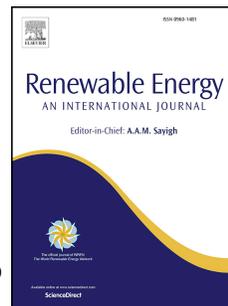


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Comparative autohydrolysis study of two mixtures of forest and marginal land resources for co-production of biofuels and value-added compounds

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1 **Comparative autohydrolysis study of two mixtures of forest and marginal land**  
2 **resources for co-production of biofuels and value-added compounds**

3  
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14 **Abstract**

15 This work was focused on evaluating two mixtures of lignocellulosic feedstock, forest  
16 and marginal land resources, in order to co-produce solid biofuel, oligosaccharides, and  
17 glucose under a biorefinery concept. The selection of renewable bio-mixtures was based  
18 on different criteria, namely, territorial distribution, fire risk during summer months and  
19 total sugar content. The two mixtures were submitted to autohydrolysis pretreatment  
20 under non-isothermal conditions (in the range of 190 °C - 240 °C corresponding to  
21 severity of 3.71 to 4.82). Both mixtures were compared in terms of fractionation  
22 (cellulose and lignin recoveries and hemicellulose solubilization), analyzed for thermal  
23 properties (high heating values) and for enzymatic susceptibility of cellulose. The  
24 highest xylan recoveries (62 and 69%), as xylose and xylooligosaccharides, were  
25 achieved for both mixtures in the liquid phase at 206 °C. Autohydrolysis pretreatment  
26 increased the high heating values of the two mixtures presenting an alternative use of  
27 solid fraction as solid biofuel. Moreover, enzymatic susceptibility of these pretreated  
28 mixtures was also improved from 45 to 90 % of glucose yield by increasing  
29 pretreatment severity. This comparative study of autohydrolysis showed a suitable  
30 process for the valorization of both mixtures within a biorefinery concept.

31

32

33 **Keywords:**

34 Multi-supply lignocellulosic biomass; Autohydrolysis; Enzymatic hydrolysis;

35 Biorefinery; Solid biofuel

36

## 37 1. Introduction

38 In Portugal, the territory is divided in wood and uncultivated land (22 %); forest of pure  
39 and mixed stands of *Pinus pinaster* and *Eucalyptus globulus* (21 %); and farm land  
40 (33 %), mainly composed by olive groves, vineyards and orchards, that generate  
41 significant amount of lignocellulosic biomass (LCB) [1]. Approximately half of the  
42 national territory (40 % to 50 %) consists of poor soils with no potential for profitable  
43 agricultural use. On average, 60 % to 70 % of the total fires take place in forested and  
44 uncultivated areas, resulting in a loss of roughly 800 million Euros annually [2]. So far,  
45 there is no sustainable alternative for the use of this territory and no viable solution for  
46 forest biomass valorization [2]. Social and economic benefits could be achieved from  
47 the utilization of these raw materials in order to develop the so-called bioeconomy,  
48 which would boost the creation of new rural jobs [3].

49 Forest and agricultural residues are the most important sources of lignocellulosic  
50 biomass [4]. LCB are the most abundant renewable resource in the world, generated at  
51 high rate [5] and suitable for production of energy, biofuels, chemicals, paper,  
52 pharmaceuticals and biomaterials [3, 6]. LCB are composed by 50 % to 60 %  
53 carbohydrates, namely, cellulose and hemicellulose and 10 % to 30% of lignin, together  
54 with non-structural components (including ashes, extractives, pectin and proteins) in  
55 lower proportions [7-9]. Nevertheless, the conversion of LCB into chemicals is one of  
56 the main challenges for biomass processing due to their complex three-dimensional  
57 structure, requiring multidisciplinary approaches to achieve their integrated benefit [5].

58 The chemical utilization of LCB can be carried out using two different approaches: (i)  
59 utilization as a whole (for example combustion, gasification or pyrolysis), or (ii) using  
60 methods based on the selective separation of its components (cellulose, hemicellulose,  
61 lignin) [3]. The latter process can be based on multistep processing, starting with

62 separation of easily recovered fractions (extractives and hemicellulose) from the more  
63 resistant ones (cellulose and lignin), which could be further fractioned by means of  
64 more aggressive treatments [5]. Hydrothermal pretreatment has been successfully  
65 applied to LCB. This eco-friendly process, also known as autohydrolysis, requires no  
66 other reagents than water and high temperature, which enables a wide variety of  
67 reactions without the need of a catalyst [10, 11]. Autohydrolysis provides several  
68 advantages, such as: i) high hemicellulose recoveries; ii) no catalyst is necessary; iii) no  
69 equipment corrosion problems are expected; iv) stages of sludge handling and acid  
70 recycling are avoided; v) enzymatic susceptibility of cellulose is improved [9]. The  
71 autohydrolysis reaction solubilizes selectively hemicellulose into oligosaccharides and  
72 promotes lower liberation of compounds derived from lignin and cellulose, as well as  
73 hemicellulose degradation products [12]. The main compounds found in the remaining  
74 solid fraction are cellulose and sulfur-free lignin. Cellulose can be subjected to  
75 enzymatic hydrolysis to produce glucose, an important input for biofuels [13] [12].

76 The use of feedstock mixtures rather than a single raw material can minimize the  
77 problems related to biomass availability, seasonality, price volatility and storage. In this  
78 work, broom (*Cytisus sp.*), carqueja (*Genista tridentate*), mimosa (*Acacia dealbata*),  
79 rockrose (*Cistus ladanifer*), eucalyptus (*Eucalyptus globulus*) and pine (*Pinus pinaster*)  
80 were identified as the most important sources of forest fire cases in Portugal. Since the  
81 security supply for biorefineries and the sustainability of exploration are key factors to  
82 ensure the industrialization of these systems, the aim of this study was to evaluate  
83 feedstock mixtures fractionation to supply a biorefinery throughout the year to produce  
84 bioenergy and value-added compounds. Two feedstock mixtures were selected and  
85 subjected to autohydrolysis treatment in the range of 190 °C – 240 °C, in order to  
86 evaluate and compare the pretreatment effect on fractionation of feedstock mixtures by

87 hemicellulose solubilization. Besides oligosaccharides, two other alternatives were  
88 evaluated for valorization of pretreated feedstock mixtures: solid biofuel production and  
89 enzymatic saccharification of cellulose into glucose.

90

## 91 **2. Materials and Methods**

### 92 **2.1. Raw materials and criteria of feedstocks mixture**

93 Lignocellulosic biomass collecting through the year was divided into four quarters,  
94 considering the biomass from 1<sup>st</sup> and 4<sup>th</sup> quarters (winter months) as mixture 1-4 (M1-4)  
95 and from 2<sup>nd</sup> and 3<sup>rd</sup> quarters (summer months) as mixture 2-3 (M2-3). The M1-4 and  
96 M2-3 were set with different lignocellulosic biomasses from forest ecosystems (A)  
97 (namely, eucalyptus and pine) and from marginal land (B) (namely, broom, carqueja,  
98 mimosa and rockrose). The criteria for the formulation of (A) and (B) were taking into  
99 account their proportion of territorial occupation, based on the National Portuguese  
100 Forest Inventory [14]. These percentages were considered to establish the proportion of  
101 lignocellulosic biomass for M2-3. The collection of biological resources from marginal  
102 land (B) during winter months (1<sup>st</sup> and 4<sup>th</sup> quarters) provide reduction of fire risks in the  
103 summer months (2<sup>nd</sup> and 3<sup>rd</sup> quarters) therefore, a preference factor of 2:1 of biomass  
104 from marginal land (B) to forest ecosystem (A) was considered in order to establish the  
105 M1-4 (Table 1). The percentage of eucalyptus and pine present in M1-4 and M2-3 was  
106 also based on area in the Portuguese territory for these species [15]. For the percentages  
107 of biomass from marginal land, the weight ratio among broom, mimosa, carqueja and  
108 rockrose was calculated as a function of total sugars content [1], since there is no  
109 information available regarding territorial distribution of biological resources from  
110 marginal land.

111 The mixtures M1-4 and M2-3 were prepared, homogenized and then characterized as  
 112 described below. The raw materials resulting from forest management practices,  
 113 namely, broom, carqueja, mimosa, rockrose, eucalyptus and pine, were collected in the  
 114 Center Region of Portugal, from a location with the same type of soil. The  
 115 lignocellulosic biomasses included branches and twigs with barks and leaves. The raw  
 116 materials were air-dried until moisture lower 5 %, milled and sieved between 0.25 to  
 117 0.40 mm using a vibratory sieve shaker (40 and 60 mesh), and stored in dry conditions  
 118 to avoid the self-biodegradation of the material. After that, the samples were  
 119 homogenized in a single lot to avoid compositional differences among aliquots and  
 120 were stored in polypropylene bags at room temperature.

