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Additional Information

1 **Ultrasound-assisted acid hydrolysis of cassava (*Manihot esculenta*) bagasse: kinetics,**  
2 **acoustic field and structural effects**

3

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19

20 **Abstract:** Improving the actual acid hydrolysis of cassava bagasse (CB) with the assistance  
21 of high-intensity ultrasound (US) was aimed in comparison with mechanical agitation (AG).  
22 The kinetics of reducing and total sugar release were mathematically modeled. The acoustic  
23 field characterization and apparent viscosity of the suspensions were correlated. Moreover,  
24 microscopic analyses (visible, fluorescence and polarized light) were carried out to identify  
25 changes produced by the treatments. Both AG and US-treatments showed themselves to be  
26 effective at hydrolyzing CB. However, US-experiments reached equilibrium in the reducing  
27 sugar release process earlier and obtained slightly higher values of total sugars released. The  
28 Naik model fitted the experimental data with good accuracy. A greater loss in the  
29 birefringence of the starch granules and the degradation of lignocellulosic matter was also  
30 observed in US-assisted hydrolysis. The actual acoustic power applied was reduced after  
31 hydrolysis, probably due to the increase in the apparent viscosity of the resulting suspensions.  
32 **Keywords:** bioethanol, hydrolysis kinetics, reducing sugar, total sugar, birefringence.

33

## 34 1. Introduction

35 Ethanol has been considered one of the most suitable secondary biofuels with which to  
36 replace fossil fuels [1]. It can be produced through the fermentation of the sugars that  
37 compose the carbohydrates of some food industry residues, such as those generated from the  
38 processing of sugarcane, corn, or potato. In this sense, cassava (*Manihot esculenta*) bagasse  
39 represents an abundant source of agro-industrial waste in Brazil. The development and design  
40 of alternative treatments for this waste have become a must. Its residual starch content can  
41 range from 35 to 85% db as a consequence of the low yield of the cassava starch extraction  
42 process [2]. Moreover, this byproduct also includes a higher content of fiber (10–65% d.b).  
43 The release of sugars of low molecular weight from this material is dependent on adequate  
44 pretreatments and hydrolysis. So, studies into alternative and eco-friendly processes have  
45 become worthy of research [3]. The pretreatment step, commonly carried out by chemical or  
46 physical methods or a combination of them, is responsible for breaking and exposing the  
47 structure of lignocellulose and starch granules, making them more accessible to enzymes and  
48 chemicals [4, 5]. An efficient pretreatment can provoke simultaneous alterations in both the  
49 structure and chemical linkages, leading to a partial hydrolysis and enhanced further  
50 enzymatic hydrolysis [6]. However, the differences of using of one method or other are more  
51 accurately evaluated if the mechanisms of sugar dissolution are differentiate from the sugar  
52 production during the actual hydrolysis [7].

53 Of the chemical methods, alkaline treatments aim to solubilize the lignin and increase  
54 cellulose digestibility [8]. On the other hand, the use of acids, such as phosphoric acid, has  
55 demonstrated to be an effective means of modifying the structure of both lignocellulose and  
56 starch granules [7, 9-11]. Phosphoric acid is reported to be less aggressive, producing less

57 sugar degradation compounds as furfural and acetic acid at lower costs [12]. Gámez et al.  
58 [12] also highlights the potential use of sodium phosphate, obtained after phosphoric acid  
59 neutralization with sodium hydroxide, as nutrient for microbial growth during sugar  
60 fermentation. Although it can be used in concentrated solutions, diluted and non-aggressive  
61 acids are preferred in this kind of pretreatments to avoid equipment corrosion and make acid  
62 recovery easier [13].

63 During the acid pretreatment, biomass suspensions can be stirred by conventional agitation  
64 at controlled temperatures to make it easy to transfer the acid to the internal matrix. On the  
65 other hand, alternative physical methods can be applied to enhance the effects of the acid  
66 solution on biomass and to allow the use of milder conditions of temperature, catalyst  
67 concentration, pressure and processing times commonly applied in literature [14, 15]. High-  
68 intensity (or power) ultrasound has appeared as an interesting non-conventional technology  
69 for this purpose [16]. The sonochemical effects are linked to certain mechanisms produced  
70 when ultrasound travels across a medium with enough energy: cavitation, micro-agitation or  
71 heating. Cavitation occurs in liquid media caused by the collapse of the cavitation bubbles,  
72 creating molecular displacement throughout the successive zones of high and low pressure.  
73 The intensity of the collapse is able to cause the erosion of polymeric materials through the  
74 formation of micro-jets and enhance the mass transfer between liquid and solid media [17].  
75 Ultrasound can also promote micro-agitation inside the particles and acoustic streaming at  
76 interfaces [18]. Moreover, heating is a consequence of the resistance forces against acoustic  
77 vibration. This effect is useful for measuring the real acoustic power applied and for  
78 determining the proportion of acoustic energy transferred to the medium, this being important

79 information for the purposes of reproducing similar conditions and scaling up the process  
80 [19].

81 Therefore, the main goal of this paper was to study the ultrasonically assisted acid treatment  
82 of cassava bagasse under mild conditions of temperature and phosphoric acid concentration  
83 to evaluate their influence on the kinetics of sugar release and the structural effects.

84

## 85 **2. Materials and Methods**

### 86 **2.1. Sample preparation**

87 In this study, the residue from the cassava (*Manihot esculenta* C.) starch extraction process  
88 was used. Wet bagasse was provided by TechnoAmido (São Pedro do Turvo, Brazil), a starch  
89 extraction industry. Thus, the wet bagasse was dried at 55 °C for 48h. The resulting coarse  
90 powder was milled in a domestic grinder to be, then, sieved to obtain samples with a particle  
91 size of under 1 mm. This material presented a moisture content of  $4.18 \pm 0.22\%$  (w. b.),  
92 determined in triplicate in a vacuum oven at 60 °C until constant weight (about 24h). The  
93 chemical composition of the dried matter was characterized in a previous study [20].

94

### 95 **2.2. Experimental plan**

96 The experimental plan included two sets of experiments. The first one studied the dissolution  
97 of the free sugars present in cassava bagasse. In the second one, an analysis of the acid  
98 hydrolysis treatment was carried out. In the latter, mild conditions were tested in order to  
99 avoid the use of corrosive acids and temperatures that might cause the gelatinization of the  
100 suspensions, creating difficulties in the mass transfer. For this reason, the temperature of the  
101 test was maintained at 50 °C to enhance the acid actuation, but keeping the temperature below

102 the gelatinization temperature of cassava starch [21] without significant impairment in the  
103 cavitation activity [22]. All of the experiments as well as the different chemical  
104 determinations were performed in triplicate.

