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Title: A biorefinery based on brewer`s spent grains: arabinoxylans recovery by microwave assisted pretreatment integrated with butanol production

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Keywords: brewing industry waste; microwave pretreatment; xylooligosaccharides; ABE fermentation; biorefinery

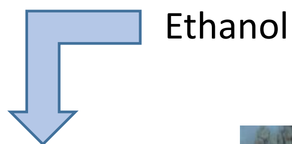
Abstract: This study proposes an integrated biorefinery of brewer's spent grain (BSG) based on the application of a microwave pretreatment to obtain bioproducts, such as arabinoxylans (AX) and an advanced biofuel, such as biobutanol. A prehydrolysate with 17 g/L AX was obtained at 172 °C and 0.38 M NaOH, which were subsequently precipitated using an organosolvent process, recovering 133 kg AX/t BSG. The subsequent enzymatic hydrolysis with endo-xylanases significantly increased the concentration of oligomers with 2-6 units of xylose. The presence of oligosaccharides with a degree of polymerization (DP) from 3 to 27 was confirmed by MALDI-TOF MS. On the other hand, the pretreated solid residue obtained in the microwave assisted alkaline pretreatment was enzymatically hydrolyzed with cellulases and fermented by Clostridium beijerinckii, producing 9.9 g/L butanol (28 kg butanol/t BSG and 37 kg ABE/t BSG). Thus, the potential for the efficient use of BSG in an integrated biorefinery was demonstrated.

Graphical Abstract

BSG

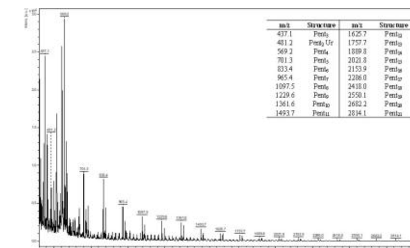
Microwave assisted sodium hydroxide pretreatment

Ethanol



Arabinoxylans precipitation

Endo-xylanases enzymatic hydrolysis



ENRICHED ARABINOXYLANS SOLUTION

LOW DEGREE OF POLIMERIZATION (DP2-DP6)

POTENTIAL PREBIOTIC PROPERTIES

Cellulases enzymatic hydrolysis

ABE fermentation

C. beijerinckii

BUTANOL

ADVANCED BIOFUEL

BUILDING BLOCK

HIGHLIGHTS

- Integration of AX recovery with butanol production to use BSG in a biorefinery
- AX production of 133 kg/t BSG by microwave assisted alkaline pretreatment
- AX with a degree of polymerization from 3 to 27 was observed
- 9.9 g/L butanol after ABE fermentation of the microwave alkaline pretreated solid
- ABE fermentation yield of 37 kg/t BSG, of which 28 kg/t are butanol

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1 **A biorefinery based on brewer`s spent grains: arabinoxylans recovery by**
2 **microwave assisted pretreatment integrated with butanol production**

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24 ABSTRACT

25 This study proposes an integrated biorefinery of brewer's spent grain (BSG) based on
26 the application of a microwave pretreatment to obtain bioproducts, such as
27 arabinoxylans (AX) and an advanced biofuel, such as biobutanol. A prehydrolysate with
28 17 g/L AX was obtained at 172 °C and 0.38 M NaOH, which were subsequently
29 precipitated using an organosolvent process, recovering 133 kg AX/t BSG. The
30 subsequent enzymatic hydrolysis with endo-xylanases significantly increased the
31 concentration of oligomers with 2-6 units of xylose. The presence of oligosaccharides
32 with a degree of polymerization (DP) from 3 to 27 was confirmed by MALDI-TOF MS.
33 On the other hand, the pretreated solid residue obtained in the microwave assisted
34 alkaline pretreatment was enzymatically hydrolyzed with cellulases and fermented by
35 *Clostridium beijerinckii*, producing 9.9 g/L butanol (28 kg butanol/t BSG and 37 kg
36 ABE/t BSG). Thus, the potential for the efficient use of BSG in an integrated
37 biorefinery was demonstrated.

38 **Keywords:** brewing industry waste; microwave pretreatment; xylooligosaccharides;
39 ABE fermentation; biorefinery.

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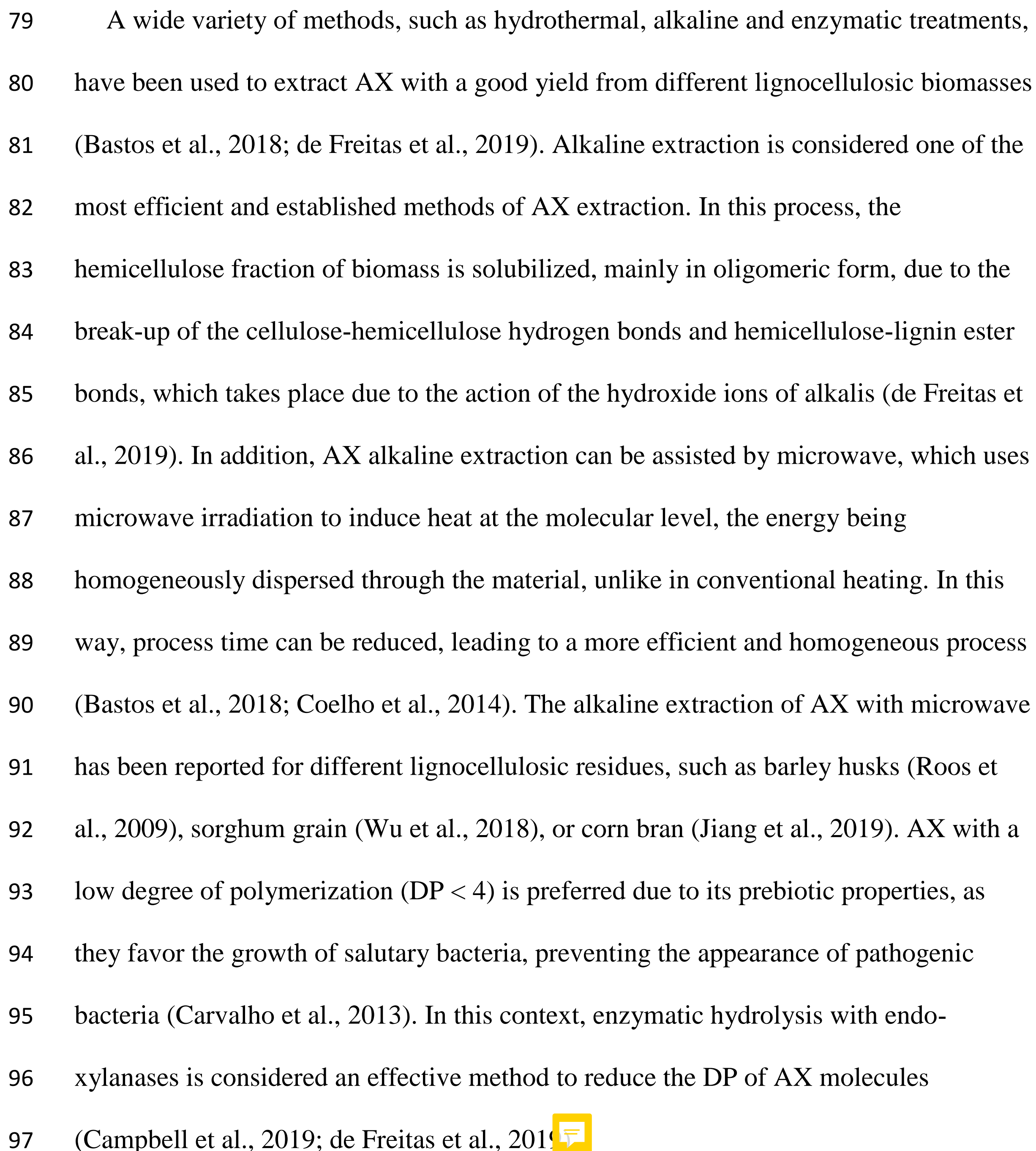
49 **1. Introduction**

50 The concept of biorefinery has become increasingly important in substituting the
51 current fossil resources. A varied quantity of products, such as energy, chemicals,
52 materials and in general different added value products, can be obtained integrally by
53 using biomass as the raw material, making the process competitive and feasible
54 (European Union, 2018).

55 The use of lignocellulosic biomass is of great importance, as it is not expensive, it is
56 both abundant and environmental-friendly, it lacks application and necessarily has to be
57 eliminated (Dias et al., 2013; Hosseini Koupaie et al., 2019). In this context, a
58 promising lignocellulosic residue is BSG, since it has a very restricted use for cattle
59 feed and a suitable composition in carbohydrates and lignin (32-50 and 12-28%,
60 respectively) for its valorization in the framework of biorefineries (Lynch et al., 2016).
61 37.4 and 180.3 million tonnes of beer are produced in the European Union and the
62 world, respectively (FAOSTAT, 2019), a generation of 20 kg of wet BSG/100 L beer
63 being estimated.