121 **Table 1-** Proportion of the two mixtures from lignocellulosic biomass established by  
 122 quarter

Type Lignocellulosic resources		Mixtures proportion (%)	
		M1-4	M2-3
(A) forest ecosystems	Pine ( <i>Pinus pinaster</i> )	16.6	24.6
	Eucalyptus ( <i>Eucalyptus globulus</i> )	18.8	27.9
	<b>Total</b>	<b>35.4</b>	<b>52.5</b>
(B) biological resources from marginal land	broom ( <i>Cytisus sp.</i> )	18.8	13.8
	mimosa ( <i>Acacia dealbata</i> )	17.0	12.5
	carqueja ( <i>Genista tridentata</i> )	14.6	10.7
	rockrose ( <i>Cistus ladanifer</i> )	14.2	10.4
	<b>Total</b>	<b>64.6</b>	<b>47.5</b>

123

124

## 125 **2.2. Analysis of raw material**

126 Analytical assays were performed according to the procedures provided by National  
127 Renewable Energy Laboratory (NREL), appropriated for bioenergy production [15]:  
128 moisture (NREL/TP-510-42621), ash content (NREL/TP-510-42622), extractives  
129 (NREL/TP-510-42621) and quantitative acid hydrolysis with 72% w/w sulphuric acid  
130 (NREL/TP-510-42618). The hydrolysates from acid hydrolysis were analyzed by high  
131 performance liquid chromatography (HPLC) for sugars (glucose, xylose and arabinose)  
132 and acetic acid using the column Aminex HPX-87H (conditions: refractive index  
133 detector; flow rate of 0.6 mL/min at 60 °C; 0.005 M H<sub>2</sub>SO<sub>4</sub> as mobile phase) and HPX-  
134 87P column for mannose and galactose analysis (conditions: refractive index detector;  
135 flow rate of 0.6 mL/min at 85 °C; H<sub>2</sub>O as mobile phase). The concentrations of sugars  
136 and acetic acid were employed to calculate the contents of cellulose and hemicellulose.  
137 The Klason lignin content was gravimetrically measured from the insoluble solid  
138 residue obtained after the quantitative acid hydrolysis. Analyses were carried out in  
139 triplicate. The analytical methods used in this work and the scheme of the whole process  
140 are shown in Figure 1

## 141 **2.3. Non-isothermal autohydrolysis pretreatment of the lignocellulosic mixtures** 142 **(M1-4 and M2-3): solid and liquid phases composition**

143 The autohydrolysis pretreatment was performed in a 2 L stainless steel reactor (Parr  
144 Instruments Company, Moline, Illinois, USA) equipped with a Parr PID temperature  
145 controller (model 4848). Each sample (M1-4 or M2-3) was mixed at liquid to solid ratio  
146 (LSR) of 8 kg of water/kg of oven-dry raw material. In autohydrolysis experiments, the  
147 reaction media was stirred at 150 rpm and heated by an external jacket, following the  
148 standard heating temperature-time profile to reach the desired maximum temperature,

149 and the reactor was rapidly cooled-down through water recirculation by an internal loop  
150 (Figure 2).

151 For each mixture, several non-isothermal conditions were tested, reaching final  
152 temperatures ( $T_{MAX}$ ) of 190, 196, 206, 216, 226 and 240 °C (pressure from 13 to 34  
153 bar). The autohydrolysis experiments were carried out by duplicate. Once the target  
154 temperature was reached, the media were immediately cooled and filtered.

155 The intensity of autohydrolysis pretreatments can be expressed in terms of “severity”  
156 ( $S_0$ ), defined as the logarithm of  $R_0$  [16], which was calculated using the expression,  
157 Equation (1): **Figure 1-** Flow chart of whole process and analytical methods used in this  
158 work.(1)

$$\begin{aligned}
 S_0 &= \log R_0 = \log [ R_{0 \text{ HEATING}} + R_{0 \text{ COOLING}} ] \\
 &= \log \left[ \int_0^{t_{MAX}} \exp \left( \frac{T(t) - T_{REF}}{\omega} \right) dt \right] \\
 &\quad + \log \left[ \int_0^{t_F} \exp \left( \frac{T'(t) - T_{REF}}{\omega} \right) dt \right]
 \end{aligned}$$

164 According to this equation,  $S_0$  includes the combined effects of temperature and  
165 reaction time along the periods of heating and cooling. In Eq. (1),  $t_{MAX}$  (min) is the time  
166 needed to achieve  $T_{MAX}$  (K),  $t_F$  (min) is the time needed for the whole heating-cooling  
167 period, whereas  $T(t)$  and  $T'(t)$  represent the temperature profiles in heating and cooling  
168 (Figure 2), respectively. Calculations were made assuming the values reported in  
169 literature for  $\omega$  and  $T_{REF}$  (14.75 K and 373.15 K, respectively). The range of studied  
170 temperatures was 190 °C to 240 °C corresponding to severities ( $S_0$ ) of 3.71 and 4.82,  
171 respectively.

172 Operational conditions were evaluated to maximize the concentration of hemicellulose  
173 derived compounds in liquid fraction and to improve enzymatic susceptibility of the  
174 glucan present in solid fraction.

175 The solid fraction was washed with distilled water and used to measure the solid yield  
176 of the autohydrolysis stage ( $SY$ ,  $\text{kg}_{\text{autohydrolyzed mixture}}/100 \text{ kg}_{\text{raw material}}$ , oven-dry basis)  
177 and analyzed for chemical composition as described in Section 2.2. An aliquot of  
178 autohydrolysis liquid phase was filtered through  $0.2 \mu\text{m}$  membranes and used for direct  
179 HPLC determination of glucose, xylose, arabinose, acetic acid, hydroxymethyl (HMF)  
180 and furfural (F), using the same method specified above. A second aliquot was  
181 subjected to quantitative acid posthydrolysis (4 % w/w sulphuric acid at  $121 \text{ }^\circ\text{C}$  for 20  
182 min), filtered through  $0.2 \mu\text{m}$  membranes and analyzed in HPLC for oligosaccharides  
183 quantification.

#### 184 **2.4. Enzymatic hydrolysis of solid fraction from autohydrolysis pretreatment**

185 Enzymatic hydrolysis (EH) of autohydrolyzed mixtures were carried out at  $50 \text{ }^\circ\text{C}$  and  
186 pH 4.85 (0.05 N sodium citrate buffer) in 100 mL Erlenmeyer flasks with 50 mL of  
187 volume in orbital agitation (150 rpm) using Cellic CTec2 (Novozymes, Bagsvaerd,  
188 Denmark). The enzyme activity was 120 FPU/mL. (measured as described by Ghose  
189 [17]). The conditions employed were 5 % of oven-dry autohydrolyzed mixtures,  
190 enzyme to substrate ratio, denoted as  $ESR = 20 \text{ FPU/g}_{\text{autohydrolyzed mixture}}$  on dry basis.  
191 The reaction time of enzymatic hydrolysis ranged from 0 h to 72 h. At selected times,  
192 samples were withdrawn from the media, centrifuged, filtered and analyzed by HPLC  
193 for glucose and cellobiose. The results achieved in the EH were expressed in terms of  
194 glucose yield ( $Y_G$ ) (%), calculated using the following Equation (2):

195 (2)

$$\% Y_G = \frac{[Glucose] + 1.053 [Cellobiose]}{1.111 f[Biomass]} 100$$

196

197 Where [Glucose] is the glucose concentration (g/L), [Cellobiose] is the cellobiose  
198 concentration (g/L), [Biomass] is the dry biomass (or LCB) concentration (g/L),  $f$  is  
199 cellulose fraction in dry biomass (g/g), the multiplication factor, 1.053, converts  
200 cellobiose to equivalent glucose. In all experiments, cellobiose was not detected.

## 201 **2.5. Determination of higher heating values of autohydrolyzed mixture**

202 Samples of dried biomass were analyzed for Higher Heating Values (HHVs) using an  
203 automatic adiabatic bomb calorimeter (Parr calorimeter Type 6200), in accordance with  
204 Jessup et al. [18]. The interior surface of the bomb was washed with distilled water and  
205 collected in a beaker. The bomb washings were titrated with a standard sodium  
206 carbonate solution (0.0709 N).