105

### 106 **2.2.1. Sugar dissolution experiments (SD)**

107 For this set of experiments, 5% cassava bagasse suspensions in distilled water (g of dried  
108 cassava bagasse per 100 g of suspension) were prepared. This biomass concentration was  
109 established because it was the maximum concentration that allowed an adequate mixing and  
110 coupling between the sample-sonotrode during the ultrasound-assisted process. Above this  
111 concentration, the suspensions assume non-Newtonian behavior and deviations from  
112 linearity should be also considered [20]. After that, the suspensions (150 g each one) were  
113 subjected to conventional agitation for 5 h at 50 °C in order to determine the amounts of total  
114 and reducing sugar in the cassava bagasse that were directly soluble (experiments B5AG0).  
115 At the preset times, the samples were extracted and total and reducing sugar determinations  
116 were carried out to obtain the kinetics of the process.

117

### 118 **2.2.2. Acid hydrolysis experiments (AH)**

119 These experiments were carried out in two steps. Firstly, 5% (g of cassava bagasse per 100  
120 g of suspensions) cassava bagasse suspensions were agitated for 4 h at 50 °C in the same way  
121 as that described for SD experiments. This was long enough to ensure the total dissolution of  
122 the free soluble sugars present. After that, metaphosphoric acid (GPR Rectapur, VWR  
123 Chemicals, Lutterworth, UK) was added to the aqueous suspensions to obtain an acid solution  
124 of 10% (g of acid per 100 g of solution). This concentration was based on previous studies

125 that concerned the hydrolysis of starchy and lignocellulosic feedstock [7, 9], aiming at  
126 obtaining interesting effects without acid over-dosage by working below the gelatinization  
127 temperatures. These suspensions (150 g by sample) were maintained under conventional  
128 agitation (B5AG10-A experiments) or high-intensity ultrasound application (B5US10-A  
129 experiments) for 1 h. Samples were extracted at preset times and total and reducing sugar  
130 determinations were performed.

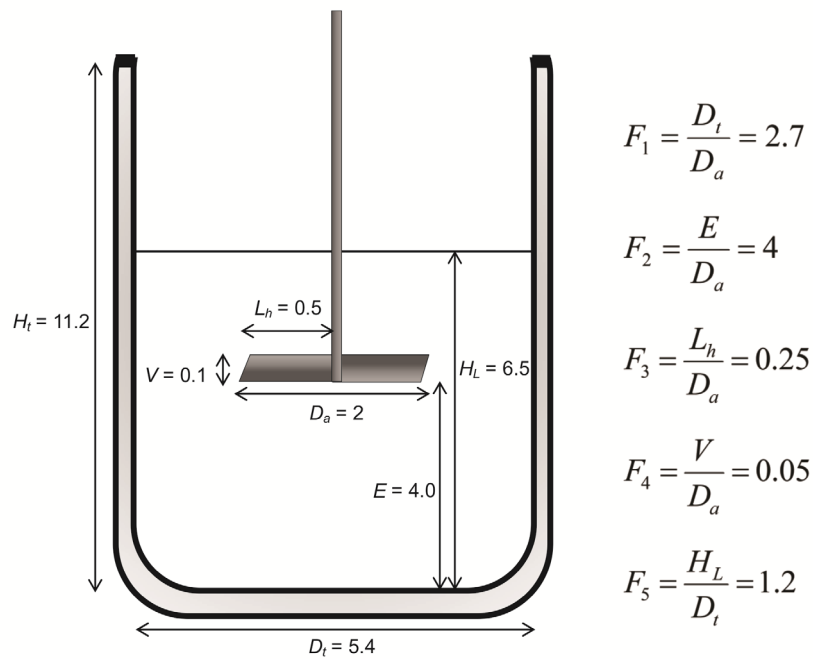
131

### 132 **2.3. Conventional agitation (AG) system**

133 Acid treatments under conventional agitation were performed in a jacketed vessel using an  
134 agitator (Heidolph RZR1; Heidolph Instruments GMBH & Co., Schwabach, Germany). This  
135 equipment was adjusted to work at 1000 rpm in order to obtain an intense stirring in the  
136 suspensions. The suspensions were maintained at 50 °C during the treatments by placing the  
137 reaction vessel into a thermostatic bath (Frigedor, J.P. Selecta, Barcelona, Spain). Figure 1  
138 shows some shape parameters ( $F_1$ ,  $F_2$ ,  $F_3$ ,  $F_4$  and  $F_5$ ) that characterize the agitation of the  
139 suspension induced by the system.

140





141

142 **Figure 1.** Set-up used in the experiments under conventional agitation.

