



## Enhancing the biorefinery of chestnut burrs. Part I. Study of the pretreatment with choline chloride urea diluted deep eutectic solvent

Iván Costa-Trigo<sup>a</sup>, Alicia Paz<sup>a</sup>, María Guadalupe Morán-Aguilar<sup>a</sup>, Nelson Pérez Guerra<sup>b</sup>, Ricardo Pinheiro de Souza Oliveira<sup>c</sup>, José Manuel Domínguez<sup>a,\*</sup>

<sup>a</sup> Industrial Biotechnology and Environmental Engineering Group “BiotecnIA”, Chemical Engineering Department, University of Vigo (Campus Ourense), As Lagoas s/n, Campus del Agua, Rúa Canella da Costa da Vela, 12, 32004, Ourense, Spain

<sup>b</sup> Department of Analytical and Food Chemistry, Faculty of Sciences, University of Vigo (Campus Ourense), As Lagoas s/n, 32004, Ourense, Spain

<sup>c</sup> Biochemical and Pharmaceutical Technology Department, Faculty of Pharmaceutical Sciences, São Paulo University, Av. Prof. Lineu Prestes, 580, Bl 16, São Paulo, 05508-900, Brazil

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### ABSTRACT

Chestnut burrs (CB) are agro-industrial wastes produced in large amounts during chestnut processing. Despite their high value composition, this type of biomass has been scarcely studied, becoming an important material to be valorized. In this work, a green technology based on the use of diluted choline chloride urea (ChCl:U) deep eutectic solvent (DES) was postulated to deconstruct the lignocellulosic structure. The pretreatment was evaluated using untreated CB and CB previously processed (washed CB or the solid residue obtained after prehydrolysis). Following a biorefinery concept, the samples obtained in this work will be further enzymatically hydrolyzed in Part II to reach fermentable sugars-containing solutions. Several operational parameters such as time (4, 8 and 16 h), temperature (60, 80, 100 and 120 °C), and liquid-solid ratio (LSR) (10:1, 15:1 and 20:1) were studied to improve CB deconstruction. Physicochemical transformations were investigated by ATR-FTIR and SEM analysis. The best delignification rate (~40%) was achieved applying the DES pretreatment in untreated CB at 100 °C for 16 h and 20:1 (w/w) LSR, being corroborated by physicochemical changes observed by FTIR and SEM.

### 1. Introduction

Circular bioeconomy aims to use biomass to produce bio-products, biochemicals, and bioenergy from an economically and environmentally sustainable point of view. This concept emerges as an answer to the society, which is turning to “reuse and recover” resources to attain “healthy environment-healthy human being” and “socio-economic prosperity” [1,2]. In this sense, biorefineries can transform efficiently lignocellulose into biofuel, biochemical, bioenergy and other value-added products through different integrated technologies, such as thermochemical, biochemical, combustion, or biological processes [2]. Forest, agricultural and food industries generate huge amounts of biomass, whose reutilization could reduce processing costs and minimize environmental impacts [3]. In addition to its high polysaccharide content, the use of lignocellulosic biomass is advantageous due to its low cost and high availability [4]. This work is focused on using wastes from the chestnut processing, because it is an emerging sector in the European

Union, with an increase of harvested area and production from 104,434 ha to 122,819 t in 2014 to 129,730 ha to 301,370 t in 2019 [5]. Spain, with 188,930 t in 2019 was the main EU producer, followed by Italy (39,980 t), Portugal (35,830 t), Greece (28,980 t), and France (7350 t) [5].

Nowadays, DESs have emerged as effective solvents for the pretreatment of biomass in detriment of ionic liquids (ILs). The latter have some limitations for their industrial applicability, such as high relative price and toxicity. DESs could overcome these issues because they are affordable, non-toxic, and easy to synthesize. Besides, DESs present other advantages including biodegradability, recyclability, as well as non-volatile and non-flammable nature. DESs are defined as a combination of different molar ratios of two or more components (a hydrogen bond acceptor, HBA; and a hydrogen bond donor, HBD), whose mixture has lower melting point than the individual compounds [6,7].