## 207 **2.6. Statistical analysis**

208 Linear Discriminant Analysis (LDA) was computed using STATGRAPHICS Centurion  
209 XVII, with a significance level of 5 %. The sources of variation for the raw material  
210 composition were cellulose (as glucan), xylan, arabinan, acetyl groups, mannan and  
211 galactan (as hemicellulose), Klason lignin, ashes and extractives for M1-4 and M2-3.

212

## 213 **3. Results and Discussion**

### 214 **3.1 Raw material mixture criteria**

215 Several lignocellulosic biomasses have been individually studied to be used as raw  
216 material for biorefineries, nonetheless, the availability, seasonality, variability, price  
217 volatility and storage of biomass supply may be the major constraints on the use of  
218 these raw materials [12, 19]. In this work, the use of different biomass sources may

219 overcome these problems. Thus, the analysis of availability, security supply and  
220 seasonality (based on fire prevention) of lignocellulosic biomasses in Portugal were  
221 considered for the mixture definition.

222 The total territorial area generating residues [14] within the work focus corresponds to  
223 52.5% of forest ecosystems (A) and 47.5% of biological resources from marginal land  
224 (B). Since eucalyptus and pine are the main ecosystems in Portugal that generate  
225 residues with high potential for the biorefineries, these two species were selected to  
226 represent (A) [20]. Whereas their distribution is according to territorial area [14], where  
227 725 thousands (46.9%) of hectares correspond to pine and 820 thousands (53.2%) of  
228 hectares to eucalyptus. Regarding (B), the selection criteria was based on total sugars  
229 content of broom (66.7%), carqueja (51.7%), mimosa (60.4%) and rockrose (50.2%)  
230 [1]. Based on the different criteria, two different mixtures, M1-4 and M2-3, were  
231 established taking into account the higher fire risk in Portugal during the summer  
232 months (2<sup>nd</sup> and 3<sup>rd</sup> quarters) and the importance of collecting biological resources from  
233 marginal land during winter months (1<sup>st</sup> and 4<sup>th</sup> quarters). In general, 60 to 70% of forest  
234 fires occurs in woods and uncultivated areas [1]. Therefore, Table 1 shows the final  
235 proportions of biomasses assembled for M1-4 and M2-3.

236 The consideration of feedstock mixtures have been previously studied only in few  
237 works [12, 21, 22]. In some of these cases, the criteria of mixture were based on the  
238 importance of these raw materials for the region, such as mixture of eucalyptus, wheat  
239 straw and olive tree pruning in Southern Europe, prepared in different combinations to  
240 be tested [12]. In other cases, the mixture was prepared in equivalent amounts (clover  
241 and ryegrass) [23] due to its importance in many agroecosystems [23], as well three  
242 relative proportions of wheat straw and clover-grass were studied [22]. Moreover, the  
243 consideration of more than one species of crops stands and/or forest feedstock

244 supplemented with energy crops (such as switchgrass) to increase biomass yield have  
245 been previously studied by Jensen et al., where five species were mixed (50/50 wt.%) in  
246 10 possible combinations [21].

247

### 248 **3.2. Chemical characterization of M1-4 and M2-3 mixtures**

249 Chemical characterization (Table 2) of the two mixtures proposed was carried out  
250 revealing a very similar composition, although the content of each fraction slightly  
251 varied according to the contribution of the predominant feedstock (described in Table  
252 1).

253 Glucan was the polysaccharide found in higher amount and similar concentrations were  
254 found in both mixtures. Among the hemicellulose components, the xylan was found in  
255 the highest amount in the two mixtures, reaching 16.58 g/100g<sub>raw material</sub> of M1-4 and  
256 17.48 g/100g<sub>raw material</sub> of M2-3.

257 **Table 2-** Chemical composition of feedstock mixtures (M1-4 and M2-3) (expressed in  
258 g/100g of raw material in oven-dry basis  $\pm$  standard deviation on three replicate  
259 determinations).

Components	Feedstock mixtures	
	M1-4	M2-3
Cellulose (as Glucan)	34.17 ± 1.14	34.63 ± 0.18
Hemicellulose		
Xylan	16.58 ± 0.62	17.48 ± 0.45
Arabinan	1.36 ± 0.21	1.27 ± 0.03
Acetyl groups	2.13 ± 0.01	1.95 ± 0.29
Mannan	3.19 ± 0.03	3.31 ± 0.01
Galactan	1.43 ± 0.02	1.26 ± 0.03
Klason lignin	1	31.76 ± 0.00
Ash	1.29 ± 0.08	1.19 ± 0.03
Extractives	10.51 ± 0.10	9.23 ± 0.31

260

261 Arabinan and acetyl groups were identified, although in lower proportions in both  
262 mixtures, approximately 2 g/100g<sub>raw material</sub>. Mannan showed concentration around 3  
263 g/100g<sub>raw material</sub> for both mixtures and galactan was detected in low concentrations  
264 approximately 1 g/100g<sub>raw material</sub>. Klason lignin was the second highest fraction in the  
265 mixtures, namely 30 % for M1-4 and 32 % for M2-3. Ashes were quantified and  
266 correspond to about 1 % for both mixtures. Extractives correspond approximately to 10  
267 % in both mixtures. As seen in Table 2, there were no significant differences regarding  
268 chemical composition between M1-4 and M2-3 ( $p$ -value > 0.05).

269 Nevertheless, the study of mixture of different species is still scarce. Previous reports  
270 already studied the species that compose M1-4 and M2-3 individually, namely, *Acacia*  
271 *dealbata* [24], *Cytisus sp.* [25], *Pinus pinaster* [5] and *Eucalyptus globulus* [26]. In  
272 these studies, the cellulose content (as glucan) was higher than 40 %, while M1-4 and

273 M2-3 presented lower content of cellulose, around 35 %. This fact can be explained due  
274 to extractives content, as the mixtures comprised branches and twigs with bark and  
275 leaves [27] , since the aim of this work was the integral valorization of these  
276 lignocellulosic resources.

277 Despite the similar composition of M1-4 and M2-3, the outcome of pretreatment could  
278 be different due to their diverse origin (hardwood, softwood and bush), making it  
279 necessary to analyze the pretreatment effect on both mixtures.

280

### 281 **3.3. Effect of autohydrolysis pretreatment on fractionation of M1-4 and M2-3** 282 **mixtures**

283 The conditions of pretreatment (190 °C to 240 °C) were chosen based on reported data  
284 by Romaní et al. [9] and Silva-Fernandes et al. [12]. For integral valorization of  
285 biomass, all fractions should be considered [3]. Thus, in this work, fractionation of two  
286 mixtures was evaluated in order to recover the hemicellulose as oligosaccharides and to  
287 improve the enzymatic saccharification of cellulose and/or use the solid fraction as solid  
288 biofuel.

#### 289 *3.3.1 Solid phase composition after autohydrolysis pretreatment*

290 Chemical composition of solid phase after autohydrolysis pretreatment is shown in  
291 Table 3. The solid yield (SY) decreased with severity increase and varied from 62.37 -  
292 75.25 g /100 g <sub>raw material</sub> for M1-4 and 62.33 - 76.70 g/100 g <sub>raw material</sub> for M2-3, which is  
293 in agreement with previous works under similar conditions for other hardwoods [11,  
294 26].

295 The glucan content varied in the range of 40.96 – 47.00 g<sub>glucan</sub>/100 g<sub>autohydrolyzed mixture</sub>  
296 (on dry basis) for pretreated M1-4 and 41.07 - 48.47 g<sub>glucan</sub>/100 g<sub>autohydrolyzed mixture</sub> (on  
297 dry basis) for pretreated M2-3. Thus, the percentage of glucan that remained in the solid  
298 fraction was very similar after pretreatment for both mixtures, presenting an average of  
299 glucan recovery about 84.86 % and 88.68 % (expressed as g of glucan per 100 g of  
300 autohydrolyzed mixture) for M1-4 and M2-3, respectively, which reveals the selectivity  
301 of this pretreatment.

302 In addition, the content of lignin after pretreatment varied in the range of 35.76 - 47.82  
303 and 37.04 - 44.56 g<sub>lignin</sub>/ 100 g<sub>autohydrolyzed mixture</sub> of M1-4 and M2-3, respectively. The  
304 average recovery was high for the two mixtures, 96.36% and 90.36% (expressed as g of  
305 lignin per 100 g of autohydrolyzed mixture) for M1-4 and M2-3, respectively.