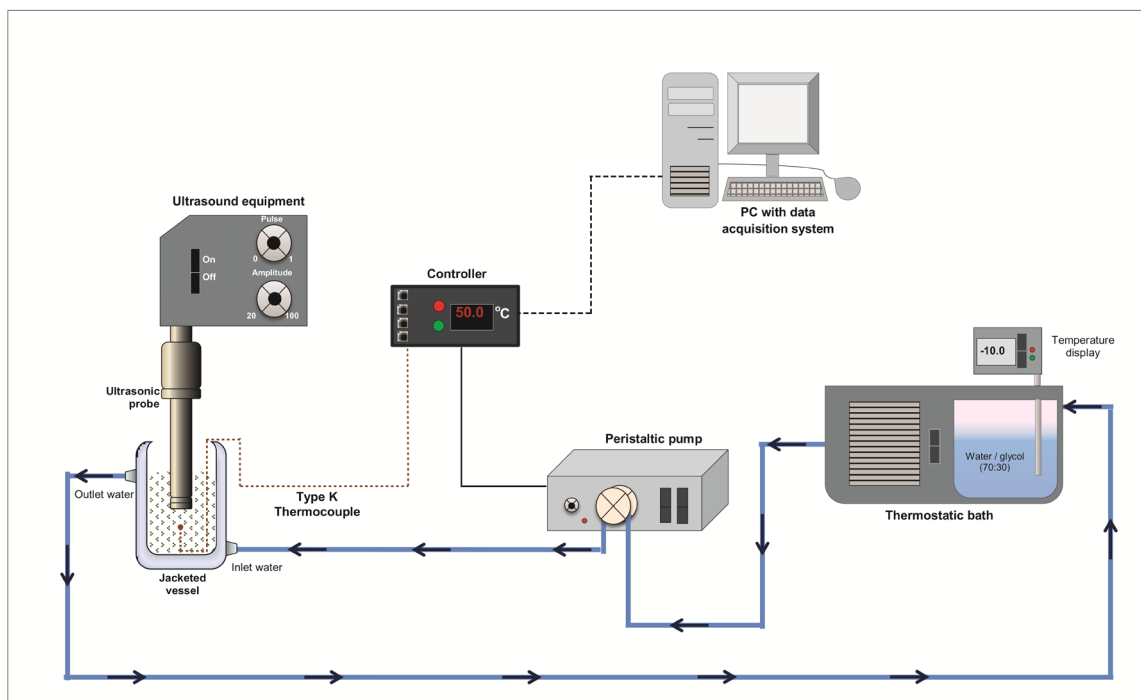
143

144 **2.4. High-intensity ultrasound (US) system**

145 A scheme of the experimental set-up used in the ultrasound-assisted hydrolysis experiments  
 146 is presented in Figure 2. The equipment consisted of an ultrasonic probe-type system  
 147 (UP400S, Dr. Hielscher, Teltow, Germany) supplied with a 22 mm diameter emitter probe  
 148 (irradiation surface of 3.8 cm<sup>2</sup>). The probe was immersed 1 cm into the acid suspensions,  
 149 which were placed in a jacketed vessel. The tests were carried out at the maximum electrical  
 150 power input of the system (400 W) in pulsed mode (0.6s on - 0.4 s off). The temperature was  
 151 maintained constant ( $50 \pm 2$  °C) during the experiments to prevent overheating. To this end,  
 152 the temperature of the suspensions was recorded with a K type thermocouple placed 2 cm  
 153 below the probe. The thermocouple was connected to a process controller (E5CK, Omron,  
 154 Hoofddorp, Netherlands) which drove a peristaltic pump (302 S, Watson-Marlow, Postfach,

155 Germany) to recirculate a 30% glycol solution at  $-10\text{ }^{\circ}\text{C}$  from a refrigerated bath (Frigedor,  
 156 J.P. Selecta, Barcelona, Spain) through the jacketed recipient containing the suspensions  
 157 (Figure 2).

158



159

160 **Figure 2.** Scheme of the set-up used in the ultrasound-assisted hydrolysis.

161

## 162 2.5. Sugar determination

163 Aliquots of approximately 3 mL were taken every 60 min in the case of the B5AG0  
 164 experiments (0, 60, 120, 180, 240 and 300 min), and every 15 min in the case of the B5AG10  
 165 and B5US10 experiments (0; 15; 30; 45 and 60 min). The acid aliquots were neutralized with  
 166 NaOH 2.5 M, to be subsequently centrifuged and filtered in PTFE micro filters (0.45  $\mu\text{m}$ , 25  
 167 mm diameter; LabBox, Barcelona, Spain). The supernatant was used for the different sugar  
 168 content measurements. Thus, the reducing sugars ( $S_R$ ) were determined in triplicate using the

169 DNS method, as proposed by Kassaye et al [3]. This is a rapid and practical method, which  
 170 is based on the reduction, in alkaline medium, of 3,5-dinitrosalicylic acid. The absorbance of  
 171 the resulting compound was measured at a wavelength of 540 nm and the reducing sugar  
 172 content was quantified by using a standard curve of anhydrous glucose, previously  
 173 determined. The data were expressed as g of reducing sugar (glucose equivalent) per 100 g  
 174 of dried cassava bagasse.

175 The total amount of hydrolysable sugars ( $S_T$ ) was determined by the same method but  
 176 including a previous hydrolysis step [15]. Thus, samples were placed into HCl 2 M solution  
 177 and maintained under boiling for 30 min. After that, they were cooled and neutralized with  
 178 NaOH 2.5 M. Then, the hydrolyzed samples were submitted to the DNS method to determine  
 179 the total amount of fermentable sugar (glucose equivalent) per 100 g of dried cassava  
 180 bagasse.

181 The values obtained by both methods were used to calculate the relative sugar release,  $Y$   
 182 (Equation (1):

$$183 \quad Y = \frac{([S]_t - [S]_{t=0})}{[S]_{t=0}} \quad (1)$$

184 Where  $[S]_t$  represents the sugar released at time  $t$  and  $[S]_{t=0}$  the sugar released at the very  
 185 beginning of the experiment.

186

## 187 **2.6. Mathematical modeling**

188 The Naik model [23], given by Equation (2), was used to fit the kinetics of sugar release.  
 189 This model is reported to represent not only the extraction kinetics of food compounds [24]  
 190 but also the sugar release during hydrolysis of byproducts [7] with good accuracy:

191 
$$Y = \frac{Y_{\infty} t}{(B + t)} \quad (2)$$

192 where  $t$  is the treatment time (min),  $Y_{\infty}$  is the relative sugar release at equilibrium and  $B$  is  
 193 the time needed (min) to reach half of  $Y_{\infty}$ , which means a measurement of the process  
 194 kinetics. The goodness of the fitting procedure was evaluated by the adjusted determination  
 195 coefficient ( $R_{adj}^2$ ) and the root-mean-square error ( $RMSE$ ), given by Equations (3) and (4),  
 196 respectively.

197 
$$R_{adj}^2 = 1 - \frac{n-1}{n-(k+1)}(1-R^2) \quad (3)$$

198 
$$RMSE = \sqrt{\frac{\sum_{n=1}^n (y_p - y_{exp})^2}{n}} \quad (4)$$

199 where  $n$  is the number of observations,  $k$  is the number of parameters in the model (excluding  
 200 the constant),  $R^2$  is the determination coefficient,  $y_p$  is the predicted value and  $y_{exp}$  is the  
 201 experimental value.

202

## 203 **2.7. Apparent viscosity**

204 The apparent viscosity of the suspensions before and after the acid hydrolyses was measured  
 205 under steady-state flow in a rotational rheometer AR-G2 (TA Instruments, USA) coupled  
 206 with the Starch Pasting Cell (SPC) geometry to avoid particle sedimentation. The shear stress  
 207 was obtained over an interval of shear rate between 1 and 265.5  $s^{-1}$ , maintaining the  
 208 temperature at 50 °C by means of the thermostatic bath of the equipment itself. The flow  
 209 curves were analyzed to determine the apparent viscosity over the range of shear rate.

