Antioxidant activity of xylooligosaccharides produced from glucuronoxylan by Xyn10A and Xyn30D xylanases and eucalyptus autohydrolysates

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ABSTRACT

Antioxidant activity of xylooligosaccharides (XOS) released from beechwood and birchwood glucuronoxylans by two different xylanases, one from family GH10 (Xyn10A) and another from family GH30 (Xyn30D) was examined. The ABTS (2, 2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) method was used, since it resulted more accurate for the antioxidant activity determination of XOS. Thin layer chromatography and MALDI-TOF MS analysis showed that Xyn10A produced a mixture of neutral and acidic XOS whereas the XOS produced by Xyn30D were all acidic, containing a methylglucuronic acid (MeGlcA) ramification. These acidic XOS, MeGlcA substituted, showed a strongly higher antioxidant activity than the XOS produced by Xyn10A (80% *vs.* 10% respectively, at 200 μg mL^{-1}). Moreover, the antioxidant activity increased with the degree of polymerization of XOS, and depended on the xylan substrate used. The antioxidant capacity of eucalyptus autohydrolysates after xylanase treatment was also analysed, showing a decrease of their antioxidant activity simultaneous with the decrease in XOS length.

Keywords:

Xyn10A, Xyn30D, antioxidant activity, xylooligosaccharides, glucuronoxylan, eucalyptus autohydrolysate

1. INTRODUCTION

New interest has been aroused to search natural and safe antioxidant agents from natural sources. An antioxidant compound can be defined as a substance whose action can inhibit oxidation rate of a free radical. Although synthetic antioxidants seem to be promising, their toxicity and side effects rule out their extensive use. Plant biomass, which is the main source of renewable materials on earth, consists largely of two polysaccharides (cellulose and hemicelluloses) and an aromatic polymer (lignin). Hemicelluloses account for 15-25% of all lignocellulose. In hardwood species, grasses and agro-industrial by-products (cereal straws, sugarcane bagasse, corn stover and sisal, among others), xylans are the most abundant hemicelluloses (Aracri & Vidal, 2011; Valls, Gallardo, et al., 2010; Valls, Cadena, & Roncero, 2013) whereas in softwoods, mannans are more abundant (Scheller & Ulvskov, 2010). The term arabinoxylan is used to describe xylan of cereals and grasses which shows abundant decorations of arabinose and ferulic acid (Ebringerová & Heinze, 2000). On the other hand, xylan from hardwood species is denominated glucuronoxylan as is highly substituted with methylglucuronic acid decorations (MeGlcA), and can be heavily acetylated while it does not contain arabinose and ferulic acid decorations (Teleman, Tenkanen, Jacobs, & Dahlman, 2002).

Endoxylanases (EC 3.2.1.8) randomly hydrolyse the β-(1,4) glycosidic bonds of the xylose backbone of xylan (Biely, Vrsanská, Tenkanen, & Kluepfel, 1997; Pollet, Delcour, & Courtin, 2010). These enzymes are glycosyl hydrolases (GH) grouped in families GH10, GH11 and GH30 (previously classified in family GH5) (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014). The

hydrolysis products of these enzymes are a mixture of xylooligosacccharides (XOS) of different degrees of polymerisation and substitution, depending on the xylanase GH family. XOS show a structure of around 2–7 xylose units linked by β-(1,4) bonds, variably decorated with different substituents depending on the source. For example, XOS from hardwoods contain acetyl groups and MeGlcA ramifications whereas XOS from agricultural residues contain arabinose units and ferulic acid. While XOS released from glucuronoxylans by GH10 and GH11 xylanases are a mixture of linear (neutral) and MeGlcA substituted (acidic) oligomers, XOS released by xylanases of family GH30 are all MeGlcA substituted xylooligomers (Kolenova, Vrsanska, & Biely, 2006; Vršanská, Kolenová, Puchart, & Biely, 2007). Antioxidant activity of XOS from arabinoxylans has been reported in several articles (Bian et al., 2013; Gowdhaman & Ponnusami, 2015; Mandelli et al., 2014; Veenashri & Muralikrishna, 2011), although in these works the activity of different types of XOS has not been evaluated.

Xylanases from families GH10 (Xyn10A), and GH30 (Xyn30D) have been previously isolated from *Paenibacillus barcinonensis* and characterized (Valenzuela, Diaz, & Pastor, 2012; Valenzuela, Díaz, & Pastor, 2010). These xylanases were previously tested for their bleach boosting ability on pulp from eucalyptus and agricultural fibres, showing different effectiveness (Valenzuela et al., 2013; Valls, Vidal, et al., 2010). We have applied Xyn10A and Xyn30D on glucuronoxylans for XOS production in order to analyse their antioxidant effect. A comparison between the antioxidant activity of XOS produced by xylanases of different GH families has been evaluated in this work for the first time.

Additionally we have analysed the antioxidant activity of XOS released from eucalyptus, the most important raw material for pulp production in Spain, a process that generates a large amount of residues that can be upgraded to added value products.

2. MATERIALS AND METHODS

2.1. Raw material

Birchwood, oat spelt xylans, D-Glucuronic acid, gallic acid and Trolox were purchased from Sigma Aldrich (Germany). Beechwood xylan was purchased from Roth (Germany). Autohydrolysates from eucalyptus wood were a gift of J. Parajó (Gullón et al., 2011).

2.2. Xylanases

The xylanases assayed were Xyn10A from family GH10 (Valenzuela et al., 2010) and Xyn30D from family GH30 (Valenzuela et al., 2012). Xyn10A is a single-domain enzyme and thus comprised of a sole catalytic module whereas Xyn30D is a modular enzyme comprised of a GH30 catalytic module linked to a CBM35 carbohydrate binding module. These xylanases were recombinant enzymes from *P. barcinonensis* that were previously cloned in *Escherichia coli* and characterized. They were purified as described (Valenzuela et al., 2012; Valenzuela et al., 2010).

Protein concentration was determined using the Bradford method (Bradford, 1976). Xylanase activity was assayed by measuring the amount of reducing sugars released from xylan hydrolysis by the dinitrosalicylic (DNS) reagent method (Miller, 1959). DNS was purchased from Sigma Aldrich

(Germany). The 100 µl volume standard assay contained the enzyme samples in 50mM sodium-phosphate buffer pH 6.5 with 1.5% birchwood xylan, final concentration. The reaction mixtures were incubated at 50º for 15 min. Then, temperature was increased at 100ºC for 2 min to inactivate the enzymes. To quantify the reducing sugars released from substrate, samples were chilled, 100 µL of DNS reagent were added and the reaction mixtures were incubated for 5 min at 100ºC. Finally, 40 µL of samples were mixed with 260 µL of distilled water in ELISA plates and the absorbance at 540 nm was measured. A standard curve of xylose was used to calculate activity units. One unit of xylanase activity was defined as the amount of enzyme that releases 1 µmol of xylose reducing sugar equivalent per min under the assay conditions described. All determinations of enzyme activity were made in triplicate.