64 Arabinoxylans (AX) are one of the main products that can be obtained in a BSG
65 biorefinery. The pentoses, xylose and arabinose, make up AX, ($\beta 1 \rightarrow 4$)-linked D-
66 xylopyranosyl residues being the linear backbone chain and α-L arabinofuranosyl
67 residues linking to these (Bastos et al., 2018; de Freitas et al., 2019). AX stand out for
68 their prebiotic activity; it is defined as an ingredient which has been specifically
69 fermented and is able to selectively change the growth and/or activity of the flora
70 contained in the colon, therefore enhancing the host's health (de Freitas et al., 2019).
71 Thus, AX are considered to be very important by the food industry. Moreover, they are
72 able to increase calcium levels as well as decrease cholesterol levels in the body and
73 prevent gastrointestinal infection. Furthermore, they contain other many properties,

such as having antiallergic, anti-inflammatory, anticarcinogenic, immunological and antimicrobial properties, which means that AX can be used in the pharmaceutical industry (Carvalho et al., 2013). AX can also be used in the cosmetic industry (for example, in skin care products) because of their antioxidant properties; or in the agricultural industry to enhance crop maturation and yield (Moure et al., 2006).



A wide variety of methods, such as hydrothermal, alkaline and enzymatic treatments, have been used to extract AX with a good yield from different lignocellulosic biomasses (Bastos et al., 2018; de Freitas et al., 2019). Alkaline extraction is considered one of the most efficient and established methods of AX extraction. In this process, the hemicellulose fraction of biomass is solubilized, mainly in oligomeric form, due to the break-up of the cellulose-hemicellulose hydrogen bonds and hemicellulose-lignin ester bonds, which takes place due to the action of the hydroxide ions of alkalis (de Freitas et al., 2019). In addition, AX alkaline extraction can be assisted by microwave, which uses microwave irradiation to induce heat at the molecular level, the energy being homogeneously dispersed through the material, unlike in conventional heating. In this way, process time can be reduced, leading to a more efficient and homogeneous process (Bastos et al., 2018; Coelho et al., 2014). The alkaline extraction of AX with microwave has been reported for different lignocellulosic residues, such as barley husks (Roos et al., 2009), sorghum grain (Wu et al., 2018), or corn bran (Jiang et al., 2019). AX with a low degree of polymerization (DP < 4) is preferred due to its prebiotic properties, as they favor the growth of salutary bacteria, preventing the appearance of pathogenic bacteria (Carvalho et al., 2013). In this context, enzymatic hydrolysis with endo-xylanases is considered an effective method to reduce the DP of AX molecules (Campbell et al., 2019; de Freitas et al., 2019

98 The AX market can only be feasible in terms of production costs if the AX are co-
99 produced in a biorefinery with other products. Thus, AX could be sold at a price of 7.77
100 $/kg (Sadhukhan et al., 2008). In this context, after AX production by alkaline
101 pretreatment, a pretreated solid remains, with all unbroken cellulose contained in BSG,
102 and this can be valorized for the production of other products, such as biobutanol by
103 acetone-butanol-ethanol (ABE) fermentation. Biobutanol is considered an advanced
104 biofuel with better fuel properties than bioethanol in terms of energy density and
105 hygroscopicity, its selling price being 1.06 $/L (Daroch et al., 2017). Butanol is also
106 regarded as a chemical building block, since it can be transformed into other secondary
107 chemicals, such as solvents, polymers, coating materials, biodiesel, or jet fuel (Cheng et
108 al., 2019). Therefore, there are good prospects to develop processes that are able to take
109 advantage of all the carbohydrates contained in lignocellulosic residues, such as BSG,
110 in an integrated approach.

111 This study evaluates the use of BSG in an integrated biorefinery framework to obtain
112 value added bioproducts and advanced biofuels. With this purpose, the recovery of AX
113 in the prehydrolysates from BSG was considered as the evaluation criteria, using a
114 microwave assisted alkaline pretreatment; the effect of temperature and NaOH
115 concentration being the analyzed factors. The recovery of the AX from the
116 prehydrolysate, using an organosolvent process, was also assessed. A subsequent
117 enzymatic hydrolysis step with endo-xylanases was used to reduce the DP of the
118 molecules recovered. In addition, the DP and structure of the resulting AX was
119 evaluated using different techniques. Furthermore, the solid residue enriched in
120 cellulose obtained in the microwave assisted alkaline pretreatment was used for butanol
121 co-production.

122

2. Materials and methods

2.1. Raw material

Brewer's spent grain, which was provided by a local brewery, was kept at −20°C. Then, a washing and drying process at 50 ºC was applied to the BSG, 3% (w/w) being its final moisture. Then, a coffee grinder (Moulinex, A505, France) was used to grind the BSG, getting a particle size < 1 mm. In this way, the BSG composition was (% w/w): cellulose, 17.9 ± 0.3; hemicellulose, 28.7 ± 0.8 (xylan, 20.7 ± 0.4; arabinan, 8.0 ± 0.4); acid-insoluble lignin (AIL), 19.4 ± 1.2; acid-soluble lignin (ASL), 6.4 ± 0.1; extractives, 2.3 ± 0.1 (glucose in extractives, 1.3 ± 0.1); ash, 2.7 ± 0.1; acetyl groups, 1.0 ± 0.1; and starch, 2.1 ± 0.0 (López-Linares et al., 2019).

2.2. Microwave assisted sodium hydroxide pretreatment

The BSG was subjected to a microwave assisted sodium hydroxide pretreatment, in a closed reactor (Multiwave PRO SOLV 16HF100, Anton Paar GmbH, Austria, Europe) being the maximum microwave power of 1800 W (for more details, see López-Linares et al. (2019)).

The solid loading used was 10% (w/v), mixing 5 g dry weight BSG and 50 mL of sodium hydroxide solution. The temperature of each run was reached through the setting of the reactor power input. Once the desired temperature in each run had been achieved, the pretreatment time began. Finally, the reactor was cooled to 50 ºC.

In order to separate the solid and liquid phases, centrifugation was carried out (10500 rpm for 10 min). Then, the pretreated solid was dried at 40 ºC and weighed. In this way, it was possible to determine the solid recovery in terms of the relation between the grams of pretreated solid and 100 grams of BSG. Moreover, the composition (structural carbohydrates, lignin and ash) of the pretreated solid was analyzed. The liquid fractions

148 (or prehydrolysates) were measured for their content in monosaccharides, AX,
149 degradation products (formic acid, acetic acid, furfural and hydroxymethylfurfural
150 (HMF)) and Total Organic Carbon (TOC) content. The recoveries of AX in the
151 prehydrolysates (AXR) were determined (as a percentage of the AX content in the
152 untreated BSG).
153
154 ***2.3. Experimental design***
155 In order to select the optimum conditions for AX extraction with the microwave
156 assisted sodium hydroxide pretreatment, a central composite experimental design was
157 proposed ($\alpha = 1.414$). The factors selected were the temperature (170-210 ºC) and the
158 sodium hydroxide concentration (0.1-0.4 M). The intervals were selected from previous
159 results (data not shown). In this way, a total of 13 experiments were performed in
160 random order, including one point and four replicates at the center of the domain
161 selected for each factor under study. The pretreatment time was kept at 2.5 min. Table 1
162 shows the values of the factors in the experimental design. The experimental data were
163 analyzed by the commercial software Statgraphics Centurion XVIII.

164 **Table 1.** Sugar composition (g/L) and arabinoxylans recovery (AXR, %) of the

165 prehydrolysates after microwave assisted sodium hydroxide pretreatment.