210

211

## 212 **2.8. Acoustic field characterization**

213 A calorimetric method was employed to characterize the actual acoustic power applied in  
214 each case, as seen as the use of hydrophone for measuring acoustic pressure was unfeasible  
215 due to their sensitivity to acids and interference of the suspended particles. For this purpose,  
216 the temperature increase in the first 60 s of ultrasound application [22] was recorded with a  
217 K type thermocouple placed in the suspensions 2 cm below the probe and connected to a data  
218 acquisition system (LabVIEW Run-Time Engine 7.0, National Instruments, USA). The  
219 measurements were taken in triplicate before and after 60 min of hydrolysis treatment in  
220 order to detect any influence of the treatment on the actual ultrasonic power applied. The  
221 temperature records were taken using the same experimental set-up used for US-assisted  
222 hydrolysis experiments. So, the acoustic power applied can be calculated by Equation (5):

$$223 \quad P = mc_p \frac{dT}{dt} \quad (5)$$

224 where  $P$  is the acoustic power (W),  $m$  is the mass of the suspension (kg),  $c_p$  is the specific  
225 heat capacity of the acid solution ( $4064.8 \text{ J}\cdot\text{kg}^{-1}\cdot\text{°C}^{-1}$ , determined by differential scanning  
226 calorimetry) and  $dT/dt$  is the heating rate ( $\text{°C}\cdot\text{s}^{-1}$ ). The acoustic intensity ( $I$ ,  $\text{W}\cdot\text{cm}^{-2}$ ) was  
227 calculated by considering the acoustic power per area of the emitter surface; the acoustic  
228 density per treated volume ( $D$ ;  $\text{W}\cdot\text{mL}^{-1}$ ) and the acoustic load per treated mass of particles  
229 ( $\text{W}\cdot\text{g}^{-1}$  of dried biomass).

230

## 231 **2.9. Microscopic analysis**

232 The morphology of the raw cassava bagasse and the product obtained after the hydrolysis  
233 treatments were microscopically analyzed in order to identify changes attributed exclusively

234 to the acid treatment. To separate and to identify the effects on the fibrous and starchy fraction  
235 different methodologies were considered to avoid erroneous conclusions. For this purpose,  
236 a Nikon Eclipse E80i (Nikon, Tokyo, Japan) optical microscope was used. To evaluate any  
237 possible alterations in the fibrous particles of the bagasse, untreated and treated samples were  
238 placed on a glass slide and stained with toluidine blue (0.1%) to be observed using bright  
239 field mode. Non-stained samples were observed by bright field, fluorescence and polarized  
240 light microscopy in order to evaluate possible alterations to the starch granules' structures  
241 and to their birefringence.

242 All of the images were captured and stored by NIS-Elements F software (Version 4.0, Nikon,  
243 Tokyo, Japan).

244

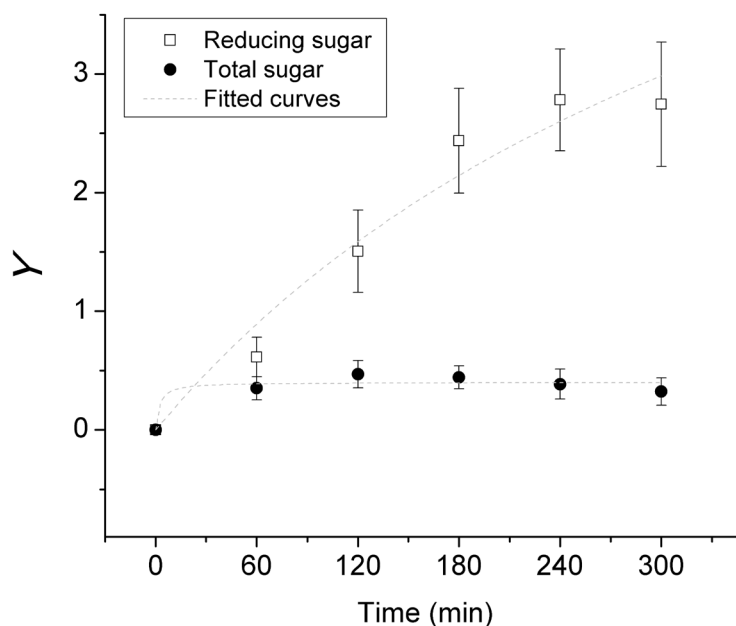
### 245 **3. Results and discussion**

#### 246 **3.1. Sugar dissolution**

247 The SD experiments allowed the kinetics of reducing and total free sugar release in aqueous  
248 medium to be quantified. The results showed significant amounts of free sugars remaining in  
249 cassava bagasse, which may be explained because the main goal of cassava industrialization  
250 is the extraction of starch. These compounds are easily fermented for the purposes of  
251 producing bioethanol. As expected, the amount of total sugars was greater than that of  
252 reducing sugars. As regards the kinetics, the maximum total sugar content was reached after  
253 2 hours of treatment (Figure 3). On the contrary, the reducing sugars needed 4 hours to  
254 achieve asymptotic behavior. Therefore, 4 hours of agitation was considered to be enough to  
255 attain dissolution equilibrium for both. After this period of time, cassava bagasse released  
256  $3.30 \pm 0.26$  g of reducing sugars per 100 g of dried CB and  $4.87 \pm 0.04$  g of total sugars per

257 100 g of dried CB, which correspond to a relative release of  $2.78 \pm 0.43$  and  $0.39 \pm 0.13$ ,  
 258 respectively (Figure 3).

259



260

261 **Figure 3.** Relative release of total and reducing sugar concentration over the agitation of  
 262 cassava bagasse in aqueous medium.