306

307

308 **Table 3-** Solid yield and chemical composition of pretreated solids and liquid fraction of mixtures M1-4 and M2-3 after autohydrolysis  
 309 pretreatment

Yield and Components	Temperature (°C) or S <sub>0</sub>											
	190 or 3.71		196 or 3.93		206 or 4.15		216 or 4.38		226 or 4.60		240 or 4.82	
	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3	M1-4	M2-3
Solid yield	75.25 ± 0.37	76.70 ± 1.31	73.93 ± 2.54	75.8 ± 0.33	67.61 ± 0.32	71.24 ± 0.59	62.15 ± 3.7	65.04 ± 2.22	62.43 ± 4.11	66.78 ± 2.09	62.37 ± 3.4	62.33 ± 2.09
<i>Solid phase composition (g/100 g autohydrolyzed mixture on oven-dry basis)</i>												
Glucan	40.96 ± 0.78	41.07 ± 1.80	41.96 ± 2.20	40.59 ± 1.83	42.12 ± 0.46	41.15 ± 0.49	47.0 ± 1.14	46.69 ± 1.64	44.09 ± 0.09	48.47 ± 0.34	43.13 ± 0.40	48.03 ± 0.06
Xylan	6.97 ± 0.93	6.64 ± 1.01	4.1 ± 0.13	2.59 ± 0.25	1.26 ± 0.16	2.30 ± 0.13	1.52 ± 0.01	2.72 ± 0.16	-	0.72 ± 0.10	-	-
Klason lignin	35.76 ± 2.63	37.04 ± 1.27	40.72 ± 1.67	39.15 ± 2.27	47.44 ± 0.54	43.17 ± 1.06	44.7 ± 0.48	40.75 ± 0.00	44.09 ± 0.96	43.53 ± 0.45	47.82 ± 0.02	44.56 ± 0.09
<i>Liquid phase composition (g/L)</i>												
GOS	2.36 ± 0.16	1.35 ± 0.16	0.47 ± 0.14	1.51 ± 0.15	-	1.72 ± 0.04	-	1.62 ± 0.11	0.08 ± 0.02	0.87 ± 0.03	0.89 ± 0.01	0.8 ± 0.01
XOS	10.82 ± 0.52	10.51 ± 0.06	10.15 ± 0.29	13.59 ± 0.25	9.35 ± 0.09	14.33 ± 0.19	5.68 ± 0.15	10.37 ± 0.69	0.23 ± 0.03	0.95 ± 0.01	0.33 ± 0.00	0.49 ± 0.01
ArOS	2.92 ± 0.12	0.92 ± 0.05	-	0.09 ± 0.01	-	-	-	-	-	-	-	-
AcGOS	4.12 ± 0.12	2.09 ± 0.00	2.81 ± 0.03	2.31 ± 0.33	3.14 ± 0.14	2.17 ± 0.08	3.72 ± 0.01	1.54 ± 0.03	3.7 ± 0.11	0.52 ± 0.03	-	-
Glucose	1.18 ± 0.01	0.98 ± 0.02	2.41 ± 0.02	1.13 ± 0.02	3.06 ± 0.00	1.07 ± 0.00	2.87 ± 0.00	0.97 ± 0.02	2.11 ± 0.01	1.23 ± 0.00	0.63 ± 0.23	0.6 ± 0.00
Xylose	0.51 ± 0.03	0.50 ± 0.01	2.98 ± 0.02	1.63 ± 0.04	4.72 ± 0.01	2.11 ± 0.02	7.17 ± 0.00	3.45 ± 0.10	7.94 ± 0.01	2.06 ± 0.00	0.28 ± 0.09	0.41 ± 0.00
Arabinose	0.66 ± 0.02	0.93 ± 0.01	2.83 ± 0.01	1.59 ± 0.00	4.09 ± 0.00	1.76 ± 0.01	2.22 ± 0.00	1.27 ± 0.03	1.08 ± 0.00	0.10 ± 0.00	0.12 ± 0.02	0.07 ± 0.00
Acetic acid	-	-	0.17 ± 0.00	0.85 ± 0.05	0.36 ± 0.02	1.15 ± 0.01	0.57 ± 0.01	2.15 ± 0.01	0.93 ± 0.01	4.68 ± 0.01	4.85 ± 0.66	4.42 ± 0.01
HMF	0.06 ± 0.01	0.01 ± 0.00	0.08 ± 0.01	0.11 ± 0.05	0.19 ± 0.07	0.14 ± 0.05	0.32 ± 0.01	0.17 ± 0.03	0.58 ± 0.02	0.21 ± 0.08	1.13 ± 0.05	1.12 ± 0.05
Furfural	0.05 ± 0.31	0.05	0.06 ± 0.02	0.19 ± 0.06	0.30 ± 0.03	0.21 ± 0.08	0.81 ± 0.07	0.31 ± 0.07	1.77 ± 0.06	1.09 ± 0.06	2.35 ± 0.07	2.91 ± 0.04

310

311 \* GOS: glucooligosaccharides; XOS: xylooligosaccharides; ArOS: arabinooligosaccharides; AcGOS: acetyl groups; HMF: hydroxymethylfurfural

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312 However, lignin content followed a typical pattern for both mixtures, up to a  
313 temperature of 206 °C the remaining lignin in the solid fraction increased, from this  
314 temperature forward, lignin content decreased and it increased again under the most  
315 severe condition (240 °C). This behavior was also studied by Moniz et al. with  
316 autohydrolyzed rice straw where until 210 °C the remaining lignin was close to 100 %  
317 of the initial amount, from 210 °C onwards showed a decreased around 30 % and it  
318 increased again for the most severe conditions [28]. The lignin increase is common of  
319 autohydrolysis pretreatment due to condensation reactions between lignin, sugars and  
320 degradation products (HMF and F) leading to the formation of insoluble compounds  
321 that are quantified as Klason lignin [9, 29].

322 Under most severe conditions ( $S_0=4.15$ ) glucan and lignin represent more than 84% of  
323 the solid fraction, and the combined amounts of these fractions matched the one  
324 contained in the raw material. These results are comparable with the results obtained by  
325 Silva-Fernandes et al. in which at the same conditions glucan and lignin contain 85% of  
326 the solid fraction [12].

327 The hemicellulose in the pretreated mixtures, namely, xylan showed a steadily decrease  
328 with the severity of pretreatment and it was the most solubilized fraction, since it was  
329 totally solubilized for both mixtures at temperatures higher than 226 °C. The same was  
330 reported by Silva-Fernandes et al. in which under most severe conditions 93-95% of  
331 xylan was solubilized in liquid phase [12]. Patel et al. studied a different pretreatment  
332 (dilute acid pretreatment) to solubilize the hemicellulose fraction in which revealed that  
333 almost, all the hemicellulose content was hydrolyzed, obtained only 0.4 % in the solid  
334 fraction. However, this pretreatment is not ecofriendly, since it requires an additional  
335 detoxification step, increasing the process cost [13].

336 The data described above indicate that autohydrolysis pretreatment in an appropriate  
337 process for the selective fractionation of both mixtures in which showed high  
338 hemicellulose solubilization, directly proportional to autohydrolysis severity, while  
339 cellulose and lignin were usually retained in the solid fraction.

### 340 *3.3.1 Composition of liquid phase resulting from autohydrolysis pretreatment*

341 Autohydrolysis process allows substantial fractionation of components, namely  
342 oligosaccharides, monosaccharides, acetyl groups from hemicellulose, and degradation  
343 products of released sugars as furfural (F) and hydroxymethylfurfural (HMF).

344 The liquid phase composition of the two mixtures (M1-4 and M2-3) is presented in  
345 Table 3, in which the products recovered were represented in three groups:  
346 oligosaccharides (OS), including glucooligosaccharides (GOS), xylooligosaccharides  
347 (XOS), arabinooligosaccharides (ArOS) and acetyl groups (AcGOS); monosaccharides,  
348 as well as glucose, xylose and arabinose; and other by-products such as organic acids  
349 (acetic acid) and furans (HMF and F).

350 Based on the previous reports [9], the concentrations (in g/L) of the liquid phase derived  
351 principally from hemicellulose fractions. Hence, the main compounds were XOS and  
352 xylose. The maximal XOS concentrations (10.8 g/L and 14.3 g/L) were obtained at 190  
353 °C and 206 °C for M1-4 and M2-3, respectively, representing 47.7 % and 58.1 % of the  
354 total compounds presented in the liquid phase. Therefore, at these conditions of  
355 autohydrolysis pretreatment, 47.4 % and 59.8 % of xylan solubilization was recovered  
356 as XOS for M1-4 and M2-3, respectively. These results can be compared with reported  
357 data in literature using single biomass as fast-growing Paulownia hardwood in which 60  
358 % of the identified compounds in the liquid phase were XOS, achieved at maximal  
359 concentration, at  $S_0$  of 3.99 [11], and using a mixture of biomasses (eucalyptus, wheat

360 straw and olive pruning) in which 63-68 % of xylan was recovered as  
361 xylooligosaccharides [12]. In addition, higher xylose concentration was obtained from  
362 the hydrothermal treatment of mixture of lignocellulosic materials (Bermuda grass,  
363 Jasmine hedges and Date palm fronds) at 200 °C than individual treatment of  
364 lignocellulosic biomasses (Bermuda grass or Date palm fronds) [13]. Nevertheless,  
365 direct comparison with the literature is not straightforward since composition and nature  
366 of biomass sources are not same.