2.3. Enzymatic treatments

XOS were obtained incubating 2% xylans (wt/vol) (2.22 mL of a xylan solution at 4.5%) with Xyn10A or Xyn30D at 10 U g^{-1} of substrate in 50 mM sodium-phosphate buffer at pH 6.5 for 2 h at 50ºC in a final volume of 5 mL. Incubations were performed in a thermostatic bath. These conditions correspond to the optimum pH for the activity of both enzymes and optimum temperature for Xyn30D activity. Xyn10A shows optimal activity at 60ºC while at 50ºC shows more than 85% of maximum activity (Valenzuela et al., 2012; Valenzuela et al., 2010). After incubation, unhydrolysed and partially hydrolysed xylans were separated from XOS by step precipitation with three volumes of ethanol. For this purpose, 5 mL of ethanol were added to the 5 mL volume

140 reaction and samples were centrifuged at 4500 rpm (revolutions per min) for 10
141 min. The supernatants were collected, mixed with additional 10 mL of ethanol
142 and centrifuged again at 4500 rpm for 10 min. The resulting supernatants of this
143 second centrifugation were collected, ethanol was eliminated by evaporation,
144 and dried samples obtained were considered as purified XOS. They were
145 dissolved in 1.5 mL of 200 mM sodium-phosphate buffer pH 6.5, analysed for
146 their content in reducing sugars, and kept for the determination of antioxidant
147 properties.

148 **2.4. Determination of reducing sugar**

149 The reducing sugar content of the XOS released from xylan hydrolysis
150 was measured according to the dinitrosalicylic (DNS) reagent method (Miller,
151 1959). 100 µl of DNS were added to 100 µl of XOS samples and mixtures were
152 incubated at 100ºC during 5 min. Then, 40µL of reaction samples were placed
153 in ELISA plates, 260 µL of distilled water were added and the absorbance at
154 540 nm was measured. Samples were analysed by triplicate. A standard curve
155 of xylose was used to calculate the xylose reducing sugar equivalent of the
156 different samples.

157 **2.5. Antioxidant activity (%)**

158 The antioxidant activity was assessed by a procedure consisting in the
159 quantification of the $ABTS^{\cdot+}$ radical decoloration described by several authors
160 (Cusola, Valls, Vidal, & Roncero, 2015; Re et al., 1999; Valls & Roncero, 2013)
161 with some modifications. The method consists in the addition of the antioxidant
162 compound to a pre-formed $ABTS^{\cdot+}$ radical solution and quantifying the

163 remaining $ABTS^{\cdot+}$ after a fixed time period, by means of UV spectrophotometry.
164 Firstly, the $ABTS^{\cdot+}$radical was pre-formed adding 44.5 μL of 140 mM potassium
165 persulfate to 2.5 mL of 7 mM ABTS and keeping at darkness for 16h. Due to
166 the precipitation of XOS of high molecular weight with ethanol, $ABTS^{\cdot+}$ was
167 dissolved in water instead of in ethanol until the absorbance was 0.7±0.1 at 730
168 nm. 900 μL of the $ABTS^{\cdot+}$ solution and 100 μL of XOS (at several
169 concentrations) were mixed in a methacrylate cuvette. The reaction was left in
170 the darkness during 10 min. Then, the final absorbance was measured at 730
171 nm. Complete decoloration meant 100% of antioxidant activity, while the
172 percentage of decoloration was equivalent to the % of antioxidant activity. It was
173 calculated as inhibition % as follows:
174 $ABTS^{\cdot+}{}_{inhib.}\ (\%) = 100\ [(A_i - A_f)/A_i]$
175 where A_i is the $ABTS^{\cdot+}$ absorbance value of the blank, and A_f is the $ABTS^{\cdot+}$
176 absorbance value after contact with the antioxidant compound. The % of
177 inhibition or decoloration of ABTS by a compound was considered the % of its
178 antioxidant activity. As positive control, the potent antioxidant agents Gallic acid
179 and Trolox were used. Experiments were performed by triplicate on a T92+UV
180 Spectrophotometer (PG Instruments).
181 **2.6. Thin-layer chromatography**
182 Purified XOS were analyzed by thin-layer chromatography (TLC) as
183 previously described (Gallardo et al., 2010). 10-15 μL of XOS were applied on a
184 silica gel plate (Merck, Germany) constituting the solid phase. 10 μL of an
185 oligomer standard mixture containing neutral xylooligosaccharides (Megazyme,
186 Ireland) at a concentration of 20 mg mL^{-1} were applied as migration standards.

The mobile phase was a mixture of chloroform, acetic acid and H_2O in a 6:7:1 ratio, respectively. The migration was repeated twice and the silica gel plate was then sprayed (Fungilab S.A., Spain) with a developing solution, consisting of 5% H_2SO_4 in ethanol. Finally, the plate was heated in the oven at 100ºC for 5 min, where the spots corresponding to the different XOS were visualized.

2.7. MALDI-TOF MS

For the analysis by MALDI-TOF MS, 1 µl of XOS was mixed with 1 µl of matrix solution (10 mg/ml 2, 5- dihydroxybenzoic acid dissolved in acetonitrile-water [1:1, vol/vol], 0.1% [wt/vol] trifluoroacetic acid). One microliter of the mixture was spotted onto the MALDI-TOF MS plate and allowed to dry before the analysis. Positive mass spectra were collected with a 4800 Plus MALDI TOF/TOF (ABSciex 2010) spectrometer with an Nd:YAG 200-Hz laser operated at 355 nm.