263

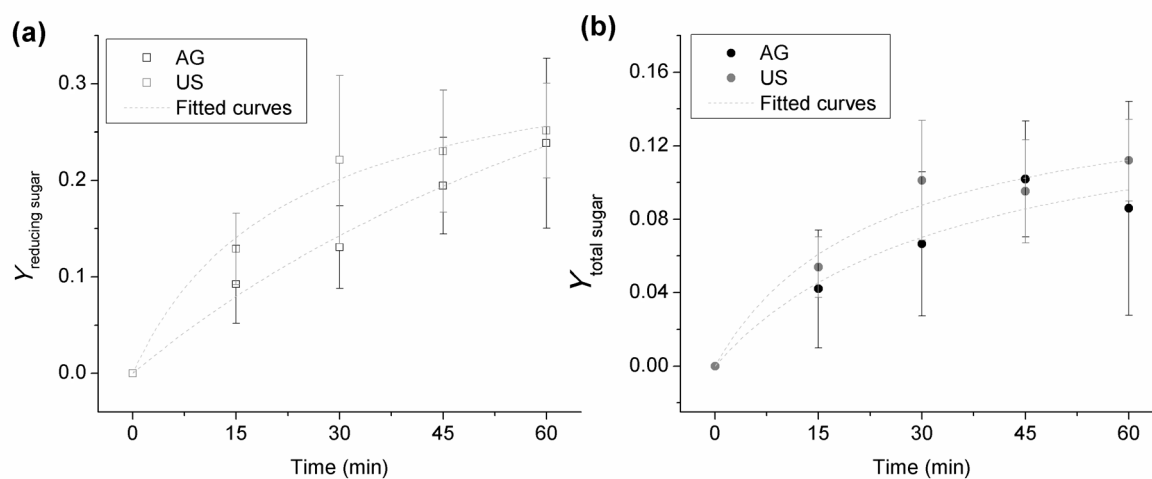
### 264 3.2. Acid hydrolysis

265 The results obtained in SD experiments showed that there was a significant amount of soluble  
 266 sugars, total and reducing, in CB. For this reason, for AH experiments, the CB was previously  
 267 treated for 4 hours in a water suspension. Thus, soluble sugars were extracted from the CB  
 268 matrix prior to the acid treatment and, consequently, it was possible to quantify the actual  
 269 effect of acid treatment on the CB. The experimental kinetics of the relative production of

270 reducing and total sugars during AH experiments carried out under agitation (AG) or with  
 271 ultrasound application (US) are shown in Figure 4.

272 Thus, the AH treatment demonstrated that it was relatively efficient at hydrolyzing cassava  
 273 bagasse, with a continuous release of reducing and total sugars throughout the time tested.  
 274 Phosphoric acid acts on the  $\alpha$ -1,6 bonds when hydrolyzing starch-based feedstock [14],  
 275 debranching and opening up the starch structure.

276



277

278 **Figure 4.** Relative production of (a) reducing and (b) total sugars during the acid hydrolysis  
 279 of cassava bagasse (CB) assisted by conventional agitation (AG) or by ultrasound application  
 280 (US).

281

282 The experimental kinetics showed that for reducing sugars, the process was slightly faster in  
 283 the case of US-assisted experiments (B5US10-A) than when using conventional agitation  
 284 (B5AG10-A). In fact, after 30 min of hydrolysis, the release of reducing and total sugars was  
 285 respectively 52.0 and 69.3% higher for the experiments with ultrasound than with mechanical  
 286 agitation. In addition to the current hydrolysis and possible side effects on the biomass



287 structure, Li et al. [25] found that the ultrasound pretreatment was able to enhance the  
288 liquefaction and saccharification of starch-based materials. The enhancement of hydrolysis  
289 by US could be attributed to the disruption of the rigid hydrophobic protein matrix and the  
290 amylose-lipid complex surrounding the granules [26]. However, the relative release of  
291 reducing sugars by US-assisted (B5US10-A) and conventionally agitated (B5AG10-A)  
292 experiments reached similar values after 60 min of hydrolysis, around 25% of the initial  
293 reducing sugar content for both processes. Although both technologies could hydrolyze  
294 cassava bagasse, ultrasound is considered a useful tool for reducing the processing time and  
295 increasing productivity.

296 In the case of total sugar release after 60 min of treatment, a 30% higher yield was observed  
297 in B5US10-A experiments (11.2%) than in B5AG10-A (8.6%) ones. On the contrary, after  
298 hydrolyzing yam with 5% phosphoric acid at 50 °C for 300 min, Ramón et al. [9] found a  
299 14% greater release of fermentable matter in experiments carried out in an ultrasonic bath  
300 when compared to non-ultrasonically assisted experiments. It is in agreement to the  
301 observation that ultrasound accelerated the acid hydrolysis, but prolonging the irradiation  
302 cannot be feasible due to increased energetic costs [27].

303 The values for total sugars are lower than the ones found for reducing sugars. This may be  
304 expected because the hydrolysis of the total sugars in solution into reducing sugars is faster  
305 than the hydrolysis of the fermentable matter trapped in the cassava bagasse structure into  
306 soluble total sugars. The fact that the differences observed between US and AG treatments  
307 were not significant is likely to be due to the variability in the sugar content of the raw matter.  
308 The post-harvest physiological deterioration of the cassava roots occurs rapidly, within 24 h,  
309 which can impair the homogeneity of the resulting samples [28, 29].

310

311

312 **3.3. Mathematical modeling**

313 The Naik model was fitted to the kinetics of sugar release by dissolution in aqueous medium

314 (SD experiments) and by acid hydrolysis (AH experiments). The fitting parameters for the

315 reducing and total sugars are shown in Table 1. A close correlation between the experimental

316 and those calculated with the model data were found, the  $R_{adj}^2$  being higher than 0.8715 and317 the low root-mean-square error ( $RMSE$ ) lower than 0.2074.

318

319 **Table 1.** Fitting parameters of the Naik model for the different treatments.

	Reducing sugars			Total sugars		
	B5AG0	B5AG10-A	B5US10-A	B5AG0	B5AG10-A	B5US10-A
$Y_{\infty}$	7.2051	0.6780	0.3520	0.4009	0.1556	0.1525
$B$	424.2585	112.4069	22.4413	2.0547	23.2993	35.1955
$R_{adj}^2$	0.9533	0.9879	0.9819	0.8715	0.9551	0.9171
$RMSE$	0.2074	0.0008	0.0109	0.0498	0.0076	0.0089

320

321 As for the parameters identified for the acid hydrolysis experiments, the higher release yield

322 ( $Y_{\infty}$ ) of the reducing sugars found in the experiments carried out using suspension agitation

323 (B5AG10-A) indicated that the yield achieved with this kind of treatment could be higher

324 than when using US treatments (B5US10-A) if the process time was prolonged.

325 Mathematical modeling gives insights that ultrasound effects are weakened over the acid

326 hydrolysis likely due to changes occurring in the ultrasound actuating mechanisms and in the

327 product itself. Indeed, such changes were more deeply investigated in Section 3.4, Section

328 3.5 and Section 3.6. On the contrary, US application increased the hydrolysis kinetics, as  
329 showed by the 80% reduction of  $B$  value found in US experiments (B5US10-A) when  
330 compared to AG (B5AG10-A), which is very interesting from an industrial point of view. As  
331 far as the fitting parameters identified for total sugars are concerned, they presented similar  
332 values in both US and AG experiments.

