium carbonate before adding the substrate.

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

The concentration of protein was determined in the culture filtrate by using the Bradford reagent (Bio-Rad, Marnes-la-Coquette, France) and bovine serum albumin (Sigma-Aldrich, L'Isle d'Abeau, France) as a standard (0–25 µg/mL) **[22]**.

Electrophoresis: polyacrylamide gel electrophoresis was performed under denaturing

conditions (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)). It was carried out in a MiniProtean 3 apparatus (BioRad) using a continuous 10–20% polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate.

In-gel digestion: Bands of interest were excised from the SDS-PAGE and subjected to in-gel reduction, alkylation and trypsic digestion essentially as described [**23**].

LC–MS/MS analysis: Mass spectrometry analyses were conducted by the platform "Biopolymers-Interaction-Structural Biology" located at the INRA Center of Angers-Nantes (www.bibs.inra.fr). Nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses of the digested proteins were performed using an Ultimate U3000 RSLC system (Dionex) coupled with an LTQ-Orbitrap VELOS mass spectrometer (Thermo Fisher). Details of the analytical procedure are given in **[23]**.

Databank search and protein identification: The LC-MS/MS collected data files were processed into mgf format using Proteome Discoverer (Thermo-Fisher). The mgf files were then searched against the Uniprot databank (http://www.uniprot.org, November 2014) restricted to the taxonomy *Pseudoalteromonas atlantica* T6c (Taxon ID# 342610) using the Mascot 2.2 program (Matrix Science). Enzymatic cleavage was declared as a trypsic digestion with one possible missed cut event. The fixed modifications of Cys residues by iodoacetamide and the possible oxidation of Met residues were considered. Precursor mass and fragment mass tolerance were set at 5 ppm and 0.5 Da, respectively. Proteins were validated when they had an e-value below 0.01, and when they were identified with a minimum of two peptides of individual score above the significance threshold provided by Mascot ($p < 0.01$).

2-8 Cloning and expression and purification of rPaGH8

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

5-GTGAGCATTGATCACTTAACGCTAACAAACAAAACGCGG-3' (forward) and 5'-GCGCGGCCGCTTCAGGCTCGTTTTCATTTGGCTTATAG-3' (reverse) encompassing BamH1 and EcoR1 restriction site respectively. The gene was cloned in pET28a which contains an N-terminal fused six-histidine-tag (6His-tag) using BamH1 and EcoR1 restriction sites (pET28a-GH8 plasmid) and transformed in *E. coli* strain BL21 (DE3). *E. coli* BL21 (DE3) harbouring the pET28-GH8 plasmid was pre-cultured overnight in Luria-Bertani medium containing 50 µg/mL of kanamycin before being diluted 20 times in the same growth medium and incubated under shaking at 20°C overnight.

Cells were harvested by centrifugation, washed with 50mM Tris-HCl NaCl 100 mM (pH 7.5), and resuspended in one-tenth of the culture volume of cold Tris-HCl 50 mM pH7.5, NaCl 100 mM, imidazole 20 mM (pH 7.5) containing protease inhibitor (Pierce™ Protease Inhibitor Mini Tablets, ThermoFisher, Illkirch, France), 5 µL DNAse (Invitrogen, ThermoFisher, Illkirch, France) and 20µg/mL RNAse (Invitrogen). Cells were disrupted using a French press at 2000 bars twice for 1 min, centrifuged 10 min at 4°C to recover a supernatant and a pellet.

The supernatant was loaded on a HisTrap™ FF column (1 mL) mounted on a ÄKTA Primer system (GE Healthcare) to purify the His-tag GH8 protein. The recombinant protein was eluted with 20 mM Tris-HCl buffer pH 8 containing 200 mM NaCl, 300 mM imidazole. The supernatant and pellet from the French press lysate, and the fractions eluted from the HisTrap column were subjected to a polyacrylamide gel electrophoresis under denaturing conditions (sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)), together with a PageRuler™ Unstained Protein Ladder (Fisher). SDS-PAGE was carried out into a MiniProtean 3 apparatus (BioRad) using a continuous 10% w/v polyacrylamide gel in the presence of 0.1% sodium dodecyl sulfate that was further stained with Coomassie-HCL **[24]**. The fractions containing the purified rPaGH8 were pooled, dialyzed against Tris-HCl 50 mM pH 8, and concentrated twenty times using a VIVASPIN 500 concentrator (PES membrane 10 kDa, Sartorius).

2-9 Analysis of the oligosaccharides produced by rPaGH8

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

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

and infused at 5 µL/min in the instrument. The instrument was operated in negative ionization mode in the so-called 'sensitivity' mode, with an ESI capillary voltage of 2.2 kV and a sampling cone voltage of 60 V. Data acquisition was carried out using MassLynx software (V4.1).

