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1 **IONIC LIQUID-WATER MIXTURES ENHANCE PRETREATMENT AND**
2 **ANAEROBIC DIGESTION OF AGAVE BAGASSE**

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18

ABSTRACT

19

20 Agave bagasse (AB), a byproduct of tequila production, is a waste generated in large
21 quantities and an emerging alternative for biofuels production. Nevertheless, the use of
22 lignocellulosic biomass for this purpose requires a pretreatment that maximizes its
23 degradation and utilization. Although there are several pretreatment types, using ionic
24 liquids (IL) has become attractive due to its ability to dissolve the lignocellulosic biomass
25 under mild conditions of time and temperature. As a drawback are the high cost of IL's and
26 their high viscosity; thus mixing them with water is a way to overcome these hindrances.
27 This work aimed to evaluate the effect of pretreatment with three diluted ILs on the *A.*
28 *tequilana* bagasse structure and sugars production, as well as the potential of the enzymatic
29 hydrolysate to produce methane in batch mode. The evaluated ILs were: 1-ethyl-3-
30 methylimidazolium acetate [Emim][OAc], choline lysinate [Ch][Lys] and ethanolamine
31 acetate [EOA][OAc]. The results showed that the IL with the best performance was
32 [Ch][Lys], which not only offered the highest yields of sugar production (0.57 ± 0.03 g total
33 sugars / g bagasse) but also, it is possible to use it at 30% in a mix with water obtaining
34 similar yields as with pure IL. Enzymatic hydrolysis of IL pretreated AB achieved 50.7 kg
35 sugars and 49.3 kg of sugars for 90%-[Ch][Lys] and 30%-[Ch][Lys], respectively per 100
36 kg of untreated biomass. The enzymatic hydrolysate from the 30%-[Ch][Lys] pretreated
37 AB was able to achieve 0.28 L CH₄/g COD_{fed}, demonstrating that both sugar and methane
38 yields are maintained when pure or 30%-[Ch][Lys] are used for pretreatment, representing
39 a step forward towards process feasibility.

40

41 **Keywords**

42 Agave bagasse; bionic liquid, biogas; methane; saccharification

43 1. INTRODUCTION

44 Current world energy demand and concern about the environmental problems generated by
45 the use of fossil fuels motivates the search for alternative energy sources that are capable of
46 supplying the requirements of human society along with a lower environmental impact.
47 Biofuels and bioproducts generation from lignocellulosic biomass has been considered a
48 sustainable alternative as this is the most abundant plant material on the planet. Worldwide
49 generation of lignocellulosic biomass reaches 1300 million tons per year as a waste product
50 of multiple human activities such as agriculture, livestock, and industry (Baruah et al.,
51 2018).

52 The production of commercially valuable chemicals and biofuels using lignocellulose-
53 based processes has the potential to decrease greenhouse gas emissions, bring benefits to
54 rural economies, and promote energy security (Bhatia et al., 2020). Likewise, the multi-step
55 valorization of low-cost lignocellulosic biomass into a variety of chemicals, fuels and bio-
56 products in a biorefinery concept will have a great social and economic impact at regional
57 level through maximizing local resources to promote industry development, generate
58 added-value and create employment (Da Costa Lopes et al., 2018; Manzanares, 2020).

59 Agave is considered to be an energy crop with features suitable for commercial application,
60 among which stands out its high biomass productivity even in arid and semi-arid lands that
61 are typically not suitable for crop cultivation due to their excellent water use efficiency in
62 comparison to traditional bioenergy feedstocks (e.g., corn stover) (Pérez-Pimienta et al.,
63 2017a). An emerging strategy for the whole valorization of agave is the use of the
64 lignocellulosic fiber (agave bagasse, AB) for biogas production through the anaerobic
65 digestion process; however, the use of lignocellulosic biomass for this purpose has not been
66 widely adopted because the complex structure of the cell wall of plant tissue makes it

67 resistant to microbial attack (Breton-Deval et al., 2018; Weber et al., 2020). Therefore, the
68 use of biomass pretreatments to maximize degradation is crucial. Among the different
69 pretreatments reported on AB, ionic liquid (IL) pretreatment has demonstrated to achieve
70 desirable sugar conversion (>90% glucan and >85% xylan conversion) after the enzymatic
71 saccharification step, while during biofuels generation has been able to achieve 125 kg
72 methane and 121 kg ethanol per Ton of untreated AB as shown in different reports (Pérez-
73 Pimienta et al., 2020, 2017c).

74 ILs are organic salts with low melting points constituted of organic cations and inorganic or
75 organic anions. Some of these ILs are powerful solvents for lignocellulosic materials,
76 which is due to the basicity of the IL anion, which establish hydrogen bonds with the
77 biomass polymers, dissolving the cellulose under mild conditions of time and temperature
78 (Da Costa Lopes et al., 2018; Sun et al., 2016). In addition, ILs can be recovered for further
79 reuse. However, their main disadvantages are the high cost and viscosity, thus using
80 aqueous solutions of ILs could be an alternative that reduces both inconveniences. We
81 previously reported that when AB is pretreated with mixtures of imidazolium-based ILs (1-
82 Ethyl-3-methylimidazolium acetate [Emim][OAc] and 1-butyl-3-ethylimidazolium acetate
83 [C4C1Im][OAc]) and water, a decrease in pretreatment efficiency was only found in
84 systems with a water content above 40% (Pérez-Pimienta et al., 2017b).

85 Recently, ILs containing choline cations and amino acid anions (e.g., cholinium lysinate
86 [Ch][Lys]), also named bionic liquids or bio-derived ILs have caught the attention of
87 researchers. These IL are less toxic to enzymes and microorganisms than imidazolium-
88 based ILs while maintaining high delignification and sugar production yields (Sun et al.,
89 2014; Sun et al., 2016). Using pure [Ch][Lys] in AB at optimized pretreatment conditions
90 (124 °C, 205 min, and 20% solids loading) demonstrated to achieve a high sugar yield of

91 0.514 g total sugars per g AB (Pérez-Pimienta et al., 2020). Another generation of
92 economic and easily synthesized ILs called protic ILs (PILs), formed in a one-step reaction
93 where a Bronsted-Lowry acid transfers a proton to a Bronsted-Lowry base, has recently
94 emerged (Greaves and Drummond, 2015). Ethanolamine acetate [EOA][OAc] is a PIL that
95 has been effective to pretreat a wide range of feedstocks from grasses (switchgrass) to
96 woody biomass (eucalyptus and pine) capable of maintaining its sugar yield in the presence
97 of [EOA][OAc] at 10 wt%, which is a great advantage when compared to imidazolium-
98 based ILs that required multiple water-washes to remove it (Li et al., 2011; Sun et al., 2017).
99 Using IL-water mixtures as pretreatment agents could reduce viscosity, eliminate gel
100 formation during pretreatment and could significantly reduce the energy requirements and
101 costs associated with IL recycling. Furthermore, the improved biocompatibility and lower
102 synthesis costs of certain ILs such as [Ch][Lys] and [EOA][OAc] when compared to
103 [EOA][OAc] provides a potential way to improve the economy of a biorefinery (Gschwend
104 et al., 2020; Liu et al., 2018).

105 To the best of our knowledge, there are no reports in the current literature comparing the
106 effect of these different classes of ILs on the pretreatment of AB using IL-water mixtures
107 that allow reducing IL usage and cost. Thus, the objective of this work was to evaluate the
108 effect of different IL-water mixtures using different class of ILs ([Emim][OAc], [Ch][Lys],
109 and [EOA][OAc]) on the pretreatment of AB. Compositional analysis and different
110 characterization techniques were applied on the untreated and pretreated biomass to
111 evaluate the impact of pretreatment on the cell wall structure. Subsequent anaerobic
112 digestion of the enzymatic hydrolysates obtained at the best conditions was also evaluated
113 to estimate the methane production potential.