3. RESULTS AND DISCUSSION

3.1. Xylooligosaccharides (XOS) production

Firstly, we analyzed the production of XOS from oat spelt arabinoxylan by a xylanase of GH10 family, Xyn10A from *Paenibacillus barcinonensis*. Xylan concentration was set at 2% according to Akpinar, Erdogan, Bakir, & Yilmaz, 2010 and Bian et al., 2013, which showed that higher substrate concentration decreased the yield of XOS, due to the increase in viscosity and density of the reaction mixture. We treated with several enzyme doses (0.1, 1, 10 and 40 Xylanase Units g^{-1} of xylan) at pH 6.5 and 50ºC for 2h and analyzed the production of XOS. To quantify them, a mixture of oligomers of different

polymerization degree, we determined the release of reducing sugars. As xylooligomers have a unique reducing group in their terminal xylose residue, we assumed that one mol of xylose reducing sugar would be equivalent to one mol of released XOS. We believe that this is a more accurate way to quantify XOS that measuring in g the amount of products released from xylan, comprised by XOS of quite different size. The doses of 0.1 and 1 U g^{-1} of xylan produced low amount of XOS, while with 10 U g^{-1} the XOS production was increased until 2.1 g of xylose reducing equivalent L^{-1}. Higher enzyme concentrations (40 U g^{-1}) gave rise to lower XOS production, probably because of poor xylan conversion by high concentrations of enzyme and substrate. For this reason, the xylanase dose of 10 U g^{-1} was chosen for the following experiments.

A different type of xylan, beechwood glucuronoxylan, was tested for XOS production. Beechwood xylan was treated with xylanase Xyn10A or with a family GH30 xylanase, Xyn30D from *Paenibacillus barcinonensis*, in the same conditions as above and the release of XOS at different intervals of incubation was analyzed. Both xylanases released a similar amount of XOS (around 500 mg L^{-1}) at short incubation time (5 min), in accordance to their similar specific activity. However, in prolonged incubations (120 min), while the amount of XOS released by Xyn30D remained constant, higher XOS production (up to 1745 mg L^{-1}) was obtained with Xyn10A, probably reflecting the higher thermostability of this enzyme (Valenzuela et al., 2012; Valenzuela et al., 2010). To calculate the yield of enzymatic treatments, the dry weight of the XOS produced by the enzymes was determined. Treatment for 120 min with Xyn10A gave rise to 0.22

g of XOS from 1 g of birchwood xylan (22% yield) while Xyn30D gave rise to a lower production (13%).

The XOS released from beechwood xylan were purified and analyzed by thin layer chromatography (TLC). They showed a different pattern of oligomers (Fig. 1). Hydrolysis products of Xyn10A (XOS_10) contain a mixture of oligomers with the mobility of neutral XOS, such as xylotriose (X3), xylotetraose (X4) xylopentaose (X5) and xylohexaose (X6), accompanied by oligomers of intermediate mobility, indicating they are methylglucuronic acid (MeGlcA) substituted oligomers (acidic XOS). Moreover, longer XOS were found, which were less abundant in long term incubations (120 min). On the contrary, all oligomers produced by Xyn30D (XOS_30) show mobility corresponding to acidic XOS, in accordance to the mode of action of GH30 xylanases, that exclusively release MeGlcA substituted xylooligomers from glucuronoxylans (Kolenova et al., 2006; Vršanská et al., 2007). Moreover, no differences in oligomer size was found in long term incubations with Xyn30D.

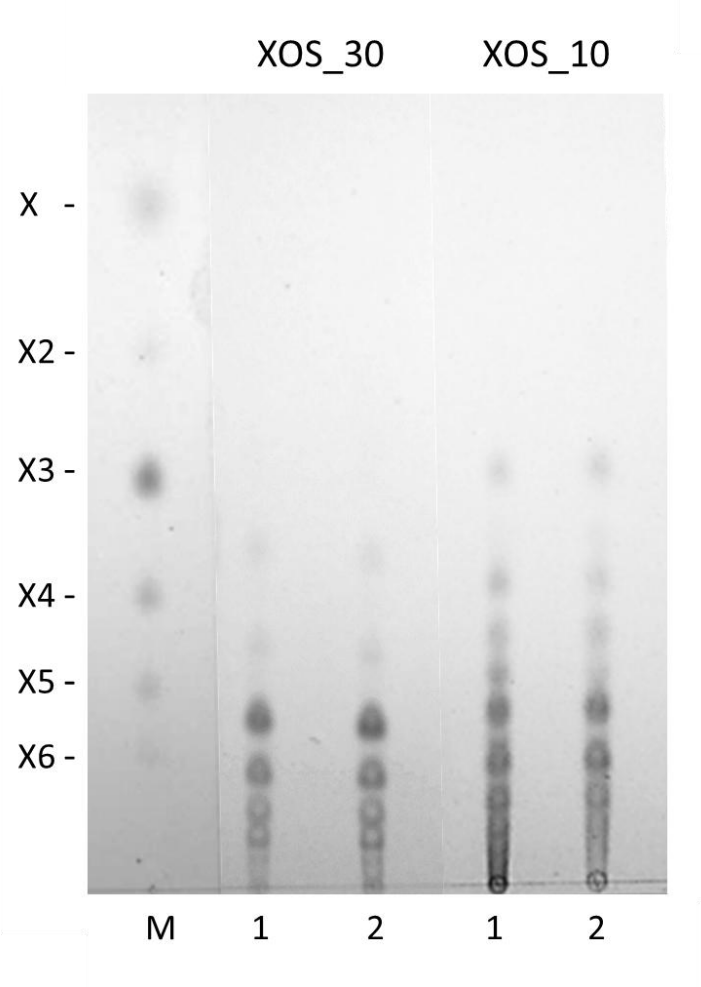


Figure 1. TLC analysis of XOS released from beechwood xylan by Xyn10A (XOS_10) or Xyn30D (XOS_30). Xylan was incubated with enzymes for 5 min (1) or 120 min (2). M) size markers of xylose (X), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6)

In order to better elucidate the structure of the XOS obtained, they were analysed by MALDI-TOF MS (Fig. 2). The mass spectrum showed the presence of molecular ions of linear and substituted oligomers identified as sodium and potassium adducts (Valenzuela, Lopez, Biely, Sanz-aparicio, & Pastor, 2016).

XOS released by Xyn10A were linear oligomers consisting of 5 to 10 xylopyranosyl (X5-X10) residues (Fig. 2), although X3 was also found. Moreover, acidic XOS containing 3 to 8 xylopyranosyl residues and a single MeGlcA residue (X3_MeGlcA to X8_MeGlcA), were also observed. In long term incubations xylobiose (X2) was also detected. The pattern found is in accordance with products pattern of family 10 xylanases, where the smaller products obtained are X2 and X3_MeGlcA (aldotetraouronic acid) (Biely, Singh, & Puchart, 2016).