3- Results

3-1 Enzymatic screening after cultivation in the presence or not of polysaccharides

To highlight enzymes active on various polysaccharides, a preliminary screening was conducted on four bacteria from marine and terrestrial origin whose genome carried at least 10 sequences of hemicelluloses-degrading enzymes. As a larger number of sequences did not necessarily give more positive responses for substrate degradation, *P. atlantica* was selected from this screening since it offered the best performance and the greatest diversity on polysaccharides. Its genome contains genes coding for GH enzymes specific for hemicellulose backbone, *i.e.* β-glucanase, xyloglucanase, β-mannanase and β-xylanase (GH3, 5, 8, 10, 16 and 43, Table 1).

P. atlantica was first grown in the basal medium recommended (Table 1) and in the absence of added polysaccharide. Forty-two terrestrial or marine polysaccharides of diverse origins and structures (Table 2) were tested as substrates to screen enzymatic activities in the culture supernatant and in the cell lysate (Table 3). By comparing the results, it was obvious that the culture filtrate was more active and more diverse than the cell lysate. The culture filtrate of *P. atlantica* was highly, moderately or slightly active on 15 substrates among the 42 tested, including 10 hemicellulose-like substrates, while the cell lysate was highly active on only 2 substrates and slightly active on 4 substrates.

To enhance the enzyme production, different hemicelluloses were added to the culture medium, as the addition of polysaccharides can play an important role in the induction of

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

The improvement of the secretion of hemicellulolytic enzymes by adding polysaccharide to *P. atlantica* culture broth was further investigated.

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

Growth of *P. atlantica* without any added polysaccharide was followed over 48 h (Figure 1). Growth was slow for the first 5 h and increased rapidly to reach a plateau after 20 h culture. Adding konjac GlcM did not lead to a significant change in the bacterial growth in comparison to culture in the absence of added polysaccharide. Conversely beechwood GuX positively affected the growth rate of *P. atlantica* as it induced faster growth from 10 to 20 h.

The culture filtrates were recovered from each culture and tested for their enzymatic activity on 12 different hemicelluloses and 8 nitrophenyl-derivatives at pH 5.2 and 8.5 to identify the secreted activities (Figure 2). The culture filtrates obtained without added polysaccharide and in the presence of konjac GlcM exhibited roughly similar activity profiles on hemicelluloses (Figures 2A and 2C, respectively). Activities on xylan-like polysaccharides were favoured at pH 8.5. Also the activities towards nitrophenyl-derivatives were similar in the two culture filtrates (Figures 2B and 2D). The activities towards *o*-NP β-D-xylopyranoside, *p*-NP α-L-arabinofuranoside and *p*-NP acetate were higher at pH 5.2 than at pH 8.5. Comparison of Figures 2A and 2B and Figures 2C and 2D demonstrated that adding konjac GlcM in the

culture medium did not improve the production of hemicellulases or glycosidases. This result agreed with the growth kinetics study, which showed that konjac GlcM did not significantly enhance the bacterial growth.

In the culture filtrate produced in the presence of beechwood GuX (Figures 2E and 2F), the activities on heteroxylans were reinforced, mostly at pH 8.5. Conversely to what was observed in the other culture conditions, the activity on *o*-NP xylopyranoside was much higher at pH 8.5.

3-3 Fractionation of culture filtrate obtained on beechwood glucuronoxylan

The culture filtrate obtained in the presence of beechwood GuX was fractionated by ion exchange chromatography. The activities were evaluated in the collected fractions on konjac GlcM, CMC, beechwood GuX and wheat AX, and also on *o*-NP β-D-xylopyranoside and *p*-NP α-L-arabinofuranoside (Figure 3). The results suggested that enzymes active on konjac GlcM and CMC (Figure 3A) eluted mainly with xylosidase activity (Figure 3B), whereas enzymes active on beechwood GuX and wheat AX eluted later in the gradient (Figure 3A).

The gel electrophoresis of the fractions eluted between 7 and 13 mL elution volume of the ion exchange chromatography (Fractions B2 to B12, Figure 4A) showed that the major band in fractions B4 to B6 stand around 45 kDa and could correspond to the GH5 β-glucanase Patl_1404. These fractions also contained a slight band around 55 kDa and a band at 72 kDa possibly corresponding to the GH43 arabinofuranosidase Patl_0842 and the GH3-like protein Patl_4162, respectively. In the fractions B7 to B9, the major band was around 42 kDa and could correspond to the GH10 β-xylanase Patl_2657. Thus the electrophoresis demonstrated the proteins associated with the enzyme activities recovered from the chromatography and is an additional element to suggest the β-glucanase activity of GH5 Patl_1404, the arabinofuranosidases activity of GH43 Patl_0842 and the β-xylanase activity of GH10 Patl_2657.