333

#### 334 **3.4. Apparent viscosity**

335 After the hydrolysis experiments, the suspensions passed from liquid-like to paste-like in  
336 appearance. Because of this reason, working with higher biomass concentration lead to bad  
337 coupling between the sonotrode and the sample and poor ultrasound propagation. During the  
338 treatments, some compounds could pass from the biomass structure into the solution. Thus,  
339 the solubilization of these compounds may be responsible for the increase in the apparent  
340 viscosity of the suspensions. The faster equilibrium attained in US-assisted hydrolysis could  
341 also be linked to a reduction in the actual acoustic power throughout the treatment produced  
342 by this increase in the apparent viscosity of the suspension. In fact, Polachini et al. [7]  
343 observed an enhancement in the ultrasound effects when decreasing the biomass  
344 concentration of the suspensions – and, consequently, their viscosity – during acid hydrolysis  
345 of artichoke residues. To confirm this hypothesis, rheological measurements were taken to  
346 quantify the apparent viscosity before and after the acid treatment under agitation or under  
347 ultrasound application (Table 2). Flow curves indicated that apparent viscosity was  
348 practically constant in the range of shear rate applied ( $R_{adj}^2 > 0.9986$ ), characterizing  
349 Newtonian behavior in the conditions being studied.

350 The measurements indicated that the suspensions hydrolyzed in the presence of ultrasound  
 351 led to higher viscosity values when compared to those mechanically agitated. This pointed  
 352 to the fact that changes in the cassava bagasse structure occurred more intensely in US-  
 353 assisted treatments. Yang et al. [30] reported that ultrasound can increase the surface  
 354 roughness of the starch granules, which leads to higher friction and, consequently, to an  
 355 increase in the viscous properties of the suspensions.

356

357 **Table 2.** Apparent viscosity of the acid suspensions before and after acid hydrolysis.

Experiment	Apparent viscosity (mPa·s)	
	Before Hydrolysis	After Hydrolysis
B5AG10-A	$1177 \pm 9^c$	$1341 \pm 10^b$
B5US10-A		$1746 \pm 3^a$

358 Means and standard deviation followed by the same lowercase letters signify there are no significant  
 359 differences between the measurements according to the Fisher test at the 95% confidence level.  
 360

361 In this sense, it is important to state that US-assisted hydrolysis needs to be properly mixed  
 362 for the suspensions to be able to provide homogenous treatment to the biomass particles  
 363 throughout a certain period of processing; this mixing energy is supplied by acoustic  
 364 agitation.

365 It is useful to characterize the evolution of the apparent viscosity throughout hydrolysis, not  
 366 only as regards the release of compounds of interest, but also for the purposes of carrying out  
 367 a correct design of the unit operations and the equipment [31]. As the material becomes more  
 368 viscous, a greater amount of energy is demanded in order to promote the same degree of  
 369 agitation that would be provided to less viscous fluids. Additionally, the viscosity of the  
 370 medium can prevent cavitation from occurring. Therefore, the process should be designed

371 with a greater energy demand in mind if a similar degree of cavitation is required throughout  
 372 the process.

373

### 374 3.5. Acoustic field characterization

375 The sonochemical effects of ultrasound are related to the real acoustic power transmitted to  
 376 the medium. In order to characterize this energy, acoustic parameters were determined by the  
 377 linear increase in temperature throughout the initial periods of ultrasound application.

378 Table 3 summarizes the acoustic parameters obtained by the ultrasound-assisted hydrolysis  
 379 before and after 60 min of the acid treatment. For every studied parameter, the values found  
 380 before the treatment were higher than those found after. During acid hydrolysis, fermentable  
 381 matter is leached from the grains and from the lignocellulose. These compounds could be  
 382 dispersed in the liquid medium without much alteration in the volume of solid biomass,  
 383 contributing to the increase in the apparent viscosity (Table 2) and to the observed paste-like  
 384 appearance of the suspensions.

385

386 **Table 3.** Acoustic parameters obtained from the determinations of the acoustic fields.

Acoustic parameters	B5US10-A experiment	
	Before	After
Acoustic power (W)	148.202 ± 2.070 <sup>a</sup>	77.129 ± 19.499 <sup>b</sup>
Acoustic intensity (W·cm <sup>-2</sup> )	38.987 ± 0.544 <sup>a</sup>	20.290 ± 5.130 <sup>b</sup>
Acoustic density (W·mL <sup>-1</sup> )	0.988 ± 0.011 <sup>a</sup>	0.514 ± 0.130 <sup>b</sup>
Acoustic load (W·g <sup>-1</sup> of dried cassava bagasse)	20.586 ± 0.235 <sup>a</sup>	10.714 ± 2.709 <sup>b</sup>

387 Means and standard deviation followed by the same lowercase letters in the same line signify there  
 388 are no significant differences between the measurements according to the Fisher test at the 95%  
 389 confidence level.

390

391 The increase in the flow resistance reflects a greater interaction between molecules and more  
392 difficulty in promoting adequate cavitation [18, 32]; the milder the cavitation activity, the  
393 lower the degree of erosion on the liquid-solid interface. Thus, extending the sonication time  
394 throughout the 60 min in this system could reduce the efficiency of the energy transfer,  
395 leading to wear in the equipment and sonotrode. Montalbo-Lomboy et al. [27] also stated that  
396 longer sonication periods can make the process more onerous, with no big differences in the  
397 release of fermentable matter.