114

115 2. MATERIALS AND METHODS

116 2.1. Experimental design

117 Three different IL-water mixtures were prepared diluting each of the evaluated ILs
118 ([Emim][OAc], [Ch][Lys] and [EOA][OAc]) in water at different proportions (90-10%,
119 70-30%, 50-50%). Subsequently, the AB was pretreated using these mixtures, and after
120 pretreatment, the biomass was washed, dried, and enzymatically saccharified to determine
121 the most suitable ratio to increase sugars recovery. Based on the delignification and sugars
122 yield obtained, the IL-water mixture with the best performance was determined, and the
123 pretreated AB was characterized and compared with the untreated AB. Finally, the
124 enzymatic hydrolysate of AB pretreated with the most appropriate IL-water mixture was
125 used as a substrate for methane production in batch assays.

126 To select the best IL-water mixture, a set of unifactorial designs were used for pretreatment
127 with each ionic liquid, where the response variable was the sugar yield per gram of AB, and
128 the factor evaluated was the percentage of IL in the mixture. All the experiments were
129 carried out in duplicate and the statistical analyses (one-way ANOVA and Tukey HSD
130 post-hoc analyses at 99% confidence level ($p < 0.01$)) were performed by using
131 Statgraphics Centurion XV.

132

133 2.2. Materials

134 *Agave tequilana* bagasse was obtained from the traditional tequila production process of
135 the Casa Herradura distillery, which involves cooking AB for 24 hours at 90-100 °C and its
136 subsequent grinding and pressing to extract the fermentable juices. The bagasse generated
137 from this process was washed in order to remove residual sugars. Subsequently, it was
138 dried at 60 °C and ground using a Pulvex mill equipped with a 20-mesh screen sieve

139 (Pulvex Plastic, Mexico City, Mexico). Commercial enzymatic cocktail CTec2 with a filter
140 paper activity of 161 FPU/mL was a gift from Novozymes. Ethanolamine ($\geq 99.5\%$ w/v),
141 glacial acetic acid ($\geq 99.5\%$ w/v) were used for [EOA][OAc] synthesis and purchased from
142 Soluciones Analíticas Integrales (Mexico).

143 Aqueous choline (46% w/v), L-lysine monohydrate ($\geq 99.0\%$ w/v), and acetonitrile ($\geq 99.5\%$
144 w/v) were used for [Ch][Lys] synthesis and purchased from Sigma-Aldrich (Mexico).
145 Others chemicals including [Emim][OAc] ($\geq 95.0\%$ w/v), citric acid ($\geq 99.0\%$ w/v), sodium
146 hydroxide ($\geq 98.0\%$ w/v), D-glucose ($\geq 99.5\%$ w/v), D-xylose ($\geq 99\%$ w/v), phenol ($\geq 99\%$
147 w/v), sulfuric acid ($\geq 98.0\%$ w/v) were also purchased from Sigma-Aldrich (Mexico).

148

149 **2.3. Pretreatment with IL-water mixtures**

150 The IL-water mixtures were made at 90, 70, and 50% (by weight). Pretreatment for each IL
151 was carried out with pure IL and IL-water mixtures, except [Ch][Lys] where the maximum
152 concentration at which it was used was 90% due to its high viscosity making it challenging
153 to use at a higher concentration. Each of the IL-water mixtures was combined with AB in a
154 60 mL ACE glass pressure tube at 20% solids loading (20 g of IL-water mixture plus 5 g of
155 AB). Pretreatments were performed in a convection oven under optimal conditions for each
156 IL (Table 1) (Pérez-Pimienta et al., 2021, 2020, 2019). After the pretreatment, 40 mL of DI
157 water was added to each pressure tube, and then the pretreated bagasse was washed five
158 times with distilled water in order to ensure that excess IL was removed. Once washed, the
159 pretreated sample was dried in an oven at 45 °C for 48 h to obtain the recovery of solids by
160 weight difference with respect to the initial untreated AB.

161

162 **2.4. Enzymatic saccharification**

163 The enzymatic hydrolysis of pretreated AB was carried out by dispersing 4% (w/v) of the
164 pretreated solids in 50 mM citrate buffer solution (pH 4.8). Cellic CTec2 multienzymatic
165 cocktail was added at a concentration equivalent to 8 filter paper units (FPU) per g of
166 bagasse in dry weight, and the reaction took place in an incubator at 50 °C for 72 h
167 (Arreola-Vargas et al., 2016). The characterization of the hydrolysates was carried out by
168 quantifying total sugars (TS) (simple sugars, oligosaccharides, polysaccharides, and their
169 derivatives), hexoses, pentoses, inhibitory compounds, volatile fatty acids (VFAs), and
170 chemical oxygen demand (COD). The sugar yield was calculated as follows:

$$171 \text{ Sugar yield } \left(\frac{\text{g TS}}{\text{g biomass}} \right) = \frac{\text{TS } \left(\frac{\text{g}}{\text{L}} \right) * \text{reaction volume (L)}}{\text{g pretreated biomass}} * \% \text{recovered solids} \quad (1)$$

172

173 **2.5. Methane production**

174 After pretreatment and enzymatic hydrolysis, methane production was carried out from
175 enzymatic hydrolysate obtained at the best condition in an AMPTS II system (Bioprocess
176 Control, Lund Sweden), which consists of 15 mechanically stirred reactors (Arreola-Vargas
177 et al., 2016). During the experimental runs, the accumulated gas volume, temperature, and
178 pressure were measured in real time and were automatically recorded by the AMPTS II,
179 which generated a report with the accumulated gas and flow values. The inoculum used at
180 10g VSS (volatile suspended solids)/L was an anaerobic granular sludge from a UASB
181 reactor treating tequila vinasses at Casa Herradura distillery.

182 These experiments were carried out in triplicate with a working volume of 360 mL at 37
183 °C, with a mechanical stirring of 150 rpm and a mineral medium adjusted at pH 8 as
184 previously reported (Arreola-Vargas et al., 2016). The substrate for the anaerobic digestion
185 experiments were enzymatic hydrolysates from pretreated AB at a concentration of 5 g

186 TS/L, which were compared with glucose as a positive control. Endogenous metabolism
187 and enzyme control experiments were also run.

188

189 **2.6. Analytical methods**

190 Compositional analysis of the untreated and pretreated AB was carried in terms of soluble
191 compounds, cellulose, hemicellulose, and lignin by using a semiautomatic fiber analyzer
192 (ANKOM Technology, Macedon, NY, USA) (Soest et al., 1991). TS produced from the
193 enzymatic saccharification (simple sugars, oligosaccharides, and polysaccharides) were
194 quantified by using the methodology reported by DuBois et al. (1956). In addition, hexoses
195 and pentoses were quantified using an Agilent 1200 Series HPLC system with a refraction
196 index detector and an Aminex HPX-87P column. For VFA the column Aminex HPX-87H
197 with a diode detector was used. The column temperature was maintained at 40 °C with a
198 flow of 1.0 mL/min and a water-acetonitrile mobile phase. Glucan and xylan conversions
199 were calculated as previously described (Pérez-Pimienta et al., 2017c). The COD was
200 obtained using the standardized method of APHA 5220 using vials TNT 822 in a DRB200
201 digester and a DR2800 spectrophotometer. The performance of the bioreactors for methane
202 production was recorded automatically by the AMPTS II.