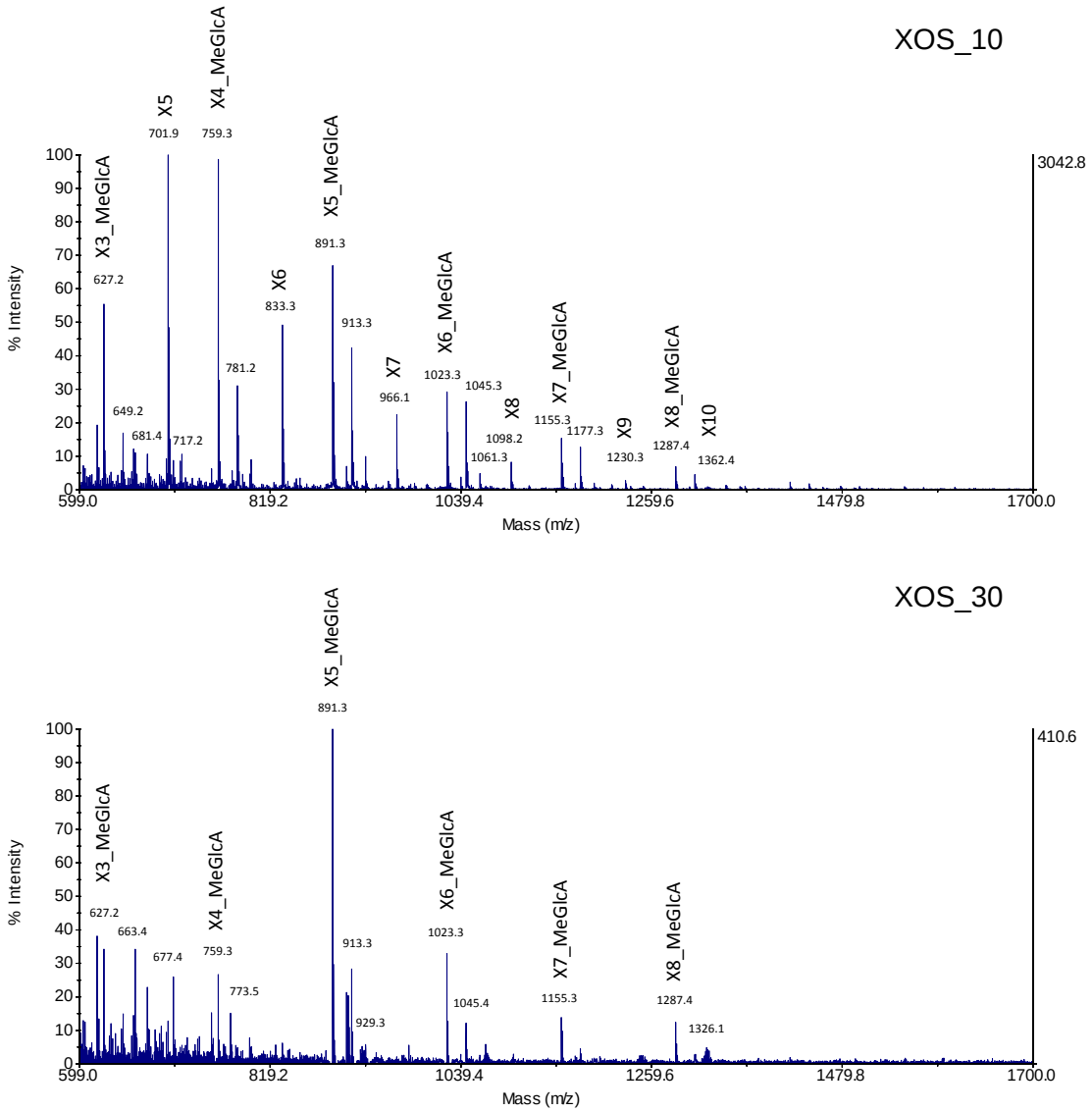


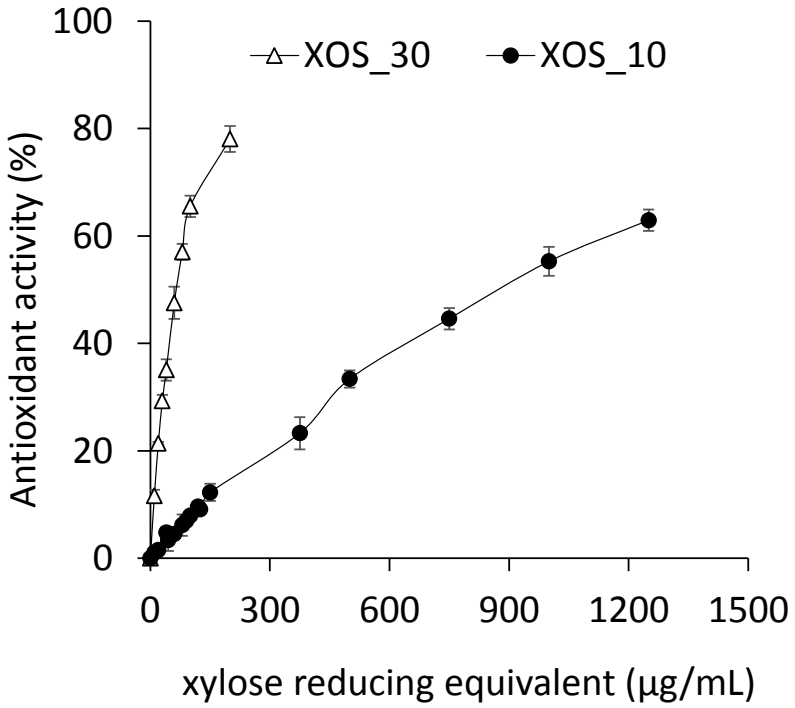
Figure 2. MALDI-TOF MS spectra of XOS_10 and XOS_30 from beechwood xylan.

The mass spectra of products released by Xyn30D showed that all of them are acidic oligomers containing a single MeGlcA residue (Fig. 2). Substituted xylooligosaccharides consisting of 3 to 8 xylopyranosyl residues (X3_MeGlcA to X8_MeGlcA) are shown, in accordance with TLC analysis. Moreover, thin layer chromatograms shown in Fig. 1 revealed that X5_MeGlcA and X6_MeGlcA are produced in higher amounts, followed by X7_MeGlcA and X8_MeGlcA. The pattern found is in agreement with the mode of action of family 30 xylanases, which have an absolute requirement of MeGlcA side chains for hydrolysis of xylan, and release XOS substituted in the penultimate xylose from the reducing end (Biely et al., 2016; Vršanská et al., 2007). The structure of the main XOS_10 and XOS_30 obtained from beechwood xylan are shown in supplementary Fig. S1.