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

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

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

3-5 Specificity and mode of action of rPaGH8

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

When beechwood glucuronoxylan was hydrolysed by xylanase M1 or rPaGH8, the later released three unidentified products absent with the former, at 17.4 min, 18.3 min and 19.0 min (Figure 5B and Table 4). When water-extractable or water-unextractable arabinoxylans from wheat (WEAX and WUAX, respectively) were submitted to xylanase M1 (Figure 5C and D), many different products appeared including X1 and X2 and many ramified oligosaccharides, of which the majority has been previously identified **[32]**. rPaGH8 produced X1 to X4 and many ramified oligosaccharides. Some of them were similar to those produced by xylanase M1, but in different amounts. Remarkably rPaGH8 released lower amounts of oligosaccharides with 1 or 2 un-substituted xylose at the reducing end (such as XA3X and XA3XX) and higher amount of oligosaccharides with 3 un-substituted xylose at the reducing end (such as XA2,3XXX). Three additional peaks appeared at 19.0 min, 19.7 min, 23.7 min. The same unidentified peaks were produced from both WEAX and WUAX, and the peak at 19.0 min was also produced from beechwood GuX (Table 4).

From all these substrates, rPaGH8 produced linear xylooligosaccharides from degree of polymerisation 1 (as traces) to 4, suggesting that the enzyme is unable to hydrolyse X4. X5 was absent (from WEAX, WUAX) or present in very minor amounts (from beechwood GuX), suggesting that it was hydrolysed in X3 + X2. X6 accumulated from *Palmaria* xylan, suggesting that rPaGH8 was not able to degrade it. As the polysaccharide contained (1,3) as well as (1,4) linkages, it is likely that this accumulating oligosaccharide contained one (1,3) linkage that prevented its degradation by rPaGH8.

The unknown peak eluting at 19.0 min (marked with a star on Figure 5) was chosen to tentatively elucidate its structure because it was released from wheat arabinoxylans and beechwood glucuronoxylan. To this aim, the peak was isolated, desalted and analysed by mass spectrometry. The MS analysis showed a m/z of 1073.35, corresponding to an oligosaccharide with a degree of polymerisation of 8 (Figure 6A). The MS/MS analysis did not allow a complete characterization of this structure. Since all hydroxyl functions could not be

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

4- Discussion

Hemicellulose-degrading enzymes contribute to the saccharification process of biomass for the production of platform molecules or second-generation bioethanol **[34 -39]**. They also have a great impact in animal feeding **[40]**. At an analytical level, polysaccharide-active enzymes are important tools to solve the fine structure of their substrates. They are produced by many microorganisms and bioinformatic tools are essential to select those potentially producing enzymes of interest for their numerous applications.

P. atlantica is a marine bacterium known to produce enzymes active on seaweeds polysaccharides such as agarases [**41**], alginate lyase [**42**] and porphyranase [**43**]. The genome annotation suggested 14 genes coding for putative hemicellulases (Table 1) [**44**]. Patl_1404 gene is the only gene from *P. atlantica* T6c coding a GH5 protein. Patl_2657 gene was annotated as belonging to GH10, which suggested it as a xylanase. These predicted endo-

glucanase and endo-xylanase could explain the presence of activities on glucan, GlcM and xylan in *P. atlantica* culture filtrate. Three sequences belong to GH43 and 4 to GH16. The substrate specificity of these proteins, together with that belonging to GH8 (Patl_1069), is less straightforwardly predictable, as these families are multispecific (5, 14, and 9 EC numbers in families GH8, GH16 and GH43, respectively). Moreover, the choice of substrates is crucial to succeed in evidencing enzymatic activities. As an example, the activities of *P. atlantica* culture filtrates were much lower on maize AX in comparison to wheat AX and beechwood xylan. In maize AX the xylan backbone can be decorated not only with arabinose but also with xylose and galactose, and the arabinose moiety can be further decorated with xylose, galactose, ferulic acid or one or more arabinose residues [**45-46**]. This gives it a more complex structure that may make it more resistant to enzymatic hydrolysis than other AX. Consequently, the hydrolysis of maize AX required a wider set of enzymes acting synergistically, some of which may be lacking in *P. atlantica* secretome.

A β-xylosidase activity was shown in *P. atlantica* culture filtrates. The genome contains two GH3-like sequences (Patl_3730, Patl_4162) whose family includes β-xylosidases, α-arabinofuranosidases and β-glucosidases. It also contains 3 putative α-arabinofuranosidases belonging to GH43 family (Patl_0810, Patl_0842 and Patl_3728) of which it is known that many characterized enzymes showed a dual-activity α-arabinofuranosidase/ β-xylosidase [**47-50**]. Thereby, there is two possible explanations for this β-xylosidase activity, either a GH3 β-xylosidase or a GH43 dual-enzyme.