367 At more severe conditions  $S_0 > 4.15$  the concentration of XOS decreased until reached  
368 0.33 g/L and 0.49 g/L for M1-4 and M2-3, respectively. XOS started to degrade into  
369 xylose, in which M1-4 achieved 7.9 g/L of maximal xylose concentration at 226 °C  
370 ( $S_0=4.60$ ) and M2-3 obtained 3.5 g/L at  $S_0= 4.38$ .

371 The highest xylan solubilization as a sum of xylose and XOS (62.2 % and 68.6 % for  
372 M1-4 and M2-3, respectively) was obtained at  $T_{MAX} = 206$  °C for both mixtures. This  
373 result is consistent with Romaní et al. [9] in which, at mild conditions ( $T_{MAX}=210$  °C)  
374 76 % of xylan can be recovered as xylose and XOS.

375 Consequently, the highest furfural concentration was 2.5 g/L for M1-4 and 2.9 g/L at  
376  $S_0= 4.16$ . The highest HMF concentration was also found at the same severity, in which  
377 1.1 g/L was obtained for both mixtures. Acetic acid raised the maximum at  $S_0 > 3.94$  of  
378 4.7 g/L and 4.9 g/L for M1-4 and M2-3, respectively. The harsher conditions of  
379 pretreatment led to an increase of inhibitor compounds, as F, HMF, and acetic acid. The  
380 concentration of F is higher than HMF, because the first is attributed to the degradation  
381 of xylose while HMF is obtained through C6 degradation, namely glucose.

382 As mentioned before, glucose was also present in low amounts varying between 0.6 to  
383 3.1 g/L for M1-4 and 0.6 to 1.2 g/L for M2-3, which represented on average 3.6 % of

384 glucan solubilization for M1-4 and 1.7 % for M2-3. The results obtained in this work  
385 are in agreement with reported data using *Paulownia tomentosa* wood in which <4 % of  
386 glucan was recovered in liquid phase [11]. As seen in Table 3, M1-4 and M2-3 showed  
387 differences in XOS and xylose concentration. This fact was probably influenced by the  
388 intrinsic features of lignocellulosic biomass, since M1-4 is composed by a higher  
389 percentage of residues from bush (as broom, mimosa, carqueja and rockrose) than M2-  
390 3. Besides oligosaccharides, sugar degradation products, acetic acid, extractives and  
391 phenolic compounds are also solubilized to liquid phase (or autohydrolysis liquor)  
392 during hydrothermal treatment [30]. The presence of these non-saccharide compounds  
393 in the hydrolysate decreased the purity of xylooligosaccharides which should be  
394 removed by physical and/or chemical means [31]. The difference observed between the  
395 two lignocellulosic mixtures could be related to a higher percentage of extractives in  
396 M1-4 than M2-3. In fact, the solubilized fraction (calculated as 100-SY) during the  
397 autohydrolysis treatment was higher in M1-4 than M2-3 (Table 3) showing differences  
398 in their fractionation. The main fractions recovered in the liquid phase, xylose and XOS,  
399 can be used for value-added compounds production as xylitol, lactic acid and ethanol  
400 obtained by fermentation and/or directly as prebiotic [32-34].

401 As previously reported, at  $T_{MAX} = 206$  °C, high percentages of hemicellulose were  
402 removed but also primary degradation products (F and HMF) were kept at relatively  
403 low levels, which could be achieved by applying pretreatment conditions of moderate  
404 severity. Although pretreatment improves enzymatic access to cellulose for further  
405 fermentation, it generates byproducts decomposition which may affect negatively  
406 fermentation [35].

407

#### 408 **3.4. Energy production of pretreated M1-4 and M2-3 mixtures**

409 HHVs of pretreated mixtures were analyzed in order to evaluate the influence of  
410 pretreatment and compare their behavior as solid fuel. These results are displayed in  
411 Figure 3. The untreated mixtures were analyzed and showed HHVs of 17.23 MJ/kg and  
412 17.26 MJ/kg for M1-4 and M2-3, respectively. HHV of pretreated samples increased  
413 with severity, achieving maximal values of 20.4 MJ/kg and 20.5 MJ/kg, for M1-4 and  
414 M2-3, respectively. The results obtained in this work are in agreement with reported  
415 data using softwood chips in which the HHV of the original wood was 17.9 MJ/kg and  
416 with temperature increase (autohydrolysis pretreatment) reached 20.5 MJ/kg [36].  
417 Leaching processes with water and acetic acid were also used to increase the HHVs of  
418 six different biomasses (fast growing timber species and oil pal biomass), achieving  
419 values in the range of 16.52-18.47 MJ/kg [37].

420 This behavior is related to the increase of lignin content in the samples (Table 3) as a  
421 consequence of temperature rise, since lignin presents higher calorific value (20.4  
422 MJ/kg) than cellulose (16.5 MJ/kg) and hemicellulose (13.9 MJ/kg) [36, 38]. The HHVs  
423 are higher for raw materials as hardwoods and softwoods than for non-wood biomass  
424 being linearly related with lignin content [39].

425 The HHV obtained in this work showed suitability of these mixtures as solid biofuels  
426 when compared with other biomasses [40]. Nevertheless the use of these mixtures as  
427 solid biofuel for energy content in combustion process or the alternative use as glucose  
428 source, for liquid biofuels production should be carefully analyzed and evaluated, in  
429 order to the overall net benefit [36].

430

#### 431 **3.5. Enzymatic saccharification of pretreated M1-4 and M2-3 mixtures**

432 Two mixtures of pretreated lignocellulosic biomass by autohydrolysis were also suitable  
433 for glucose production, the main carbon source to produce several industrial products  
434 (as bioethanol and chemicals). Thus, cellulosic fraction can be saccharified for sugar  
435 production using enzymes. Autohydrolysis pretreatment improves the enzymatic  
436 saccharification due to the structural alteration, as result of hemicellulosic fraction  
437 solubilization. In this sense, the solid fraction obtained from autohydrolysis was used as  
438 substrate in the assays of enzymatic hydrolysis in order to evaluate the susceptibility of  
439 pretreated biomass for glucose production. Time course of glucose yield for the two  
440 mixtures in the selected autohydrolysis conditions studied in this work ( $S_0$ : 3.71 - 4.82)  
441 is displayed in Figure 4 (a) and (b). As seen in Figure 4, kinetics of enzymatic  
442 hydrolysis followed a typical pattern. Therefore, values of glucose yield obtained from  
443 enzymatic hydrolysis in this set of experiments were fitted to the Holtzapfle empirical  
444 equation (3) [41]:

445 (3)

$$Y_{Gt} = Y_{GMAX} \times \frac{t}{t + t_{1/2}}$$

446 Where  $Y_{Gt}$  is the glucose yield at time  $t$ ,  $Y_{GMAX}$  is the maximum glucose yield  
447 achievable at infinite reaction time, and  $t_{1/2}$  (h) measures the reaction time needed to  
448 reach 50% of glucose yield.

449 The representation of calculated and experimental data (Figure 4) and the values of  $R^2$   
450 (Table 4) showed the goodness of adjustment to the empirical model. These results  
451 showed that the severity of pretreatment increased the glucose yield and reduced the  
452 time of hydrolysis. The reaction time needed to reach 50% of glucose yield ( $t_{1/2}$ ) was  
453 lower for M1-4 than for M2-3, in all the cases, except for the lowest (190 °C) and  
454 highest (240 °C)  $T_{MAX}$ . As evident in Figure 4, the harshness of pretreatment had a

455 positive effect on the susceptibility of pretreated biomass to enzymatic hydrolysis.  
 456 Glucose yield increased from 45.30 % to 89.94 % and from 42.46 % to 81.78 %, for  
 457 M1-4 and M2-3, respectively, at 72h of enzymatic hydrolysis (Table 4). Considering  
 458 only autohydrolyzed *Eucalyptus globulus* wood, Romani et al. [42] reported a glucose  
 459 yield of 100% at  $T_{MAX} > 210$  °C.