Run	T (ºC)	NaOH conc. (M)	Monomeric sugars (g/L)		Oligomeric sugars (g/L)		AXR (%)
			Glucose	X + Ar	Glucose	AX	
1	210	0.10	n.d.	n.d.	n.d.	12.4 ± 0.1	38.4
2	170	0.10	n.d.	n.d.	n.d.	4.7 ± 0.1	14.6
3	210	0.40	0.51 ± 0.01	n.d.	n.d.	14.1 ± 0.1	43.6
4	190	0.25	0.06 ± 0.00	n.d.	0.11 ± 0.01	18.3 ± 0.1	56.4
5	190	0.25	0.01 ± 0.00	n.d.	n.d.	14.6 ± 0.1	45.0
6	190	0.25	n.d.	n.d.	n.d.	15.7 ± 0.2	48.6
7	190	0.04	n.d.	n.d.	1.09 ± 0.05	1.4 ± 0.0	4.2
8	190	0.25	n.d.	n.d.	n.d.	14.1 ± 0.1	43.6
9	190	0.46	0.58 ± 0.02	n.d.	n.d.	16.2 ± 0.1	50.0
10	218.3	0.25	n.d.	n.d.	n.d.	16.2 ± 0.1	49.9
11	170	0.40	0.63 ± 0.01	n.d.	n.d.	17.2 ± 0.2	53.2
12	190	0.25	0.05 ± 0.00	n.d.	n.d.	14.2 ± 0.1	43.9
13	161.7	0.25	0.28 ± 0.01	n.d.	n.d.	13.0 ± 0.1	40.0

166 X: xylose
167 Ar: Arabinose
168 AX: arabinoxylans (xylose and arabinose in oligomeric form)
169 AXR (arabinoxylans recovery in liquid fractions): g arabinoxylans (xylose + arabinose as
170 oligomeric sugars) in liquid fractions/100 g arabinoxylans in BSG
171 n.d.: not detected
172

173 ***2.4. Arabinoxylans recovery experiments***

174 In order to recover AX from BSG prehydrolysates, AX recovery experiments by
175 precipitation with ethanol were carried out using the prehydrolysate obtained under
176 optimal pretreatment conditions (Fig. 1). The prehydrolysate was mixed with 96% (v/v)
177 ethanol solution using different ratios (v/v) of prehydrolysate:ethanol (1:1, 1:2 and 1:3).
178 Thus, 50 mL prehydrolysate were mixed with 50, 100 and 150 mL 96% (v/v) ethanol,
179 respectively, using 500 mL erlenmeyer flasks. The AX precipitation experiments were
180 carried out in an orbital shaker (Comecta Optic Ivymen system) at 25 ºC and 100 rpm
181 for 60 min.

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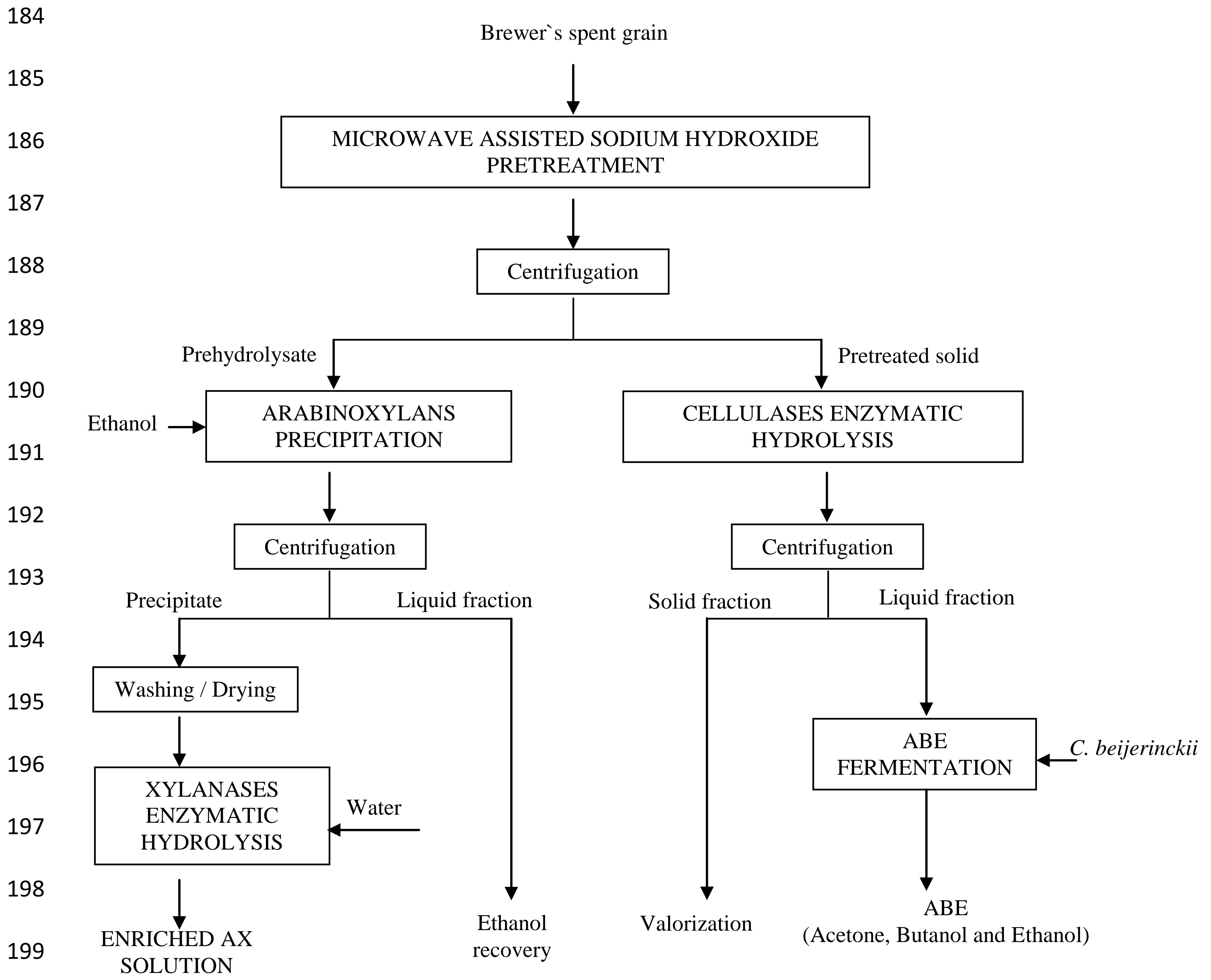


Fig. 1.

The liquid and solid phases were separated by centrifugation (3000 g for 10 min). The solid phase was washed with 96% (v/v) ethanol, dried at 60 ºC for 48 h and weighed to determinate the mass recovered. Finally, the resulting solid containing the extracted AX was suspended in water in an orbital shaker at 35 ºC and 200 rpm for 24 h. The AX suspension was analyzed for its monosaccharides and TOC content, and used for enzymatic hydrolysis essays and further analysis (such as DP and molecular weight distribution). The purity in the AX suspension was determined using TOC analysis following the equation proposed by Sánchez-Bastardo et al. (2017) (Eq. (1)).

$$\text{Purity (\%)} = \frac{\text{theoretical carbon content of (Ara + Xyl)as oligomeric sugars (g)}}{\text{total carbon content given by TOC (g)}} \text{ x } 100 \quad (1)$$

209

210 *2.5. Enzymatic hydrolysis (EH) of the AX suspension*

211 The suspension containing the precipitated AX (as described in section 2.4) was
212 enzymatically hydrolyzed using two commercial endo-xylanases (Shearzyme 2X and
213 Pentopan Mono BG), kindly provided by Novozymes A/S (Denmark).

214 The EH tests were carried out in triplicate at 45 ºC, 120 rpm, 48 h and pH 4.7, which
215 was adjusted with 1 M H_2SO_4 solution. The essays were performed in 100 mL flasks, 25
216 mL being the working volume. Two dosages of endo-xylanase were tested, 1 and 10
217 Xylanase Units (XU)/mL liquor (Fig. 1). Enzyme blanks were used to consider the
218 monosaccharides content of the commercial enzymes. In all essays, samples were
219 withdrawn at 8, 24 and 48 h, analyzed for their monosaccharides and AX content, and
220 used for further analysis (such as DP, molecular weight distribution and MALDI-TOF-
221 MS analysis).

222

223 *2.6. Butanol production from pretreated BSG*

224 The pretreated solid obtained under optimal pretreatment conditions was subjected to
225 EH with cellulases in an orbital shaker (Comecta Optic Ivymen system) in order to
226 obtain a rich sugar solution, which can be used in ABE fermentation (Fig. 1). The
227 substrate loading was 10% (w/v), 140 mL being the working volume using 500 mL
228 erlenmeyer flasks. The pretreated solid was hydrolyzed enzymatically using Cellic
229 CTec2, which is a cellulolytic complex kindly provided by Novozymes A/S (Denmark).
230 The essays were carried out in triplicate at 50 ºC, 150 rpm, 48 h, 15 Filter Paper Units
231 (FPU)/g solid of Cellic CTec2 enzyme load and pH 4.8, which was adjusted with solid
232 NaOH using water as solvent. After EH, a vacuum filtration was used to separate the

233 liquid (enzymatic hydrolysate) and solid phases. The enzymatic hydrolysate was
234 analyzed for its monosaccharides and degradation products content. Enzyme blanks
235 were used to consider the monosaccharides content of the commercial enzymes. EH
236 yield was calculated as a percentage of glucose released with respect to the structural
237 glucose (as cellulose) contained in the pretreated BSG. The enzymatic hydrolysate was
238 used as substrate for the ABE fermentation with *C. beijerinckii* DSM 6422 at 35 ºC and
239 135 rpm for 48 h (Fig. 1). All experiments were performed in triplicate in 100 mL
240 serum bottles. O_2 free nitrogen was flushed initially into the solution to create anaerobic
241 conditions. The preculture was inoculated at 10% (v/v). The initial pH of the
242 fermentation was 5.5, and it was not controlled during the fermentation (for more
243 details, see López-Linares et al. (2019).
244

245 ***2.7. Analytical methods***

246 The structural carbohydrates, lignin and ash content of the pretreated BSG were
247 determined using the National Renewable Energy Laboratory (NREL) analytical
248 methods (Sluiter et al., 2011, 2008).
249 The concentrations of sugars (glucose, xylose and arabinose), inhibitor compounds
250 (acetic and formic acids, furfural and HMF), ABE solvents (acetone, butanol and
251 ethanol) and other organic acids (lactic and butyric acids) were measured by High
252 Performance Liquid Chromatography (HPLC), using an Aminex HPX-87H column.
253 The sugars were analyzed using a refractive index detector (Waters 2414), whereas
254 furfural and HMF were measured with a photodiode array detector (DAD) at 280 nm
255 (Waters 996). 0.01 N H_2SO_4, at a flow rate of 0.6 mL/min and 30 °C (solvents) or 60 °C
256 (sugars, organic acids, furfural and HMF), was the mobile phase used. Previously, all

257 samples were centrifuged at 13400 rpm for 10 min, and filtered with 0.2 μm nylon
258 filters.