The xylanase activity shown after fractionation of *P. atlantica* secretome could be ensured by two enzymes, the GH10 Patl_2657 and the GH8 Patl_1069. GH10 xylanases are characterized by their high versatility since they hydrolyse many types of heteroxylans in which they can act near a substituted xylose residue [**51 – 53**]. GH8 family contains xylanases for which little data is available to date, and reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156), 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

polysaccharides, this observation is consistent with the previous evidence that marine bacteria secrete pectin-degrading enzymes in the presence of pectin. This was demonstrated for bacteria close to *P. atlantica* such as *P. haloplanktis* [**60**] and *Alteromonas macleodii* [**61**]. The polysaccharides tested as substrates in the present study have rather known structures. The structure of wheat AX was extensively studied and shown to vary largely in its level of arabinose di-substitution [**62**]. NMR spectroscopy revealed that beechwood GuX contained approximately one 4-O-methylglucuronic acid every 15 xylose residues [**63**]. From these polysaccharides PaGH8 produced oligosaccharides in which the arabinose substitutions were gathered at the non-reducing side of the molecule, showing that the initial polysaccharides contained such stretches of xylose residues without any substitution. These new oligosaccharides pave the way to elucidate further the chemical structure of the polysaccharides thanks to PaGH8 particular behaviour.

Altogether the present results highlighted *P. atlantica* as a source of hemicellulose-degrading enzymes. Our data showed that the use of various polysaccharides generated a wide variety of enzymes. Only those produced in the presence of glucuronoxylan were studied, which particularly revealed the new GH8 xylanase. Results obtained with one or more other polysaccharide substrates lead to the production of a wide range of enzymes that remain to be valued in the near future.

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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

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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
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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).

(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 (see Figure 5).

(**A**) Zoom of the MS spectrum with the peak corresponding to an AX DP8 (*m/z* 1073.35).

(**B**) MS/MS spectrum of the 1073.35 *m/z* species after further fragmentation.

(**C**) One possible structure of observed fragment depicted according to the nomenclature of Domon and Costello, 1998 [**33**]. The two pentoses in orange can be placed in any orange-marked position. Blue annotations: intercyclic fragments; Red annotations: intracyclic fragments; Stars: fragments with the loss of one water molecule.

Supplementary data

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

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

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

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

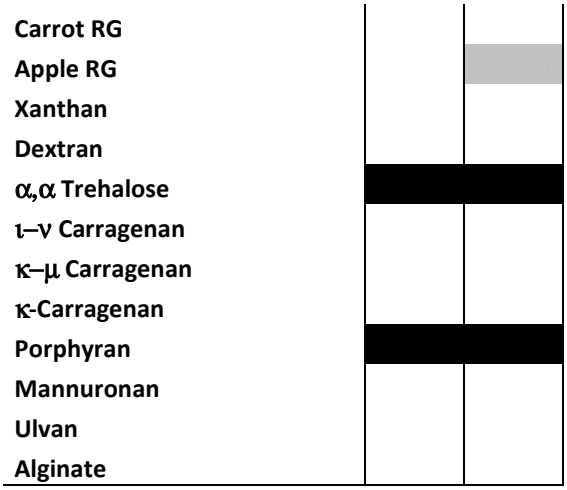
	Polysaccharide	Biological origin	Source
1	**Arabinoxylan (AX)**	Maize	Lab collection
2	**Arabinoxylan (AX)**	Rye	Megazyme
3	**Arabinoxylan (AX)**	Wheat	Megazyme
4	**Glucuronoxylan (GuX)**	Beechwood	Sigma
5	**Glucuronoxylan (GuX)**	Tomato	[**16**]
6	**Glucuronoarabinoxylan (GAX)**	Apple	[**8**]
7	**Glucomannan (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

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

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

Table 4: Hydrolysis products released by rPaGH8 from various hemicelluloses. Crosses (+) in bold indicate oligosaccharides produced exclusively by rPaGH8 and not by xylanase M1. The 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			+	+

#this peak eluted at the same time as XA3XX but corresponds to another oligosaccharide as the xylan from *Palmaria* does not contain any arabinose.