3.2. Antioxidant activity of XOS from beechwood xylan

We analyzed the antioxidant capacity of the XOS produced from beechwood xylan by Xyn10A and Xyn30D. As the methodology to evaluate antioxidant activity we used quantification of the $ABTS^{\cdot+}$ radical discoloration. This method confirmed the high antioxidant effect of well known compounds such as Trolox and gallic acid, which exhibited a powerful antioxidant activity, 100% decoloration of $ABTS^{\cdot+}$ at 20 and 10 μM respectively (supplementary Fig. S2). We measured the antioxidant activity of the XOS produced by Xyn10A and Xyn30D at several concentrations (Fig. 3). In both cases the antioxidant activity increased with XOS concentration, although the antioxidant activity of the two

292 types of XOS was clearly different. XOS_30 showed a much higher
293 effectiveness in reducing the $ABTS^{\cdot+}$ radical, much higher antioxidant activity
294 than XOS_10. At a concentration of 200 μg xylose reducing equivalents mL^{-1}
295 the antioxidant activity was around 80% in XOS_30 and 10% in XOS_10.
296



297

298 **Figure 3.** Antioxidant activity of XOS_10 and XOS_30 from beechwood xylan.

299

300 The antioxidant activity of a compound can be compared to that of
301 Trolox, obtaining the TEAC (trolox equivalent antioxidant activity) value (Cusola
302 et al., 2015; Re et al., 1999). However, this value is determined according to the
303 molar concentration of the compound. Since the evaluated XOS include
304 oligomers of different length and molecular weight, the molar concentration
305 cannot be measured. As detailed above we can associate the antioxidant

306 activity value of a XOS sample to its concentration expressed as μg mL^{-1} of
307 xylose reducing equivalents, and refer this value to the Trolox concentration
308 needed to obtain the same antioxidant activity. Thus, 100 μg mL^{-1} of XOS_30
309 produce the same antioxidant activity than Trolox at 13.2 μM whereas the
310 antioxidant activity of XOS_10 at the same 100 μg mL^{-1} concentration
311 corresponds to 3.2 μM Trolox.

312 Although the antioxidant capacity of XOS produced by xylanases has
313 been reported in several works, to our knowledge, differences in the antioxidant
314 effect of XOS obtained by different families of xylanases has not been
315 described previously. The XOS described in literature come from different raw
316 materials such as sugarcane bagasse (Bian et al., 2013; Mandelli et al., 2014),
317 corncob (Gowdhaman & Ponnusami, 2015), garlic straw (Kallel, Driss,
318 Chaabouni, & Ghorbel, 2014), wheat bran (Lasrado & Gudipati, 2014), wheat
319 aleurone (Malunga & Beta, 2015), sunflower stalk and wheat straw (Akpinar,
320 Erdogan, et al., 2010), or agricultural residues (Rashad et al., 2016). However,
321 to our knowledge this is the first report on the antioxidant properties of XOS
322 from glucuronoxylans, xylans without ferulic acid ramifications.

323 Xylanases randomly hydrolyse xylan chains resulting in XOS of varying
324 degrees of polymerisation and substitution depending on the family of GH used.
325 TLC and MALDI-TOF have demonstrated a different XOS pattern depending on
326 the xylanase used, being those produced by Xyn10A a mixture of neutral and
327 acidic XOS whereas those produced by Xyn30D only acidic XOS. Since neither
328 xylose, neutral XOS (X2-X6) or glucuronic acid, at a concentration range of 200-
329 1000 μg mL^{-1} exhibited antioxidant activity, it could be concluded that the

presence of the MeGlcA substitutions determines the antioxidant activity effect found on XOS. In fact, Rao & Muralikrishna (2006) showed that the presence of sugars with uronyl or acetyl groups impart strong antioxidant activity to cereal polysaccharides. It has been also reported that carboxyl groups increase antioxidant activity of cell wall polysaccharides (Pristov, Mitrovi, & Spasojevi, 2011). In a similar way, Malunga & Beta, (2015) reported that the antioxidant capacity of XOS obtained from arabinoxylan is correlated to their degree of ferulic acid substitution.

3.3. Effect of the XOS length on the antioxidant activity

Apart of substitution, other factors, such as XOS length, can affect the antioxidant property. XOS samples studied up to this point had been depleted of undigested or partially digested xylans by graded precipitation with three volumes of ethanol. This methodology can also be used to separate XOS of different degrees of polymerisation (Malunga & Beta, 2015). Analysis of a first precipitate obtained by the addition of one volume of ethanol, showed that it mainly contained unhydrolysed xylan and exhibited very low antioxidant activity (Fig. 4a, b). A second precipitate was obtained by the subsequent addition of two more volumes of ethanol. It mainly contained very long xylooligomers, which showed high antioxidant activity. Remarkably this XOS fraction showed considerably higher antioxidant activity than that of the supernatant, which correspond to the purified XOS fraction (XOS_30), which showed lower degree of polymerization (Fig. 4a, b). The results indicate that the antioxidant activity of XOS_30 is also affected by the degree of polymerization. Similar results were obtained by grade ethanol precipitation of XOS_10 (data not shown).