Choline chloride (ChCl) is the most widely used HBA. Several natural chemicals can act as HBD, like carboxylic acids, alcohols, polyols, or amines [8]. Particularly, ChCl-urea (ChCl:U) was the first reported DES

\* Corresponding author.

E-mail address: [jmanuel@uvigo.es](mailto:jmanuel@uvigo.es) (J.M. Domínguez).

[9] and since then, several authors studied its effect for pretreatment of biomass [10,11]. Indeed, Ong et al. [12], pointed out that sequential pretreatments with DES could be a solution to improve the efficiency of delignification.

This study is the first report concerning the treatment of chestnut burrs (CB) using a diluted DES formed by ChCl as HBA and urea as HBD. The solid samples obtained will be evaluated in a further work to produce sugar-containing solutions by enzymatic hydrolysis. The effects of temperature, time, and liquid-solid ratio (LSR) on the composition of the recovered carbohydrate-rich materials (CRM) were tested. Additionally, the effect of processing CB by washing or diluted acid hydrolysis, prior the application of DES was also analyzed. ATR-FTIR and SEM techniques were also assessed in both the CRMs and original material to determine the influence of the pretreatments.

## 2. Material and methods

### 2.1. Reagents

ChCl (>99%) and urea (>99.5%) were provided by Sigma Aldrich (Spain). ChCl was maintained in a gel desiccator between 48 and 72 h to keep moisture stable.

### 2.2. Substrates

CB were provided by local cultivars (Vilardevós, Ourense, Spain), homogenized by milling (SOGO mill, SS-5430 models, Sanysan Appliance SL, Valencia, Spain) and stored until further use.

The influence of DES was evaluated in three different substrates: the original CB and the solid samples resulting from the following processing.

#### 2.2.1. Washed CB

CB was mixed with distilled water in a glass bottle with a LSR of 8:1 (v/w) and placed in an orbital shaker (Optic Ivymen System, Comecta S. A., distributed by Scharlab, Madrid, Spain) at 50 °C and 150 rpm for 4 h. Then, the solid residue was recovered by filtration and dried at 50 °C (Celsius 2007, Memmert, Schwabach, Germany). This solid was named W-CB and analyzed according to section 2.4. Meanwhile, the liquid steam was filtered through 0.2 µm pore membranes (Sartorius, Goettingen, Germany) and analyzed by HPLC (Agilent, model 1200, Palo Alto, CA) following the methodology described by Costa-Trigo et al. [13], to quantify free sugars. In addition, total phenolic content (TPC) in this liquid phase was also quantified following as described by Singleton and Rossi [14].

#### 2.2.2. Prehydrolysis of CB

CB were pretreated with diluted H<sub>2</sub>SO<sub>4</sub> under the conditions previously optimized [15]. The solid residue obtained after prehydrolysis was washed with distilled water until neutral pH. Then, the pretreated biomass was dried at 50 °C and the solid recovered was named PreH. Additionally, TPC was also calculated in the liquid obtained following the same methodology previously cited.

### 2.3. DES

#### 2.3.1. Synthesis of DES

DES was prepared mixing ChCl and urea with a molar ratio of 1:2 (mol/mol). The mixture was placed in a water bath at 60 °C with stirring until a colorless liquid was obtained [16]. Then, the mixture ChCl:U was homogenized adding water at the ratio 80:20 DES/water (v/v) under magnetic stirring.

#### 2.3.2. Pretreatment of CB with DES

Initially, the influence of time and temperature on CB deconstruction by DES was studied using ground samples of CB (0.5 g) mixed with

diluted ChCl:U in a LSR of 20:1 (w/w) under different times (4, 8 and 16 h) and temperatures (60, 80, 100 and 120 °C). Once the reaction was finished, 50 mL of antisolvent (water) was added and maintained 30 min under stirring. The carbohydrate-rich materials (CRMs) obtained were washed with 40 mL of water and centrifuged (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 3700 rpm for 15 min, repeating this stage 4 times. Finally, CRMs were dried at 50 °C to remove water and stored until further analysis (section 2.4.). Once these conditions were optimized, the volume of DES with respect to the biomass load was analyzed. Consequently, LSR of 10:1, 15:1 and 20:1 (w/w) were assayed following the same pretreatment methodology previously described. All CRMs obtained after DES pretreatments were stored until further use.