259 The NREL method to determine the structural carbohydrates was applied to quantify
260 the oligomers or AX content in the prehydrolysates obtained after pretreatment and in
261 the enzymatic hydrolysates when endo-xylanases were used. The AX concentration was
262 calculated as the increase in sugar monomers measured by HPLC. TOC analysis was
263 carried out using a TOC-V 5000 analyzer (Shimadzu TOC-VCSH).

264 In order to establish the molecular weight distribution of the AX, High Performance
265 Size Exclusion Chromatography (HPSEC) was used, employing a refractive index
266 detector (Waters 2414) and an Ultrahydrogel 250 column (Waters, Japan) at 35 ºC. The
267 mobile phase used was ultrapure water (0.7 mL/min), the sample injection volume
268 being 50 μL. Dextran standards (Sigma-Aldrich, USA), with molecular weights of
269 1000, 5000, 12000, 50000 and 670000 Da, were used.

270 Quantitative analysis of the DP (DP2-DP6) of the AX was determined, following the
271 method described by Samala et al. (2012), by HPLC analysis with a refractive index
272 detector (Waters 2414) and an Aminex HPX-42A column (Bio-Rad, Richmond, USA)
273 at 80 ºC and with a flow rate of 0.6 mL/min, using ultrapure water as mobile phase. The
274 xylooligosaccharides (DP2–DP6), such as xylobiose (DP2), xylotriose (DP3),
275 xylotetrose (DP4), xylopentose (DP5) and xylohexose (DP6), from Megazyme
276 (Megazyme International, Ireland) were used as standards.

277 In order to thoroughly study the structural characterization of the AX, matrix-assisted
278 laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)
279 analysis was carried out in the Laboratory of Instrumental Techniques (LTI) Research
280 Facilities (University of Valladolid). The analysis was performed according to the
281 method described by Gómez et al. (2014), using an Autoflex speed workstation (Bruker

282 Daltonics, Bremen, Germany). Previously, the samples were treated with ion-exchange
283 resins (Lewatit MonoPlus S 108 H), kindly provided by Lanxess AG (Germany), at
284 room temperature for 30 min.

285 Analytical determinations were carried out in triplicate and the average results are
286 shown. Relative standard deviations were below 3%.

287

288 **3. Results and discussion**

289 ***3.1. Effect of microwave assisted sodium hydroxide pretreatment on BSG and***
290 ***optimization***

291 A microwave assisted sodium hydroxide pretreatment was used to solubilize the AX
292 contained in the BSG. The composition of the prehydrolysates obtained after
293 pretreatment are shown in Table 1. As can be seen, xylose and arabinose were detected
294 in oligomeric form in all experimental runs, ranging between 1.4 g/L (run 7) and 17.2
295 g/L (run 11). An average concentration of 15.4 g AX/L was observed around the central
296 point (190 ºC and 0.25 M NaOH). In alkaline pretreatment, the hemicellulose fraction of
297 biomass is solubilized, mainly in oligomeric form, due to the break-up of cellulose-
298 hemicellulose hydrogen bonds and hemicellulose-lignin ester bonds, which takes place
299 due to the action of hydroxide ions of alkalis (de Freitas et al., 2019). The AX
300 concentration response was modeled using a second-order polynomial equation
301 (Eq.(2)):

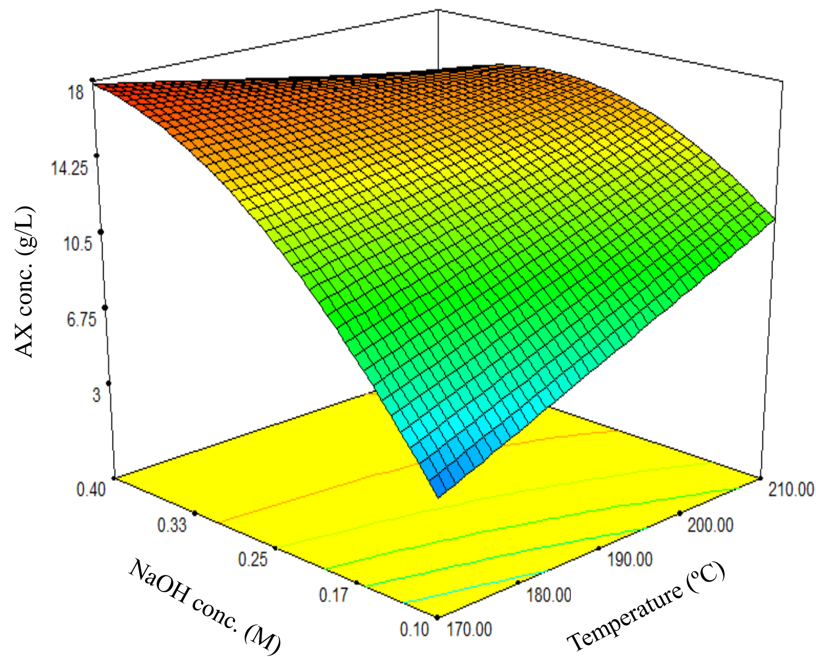
$$\mathrm{AX} = 14.71 + 1.13\,\mathrm{T} + 4.40\,\mathrm{C} - 2.70\,\mathrm{T}\,\mathrm{C} - 2.84\,C^2 \quad (2)$$

$(R^2 = 0.9689; \quad R^2 \text{ adjust} = 0.9511)$

302 where T is the temperature (°C) and C is the sodium hydroxide concentration (M). The
303 AX concentration model was predictive, as suggested by their values of R^2 and adjusted
304 R^2 (Eq. 2) and the confidence level (95%, $p < 0.05$).

305 As can be seen in Table 1 and Eq. (2), both the temperature and NaOH concentration
306 factors exerted positive effects on the AX concentration response, the influence of the
307 NaOH concentration being higher. However, a not very high negative interaction
308 between both factors was observed, which explains why the combined effect of both
309 parameters can lead to a decrease in the AX concentration, probably due to the sugar
310 degradation at high pretreatment severity. This trend can also be appreciated in Fig. 2,
311 which shows the influence of the temperature and the NaOH concentration on the AX
312 concentration, through a response surface plot. The same behavior is also reflected in
313 the arabinoxylans recovery (AXR) (Table 1). Thus, the microwave assisted sodium
314 hydroxide pretreatment is able to recover up to 53% (run 11) of the AX contained in the
315 untreated BSG.

316 Glucose was detected in monomeric form at low levels (< 0.63 g/L) for NaOH
317 concentrations equal to or higher than 0.25 M. However, only for run 7 (190ºC, 0.04 M
318 NaOH), the presence of oligomeric glucose was detected, which may be due to the low
319 NaOH concentration used in this experimental run. Therefore, this glucose probably
320 comes from the nonstructural glucose fraction (glucose in extractives and starch)
321 contained in BSG (Rojas-Chamorro et al., 2018).



322
323 **Fig. 2.**

324 The desirability function was used as optimization method (Mesa et al., 2017).
325 Considering the maximization of the AX concentration as the optimization criterion, the
326 optimal pretreatment conditions were 172 °C and 0.38 M NaOH. Under these
327 conditions, an AX concentration of 17.5 g/L could be reached in the prehydrolysate. To
328 support the proposed mathematical model, a confirmatory experimental run was
329 performed under optimal pretreatment conditions (Table 2). So a cellulose-enriched
330 solid with a significant content in xylan and arabinan was obtained. Moreover, under
331 these conditions, delignification was considerably high (87.6%), which is beneficial to
332 the subsequent enzymatic hydrolysis step of the pretreated solid, since the cellulose is
333 more accessible to enzymes (Alvira et al., 2010). The resulting prehydrolysate contained
334 just 0.3 g/L glucose (mainly in monomeric form) and an AX concentration as high as 17
335 g/L, therefore achieving a good adjustment between the predicted and experimental
336 values. Then, the microwave assisted sodium hydroxide pretreatment allowed 52.6% of
337 the AX contained in the untreated BSG to be recovered. Moreover, the content of

338 degradation products in the prehydrolysate was low, mainly formic (generated from
339 furfural and HMF) and acetic acids (from the release of acetyl groups contained in the
340 hemicellulose structure).