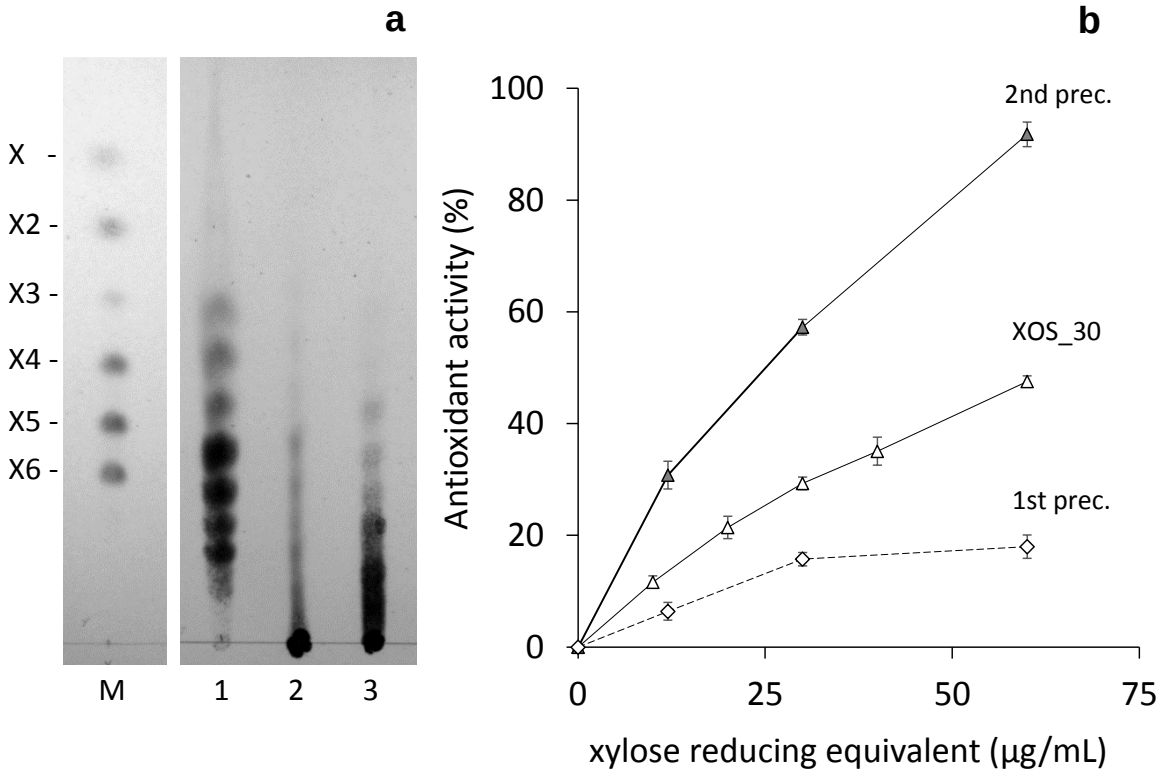


Figure 4. Analysis of XOS_30 from beechwood xylan. a) TLC analysis. 1, XOS_30; 2, first ethanol precipitate; 3, second ethanol precipitate; M, size markers of xylose (X), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6). b) Antioxidant activity of XOS_30, first and second ethanol precipitates.

The time length of the enzyme treatments can affect to the degree of polymerization of the released XOS. We analyzed the antioxidant activity of the purified XOS obtained after different incubation time (from 1 to 120 min). Since XOS content was different at each time, to better visualize the results, the antioxidant activity of the different samples was divided by the amount (µg) of xylose reducing equivalents in each of them (Table 1). The values of antioxidant

activity oscillated from 0.32 to 0.16 for XOS_10 and from 1.41 to 1.20 for XOS_30. The results confirm the higher antioxidant activity of the XOS produced by family GH30 xylanases previously found. Comparing samples of 5 and 120 min enzyme incubation, in the case of Xyn10A, antioxidant activity was reduced 50% with time. This can be caused by the diminished degree of polymerization of the XOS_10 obtained after 120 min, as it can be seen by TLC (Fig. 1). On the other hand, with Xyn30D, the antioxidant activity of XOS_30 did not show a significant variation with time. In fact, the same pattern of the XOS obtained after 5 and 120 min incubation with Xyn30D was observed by TLC (Fig.1).

Table 1 Antioxidant activity of XOS obtained after different incubation time. Beechwood xylan was treated with Xyn10A or Xyn30D and the XOS released at different incubation time were purified and analyzed.

	% Antioxidant activity μg^{-1} xylose reducing equivalent	
Time (min)	XOS_10	XOS_30
1	0.32	1.41
5	0.36	1.43
10	0.29	1.26
30	0.17	1.59
120	0.16	1.20

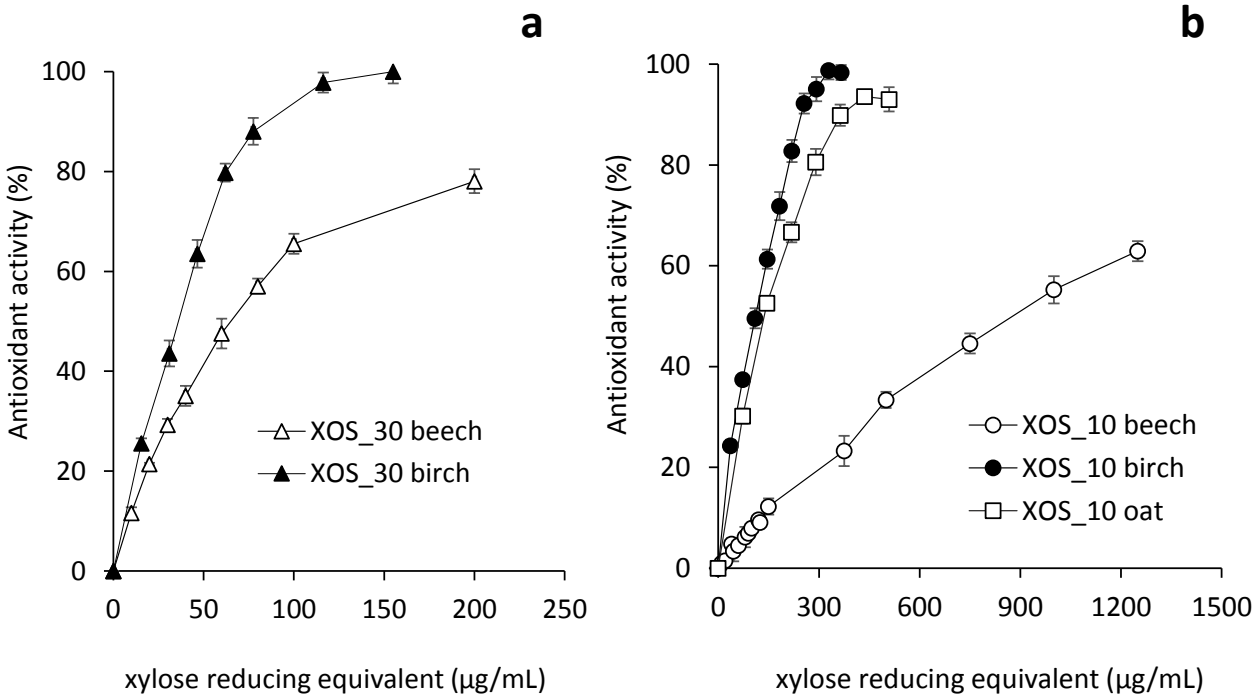
Our results clearly show that apart of ramifications, the degree of polymerization of XOS obtained from glucuronoxylans affects the antioxidant

activity. In fact, the increase of the antioxidant activity with the molecular weight had been previously reported for XOS released from arabinoxylans by Malunga & Beta, (2015) and Rao & Muralikrishna, (2006).