460 There was a greater difference of glucose yield at  $T_{MAX}$  196 °C between M1-4 (45.09%)  
 461 and M2-3 (73.70%) at 72h. On the other hand, M1-4 reached glucose yield higher than  
 462 M2-3 at  $T_{MAX} > 226$  °C.

463 **Table 4-** Glucose concentration,  $G_{72}$ , and glucose yield,  $Y_{G72}$ , at reaction time of 72 h,  
 464 maximal glucose yield,  $Y_{GMAX}$ , time needed to achieve  $\frac{1}{2}$  of  $Y_{GMAX}$ ,  $t_{1/2}$  and coefficient  
 465 of determination  $R^2$ .

$T_{MAX}$ (°C) / $S_0$	Substrate	$G_{72}$ (g/L)	$Y_{G72}$ (%)	$Y_{GMAX}$ (%)	$t_{1/2}$ (h)	$R^2$
190 or 3.71	M1-4	10.04	45.30	73.21	45.68	0.98
	M2-3	9.44	42.46	48.15	10.47	0.95
196 or 3.93	M1-4	10.26	45.09	51.86	11.67	1.00
	M2-3	16.21	73.70	100.0	39.11	0.96
206 or 4.15	M1-4	13.81	60.18	71.08	13.54	1.00
	M2-3	16.47	73.72	100.0	35.61	0.97
216 or 4.38	M1-4	19.91	77.97	85.33	5.77	1.00
	M2-3	21.15	85.54	100.0	23.45	0.97
226 or 4.60	M1-4	21.51	89.82	92.26	5.98	0.99
	M2-3	19.59	74.43	87.26	21.25	0.96
240 or 4.82	M1-4	21.11	89.94	99.87	6.07	1.00
	M2-3	21.34	81.78	81.28	1.93	0.99

466  
 467 An increase in the autohydrolysis severity ( $S_0$ ) from 3.71 to 4.82 allowed glucose  
 468 concentration to increase from 10 g/L to 21 g/L, approximately, at 72 h of hydrolysis for  
 469 the two mixtures (Table 4). The similar behavior was reported by Domínguez et al. [11],

470 using *Paulownia tomentosa* as biomass, where an increase in the autohydrolysis  
471 severity from  $S_0$  3.31 to 4.82 allowed a five-fold increment in glucose concentration to  
472 27.5 g/L at 120 h.

473 In general, enzymatic hydrolysis is an efficient process without generation of any toxic  
474 waste and does not contain fermentation inhibitors, which reveals a promising strategy  
475 to obtain higher glucose yield [43].

476

### 477 **3.6. Overall balance of M1-4 and M2-3**

478 Considering the results obtained in this study, Figure 5 compares the fractionation effect  
479 of autohydrolysis pretreatment on the two feedstock mixtures. The highest  
480 hemicellulose solubilization (as XOS and xylose) was observed at  $T_{MAX} = 206$  °C, as  
481 glucose yield of enzymatic hydrolysis higher than 60% for both mixtures (Table 4). As  
482 seen in Figure 5, the value-added compound obtained in separated streams was of 19.1  
483 kg of glucose for M1-4 and 24.1 kg of glucose for M2-3. Overall yield of glucose for  
484 M1-4 and M2-3 was 50% and 63%, respectively. These results can be compared with  
485 reported data in literature using the same pretreatment, in which 76% and 63% of  
486 glucose yield at  $S_0 = 4.13$  were achieved from brewers's spent grain and corn husk,  
487 respectively [44]. At the same condition, overall yield of xylose was 62% and 69% for  
488 M1-4 and M2-3, respectively. The results obtained for xylose yield can be favorably  
489 compared with data reported by Nitsos et al., that obtained around 60% yield ( $S_0$  3.8 -  
490 4.01) for poplar and grapevine, respectively [19]. The data described above indicate that  
491 autohydrolysis at 206 °C is an appropriate process for the selective fractionation of  
492 mixtures obtaining a solid fraction composed mainly by glucan and lignin, and high  
493 solubilization of hemicellulose into the liquid phase with minimum formation of

494 degradation products. Cellulose was subjected to enzymatic hydrolysis and could be  
495 further processed for biological conversion into biofuels, biochemical or biomaterials as  
496 single or in combination with sugars obtained from liquid phase. The remaining lignin  
497 can simply be used for co-generation of energy in a biorefinery context or exploited for  
498 other high value applications.

499

#### 500 **4. Conclusions**

501 This work provides a comparative study of two biomass mixtures in order to supply a  
502 lignocellulosic biorefinery throughout the year, showing a suitable solution for the  
503 utilization and valorization of forest and marginal land resources in Portugal.  
504 Autohydrolysis was used for the fractionation of these biomass mixtures, showing  
505 differences on hemicellulose solubilization and enzymatic hydrolysis of cellulose into  
506 glucose. Nevertheless, under selected conditions, maximal hemicellulose recovery as  
507 xylooligosaccharides and xylose was achieved for two mixtures, allowing to operate at  
508 same conditions independently of selected biomass mixture. The proposed multi-supply  
509 raw materials biorefinery increases the sustainability of the value chain, in terms of the  
510 biomass (not pressure in same renewable resources) and avoids forest fires. In addition,  
511 an environmentally-friendly pretreatment is used for the fractionation of multi supply  
512 biomass in order to coproduce solid biofuels, oligosaccharides and glucose which may  
513 be further converted to liquid biofuels or to platform chemicals.

514

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525

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## 662 **Figure captions**

663 **Figure 2-** Flow chart of whole process and analytical methods used in this work.

664 **Figure 2-** Heating and cooling temperature profiles of autohydrolysis assay carried out  
665 at  $S_0 = 3.71$  and  $4.82$ .  $T_{REF}$ : temperature of reference,  $100\text{ }^\circ\text{C}$ .

666 **Figure 3-** Calorific value (MJ/kg) of raw material and pretreated biomasses (M1-4 and  
667 M2-3) at different  $T_{MAX}$ .

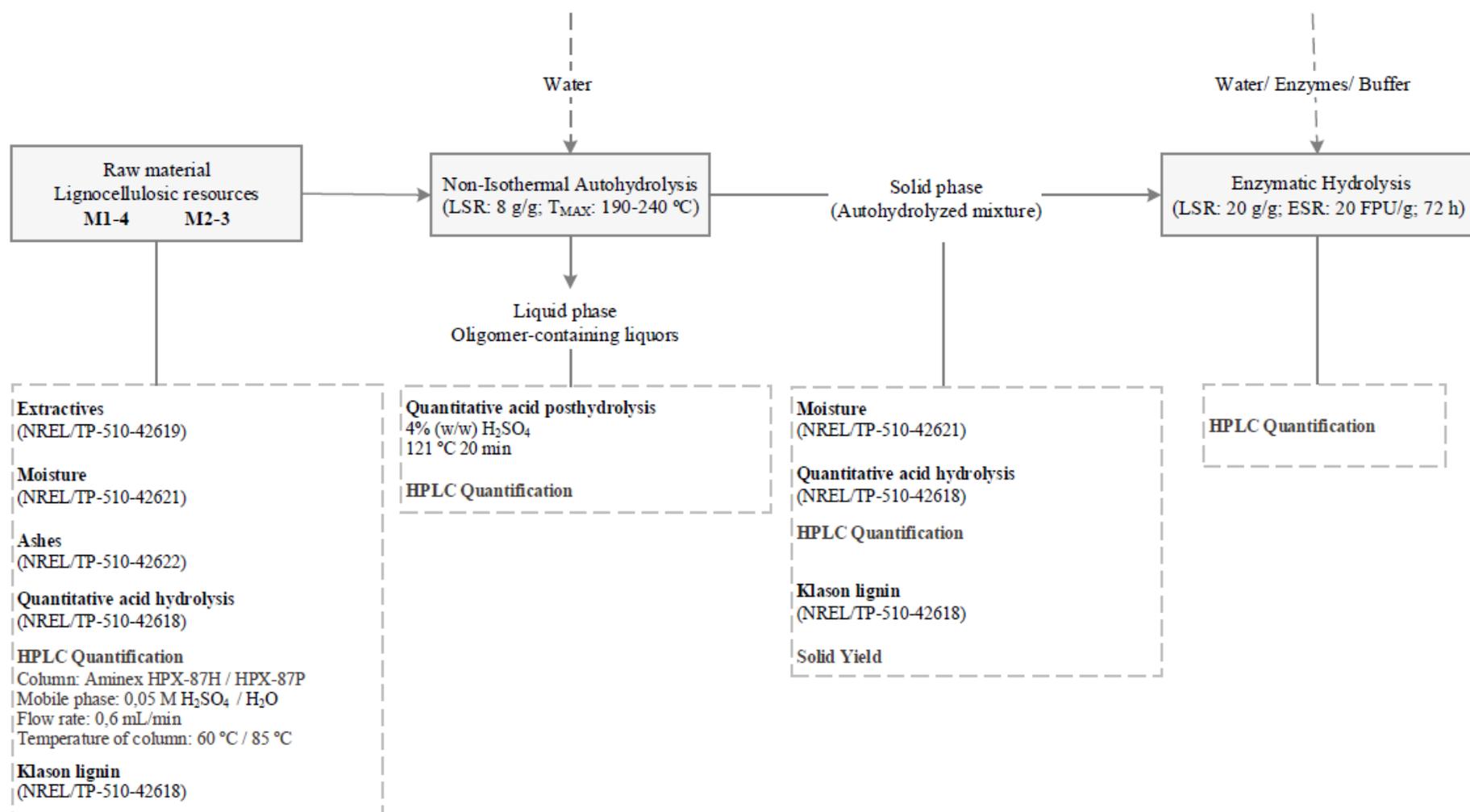
668 **Figure 4-** Yield of glucose  $Y_G$  (%) at autohydrolysis conditions ( $T_{MAX}$ ) in the range  $190$   
669  $^\circ\text{C}$  to  $240\text{ }^\circ\text{C}$  for M 1-4 (a) and M 2-3 (b).