203 The attenuated total reflectance (ATR)-FTIR spectroscopy was conducted using an infrared
204 IR2 module coupled to a microscope of a HORIBA Jobin Yvon micro-Raman confocal
205 spectrometer model LabRam HR-800. The lateral order index (LOI) was calculated by
206 using absorbance (A) ratio as shown in the following equation (Karimi and Taherzadeh,
207 2016):

$$208 \quad LOI = \frac{A_{1430}}{A_{898}} \quad (2)$$

209 A_{1430} = absorbance reading at 1430 cm^{-1}

210 A_{898} = absorbance reading at 898 cm^{-1}

211 Additionally, the total crystallinity index (TCI) was calculated as follows:

$$212 \quad TCI = \frac{A_{1378}}{A_{2900}} \quad (3)$$

213 A_{1378} = absorbance reading at 1378 cm^{-1}

214 A_{2900} = absorbance reading at 2900 cm^{-1}

215 Untreated and pretreated samples were placed on-site in a low emissivity slide in the
216 infrared over the equipment platen. Spectra from untreated and pretreated samples were
217 obtained with an average of 32 scans (in triplicates) over the range between 800 cm^{-1} and
218 4000 cm^{-1} with a spectral resolution of 4 cm^{-1} .

219 X-ray powder diffraction patterns of untreated and pretreated samples were collected with a
220 Rigaku Miniflex 600 diffractometer equipped with a high-velocity Dtex ultra detector and
221 operated at 40 kV and 15 mA using Cu- $K\alpha$ radiation ($\lambda = 1.54 \text{ \AA}$). Patterns were collected
222 in the 2θ range of 5 to 50°, the step size was 0.01°, and a velocity of 3°/min. The
223 crystallinity index (CrI) of the biomass samples were determined according to Segal et al.
224 (1959):

$$225 \quad CrI(\%) = \frac{I_{total} - I_{am}}{I_{total}} * 100 \quad (4)$$

226 where I_{total} is the scattered intensity at the main peak, whereas I_{am} is the scattered intensity
227 due to the amorphous portion.

228 Morphology of untreated and pretreated AB were analyzed using a high-resolution
229 scanning electron microscope (SEM) by a JEOL JSM-7800F equipment. The images were
230 acquired with a 1 kV accelerating voltage and analysis using 5 kV. Energy-dispersive X-ray
231 spectroscopy (EDS) was used simultaneously with SEM imaging for the determination of

232 element content. Confocal fluorescence microscopy images from untreated and pretreated
233 AB were captured using a Carl Zeiss LSM 710 NLO coupled with two laser sources (405
234 and 633 nm). All samples were labeled with Calcofluor white stain (0.1 %) for 5 min in
235 order to show microstructure based on lignin and cellulose distribution (autofluorescence).

236

237 **3. RESULTS AND DISCUSSION**

238 **3.1. Effect of pretreatment with IL-water mixtures**

239 One of the main effects of IL pretreatment is the dissolution of the plant cell wall
240 components, which affects the amount of solids recovered after pretreatment. Therefore,
241 Fig. 1 shows the pretreatment performance with IL-water mixtures in terms of solids
242 recovery, sugar production, and sugar yield. The IL that offered the highest solids recovery
243 was [Emim][OAc], with values above 90% for the 50% IL-water mixture.

244 However, the ANOVA showed that there is no statistical significance (P value=0.05) on the
245 recovery of solids from the different [Emim] [OAc]-water mixtures. Recently, we
246 demonstrated that long times and high temperatures significantly decreased the recovery of
247 solids and sugars produced during the optimization of process conditions (time, temperature,
248 and solids loading) with [Emim][OAc] (Pérez-Pimienta et al., 2019). These results indicate
249 that pretreatment severity plays a more critical role in the recovery of solids than the
250 amount of water present in the IL. On the contrary, with [EOA] [OAc] there was a
251 significant effect on the recovery of solids from the pretreated AB ($P = 0.003$), being more
252 significant when the [EOA][OAc] was at 50%. The latter suggests that fewer components
253 dissolve during pretreatment when the amount of water in the mixture increases.

254 The results obtained in saccharification show apparent differences between the three
255 studied ILs to favor the hydrolysis process (Fig. 1). The quantity of sugars produced by the

256 pretreated AB with undiluted [Emim][OAc] is consistent with the values previously
257 reported that obtained around 27 g/L (Pérez-Pimienta et al., 2016). However, the 70% and
258 50% IL-water mixtures with [Emim][OAc] had very similar production to the sugars
259 produced by untreated AB (~ 5 g/L) as shown by Pérez-Pimienta et al. (2019), which
260 indicates that this IL loses effectiveness in the presence of water and suggests that the
261 pretreatment did not produce any important change in the structure and composition.

262 For the pretreated AB with [EOA][OAc], the quantity of water does not seem to have such
263 a severe detrimental effect, since the production of sugars is very close to that of pure IL in
264 the 90 and 70% mixtures and is only significantly lower when used at 50%. The pretreated
265 AB with [Ch][Lys] not only produced the highest amount of sugars in saccharification but
266 also, no significant difference was observed in any of the mixtures ($P = 0.52$).

267 Sugar yield depends on both sugars released during the saccharification process and solids
268 recovered after pretreatment. Fig. 1 shows that [Ch][Lys] achieved the highest sugar yield
269 per gram of AB and [Emim][OAc] the lowest. In the case of [Emim][OAc], the results
270 using pure IL are consistent with a recent report that employs the same pretreatment
271 condition in AB, obtaining a sugar yield of 0.53 g TS/g biomass (Pérez-Pimienta et al.,
272 2019).

273 This IL disrupts the extended hydrogen bond network in the lignocellulosic matrix,
274 including hydrogen bonds in fibrils of highly crystalline cellulose (Li et al., 2010). The
275 regenerated material after [Emim][OAc] pretreatment exhibits high yields after
276 saccharification due to the higher accessibility of the sugar polymers and the low
277 crystallinity of cellulose. However, the statistical analysis for pretreatment with this IL
278 reveals that the amount of water in the mixture significantly affects sugar yield ($P =$
279 0.0046), which is evident by comparing the pretreatment using pure IL (0.52 g TS/g

280 biomass) vs. 50% IL-water mixture (0.17 g TS/g biomass). The significant decrease in the
281 effectiveness of [Emim][OAc] is consistent with a previous report, where it was suggested
282 that even small amounts of water present in this IL (10%) affect the proton acceptor
283 capacity of the acetate anion, a property that allows [Emim][OAc] to dissolve
284 lignocellulosic biomass (Doherty et al., 2010).

285 Previous studies have also recommended to add 10–40% water (ionosolv pretreatment) for
286 effective fractionation of lignocellulosic biomass (Brandt et al., 2011; Gschwend et al.,
287 2016).

288 In this strategy, the IL-water mixture fractionates lignocellulose through dissolving lignin
289 and hemicellulose but leaving behind the cellulose as a filterable solid. It is hypothesized
290 that a fraction of water in IL is necessary for: (1) hydrolysis reactions that are required to
291 separate the components from each other (hydrolysis of lignin ether bonds, ferulic acid
292 ester bonds, and glycosidic hemicellulose bonds), (2) to avoid sulfation reactions between
293 the hydrogen sulfate and hydroxyl groups in the biomass, and (3) to reduce the viscosity of
294 the solvent (Brandt-Talbot et al., 2017).