341 **Table 2.** Microwave assisted sodium hydroxide pretreatment of BSG under optimal
342 conditions (172 ºC, 0.38 M NaOH). Confirmatory experimental run: composition of
343 solid and liquid fractions.

Component	Concentration	
Solid fraction (%)		
Cellulose	49.6 ± 0.9	
Xylan	24.7 ± 0.1	
Arabinan	4.2 ± 0.1	
Lignin	13.4 ± 1.9	
Liquid fraction (g/L)		
	Monomeric form sugars	*Oligomeric form sugars*
Glucose	0.3 ± 0.0	0.04 ± 0.01
Xylose	n.d.	10.2 ± 0.1
Arabinose	n.d.	6.8 ± 0.1
	Degradation products	
Furfural	n.d.	
HMF	n.d.	
Formic acid	1.2 ± 0.0	
Acetic acid	1.2 ± 0.0	

344 n.d.: not detected

345 The results achieved, considering high AX recoveries and the low concentration of
346 degradation products, agree with those obtained by Sánchez-Bastardo et al. (2017), who
347 reported AX recoveries of about 50-78% when pretreating wheat bran at 180 ºC for 10
348 min using $RuCl_3$-based catalysts. A similar AX recovery (52%) was also obtained from
349 *Miscanthus* pretreated by steam explosion at 200ºC, 15 bar and 10 min (Bhatia et al.,
350 2020). AX recoveries as low as 36% were achieved by Álvarez et al. (2017) from wheat
351 straw pretreated by steam explosion at 200ºC for 4 min. Pontes et al. (2018) achieved
352 AX concentrations and recoveries of 10.8 g/L and 47.4%, respectively, from a mixture
353 of lignocellulosic feedstock (forest and marginal land resources) pretreated by
354 autohydrolysis at 190 ºC.

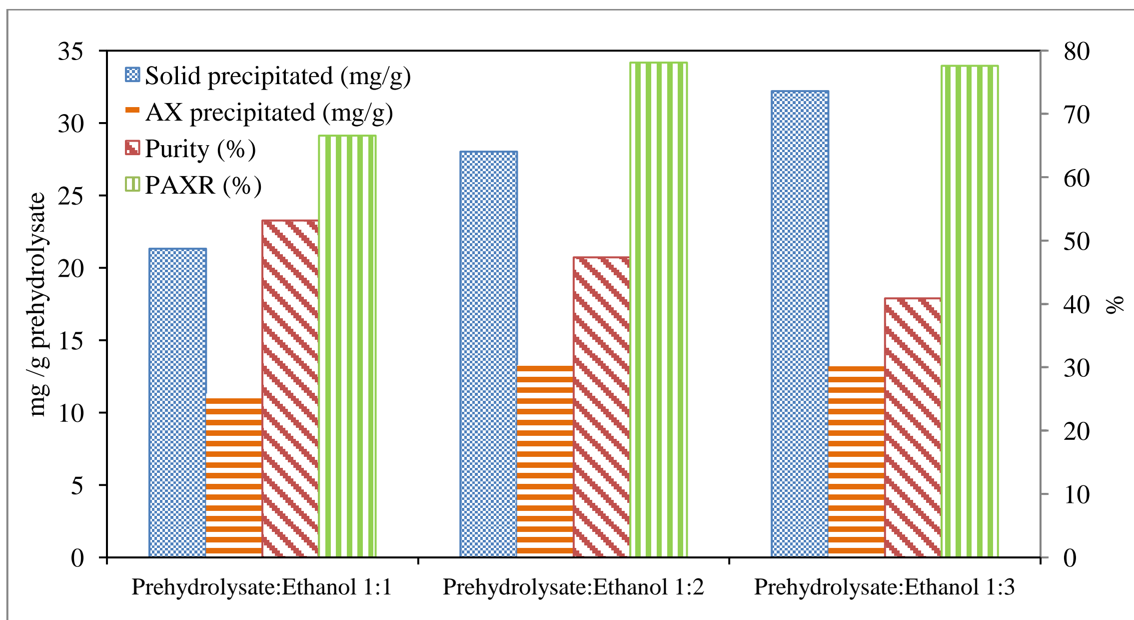
355

356 ***3.2. Arabinoxylans recovery***

357 In order to recover the AX contained in the prehydrolysate of BSG obtained under
358 optimal pretreatment conditions (172 °C and 0.38 M NaOH), a precipitation method
359 based on the addition of ethanol was carried out, using different ratios of
360 prehydrolysate:ethanol (1:1, 1:2 and 1:3 v/v). Ethanol precipitation has been considered
361 a suitable method for AX precipitation, as ethanol can be produced in ABE fermentation
362 and thus enable the economically viable production of AX in an integrated biorefinery
363 (Campbell et al., 2019). Moreover, ethanol can be recovered by distillation, unlike other
364 methods for AX purification, such as the ion-exchange resins, which can only be
365 regenerated using chemical products. In addition, ethanol is usually used mixed with
366 water, which are solvents suitable for human consumption, so AX generated in this way
367 could be used in the food industry (Skendi et al., 2018).

368 Fig. 3 shows the amount of solid and AX precipitated in each case (expressed as
369 mg/g prehydrolysate), as well as the purity of the AX in the solid precipitated and the
370 precipitated arabinoxylans recovery (PAXR, expressed as g AX recovered in the
371 resulting precipitate/100 g AX in the prehydrolysate). As can be seen, the mass of solid
372 precipitated increased in proportion to the ratio of ethanol, obtaining the highest data for
373 a prehydrolysate:ethanol ratio of 1:3 (32.2 mg solid precipitated/g prehydrolysate).
374 However, the purity of AX in the solid precipitated decreased when the
375 prehydrolysate:ethanol ratio varied from 1:1 (purity of 53.2%) to 1:3 (purity 40.9%).
376 Regarding the mass of AX precipitated, the values slightly increased when the
377 prehydrolysate:ethanol ratio was varied from 1:1 to 1:2 (11.3 to 13.3 mg AX
378 precipitated/g prehydrolysate), while no further change was observed when the
379 prehydrolysate:ethanol was set at 1:3 (13.20 mg/g). The same behavior was also
380 observed for PAXR (Fig. 3). Therefore, it can be said that a ratio of

381 prehydrolysate:ethanol of 1:2 can be considered as suitable to precipitate the AX
382 contained in the BSG prehydrolysate, recovering 78.1 g AX/100 g AX in the
383 prehydrolysate, which corresponds to a global yield of 41 g AX recovered/100 g AX in
384 raw BSG (133 kg AX/t BSG). Similar results were achieved by Coelho et al. (2014),
385 who recovered 43% of AX contained in raw BSG by pretreating the raw material by
386 microwave superheated water (210 ºC, 2 min, 6:1 liquid/solid ratio) and subsequent
387 precipitation with 70% (v/v) ethanol solution. The ethanol solution was slightly more
388 concentrated than that selected in our work (1:2 ratio of prehydrolysate:ethanol, that is,
389 67% (v/v) ethanol solution).



390
391 Solid precipitated: mg solid precipitated /g prehydrolysate
392 AX precipitated: mg AX precipitated /g prehydrolysate
393 PAXR (precipitated arabinoxylans recovery): g AX recovered in the solid precipitated/100 g
394 AX in the prehydrolysate.
395 **Fig. 3.**

396 The HPSEC technique is considered a suitable method to establish the molecular
397 weight distribution (low, medium and high) of AX (Álvarez et al., 2017). Fig. 4 shows
398 the HPSEC chromatographic profile (called "Initial") of the AX precipitated at 1:2 ratio
399 of prehydrolysate:ethanol. As can be seen, the presence of compounds of high-
400 molecular weight was identified. In this way, high concentrations of compounds with a