398

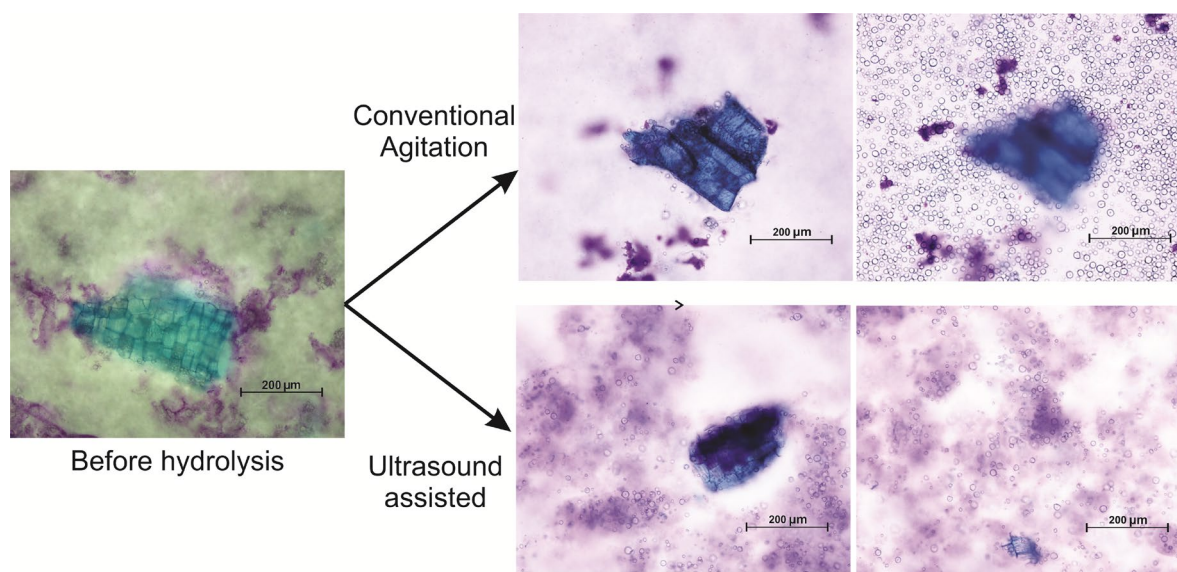
### 399 **3.6. Microscopic analyses**

#### 400 **3.6.1 Effects on the fibrous matter**

401 The morphology of cassava bagasse was qualitatively assessed by different techniques of  
402 optical microscopy. Firstly, an analysis of the general structure of raw cassava bagasse was  
403 performed in order to compare it with the treated samples. Figure 5 presented the light  
404 micrographs of both the raw material and the partially hydrolyzed cassava bagasse, with  
405 agitation and with ultrasound application. The staining technique using toluidine blue  
406 imparted metachromatic properties to the samples treated using the different technologies.  
407 Shades of blue or green indicate the presence of phenolic compounds, such as lignin [33]. It  
408 could be easily observed that the larger fibers are mainly made up of cellulose and  
409 hemicellulose with the presence of lignin. These blue/green structures apparently exhibited  
410 more integrity in the untreated cassava bagasse than in the treated, which could be related to  
411 the lower viscosity observed for the untreated suspensions. Moreover, in the US-treated  
412 samples, the fibrous structure seemed to have been subjected through a shearing process in  
413 which the surface became less irregular when compared to the untreated or the AG treated

414 samples. Small pieces of fibrous matter could also be observed in the bagasse hydrolyzed by  
 415 US, indicating that ultrasound could have an effect on biomass fragmentation. In fact, it could  
 416 be responsible for the greater increase in the viscosity of the suspensions treated with US.

417



418

419 **Figure 5.** Bright field micrographs of cassava bagasse before and after acid treatment under  
 420 conventional agitation or assisted by high-intensity ultrasound (10x).

421

422 Despite the bluish structures, regions could be seen that were purple in color. Santos et al.  
 423 [33] related these shades of purple with primary structures of cell walls, such as cellulose,  
 424 hemicellulose and pectin. In the raw sample, these regions appeared dispersed around the  
 425 fibrous particles, in all likelihood as a consequence of the milling process. This empurpling  
 426 of the material was not noticed in the bagasse hydrolyzed under agitation to the same degree  
 427 as occurs in the raw material, which gives an indication that free cellulose and hemicellulose  
 428 could be hydrolyzed by the acid. On the other hand, bagasse hydrolyzed in the presence of  
 429 ultrasound exhibited a higher concentration of empurpled materials dispersed in the sample

430 as a consequence of cell wall disruption [34]. As the yield of sugar release was similar to  
431 conventionally agitated samples, it is possible to infer that ultrasound was able, not only to  
432 hydrolyze the free polysaccharide, but also to retrieve part of the fermentable matter from the  
433 lignocellulose structure.

434

### 435 **3.6.2 Effects on the starch granules**

436 A high concentration of starch granules could be observed both around and entangled in the  
437 fibrous matter (Figure 5), reflecting the fact that cassava bagasse is a potential source of  
438 fermentable matter. It reinforces the need to evaluate the effects of both processes on the  
439 structure of starch granules.

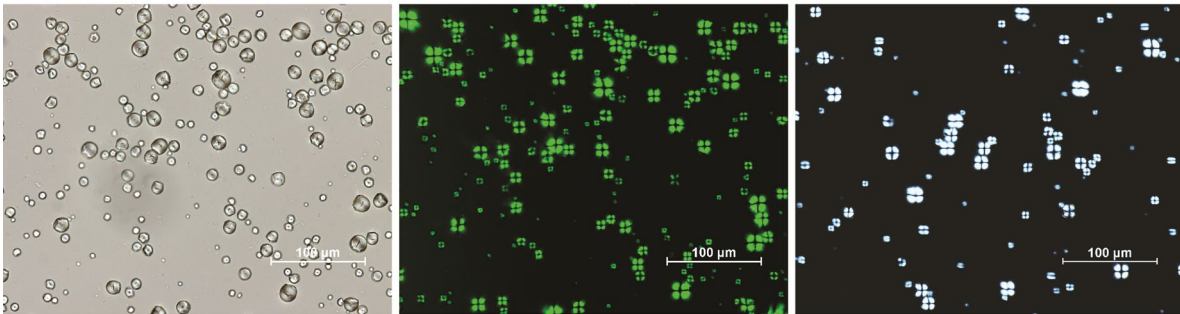
440 In Figure 6, there are images obtained by bright field, fluorescence and polarized light  
441 microscopy of the starch granules before and after acid treatments at 20x of magnitude.  
442 Starch granules in raw cassava bagasse presented regular surfaces with the typical  
443 morphology of cassava starch: oval, truncated and rounded [35]. In this sample, no significant  
444 alterations could be evidenced. The subsequent application of fluorescence and polarized  
445 light exhibited the birefringence properties of starch granules. The high light intensity of the  
446 Maltese cross in the grains was an evidence of strong birefringence, even after 4 hours of  
447 prior agitation in aqueous medium.

448 When cassava bagasse was subjected to acid hydrolysis under conventional agitation, the  
449 alterations were not easily detectable by observation under bright field mode. On the other  
450 hand, a reduction in the granules' birefringence could be observed, as indicated by white  
451 arrows. It may be stated that agitation could promote adequate contact of phosphoric acid  
452 with the bagasse, affecting the crystalline structure of the starch granules.