3.4. Antioxidant activity of XOS from birchwood and oat spelt xylans

Once made it evident the antioxidant activity of XOS obtained from beechwood xylan, XOS produced from other glucuronoxylans, such as birchwood xylan, and from arabinoxylans, such as oat spelt xylan, were also evaluated. These xylans were hydrolyzed by xylanases, the XOS obtained after 2h of treatment were purified and their antioxidant activity was analysed at increasing concentrations. As shown in Fig. 5 (a and b) the XOS obtained from birchwood xylan showed higher antioxidant activity than those obtained from beechwood xylan for both xylanases, although the difference was higher for Xyn10A. These glucuronoxylans are very similar although they differ in their number of methyl glucuronic acid ramifications, with higher content in birchwood xylan (Hespell & Cotta, 1995). Differences in antioxidant activity may also result from the different size of the XOS produced by the enzymes from the different glucuronoxylans. Since Xyn30D is a glucuronoxylan-specific xylanase, and it does not hydrolyse arabinoxylans (Valenzuela et al., 2012) this enzyme was not tested on oat spelt xylan. XOS produced from oat spelt xylan by Xyn10A showed similar antioxidant activity than those produced from birchwood xylan, and much higher than those produced from beechwood xylan. This effect can be justified by the presence of ferulic acid substitutions in arabinoxylans from oat spelt. In fact, high antioxidant activity has been reported in water-soluble arabinoxylans (feraxans) (Rao & Muralikrishna, 2006), isolated from rice and

421 ragi. The phenolic content of oligosaccharides has been proposed to have an
422 important effect in antioxidant activity (Bijalwan, Ali, Kesarwani, Yadav, &
423 Mazumder, 2016; Rashad et al., 2016). On the other hand, in beechwood and
424 birchwood XOS, since they do not contain ferulic acid, the main reason of their
425 antioxidant capacity was the presence of methyl glucuronic acid ramifications.



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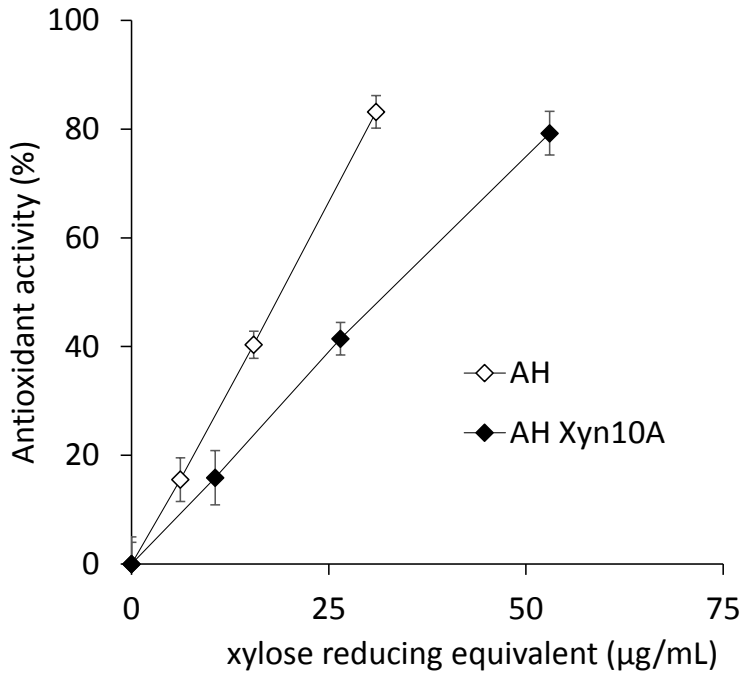
427 **Fig. 5** Antioxidant activity of XOS from beechwood, birchwood and oat spelt
428 xylans. a) XOS_30. b) XOS_10

429

430 **3.5. Antioxidant activity of XOS from eucalyptus autohydrolysates**

431 Pulping process in paper industry produces an enormous amount of wastes
432 containing an important quantity of dissolved xylans among other wood
433 components. Evaluation of the antioxidant activity of XOS derived from
434 eucalyptus xylan can make it possible its upgrading to added value products. As

435 a first approach we tested the antioxidant activity of XOS extracted from
436 eucalyptus wood by the autohydrolysis process. In this process, biomass is
437 treated with hot water or steam and hemicelluloses are converted into soluble
438 glucuronoxylan polymers and long oligosaccharides (Gullón et al., 2011).
439 Eucalyptus autohydrolysates showed high antioxidant activity, probably due to
440 the presence of long XOS together with some quantity of lignin (Fig. 6).
441 However, when autohydrolysates were treated with Xyn10A for 1 h antioxidant
442 activity was strongly decreased. A similar value of around 80% antioxidant
443 activity required twice the amount of xylose reducing equivalents when treating
444 with the enzyme. TLC analysis showed a high amount of XOS of high molecular
445 weight (higher than X6) in the initial AH whereas XOS of lower molecular weight
446 appeared after treatment with Xyn10A (data not shown). As previously stated,
447 the XOS length is an important factor for their antioxidant activity. Concerning
448 Xyn30D, the enzyme did not show noticeable enzymatic activity on eucalyptus
449 autohydrolysate, probably as a result of the heavy acetylation of xylan in the
450 autohydrolysate or to the lack of accessibility of the enzyme, of bigger size than
451 Xyn10A, to the substrate. So the effect of Xyn30D could not be tested on
452 eucalyptus hydrolysate.



453

454 **Fig. 6** Antioxidant activity of autohydrolysate liquors from eucalyptus wood.
455 Autohydrolysates without enzyme treatment (AH) or treated with Xyn10A (AH
456 Xyn10A).

457

458 To compare the antioxidant effect of XOS from eucalyptus hydrolysates
459 with that of XOS obtained by enzyme action on xylans, the antioxidant activity
460 per µg of xylose reducing equivalents of the different XOS studied was
461 determined (Supplementary Table S1). Although eucalyptus hydrolysates
462 showed the highest antioxidant activity (2.65), a rather similar activity was found
463 in XOS_30 from birchwood xylan (1.30), while XOS_10 from beechwood
464 showed the lowest activity (0.08).