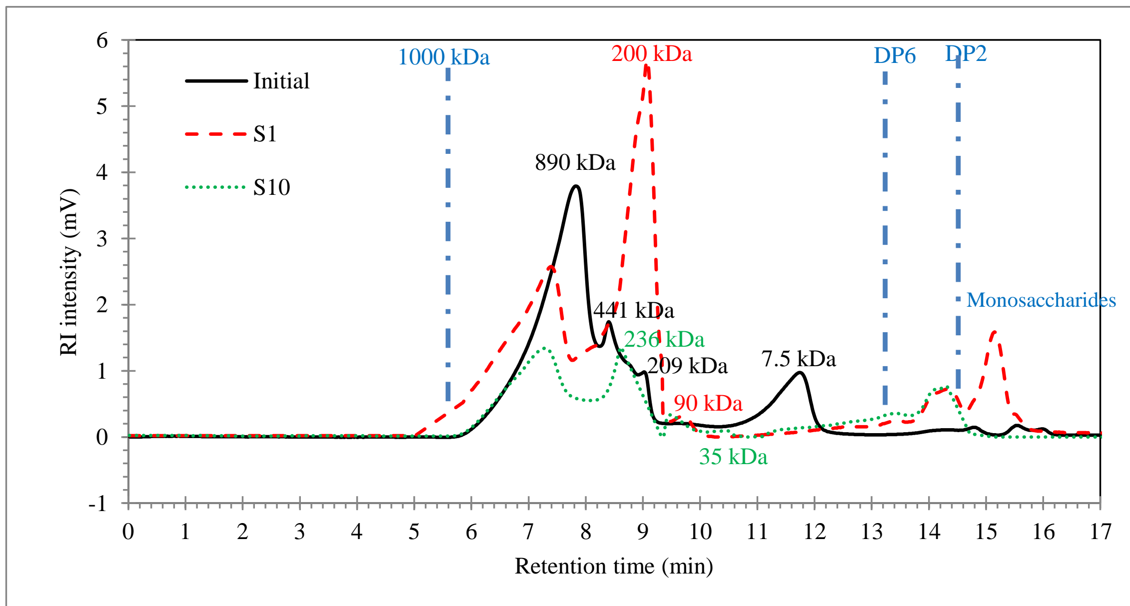
molecular weight of up to approximately 1000 kDa were appreciated, highlighting those found at approximately 890, 441 and 7.5 kDa. However, the presence of oligosaccharides with a polymerization degree from DP6 to DP2 and monosaccharides was not significant. As can be observed, the first eluted compounds were probably the high molecular weight polysaccharides, followed by oligosaccharides (DP2-DP6), monosaccharides and other secondary products (Álvarez et al., 2017).

The presence of polysaccharides (with DP>4) and impurities of high-molecular weight was also observed by Wang and Lu (2013) in the resulting solution of the microwave assisted water pretreatment of wheat bran. According to Chung et al. (2003), besides polysaccharides, these macromolecules might be starch, pectin, protein and tannin originated from BSG. Álvarez et al. (2017) also noticed the presence of high-molecular polysaccharides (up to about 40000 Da) in the prehydrolysate of wheat straw pretreated by steam explosion (200 ºC and 4 min).

3.3. Enzymatic hydrolysis and characterization of the AX suspension

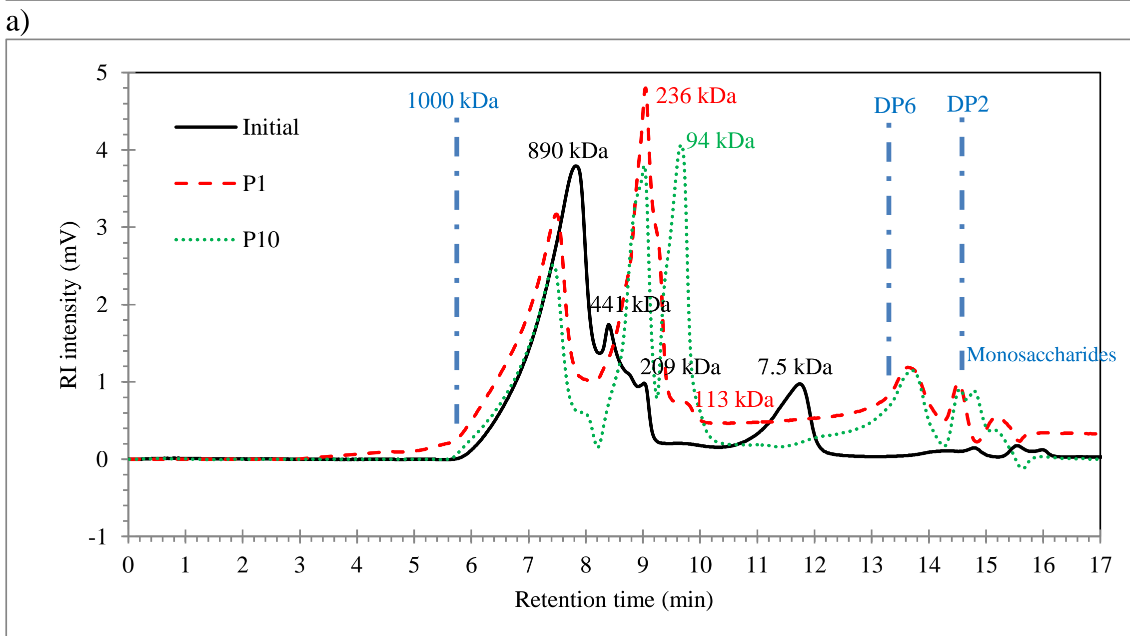
Enzymatic hydrolysis with endo-xylanases is considered an effective process to reduce the DP of AX molecules (Campbell et al., 2019; de Freitas et al., 2019). Oligosaccharides with units from DP2 to DP4 are preferred due to their prebiotic properties (Carvalho et al., 2013). In this context, to break the high-molecular weight AX, an enzymatic hydrolysis was undertaken using two commercial endo-xylanases (Shearzyme 2X and Pentopan Mono BG, identified as S and P xylanases). In addition, two enzyme dosages (1 and 10 XU/mL liquor) were tested. Then, the enzymatic essays performed were: S1, S10, P1 and P10.

424



425 a)

426



427 b)

428 **Fig. 4.**

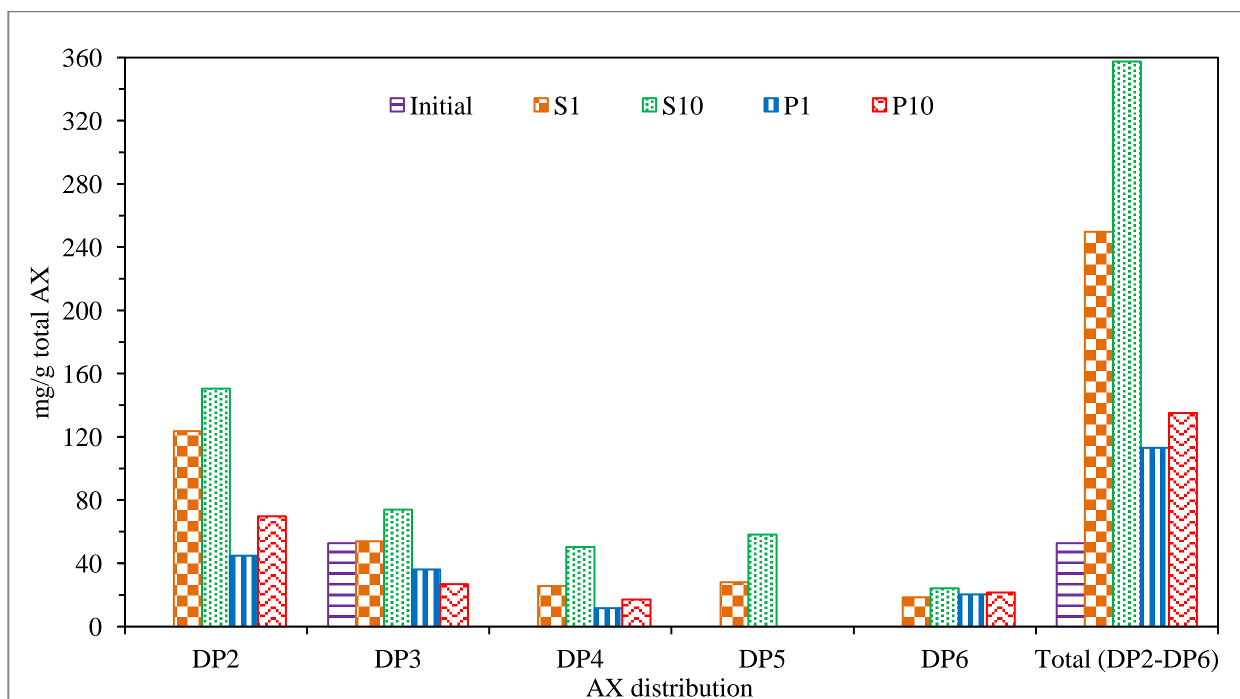
429 The HPSEC technique was applied to establish the molecular weight distribution of
430 compounds after 8 h of enzymatic hydrolysis (Fig. 4a, b). As can be seen, in all essays
431 (S1, S10, P1 and P10) the xylanases action resulted in the rupture of those highest
432 molecular weight polysaccharides, originating compounds of lower molecular weight
433 (of about 200 kDa), oligosaccharides with a polymerization degree from DP6 to DP2
434 and low concentrations of monosaccharides.