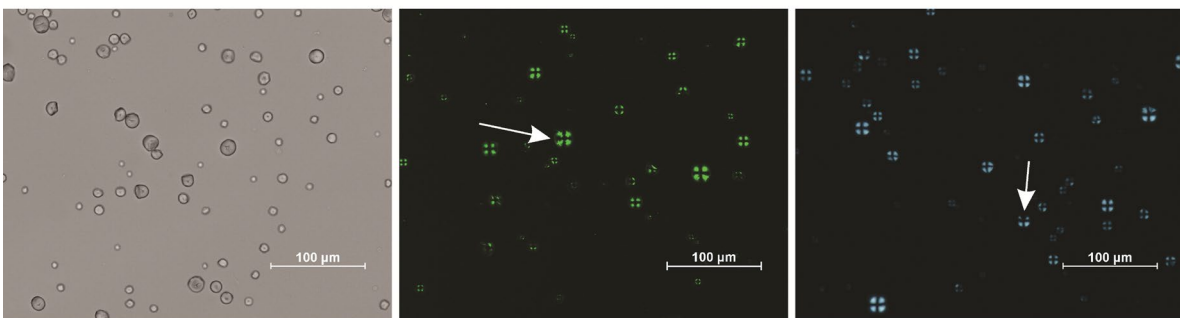


453 The application of ultrasound during the acid treatment led to a clearer degradation of the  
454 starch granules observed through bright field mode. The effects of acid seemed to be  
455 intensified by ultrasound application. There were cracks and surface irregularities in the  
456 granules, which were not observed in the raw bagasse or in the hydrolyzed samples under  
457 mechanical agitation. Monroy et al. [21] reported that ultrasound has a more pronounced  
458 effect on the surface roughness of the cassava starch than on the shape itself. The authors  
459 attributed these alterations to the collapse of the cavitation bubbles, which cause high  
460 pressure gradient and shear forces in the surrounding area to depolymerize amylose and  
461 amylopectin the longer the process carries on. This degradation generates micro channels in  
462 the granules, which are useful for enhancing digestibility by glucoamylase, for example [36].  
463 Analogously, as the interaction between enzyme and starch was reported to be enhanced by  
464 US, the same mechanism could be applied to phosphoric acid in solution. The presence of  
465 micro-pores in the granule may be responsible for intensifying acid penetration and  
466 promoting structural disorganization. Sujka and Jamroz [37] also observed the  
467 depolymerization of different starches, with the consequent changes in morphology and  
468 swelling properties caused by the increase in water absorption after ultrasonic treatment.  
469

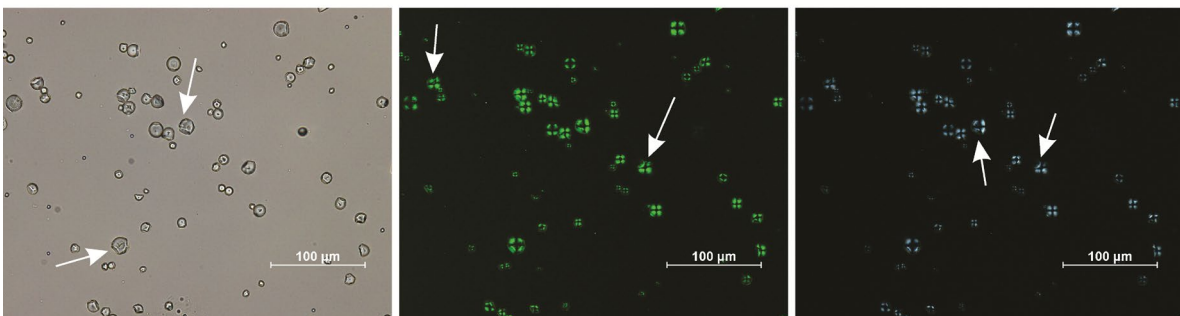
### Before Hydrolysis



### Conventional Agitation



### Ultrasound-assisted



470

471 **Figure 6.** Bright field (left), fluorescence (middle) and polarized light (right) micrographs of  
 472 starch granules from cassava bagasse before and after acid treatments under conventional  
 473 agitation or by high-intensity ultrasound (20x).

474

475 These alterations are reinforced by a greater decrease in the degree of birefringence of US-  
 476 treated bagasse when compared to that conventionally agitated, observed in fluorescence and

477 polarized light microscopies. According to Muñoz et al. [38], the granule swelling is  
478 preceded by a rapid loss of birefringence and tends to continue even after the complete loss  
479 of birefringence. The Maltese cross presented a gradual decrease from the hilum towards the  
480 surface as a consequence of the absorption of the acid solution by the granules. It is a result  
481 of the greater loss of radial organization of amylose and amylopectin chains [39]. Tao et al.  
482 [40] also observed loss of birefringence by the technique of polarized light microscopy  
483 throughout the gelatinization of starch granules.

484 These findings concur with the observations made in the stained fibrous matter and with the  
485 kinetics data of sugar release, which demonstrated a more pronounced effect of ultrasound  
486 than agitation on hydrolyzing solid particles. The degradation of the starch surface by  
487 cavitation indicates that there is a significant release of carbohydrates from the granules and  
488 better accessibility to chemicals and enzymes could be acquired in further steps of bioethanol  
489 production.

490

#### 491 **4. Conclusions**

492 Ultrasound application enhanced the hydrolysis of cassava bagasse in the presence of  
493 phosphoric acid increasing the kinetics of the process. Thus, compared to experiments carried  
494 out with agitation, the sugar release of ultrasonically assisted experiments was 52-70% higher  
495 after only 30 min of processing. The Naik model adequately fitted the experimental kinetics  
496 of sugar release indicating a decrease of ultrasound influence at the end of the process. The  
497 reduction in the acoustic power produced by the increase in viscosity could weaken the  
498 ultrasound effects.

499 Conventional agitation and US application provided a good interaction between the catalyst  
500 and the biomass, reducing the birefringence of the starch granules in both processes. In  
501 addition, US brought about a slight change in the fibrous matter and a more pronounced  
502 disorganization of the crystalline structure of the starch granules, which could enhance the  
503 later accessibility to chemicals and enzymes in further steps of bioethanol production  
504 Therefore, ultrasound technology represents an alternative means of treating biomass for the  
505 purposes of altering the structure and releasing fermentable matter. It may also account for a  
506 shortening of the processing time and a reduction in chemical usage in further steps involved  
507 in bioethanol production.

508

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514

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