465 Akpinar, Gunay, Yilmaz, Levent, & Bostanci, (2010) found that liquors
466 from autohydrolysis of agricultural residues had antioxidant activity while

467 González, Cruz, Domínguez, & Parajó, (2004) and Rivas, Conde, Moure,
468 Domínguez, & Parajó, (2013) reported antioxidant effect in liquors from
469 eucalyptus wood autohydrolysis. However, no knowledge exist concerning the
470 effect of xylanases on the antioxidant activity of eucalyptus autohydrolysates.

471 **3.6. Antioxidant activity: ABTS vs. DPPH**

472 Several methods exist for the measure of antioxidant activity. Concerning
473 the antioxidant activity of XOS, several works use the DPPH method (Akpinar,
474 Gunay, et al., 2010; Bian et al., 2013; Gowdhaman & Ponnusami, 2015;
475 Hromádková, Paulsen, Polovka, Košťálová, & Ebringerová, 2013; Kallel et al.,
476 2014; Lasrado & Gudipati, 2014; Veenashri & Muralikrishna, 2011) although
477 other reports use the ABTS method (Malunga & Beta, 2015; Rivas et al., 2013).
478 Both, DPPH and ABTS methods consist on radical scavenging assays whose
479 reaction mechanism involves transfer of electron by the reducing agent to the
480 DPPH/ABTS radical. The discoloration produced by the interaction of this
481 radical with hydrogen or electron donor species is quantified.

482 In this work the antioxidant activity of XOS produced by xylanases from
483 beechwood was also analysed by the DPPH method. However, some difficulties
484 were observed with this method. The DPPH radical has to be firstly dissolved in
485 ethanol or methanol to perform the measurements. When XOS were mixed with
486 this solution, some XOS precipitated giving an erroneous lecture of the
487 absorbance, and making the method less sensitive or not suitable for XOS
488 determination. The concentration that produced the 50% of inhibition of the
489 radicals was measured, being determined as 13157 μg of xylose reducing
490 equivalent mL^{-1} for XOS_10 with DPPH method, or 850 of μg of xylose reducing

491 equivalent mL^{-1} with ABTS. In the case of XOS_30 these values were 417 or 70
492 μg of xylose reducing equivalent mL^{-1} with DPPH or ABTS, respectively.
493 Although the higher effectiveness of XOS_30 was shown by both methods, the
494 concentration needed to inhibit DPPH was strongly higher, indicating a higher
495 sensitivity of the ABTS method for XOS studies. Rivas et al., (2013) analysed
496 the antioxidant activity of autohydrolysates liquors and also found higher
497 antioxidant activity with ABTS than with DPPH. In reported works, where the
498 antioxidant activity of XOS from arabinoxylans has been evaluated by the
499 DPPH method, 50% of inhibition has been found with 1000 μg mL^{-1} of XOS
500 from corncob (Gowdhaman & Ponnusami, 2015) or with 620 μg mL^{-1} of
501 sugarcane bagasse derived XOS. Although these reported values for producing
502 50% DPPH inhibition cannot be compared to those obtained in our work,
503 obtained from different xylan substrates, our results indicate that the important
504 antioxidant activity we have identified in MeGlcA branched XOS can be
505 compared to that of arabinoxylan derived XOS, ferulic acid branched, of high
506 potential as antioxidants for food industry.

507 **4. CONCLUSIONS**

508 XOS produced by enzymatic hydrolysis of glucuronoxylan showed high
509 antioxidant activity. XOS produced by a family GH30 xylanase (Xyn30D),
510 comprised exclusively of acidic XOS, showed higher antioxidant activity than
511 those produced by xylanase Xyn10A of family GH10, a mixture of neutral and
512 acidic XOS. The MeGlcA ramification contained in acidic XOS seems to be an
513 important determinant of the antioxidant power of xylooligomers. Besides,
514 antioxidant activity increased with XOS degree of polymerization. Eucalyptus

wood autohydrolysate showed high antioxidant activity, which was diminished by xylanase treatment. ABTS was a more accurate methodology for the antioxidant activity determination of XOS than DPPH.

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693

Supplementary data

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Graphical Abstract

XOS Enzymatic Production

MeGlcA

Xyn30D

Acidic XOS

MeGlcA

Glucuronoxylan

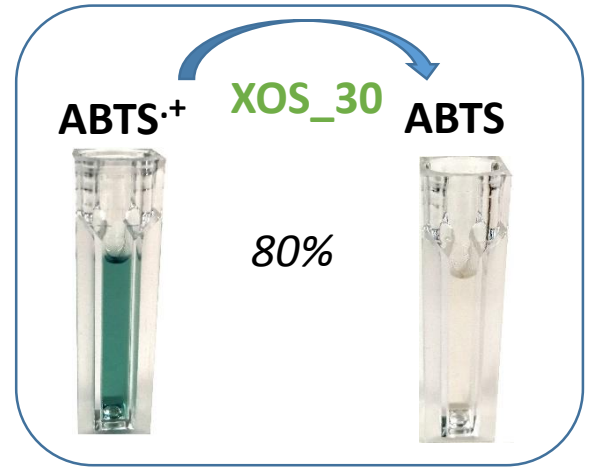
MeGlcA

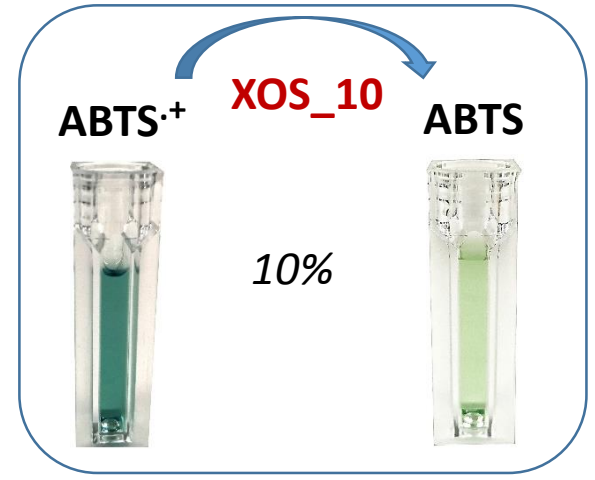
Xyn10A

Acidic + Neutral XOS

Antioxidant activity

at 200 µg xylose reducing equivalent mL^{-1}





Increased by:

- *Acidic XOS*
- *XOS length*
- *Lignin*

Eucalyptus autohydrolysates (AH)

XOS mixture ✚ *Lignin*