435 Fig. 5 displays the concentrations of oligosaccharides with 2-6 units of xylose (DP2-
436 DP6) before and after 8 h enzymatic hydrolysis (S1, S10, P1 and P10), expressed as
437 mg/g total AX. Only DP3 units (52.72 mg/g total AX) were found before enzymatic
438 hydrolysis, this solution containing 5% of DP3 and 95% of xylooligosaccharides with a
439 polymerization degree higher than DP6. However, after 8 h enzymatic hydrolysis, a
440 significant increase of xylooligomers from DP2 to DP6 was observed independently of
441 the type and dose of xylanase used, except when P xylanase was used, when no
442 generation of DP5 units was detected. Campbell et al. (2019) also reported a positive
443 effect of the use of xylanases to reduce the size of AX molecules. Regarding S xylanase,
444 the use of a higher dosage of enzyme increased the amount of xylooligosaccharides with
445 a polymerization degree from DP2 to DP6. When the xylanase dosage was increased
446 from 1 to 10 XU/mL liquor (S1 and S10, respectively), a total increase of 43% in the
447 concentration of oligomers from DP2-DP6 was observed, especially in the DP4 and
448 DP5 units. However, when P xylanase was used, the increase of enzyme dosage from 1
449 to 10 XU/mL liquor led to a slight increase (only 20%) in the total DP2-DP6
450 xylooligomers, only observing a significant increase in concentration for the DP2 and
451 DP4 units (56 and 47%, respectively) (Fig. 5). Comparing S and P xylanases, Fig. 5
452 clearly shows that S xylanase was much more effective in depolymerizing high
453 molecular weight AX, independently of the xylanase dosage employed, achieving 2.2
454 and 2.6-fold higher total concentrations of DP2-DP6 xylooligomers than when P
455 xylanase was used (for xylanase dosages of 1 and 10 XU/mL liquor, respectively).
456 Therefore, it can be concluded that S10 (S xylanase at dosage of 10 XU/mL liquor) was
457 most effective to depolymerize the high-molecular weight polysaccharides, producing
458 4% of monosaccharides, 36% of xylooligomers with DP2-DP6 (mainly DP2 and DP3)
459 and 60% of xylooligosaccharides with DP>6. It is worth noting that no significant

460 increase in the independent and total units from DP2 to DP6 took place after 8 h

461 enzymatic hydrolysis (data not shown), which was also reported by Álvarez et al.

462 (2017).



463

464 **Fig. 5.**

465 Teng et al. (2010) reported that oligomers of xylose with a polymerization degree of

466 DP2 and DP3 were the majority units (>90%) after the enzymatic hydrolysis with

467 xylanases (from *Paecilomyces themophila* J18) of steam exploded corncobs (196 ºC, 5

468 min). The total content of the DP2- DP6 units (286 mg/g total AX) was lower than that

469 found in our work for S10 (358 mg/g total AX, Fig. 5). Similar behavior was also

470 reported by Bhatia et al. (2020), who achieved an increase of up to 6-fold of DP2 units

471 after enzymatic hydrolysis with endo-xylanase (NS22083) of the prehydrolysate of

472 *Miscanthus* pretreated by steam explosion (200 ºC, 15 bar and 10 min). It is worth

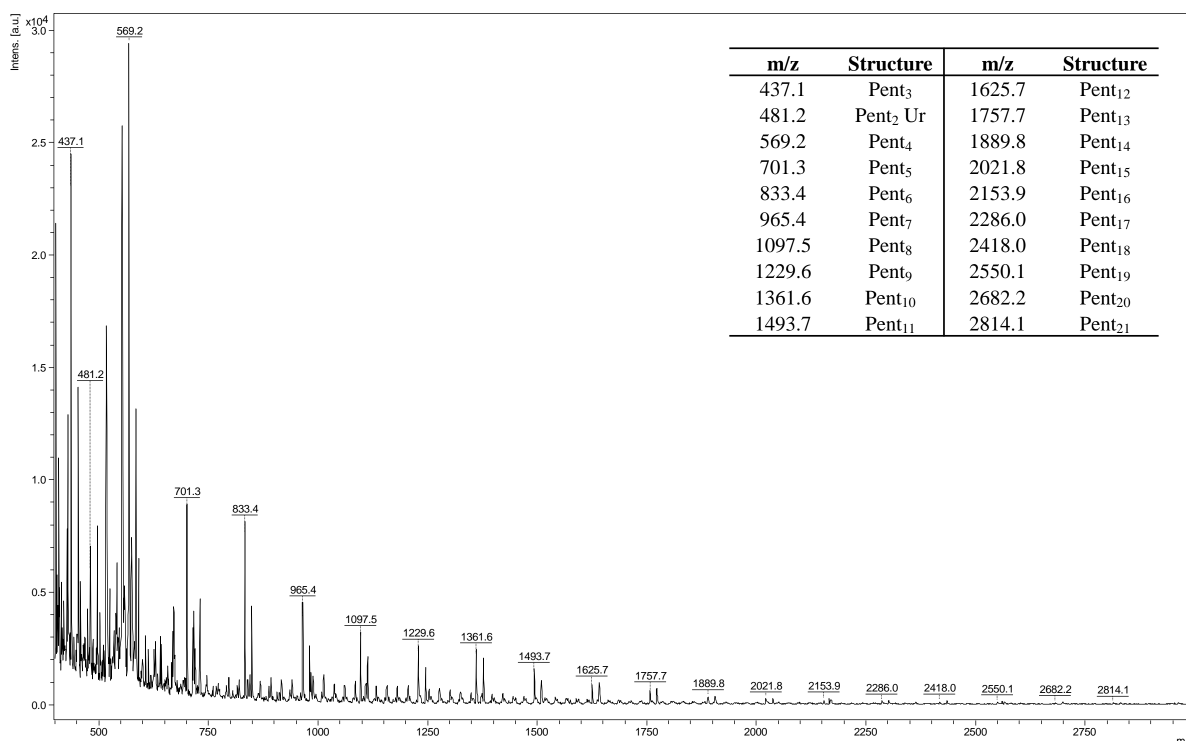
473 mentioning that the units from DP2 to DP4 are more generally preferred than other

474 oligomers in the food industry (Carvalho et al., 2013). Considering the enzyme dosage,

475 Álvarez et al. (2017) achieved higher amounts of DP2 and DP3 units when the xylanase

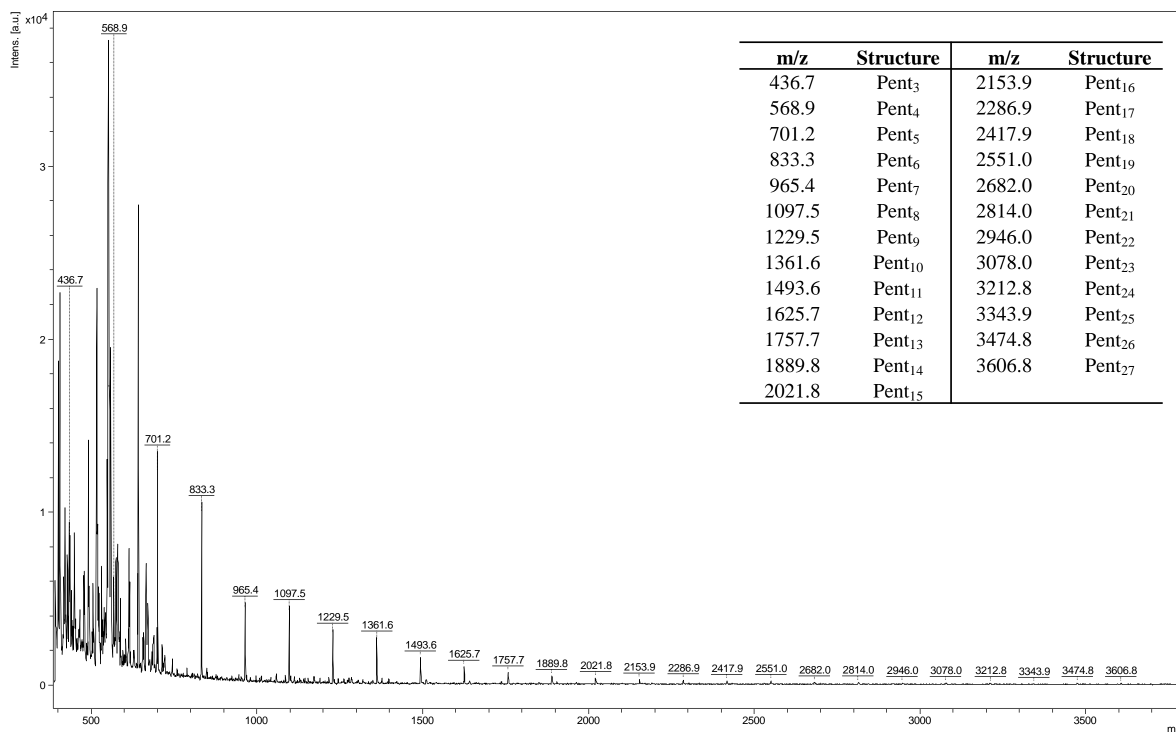
476 dosage (NS50030) was increased from 4.8 to 7.2 XU/mL of prehydrolysate of wheat
477 straw pretreated by steam explosion (200 ºC and 4 min). Wan Azelee et al. (2016) also
478 reported an increase of total AX production when the xylanase dosage increased from
479 50 to 400 XU/ml liquor, decreasing for a higher enzyme dosage.