* this peak was further identified by mass spectrometry

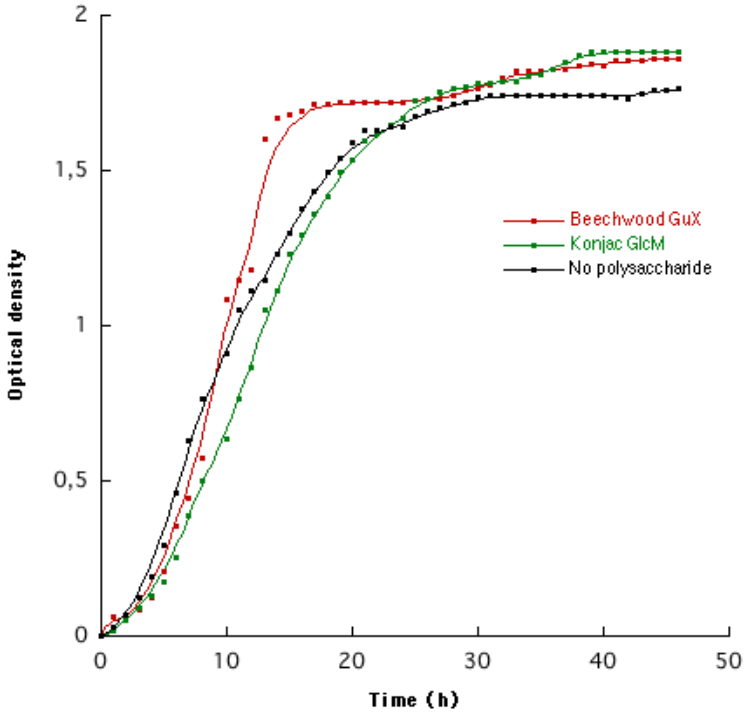


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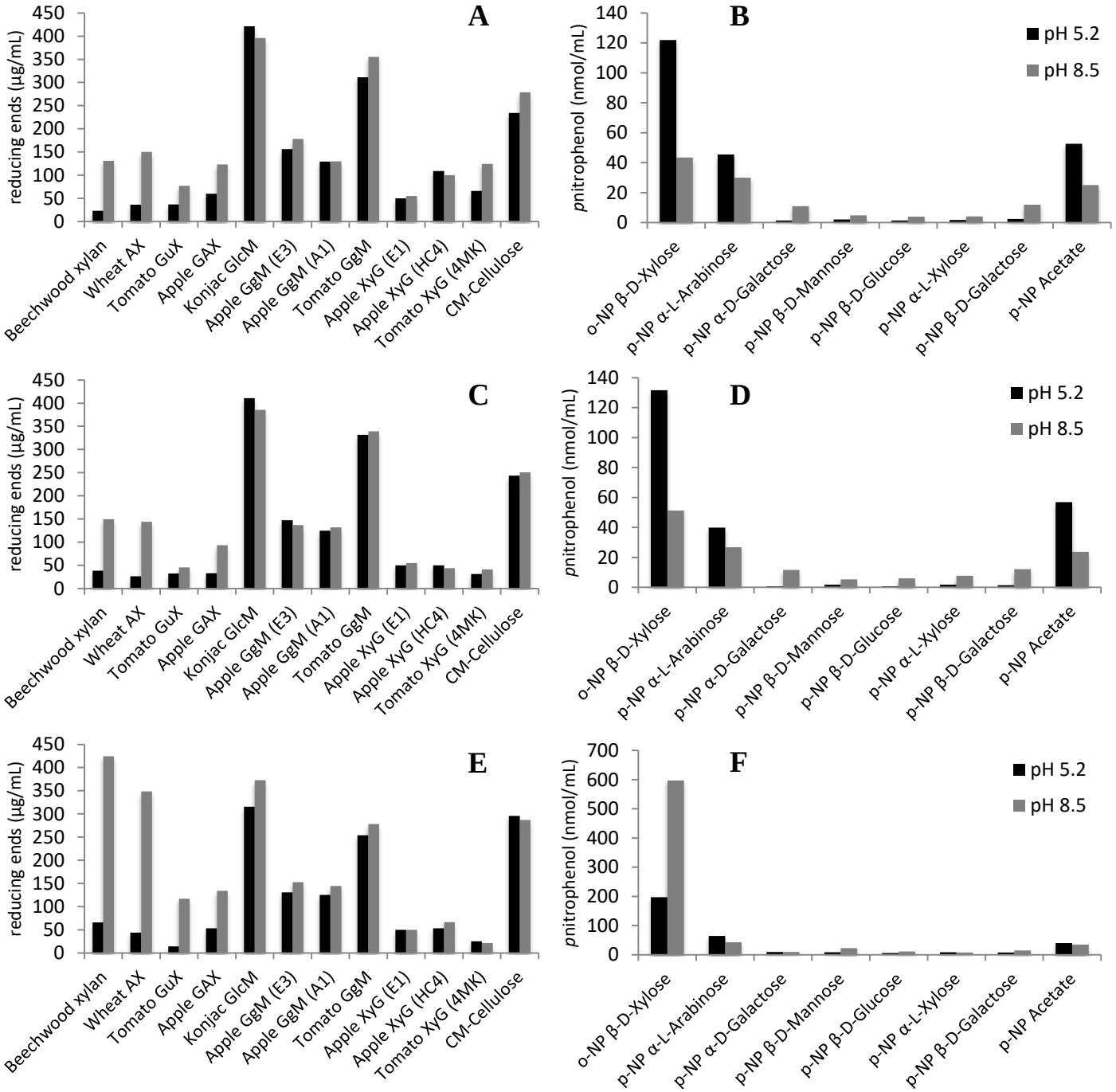