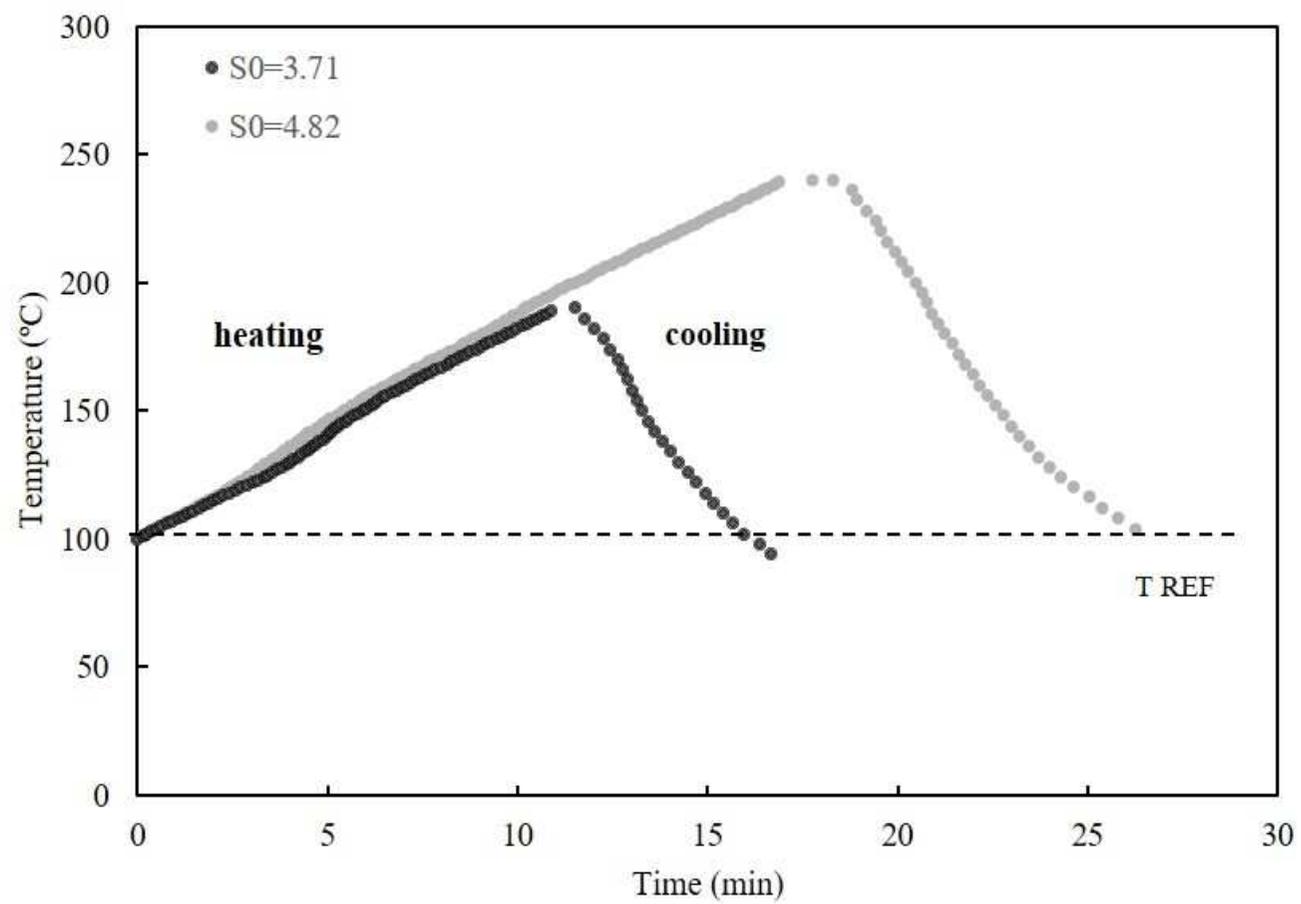
670 **Figure 5-** Overall balance of M1-4 (a) and M2-3 (b) for autohydrolysis and  
671 saccharification processing at  $T_{MAX} = 206\text{ }^\circ\text{C}$  (results expressed in kg/100kg raw  
672 material) oven dry basis.

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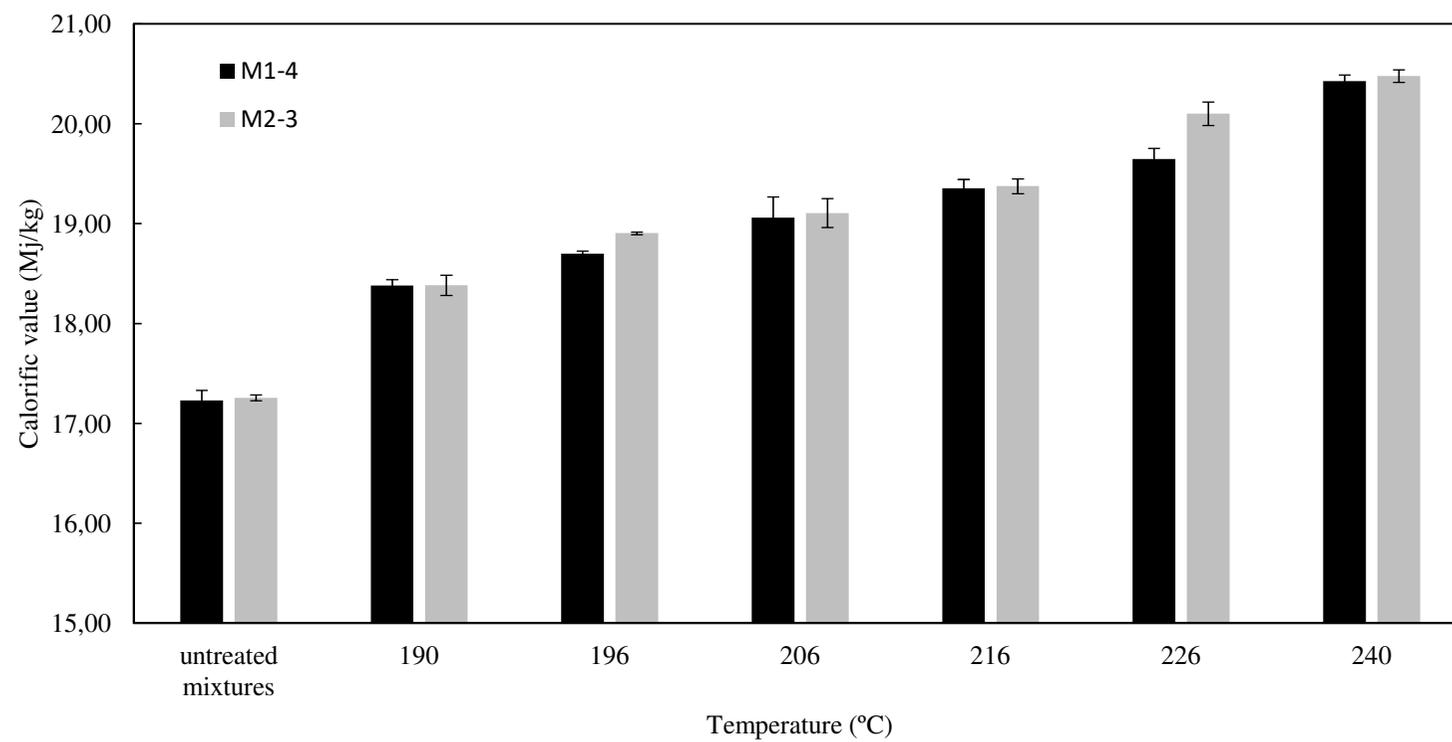
675