295 Fig. 1 also shows that the highest sugar yields for all IL-water mixtures were achieved with
296 [Ch][Lys]. Since the AB pretreatment effectiveness with [Ch][Lys] did not show any
297 significant change, and even the yield slightly increased at the lowest concentration, it was
298 decided to test two additional concentrations to evaluate up to what dilution the
299 performance of this IL is affected. The selected concentrations were at 30 and 10% IL.
300 These concentrations were chosen because the literature has already evaluated the
301 performance of [Ch][Lys] at concentrations as low as 10% in the pretreatment of sorghum
302 and corn stover, obtaining glucose yields of ~80% (Sundstrom et al., 2018; Xu et al., 2016).

303 Fig. 2 presents the sugar yields obtained from pretreated AB with [Ch][Lys] at different

304 concentrations. The sugar yield resulted significantly lower when the AB was pretreated
305 with the 10% IL-water mixture ($P = 0.0001$).

306 Despite that previous reports on [Ch][Lys] diluted to 10% showed high effectiveness in the
307 pretreatment, several factors are considered, such as the type of biomass, solids loading,
308 and time/temperature. In the case of the 30% mixture, the high solids recovery contributed
309 to an increase in the sugar yield, and, consequently, to the fact that there was no significant
310 difference between the mixtures with higher IL content ($P=0.07$). From this analysis, it is
311 concluded that it is possible to use [Ch] [Lys] mixed with water at a concentration as low as
312 30% and obtain no statistical significance when compared to more concentrated IL (90%).
313 The latter presents several advantages, such as having better mass transfer during the
314 process, a decrease in the amount of IL used, and, consequently, a lower cost of
315 pretreatment. It is important to note that all of the saccharification experiments during the
316 present study were carried out with pretreated biomass that was extensively washed to
317 remove the ILs because the main aim of the study was to determine the effect of the IL-
318 water mixtures pretreatment on the sugar yield. However, visualizing that these extensive
319 washing steps will be complex in scaled-up processes, the effect of the three ILs was also
320 evaluated on the saccharification step. Supplementary Fig. S1 shows that even at low
321 concentrations, such as 3.5% (by weight), the three ILs exert a negative impact on the
322 saccharification performance of the CTec2 enzymatic complex. In the case of [Ch][Lys],
323 the effect it exerts on saccharification does not seem to increase drastically with any
324 concentration used. Future studies are needed to integrate the pretreatment step with the
325 enzymatic hydrolysis.

326

327 **3.2. Characterization of the enzymatic hydrolysate**

328 In order to determine if there is any difference between the enzymatic hydrolysates
329 produced by the saccharification of the pretreated AB with concentrated [Ch][Lys] (90%)
330 and diluted [Ch][Lys] (30%), both hydrolysates were characterized in terms of relative
331 abundance of sugars by HPLC (Table 2). Additionally, as the most economically and
332 suitable option for downstream processes, the hydrolysate pretreated with 30% [Ch] [Lys]
333 was extensively characterized, as shown in Table S1. The similarity between
334 monosaccharides and cellobiose in the hydrolysates demonstrates that not only the
335 effectiveness of the pretreatment in terms of sugar yield (g TS/g biomass) is similar
336 between the pretreated AB with concentrated IL and that diluted to 30%, but also the
337 composition of the enzymatic hydrolysates is similar. This suggests that the IL's mechanism
338 on the biomass during AB pretreatment is similar in both cases and supports the idea that it
339 is not necessary to use the concentrated IL to pretreat AB. Similarly, a previous report
340 analyzed the composition of the enzymatic hydrolysate produced by AB pretreated with
341 [Emim][OAc], and found that it was constituted by ~47% glucose, ~25% xylose and ~28%
342 cellobiose where the same process conditions were employed as in the current study (Pérez-
343 Pimienta et al., 2019).

344 It is then possible to conclude that pretreated AB with [Ch][Lys] (either diluted or
345 concentrated) presents modifications that favor a more complete hydrolysis of cellulose,
346 since these hydrolysates have a lower amount of cellobiose and their main component is
347 glucose. Based on the data presented in Table 2, it is possible to infer that the enzymatic
348 hydrolysate produced from pretreated AB with 30% [Ch][Lys] will be a suitable substrate
349 for methane production, since it contains C₅ and C₆ monosaccharides easily consumed by
350 anaerobic digestion microorganisms and does not present relevant amounts of compounds
351 that could be inhibitory for them (Table S1). As previously mentioned, this is one of the

352 advantages of using enzymatic hydrolysis after pretreatment, since a large amount of sugar
353 is released and practically no other degradation compounds are produced.

354

355 **3.3. Mass balance and structural changes of pretreated AB with [Ch][Lys]**

356 Mass balances for two pretreatment conditions (90%-[Ch][Lys] and 30%-[Ch][Lys]) were
357 performed to develop a clear overview of the pretreatment and saccharification processes.

358 Sugar yields were normalized to a common basis of 100 kg of dry AB as starting material.

359 A simplified representation is shown in Fig. 3 to illustrate both pretreatment conditions and
360 the amounts of each major plant cell wall component, solids recovery, and sugar production.

361 In terms of solids recovery after pretreatment, the 30%-[Ch][Lys] pretreated sample
362 recovered more solids than the 90%-[Ch][Lys] pretreated sample, 80.6% and 74.8%,

363 respectively; in both cases, the recovered material contained lower levels of residual lignin.

364 Compared to the untreated sample, lignin reductions of 33.1 and 18.6% were obtained for

365 the 90% and 30% [Ch][Lys] pretreated AB, respectively. Glucan values reported in the

366 literature for untreated AB agree with our results (26-46%), while for xylan they are

367 slightly higher (15-23%) (Pérez-Pimienta et al., 2017a).

368 For both pretreatments, a relative increase in the percentage of glucan and xylan was

369 observed (Fig.3). As previously mentioned, the high sugar yields observed for both

370 pretreatments (Fig. 1) were not only due to the increase in the digestibility of the bagasse

371 but also to the small loss of the initial AB carbohydrates. Finally, per 100 kg of untreated

372 bagasse, the enzymatic hydrolysates achieved 50.7 kg sugars for 90%-[Ch][Lys] pretreated

373 AB and 49.3 kg of sugars for 30%-[Ch][Lys] pretreated AB.

374 The X-ray diffractograms of untreated and [Ch][Lys] pretreated AB are shown in Fig. 4.

375 Biomass crystallinity as one important factor of enzymatic saccharification can be

376 measured as a whole (considering cellulose, hemicellulose and lignin) using XRD while
377 FTIR analysis determines the relative crystallinity of cellulose in the cellulose-rich material
378 (Morais et al., 2016). It is possible to observe that the peak located at 22.1° in the untreated
379 AB, changed at a smaller angle (20.8°) and became wider in the pretreated AB, which is
380 indicative of a change from crystalline cellulose type I to cellulose type II (Pérez-Pimienta
381 et al., 2017c).

382 The crystallinity index (CrI) values were 41.1% for the untreated AB while for the
383 pretreated samples were 31.7% ([Ch][Lys] at 90%) and 23.0% ([Ch][Lys] at 30%). The
384 crystalline structure of cellulose is usually modified in IL pretreatment either by the
385 breaking of hydrogen bonds between the cellulose chains or by the selective removal of
386 other amorphous components of the biomass.

387 These changes in the crystallinity index are not consistent with other reports that employed
388 [Ch][Lys] in biomass pretreatment since they found that there was practically no variation
389 in the crystallinity index of the pretreated biomass, probably due to the removal of other
390 components such as lignin (Hou et al., 2017, 2012; Liu et al., 2017). However, it is
391 essential to mention that in these reports, there is not a clear correlation between the
392 crystallinity index of cellulose in the [Ch][Lys] pretreated biomass and the efficiency in its
393 saccharification (Hou et al., 2012).