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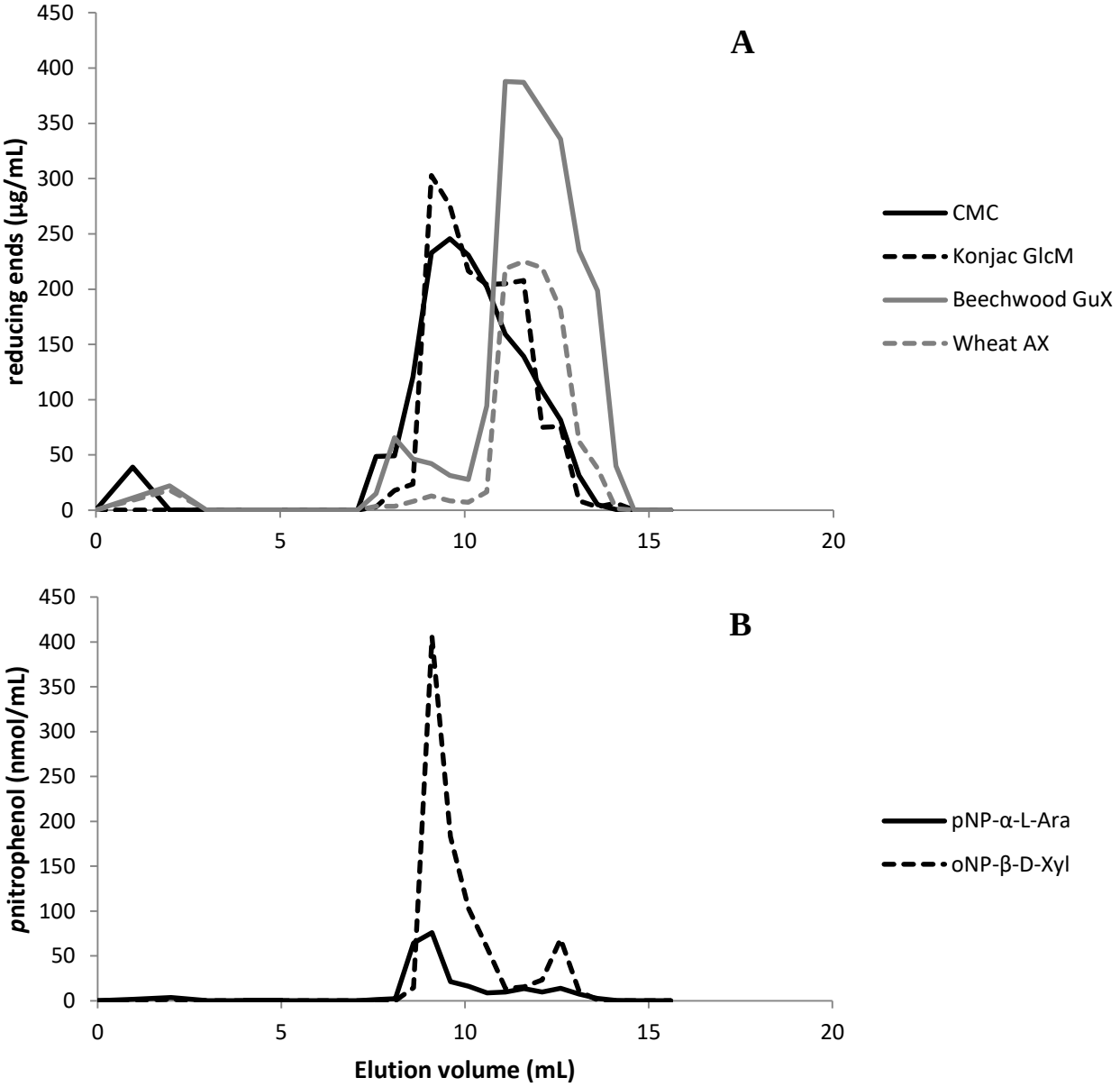