1 **Figure 1**



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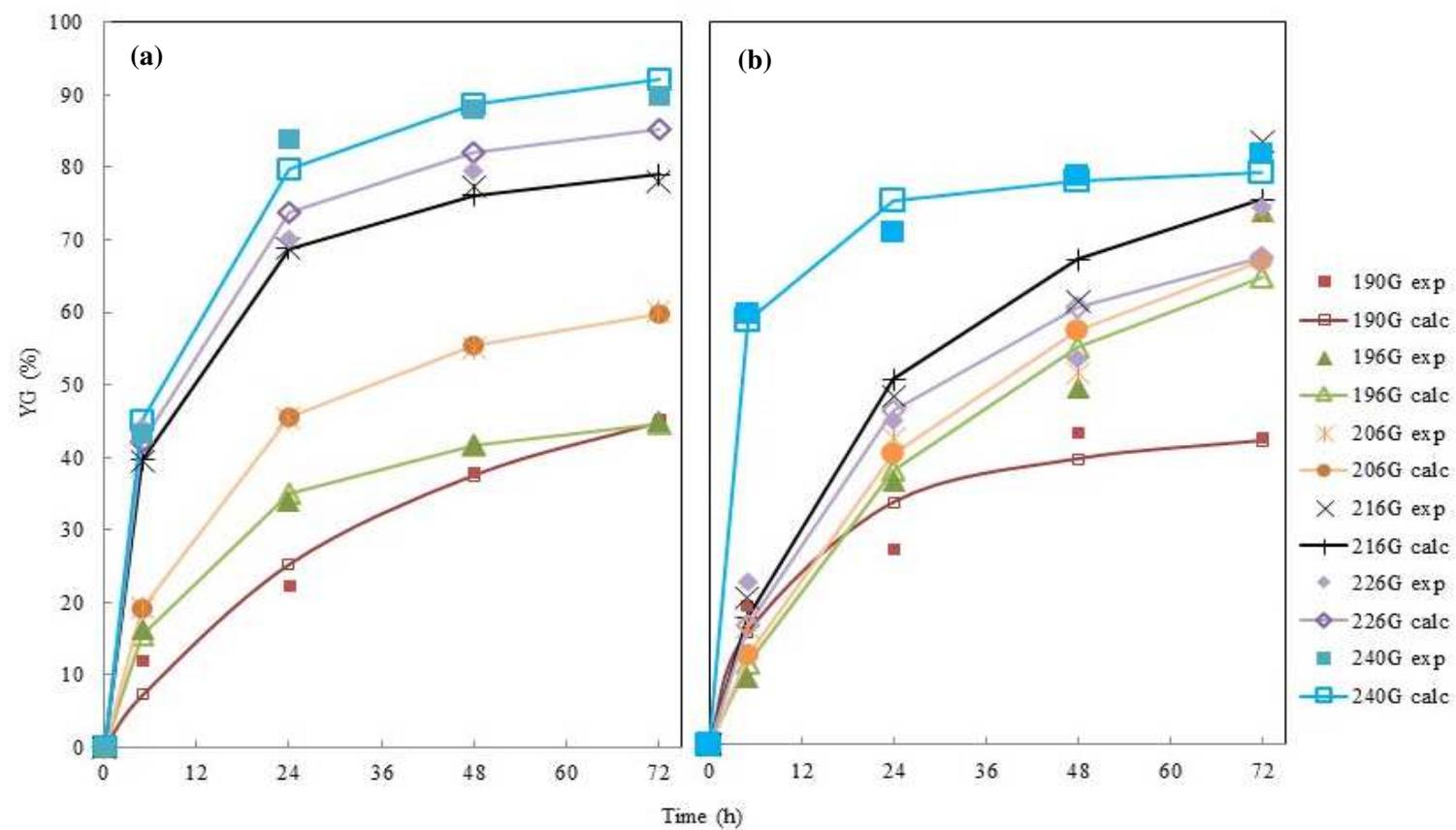
3 **Figure 2**



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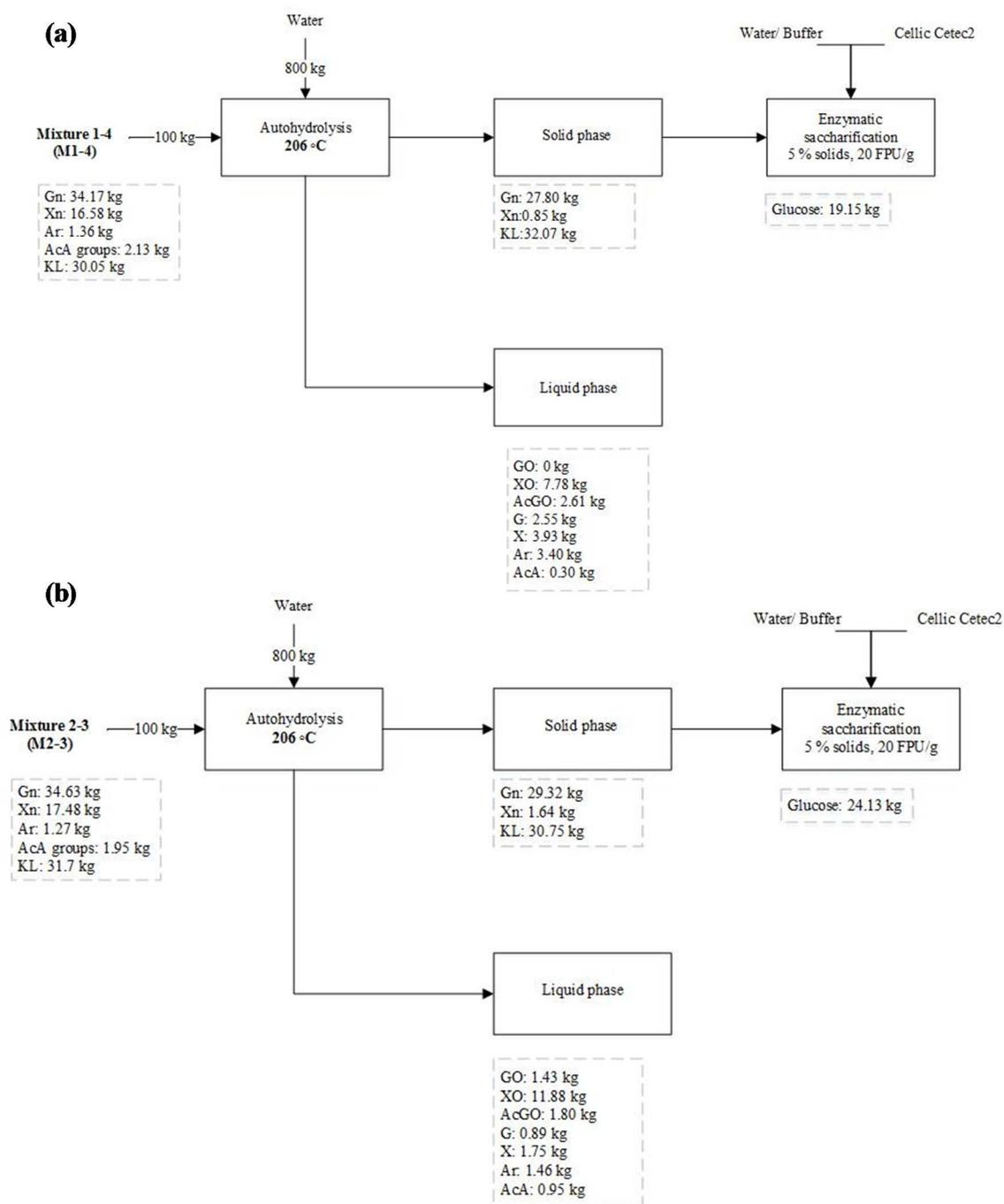
6 **Figure 3**



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9 **Figure 4**



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11 **Figure 5**

**Highlights**

- Efficient fractionation of two biomass mixtures was achieved by autohydrolysis
- The highest xylan recovery was obtained at severity of 4.15 for two mixtures
- Severity of treatment increases High Heating Values of pretreated biomasses
- The increase of severity improves the enzymatic hydrolysis of pretreated mixtures