394 In addition, LOI and TCI parameters were calculated from the untreated and [Ch][Lys]
395 pretreated AB samples. The LOI of untreated AB was 1.255 while the LOI for pretreated
396 samples were 0.990 or [Ch][Lys] at 90% and 1.415 for [Ch][Lys] at 30%. The TCI is
397 proportional to the crystallinity degree of cellulose, reflecting more disordered structure at
398 low values (Dri et al., 2013; Karimi and Taherzadeh, 2016). The TCI of untreated AB was
399 0.972 while values for pretreated samples were 0.696 ([Ch][Lys] at 90%) and 0.876

400 ([Ch][Lys] at 30%). The overall decrease in LOI and TCI of the pretreated samples when
401 compared to the untreated AB indicates that IL pretreatment weakens the biomass ordered
402 cellulose structure. Only the LOI of AB-[Ch][Lys] at 30% increased possibly due to an
403 interference of the CaOX peaks on the absorbance reading. The changes in the CrI, LOI
404 and TCI parameters confirm that IL pretreatment with [Ch][Lys] can alter lignocellulose
405 structure decreasing its recalcitrance for downstream processing.

406 Since the compositional analysis of the AB does not indicate that pretreatments with
407 [Ch][Lys] at 90 and 30% removed large amounts of hemicellulose or lignin, the decrease in
408 cellulose crystallinity was probably due to a different mechanism, where the amount of
409 water present had a significant effect. The peaks in the spectrum of untreated AB found at
410 angles 14.8° , 24.2° , 30.0° , and 38.0° correspond to monohydrated calcium oxalate, whose
411 presence has already been widely reported in this type of biomass (Pérez-Pimienta et al.,
412 2019). Calcium oxalate crystals serve essential functions within plants, such as regulation
413 of calcium in tissues, protection, and detoxification of heavy metals. In particular, plants
414 with crassulacean acid metabolism (CAM), such as cacti or agave, have higher levels of
415 calcium oxalate compared to other sources of biofuels (Pérez-Pimienta et al., 2017a).

416 The effect of pretreatment with [Ch][Lys] at 90 and 30% on the removal of calcium oxalate
417 from AB is evident in the decrease in peak intensity; and, from both pretreatments, [Ch]
418 [Lys] at 30% shows a better performance in this regard. The effectiveness of IL
419 pretreatment to remove oxalate of calcium from agave bagasse has been previously
420 reported with [Emim] [OAc] (Pérez-Pimienta et al., 2017a).

421 ATR-FTIR was also used to evaluate the modifications produced by pretreatment in AB
422 with [Ch][Lys] at 90 and 30% (Fig. 5). Seven bands were used to monitor chemical
423 changes in lignin and carbohydrates and the relationship between crystalline and

424 amorphous cellulose, plus two additional bands for variations in calcium oxalate. The
425 relative changes are summarized in Table S2. The most noticeable changes occurred in the
426 bands corresponding to calcium oxalate (1321 and 1622 cm^{-1}), where there is a significant
427 reduction, consistent with the decrease of this compound in the pretreated biomass. There
428 are differences between the two pretreatments in the bands corresponding to amorphous
429 cellulose (900 cm^{-1}) and to the bonds and hydrogen bonds of cellulose (2900 and 3348 cm^{-1}),
430 which indicates that although both had an effect on AB, it was different according to the
431 IL concentration, and may also be related to the differences in the crystallinity of the
432 pretreated samples.

433 One of the most particular peaks in lignocellulosic biomass is the one corresponding to
434 1510 cm^{-1} , attributed to the aromatic skeleton of lignin. However, it is not possible to
435 analyze it in agave bagasse due to the presence of the peak corresponding to calcium
436 oxalate at 1622 cm^{-1} , which covers that area and prevents observing the pretreatment
437 changes.(Pérez-Pimienta et al., 2017c) Despite this, the relative change in the 1745 cm^{-1}
438 peak, associated with the stretching of the carbonyl group, indicates the cleavage of lignin
439 and secondary chains.(Pérez-Pimienta et al., 2019)

440 The images obtained by scanning electron microscopy of the untreated and pretreated AB
441 with [Ch][Lys] at 90 and 30% were taken with a magnification X500 (Fig. 6 A-C) and
442 X5,000 (Fig. 6 D-F). The untreated AB exhibits a homogeneous morphology, with a
443 compact fibrillar structure and with microstructures of similar size on the surface.

444 Morphological changes resulting from pretreatment with [Ch][Lys] at 90 and 30% are
445 evident, as the formation of pores, loss of homogeneity, and swelling of the fibers are
446 observed. These results are consistent with those obtained with XRD and FTIR, and the
447 increase in the hydrolysis rate of the pretreated AB previously discussed since the IL

448 pretreatment had an apparent effect on the fibers, generating a greater surface area and
449 accessibility to the chains of carbohydrates. Additionally, it is possible to appreciate in the
450 untreated AB crystals of a more or less homogeneous size, corresponding to calcium
451 oxalate. The amount of these crystals and their uniformity also seem to be affected by both
452 pretreatments, as observed in the images with higher magnification (Fig. 6 D-F). The
453 images obtained by confocal microscopy provide an idea of the distribution of
454 carbohydrates on the surface of the analyzed fibers (Fig. S2), where the distinctive
455 fluorescence intensities for lignin (blue) and cellulose (green) are observed. In the image
456 corresponding to the untreated AB, cellulose is mainly seen in a homogeneous and compact
457 form, where the lignin located closely between the fibers is visualized. The effect of
458 pretreatment with [Ch] [Lys] at both concentrations on the AB can be clearly observed
459 since, in the untreated sample, large amounts of agglutinated lignin are seen on the
460 cellulose, while the micrographs corresponding to the pretreated bagasse, a decrease in the
461 amount of lignin that covers the cellulose portion is perceived.

462 Although the pretreatment did not achieve a very high delignification in the structure, a loss
463 of homogeneity in the fibers and delocalization of the remaining lignin is observed, which
464 could contribute to the greater digestibility of the pretreated AB. Visually, no significant
465 differences are perceived between bagasse pretreated with [Ch][Lys] at 90 and 30%, which
466 is an indication that pretreatment with both concentrations could have followed similar
467 mechanisms.

468

469 **3.4. Production of methane from enzymatic hydrolysates of pretreated AB with [Ch]**
470 **[Lys] at 30%**

471 The enzymatic hydrolysate of AB with [Ch][Lys] turned out to be a suitable substrate for
472 methane production, as shown in Fig. 7. After 14 days, 616 ± 43 mL of methane were
473 produced, a slightly higher production than the positive control (glucose), 561 ± 30 mL.
474 The contribution of methane produced by endogenous metabolism was evaluated separately
475 and subtracted from the total production of the other reactors. The fact that the hydrolysate
476 has generated a slightly higher production than the control is explained by the additional
477 COD in the enzymatic complex used during saccharification. Fig. 7 shows that the
478 enzymatic complex produces around 25% of the enzymatic hydrolysate methane production,
479 which agrees with our previous observations (Arreola-Vargas et al., 2016).

480 Although the methane yield is slightly lower in the hydrolysate compared to the positive
481 control (0.28 vs. 0.35 L CH₄/g COD_{fed}), the methane production rate in the hydrolysate is
482 higher (0.29 vs. 0.25 L CH₄/L*day), this is probably because this substrate is composed of
483 different easily assimilated sugars (C₅ and C₆) and additional trace elements, which could
484 favor the activity of microorganisms. The COD removal in the enzymatic hydrolysate was
485 $87.3 \pm 2.5\%$, and the methane yield reached close to 75% of the theoretical value (0.35 L
486 CH₄/g COD_{fed}).