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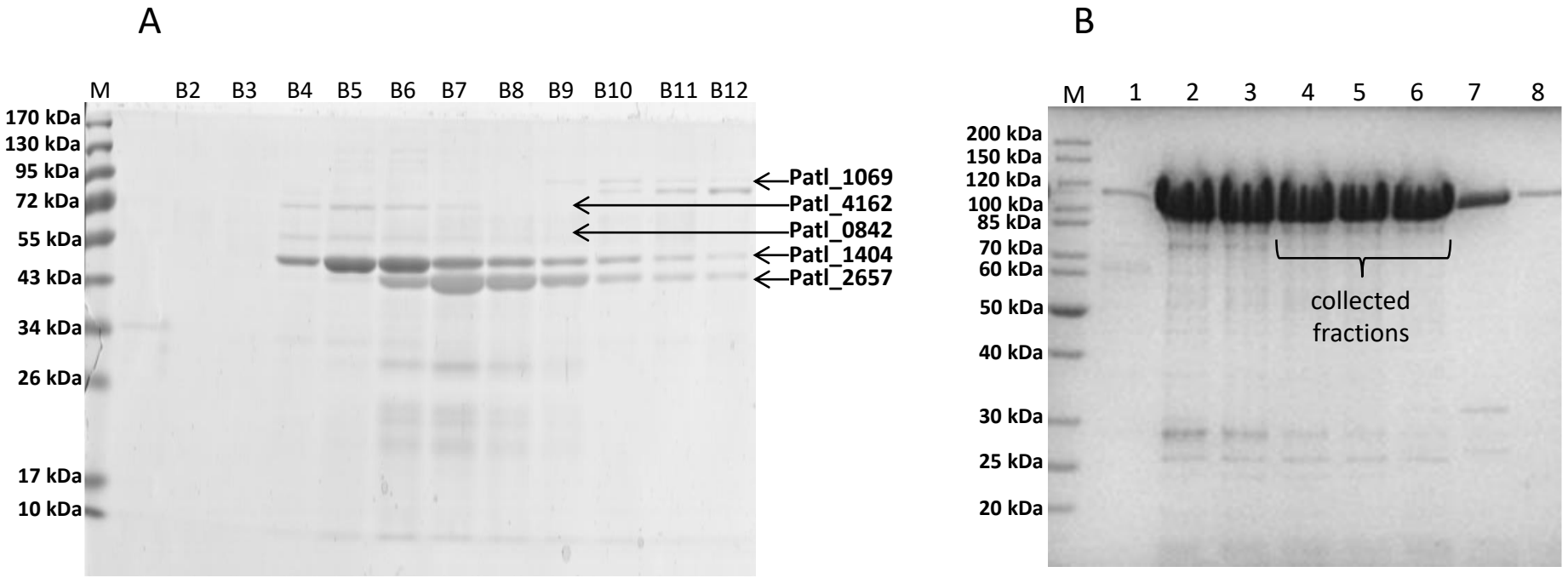


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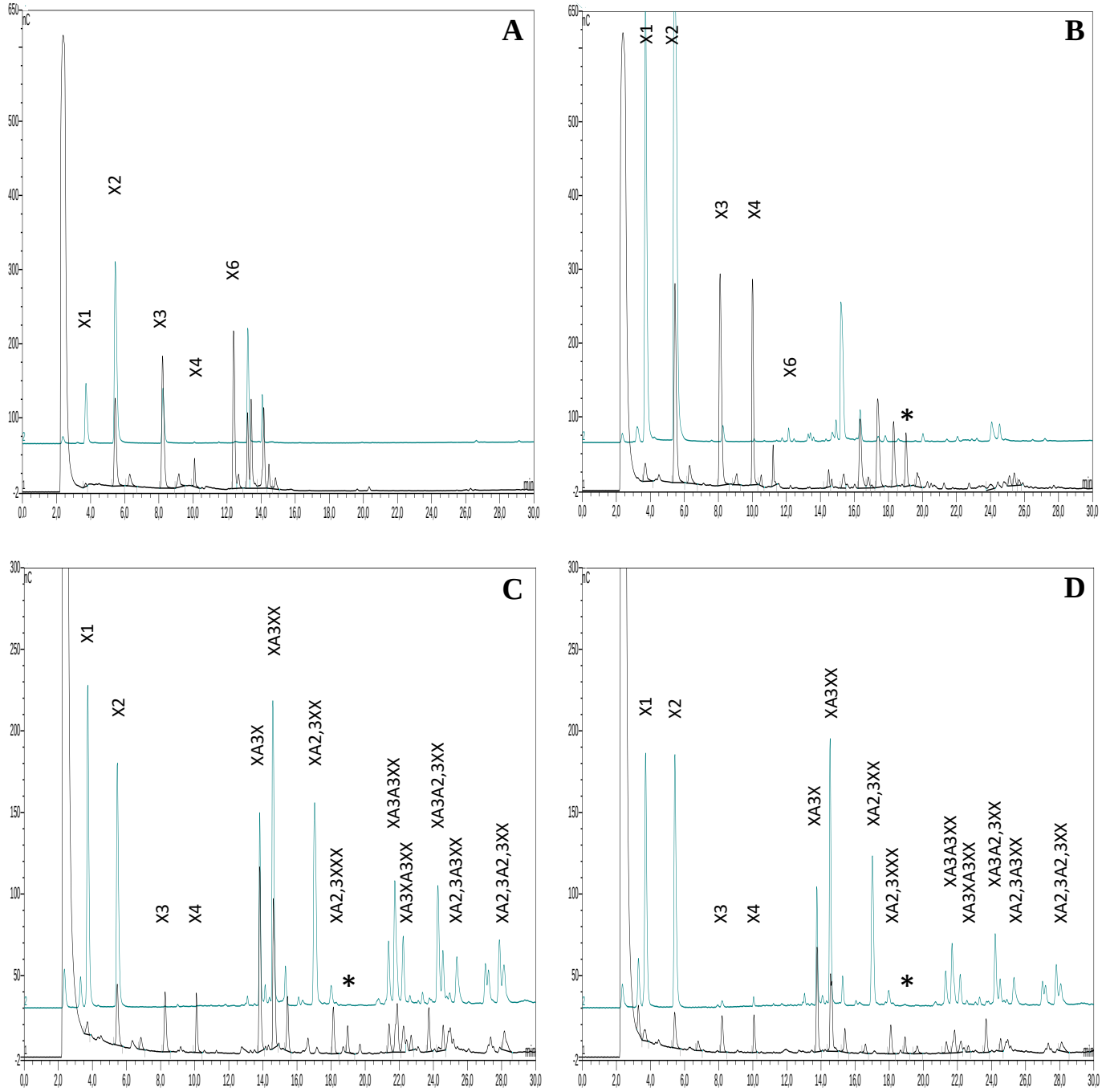