480 In order to obtain more accurate structural information about the oligomers present in
481 the hydrolysates, a MALDI-TOF-MS analysis was applied (Fig. 6). As can be seen,
482 high-intensity signals were detected at intervals of 132 m/z units in both S10 and P10
483 spectra, corresponding to pentose moieties, according to Coelho et al. (2016). In
484 general, a wide variety of DP units were observed in both cases. Thus, xylose polymers
485 with a polymerization degree from DP3 to DP21 were detected for S10 (Fig. 6a); while
486 units ranging between DP3 and DP27 were found for P10 (Fig. 6b). In addition, some
487 series of ions with more 176 m/z than the ions of the pentose moieties was appreciated
488 in S10 spectrum (Fig. 6a), which were identified as uronic acid residues (Coelho et al.,
489 2016).

490 

m/z	Structure	m/z	Structure
437.1	$Pent_3$	1625.7	$Pent_{12}$
481.2	$Pent_2$ Ur	1757.7	$Pent_{13}$
569.2	$Pent_4$	1889.8	$Pent_{14}$
701.3	$Pent_5$	2021.8	$Pent_{15}$
833.4	$Pent_6$	2153.9	$Pent_{16}$
965.4	$Pent_7$	2286.0	$Pent_{17}$
1097.5	$Pent_8$	2418.0	$Pent_{18}$
1229.6	$Pent_9$	2550.1	$Pent_{19}$
1361.6	$Pent_{10}$	2682.2	$Pent_{20}$
1493.7	$Pent_{11}$	2814.1	$Pent_{21}$

491 a)



m/z	Structure	m/z	Structure
436.7	$Pent_3$	2153.9	$Pent_{16}$
568.9	$Pent_4$	2286.9	$Pent_{17}$
701.2	$Pent_5$	2417.9	$Pent_{18}$
833.3	$Pent_6$	2551.0	$Pent_{19}$
965.4	$Pent_7$	2682.0	$Pent_{20}$
1097.5	$Pent_8$	2814.0	$Pent_{21}$
1229.5	$Pent_9$	2946.0	$Pent_{22}$
1361.6	$Pent_{10}$	3078.0	$Pent_{23}$
1493.6	$Pent_{11}$	3212.8	$Pent_{24}$
1625.7	$Pent_{12}$	3343.9	$Pent_{25}$
1757.7	$Pent_{13}$	3474.8	$Pent_{26}$
1889.8	$Pent_{14}$	3606.8	$Pent_{27}$
2021.8	$Pent_{15}$		

b)

Fig. 6.

3.4. ABE fermentation of enzymatic hydrolysate resulting from BSG pretreated at optimal conditions

The pretreated solid obtained under optimal pretreatment conditions (172 °C and 0.38 M NaOH) enriched in cellulose (as described in section 3.1), was enzymatically hydrolyzed at 10% (w/v) to obtain a rich sugar solution that can be used in ABE fermentation. Thus, an enzymatic hydrolysate rich in sugars (42.4 and 17.6 g/L glucose and xylose, respectively) was achieved, which corresponded to a recuperation of the glucose and xylose contained in the pretreated BSG of 77.8 and 63.0%, respectively.

Later, the resulting enzymatic hydrolysate was fermented with *C. beijerinckii* DSM 6422 for 48 h to analyze its fermentability. As can be seen in Table 3, most sugars contained in the enzymatic hydrolysate were consumed by *C. beijerinckii* (sugar uptake=94.5%) in just 48 h of fermentation, with only 2.8 g/L monosaccharides remaining at the end of the fermentation. In this way, butanol and ABE concentrations

510 of 9.9 and 13.3 g/L, respectively, were obtained, achieving high butanol and ABE yields
511 (0.20 and 0.27 g/g sugars consumed, respectively) (Table 3). Moreover, high butanol
512 and ABE productivities were also observed (0.240 and 0.323 g/L· h, respectively). It is
513 worth mentioning that, due to the low concentration of inhibitor compounds (0.4 g/L
514 acetic acid and 0.70 g/L phenolic compounds), no detoxification step was necessary,
515 since these concentrations were lower than the limits established for *C. beijerinckii*
516 (Bellido et al., 2018). The global butanol and ABE yields were 28 kg butanol/t BSG and
517 37 kg ABE/t BSG. The fermentation results obtained in this work were favorable when
518 compared with those achieved by Hijosa-Valsero et al. (2018) (7.6 g/L butanol and
519 0.186 g butanol/g) in the fermentation with *C. saccharobutylicum* DSM 13864 of the
520 enzymatic hydrolysate resulting from the autohydrolysis of pretreated potato peel (140
521 °C, 56 min). López-Linares et al. (2019) achieved a slightly lower butanol concentration
522 (8.3 g/L), but a higher global butanol yield (46 kg butanol/t BSG) from the enzymatic
523 hydrolysate fermentation with *C. beijerinckii* DSM 6422 of microwave assisted
524 hydrothermal pretreated BSG (192.7 °C, 5.4 min).

525 **Table 3.** ABE fermentation of the hydrolysate resulting from the enzymatic hydrolysis
526 of the pretreated solid obtained under optimal pretreatment conditions. Initial and final
527 monosaccharides concentration (g/L), butanol and ABE concentrations (g/L), and
528 butanol and ABE yields ($Y_{BUT/sugars}$, $Y_{ABE/sugars}$ expressed as g/g sugars consumed) at 48 h
529 fermentation.

Parameter	
Initial monosaccharides (g/L)	51.3 ± 04
Final monosaccharides (g/L)	2.8 ± 0.0
Butanol (g/L)	9.9 ± 0.1
ABE (g/L)	13.3 ± 0.2
$Y_{BUT/sugars}$ (g/g)	0.20
$Y_{ABE/sugars}$ (g/g)	0.27

530

531 ## 4. Conclusions

532 An integrated biorefinery based on emergent microwave assisted alkaline pretreatment
533 was developed for the whole valorization of carbohydrates contained in BSG, leading to
534 bioproducts such as AX and an advanced biofuel such as biobutanol. Under the
535 pretreatment conditions studied (172 °C, 0.38 M NaOH), 133 kg AX/t BSG were
536 recovered by ethanol precipitation. The subsequent enzymatic hydrolysis with endo-
537 xylanases led to compounds with low DP (DP2-DP6), which are molecules with
538 potential prebiotic properties. Alkaline pretreatment led to a global yield of 37 kg ABE/t
539 BSG (28 kg butanol/t BSG) from ABE fermentation of the pretreated solid residue.
540
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546 analysis.
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557 **Figure captions**

558 **Fig. 1.** Scheme of the AX recovery and biobutanol production from BSG by microwave
559 assisted sodium hydroxide pretreatment.

560 **Fig. 2.** Response surface plots representing the interactive effect of temperature and
561 sodium hydroxide concentration on the concentration of arabinoxylans (AX conc.) in
562 the liquid fraction.

563 **Fig. 3.** Solid and AX precipitated (mg/g prehydrolysate) purity (%) of AX in the solid
564 precipitated and precipitated arabinoxylans recovery (PAXR, %) from the arabinoxylans
565 recovery process at different ratios of prehydrolysate:ethanol (1:1, 1:2 and 1:3 v/v).

566 **Fig. 4.** HPSEC chromatographic profile of the AX suspension obtained at 1:2
567 (v/v) prehydrolysate:ethanol ratio, after enzymatic hydrolysis with Shearzyme 2X (S
568 enzyme) (a) and Pentopan Mono BG (P enzyme) (b) endo-xylanases at 1 and 10
569 XU/mL liquor.

570 **Fig. 5.** AX distribution (expressed as mg/g total AX) of initial and the enzymatically
571 hydrolyzed AX suspension obtained at 1:2 (v/v) ratio of prehydrolysate:ethanol. The
572 enzymatic hydrolysis was performed during 8 h with Shearzyme 2X (S enzyme) and
573 Pentopan Mono BG (P enzyme) endo-xylanases, at 1 and 10 XU/mL liquor.

574 **Fig. 6.** MALDI-TOF mass spectrum of the AX suspension obtained at 1:2 (v/v)
575 prehydrolysate:ethanol ratio, after enzymatic hydrolysis with Shearzyme 2X (a) and
576 Pentopan Mono BG (b) endo-xylanases at 10 XU/mL liquor (S10 and P10,
577 respectively).

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