487 This value is slightly higher compared to a recent study that obtained a yield of 0.26 L
488 CH₄/g COD using enzymatic hydrolysate of pretreated AB with pure [Ch][Lys].(Pérez-
489 Pimienta et al., 2020) Interestingly, in the present study no volatile fatty acids (VFAs) were
490 found at the end of the operation, which indicates that all the VFAs produced during the
491 acidogenesis stage were completely consumed for methane production. In recent years, the
492 reports on methane production from IL pretreated biomass have increased but still limited
493 when compared to others bioproducts. In a recent report by Duran-Cruz et al., (2021), the
494 authors employed steam explosion for 15 min at 142 °C for methane production from AB

495 obtaining a methane yield of 0.28 NL CH₄/g COD_{fed}. On the other hand, Gao et al. (2013)
496 studied four different feedstocks (water hyacinth, rice straw, mango leaves and spruce)
497 using [Bmim][Cl] pretreatment at 120 °C for 2 h obtaining up to 233 mL CH₄/g
498 carbohydrates in 140 days. In another report, Xie et al., (2016) carried out the simulation
499 using Aspen Plus for biogas upgrading using imidazolium based ILs ([Hmim][Tf₂N],
500 [Bmim][Tf₂N] and [Bmim][PF₆]) obtaining a 11% reduction in energy consumption in
501 [Bmim][Tf₂N], which suggest the promising potential of IL pretreatment for biogas
502 upgrading. Table 3 presents some others relevant works regarding methane production with
503 various lignocellulosic substrates subjected to different pretreatments. The methane yield
504 obtained with the AB hydrolysate pretreated with 30% IL is comparable to that obtained
505 with others feedstocks and pretreatments. The most-reported studies from AB were carried
506 out by using acid hydrolysis as pretreatment. In this sense, it is essential to note that the
507 yields obtained with IL pretreatment and subsequent saccharification have been comparable
508 or even higher. Also, some disadvantages that occur when acid hydrolysis is performed,
509 such as the production of inhibitors and corrosion, are not observed with ILs. However, and
510 as indicated in Table 3, the production of methane from AB hydrolysate pretreated with ILs
511 has only been evaluated in batch and carrying out the conventional washing step of the
512 bagasse after pretreatment. Recent reports on AB valorization have proposed the use of
513 water (Aguirre-Fierro et al., 2020) or carbon dioxide (Navarro et al., 2021) at high pressure
514 as well as others approaches (e.g. Organosolv) (Robles et al., 2018) to improve
515 pretreatment. Nonetheless, IL pretreatment has a high potential to improve AB valorization
516 including selective extraction of xylan or lignin, bioproducts generation (including new
517 lignin derived ILs) as well as tailor made ILs for AB to name a few. Therefore, further
518 studies should be carried out on the scaling-up of both IL pretreatment and anaerobic

519 digestion, especially with biocompatible ILs that could improve the robustness of current
520 reactors to apply one-pot schemes and continuous systems.

521

522 **4. CONCLUSIONS**

523 The use of IL-water mixtures proved to be effective for AB pretreatment, obtaining high
524 sugar yields comparable to pure ILs especially for diluted [Ch][Lys] at 30%. The increased
525 digestibility after pretreatment was mainly due to delignification and a decrease in cellulose
526 crystallinity.

527 On the other hand, the enzymatic hydrolysate of pretreated AB with [Ch][Lys] at 30%
528 achieved a yield of 0.28 L CH₄/gCOD_{fed}, comparable with other reports using pure IL
529 during pretreatment as well as to other feedstocks and pretreatments. This study highlights
530 the potential of employing IL-water mixtures that implicitly lead to a lower total process
531 cost while allowing easier handling due to its lower viscosity and achieving high methane
532 generation.

533

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720

721 **Table 1.** Pretreatment conditions of AB in different ILs.

IL	Solid loading (%)	Temperature (°C)	Time (min)
[Emim][OAc]	20	119	142
[EOA][OAc]	20	160	205
[Ch][Lys]	20	160	90

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724 **Table 2.** Relative abundance of glucose, xylose and cellobiose in the hydrolysates obtained
725 from AB pretreated with [Ch][Lys] at 30 and 90% concentration.

Enzymatic hydrolysate	% Glucose	% Xylose	% Cellobiose
90 %	65.8±5.2	25.7±2.1	3.3±0.1
30 %	67.5±0.5	29.5±0.4	2.9±0.1

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727

728 **Table 3.** Methane production from AB and others feedstocks.

Feedstock	Pretreatment	Reactor configuration	Yield	Reference
Apple waste and swine manure (25-75)	-	CSTR	0.24 L CH ₄ /g COD _{fed}	(Kafle and Kim, 2013)
Barley straw	NMMO*	Batch	0.23 Nm ³ CH ₄ /kg VS	(Kabir et al., 2014)
Sugarcane bagasse	Alkaline (Na ₂ CO ₃)	Batch	0.264 L CH ₄ /g COD _{fed}	(Nosratpour et al., 2018)
Woody residues	NMMO*	Batch	0.15 Nm ³ CH ₄ /kg VS	(Kabir et al., 2014)
AB	Acid hydrolysis	Batch	0.09 (single stage) and 0.24 (two stage) L CH ₄ /g COD _{fed}	(Arreola-Vargas et al., 2016)
AB	Enzymatic hydrolysis	Batch	0.18 L CH ₄ /g VS	(Arreola-Vargas et al., 2016)
AB	Steam explosion	Batch	0.28 L CH ₄ /g COD _{fed}	(Duran-Cruz et al., 2021)
AB	[Ch][Lys]	Batch	0.26 L CH ₄ /g COD _{fed}	(Pérez-Pimienta et al., 2020)
AB	[Ch][Lys]-30%	Batch	0.28 L CH ₄ /g COD _{fed}	This study

729 *NMMO: N-methylmorpholine-N-oxide.

Figures captions

No	Title
1	Effect of AB pretreatment with IL-water mixtures on solids recovery (top), sugar production (medium) and sugar yield (bottom). Error bars represent the standard deviation.
2	Sugar yield (g TS/g biomass) of pretreated AB with [Ch][Lys] at different IL-water mixtures. Error bars represent the standard deviation.
3	Mass balance for pretreatment using [Ch][Lys] at 90 and 30% concentration followed by enzymatic saccharification from 100 kg of untreated AB.
4	XRD pattern from untreated and pretreated AB with [Ch][Lys] at 90 and 30%.
5	FTIR spectra of untreated and pretreated AB with [Ch][Lys] at 90 and 30%.
6	Scanning electron microscopy images of untreated AB (A, D), pretreated AB with [Ch][Lys] at 30% (B, E) and pretreated AB with [Ch][Lys] at 90% (C, F). Images A, B and C have a magnification of X500 while images D, E and F have a magnification of X5000.
7	Batch methane production from the enzymatic hydrolysate of pretreated AB with [Ch][Lys] at 30%. Glucose was used as positive control and the enzyme control was used to measure methane generated by the enzymatic cocktail. Methane generation by endogenous metabolism was evaluated separately and subtracted from the experiments. Error bars represent the standard deviation.