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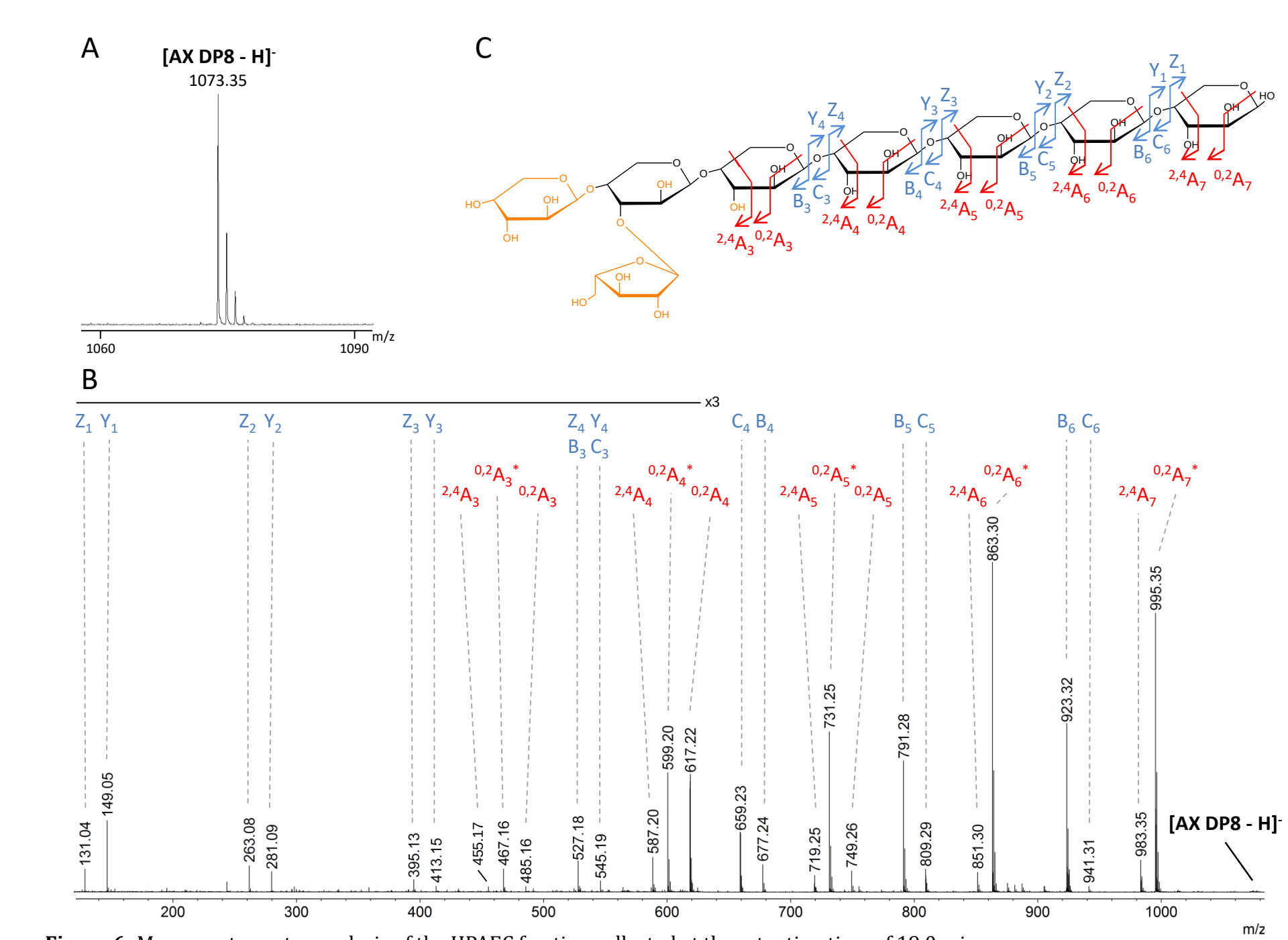


Figure 6: Mass spectrometry analysis of the HPAEC fraction collected at the retention time of 19.0 min.