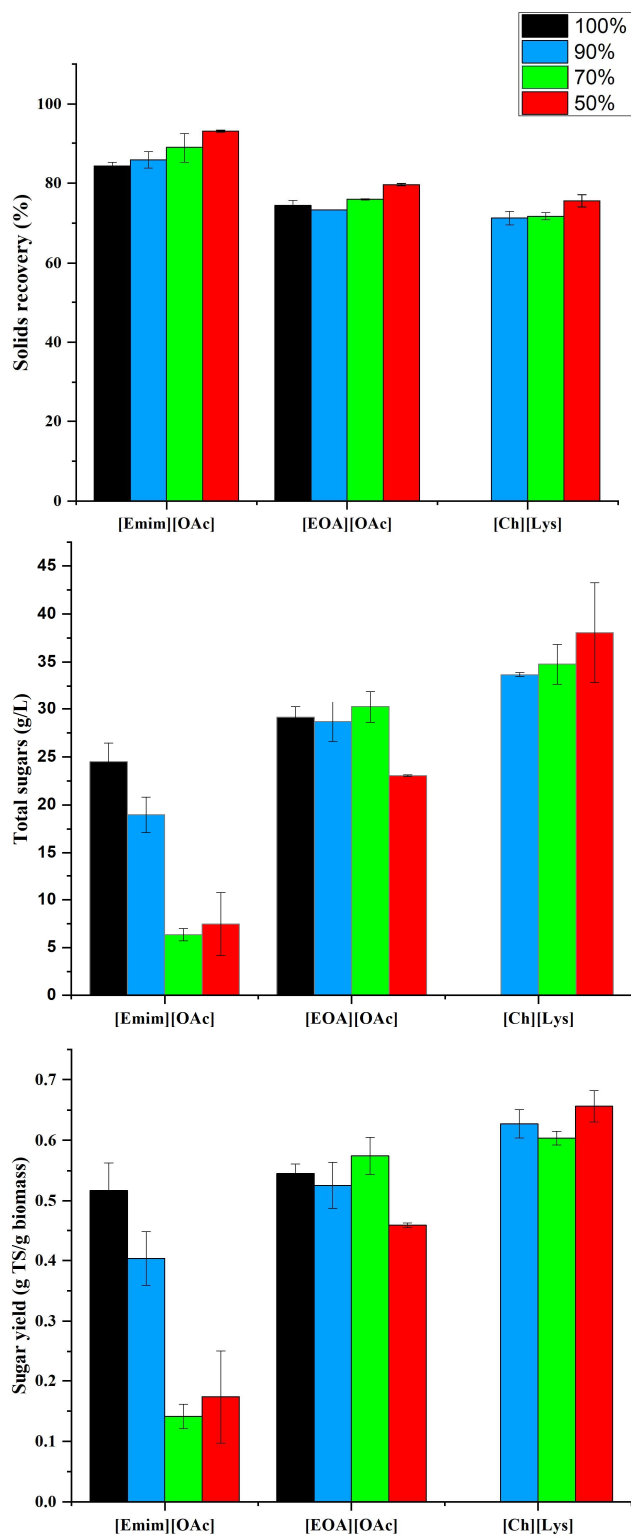


Figure 1.

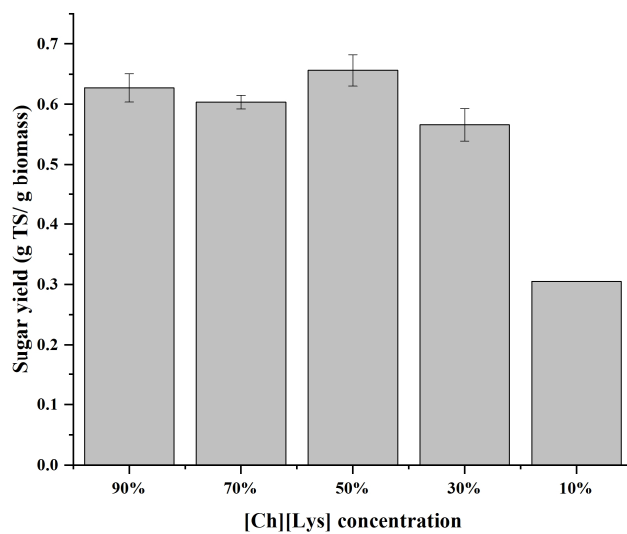


Figure 2.

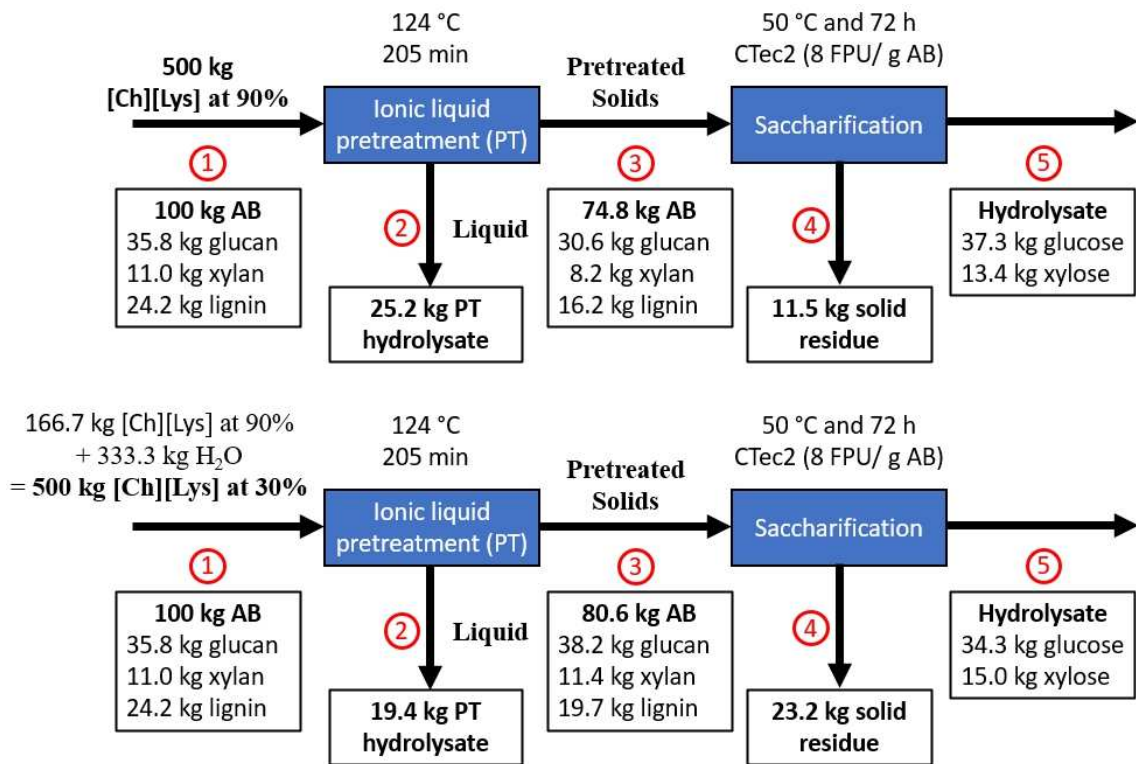


Figure 3.

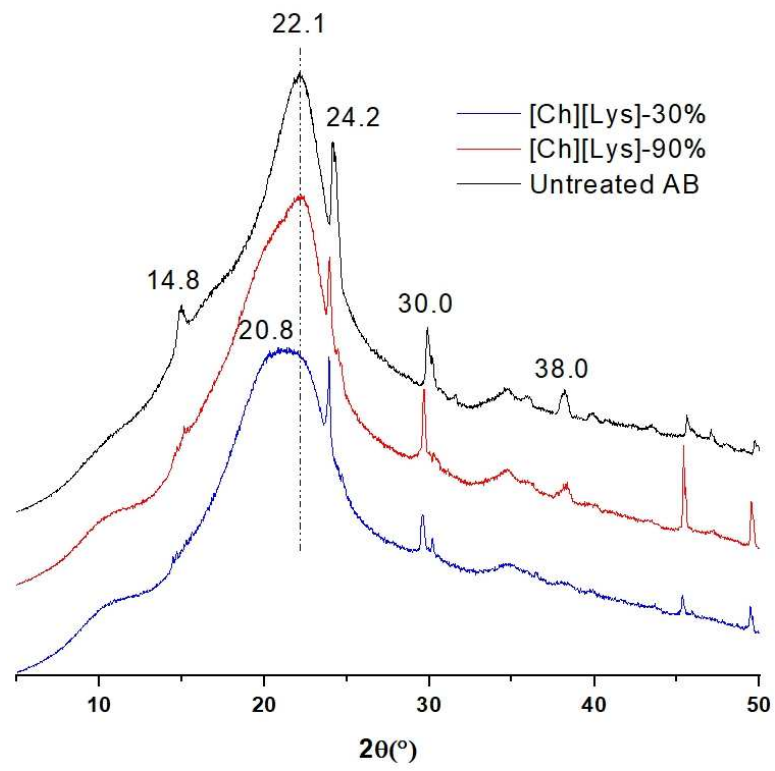


Figure 4.

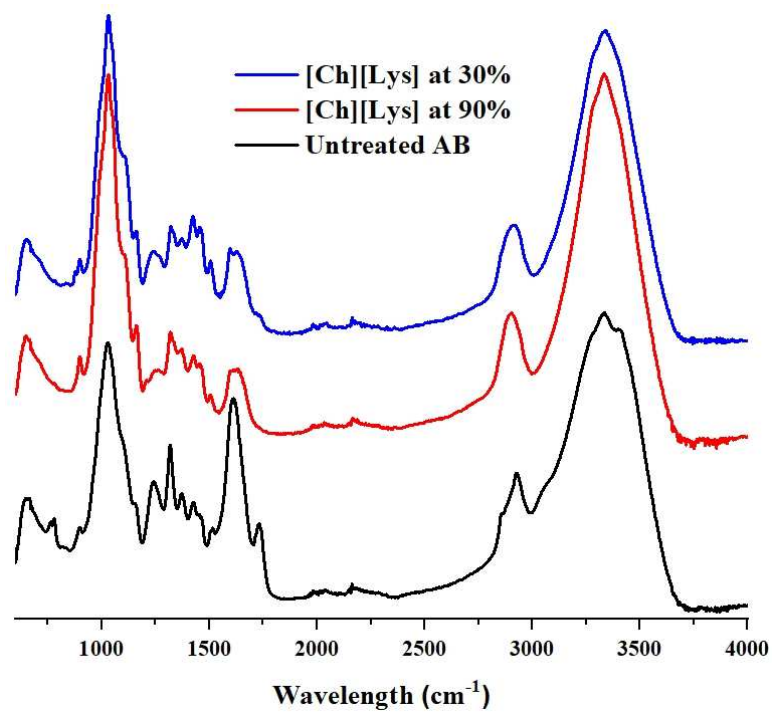


Figure 5.

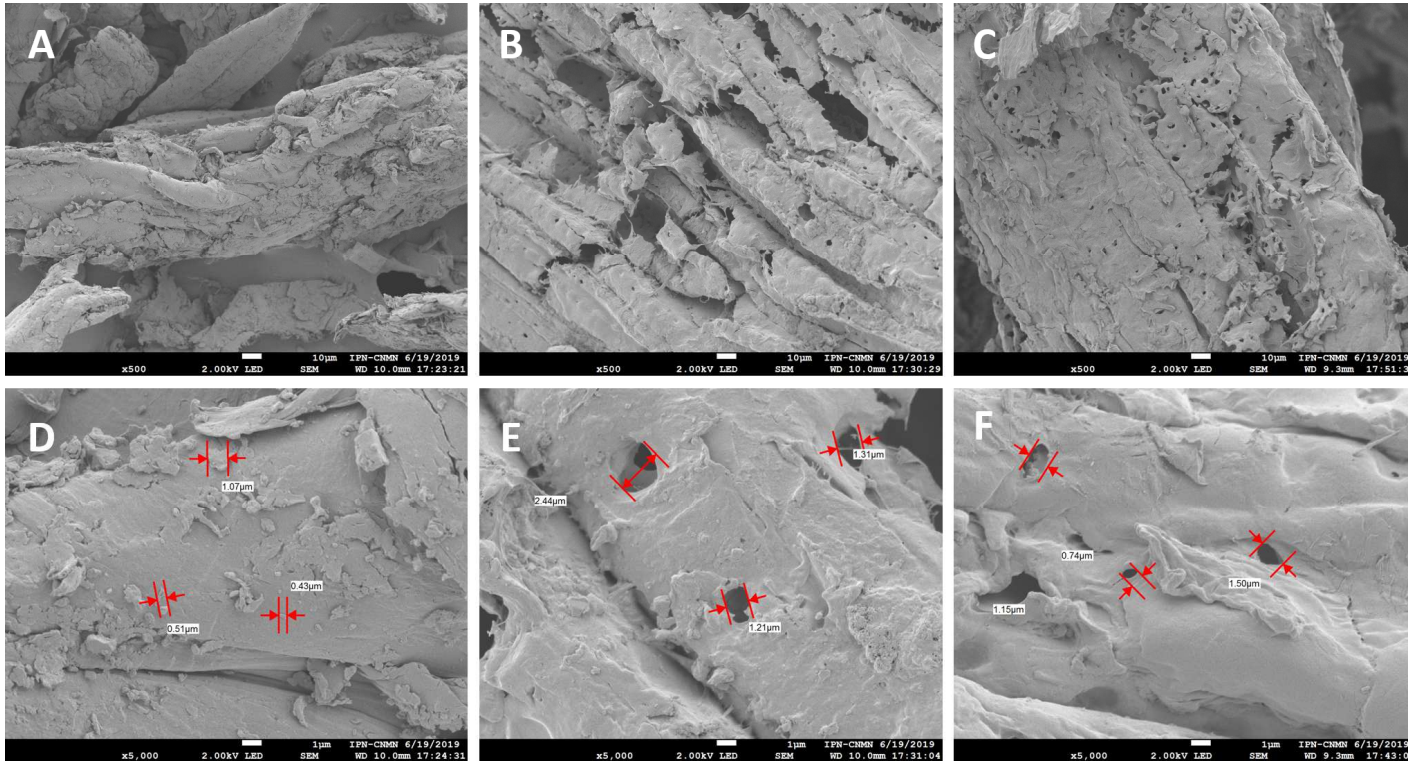


Figure 6.

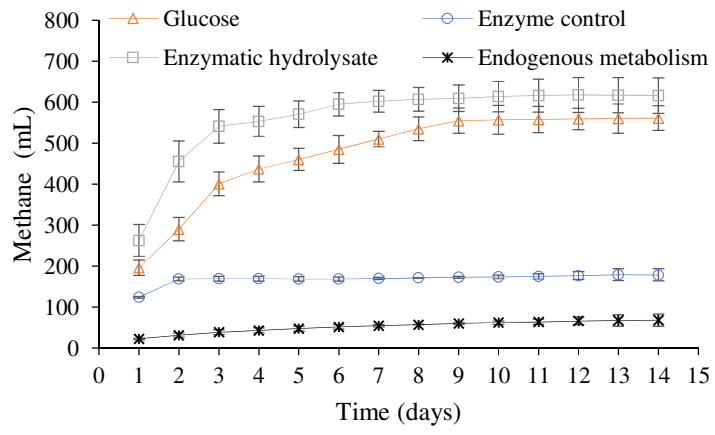


Figure 7.