#### 2.3.3. Pretreatment of processed biomass with DES

The samples obtained after prehydrolysis (PreH) or after washing CB (W-CB) were treated with DES under the conditions previously selected, to obtain the pretreated materials PreH + DES and W-CB + DES, respectively.

### 2.4. Analytical methods

#### 2.4.1. Lignocellulosic composition

Quantitative acid hydrolysis was carried out in two steps to characterize the polysaccharide and total lignin content in untreated CB and all CRMs obtained following the NREL/TP-510-42618 methodology reported by Sluiter et al., [17]. Besides, total lignin was calculated as a sum of acid soluble lignin (ASL) and acid insoluble lignin (AIL). Delignification percentage was calculated using equation (1) [18].

$$\% \text{ delignification} = 1 - \frac{\text{lignin \% in pretreated material}}{\text{lignin \% in untreated material}} \times 100 \quad \text{Eq.1}$$

Glucan (PCR), xylan (PXnR) or arabinan (PArR) recovery percentages were estimated according to equation (2).

$$\text{PCR, PXnR or PArR} = \frac{\text{g of glucan, xylan or arabinan in CRM}}{\text{g of glucan, xylan or arabinan in raw material}} \times 100 \quad \text{Eq.2}$$

#### 2.4.2. Attenuated total reflectance fourier-transform infrared (ATR-FTIR) spectroscopy

Attenuated total reflection (ATR) accessory equipped with a diamond crystal (Smart Orbit Diamond ATR, Thermo Fisher, USA) coupled to Infrared spectroscopy equipment (Thermo Nicolet 6700 FTIR Spectrometer, Thermo Fisher Scientific Inc., Madison, WI, USA) was used to characterize at 25 °C untreated CB, W-CB, PreH and all CRMs obtained after DES pretreatment. Deuterated triglycine sulfate (DTGS) KBr detector was employed to analyze dry samples with a resolution of 4 cm<sup>-1</sup> and 20 scans, in the range 4000–400 cm<sup>-1</sup>.

#### 2.4.3. Field emission scanning electron microscopy (FE-SEM)

Sputter Coater (Sputtering Emitech K550X, Quorum Technologies, Kent, UK) was used to analyze for 3 min the dry untreated material and CRMs, which were mounted onto aluminum stubs and coated with gold. The study of the morphological changes of untreated material and CRMs was obtained photographing all the materials by FE-SEM system (Model JSM-6700 F, Jeol, Japan).

### 2.5. Statistic analysis

Statgraphics statistical package (Statgraphics Centurion XVI version 16.1.11) was employed to compare all data through One-Way and Factorial ANOVA. Least standard deviation (LSD) test was used to show statistically significant differences between means with a confidence level of 95%. All experiments were conducted in triplicate and results were presented as mean ± standard deviation.

**Table 1**

Lignin, extractives and polysaccharide composition of untreated and processed CB expressed on a dry weight basis (%). Total phenolic content release after pretreatments in liquid steam was expressed as g GAE/L.

(%)	CB	W-CB	PreH
AIL	20.04 ± 0.30 <sup>a</sup>	22.76 ± 0.89 <sup>b</sup>	37.52 ± 0.25 <sup>c</sup>
ASL	11.26 ± 0.26 <sup>a</sup>	9.16 ± 0.28 <sup>b</sup>	3.68 ± 0.04 <sup>c</sup>
Total lignin	31.31 ± 0.04 <sup>a</sup>	31.92 ± 0.61 <sup>a</sup>	41.13 ± 0.29 <sup>b</sup>
Extractives	12.86 ± 0.25 <sup>a</sup>	6.82 ± 1.21 <sup>b</sup>	9.12 ± 0.12 <sup>c</sup>
Glucan	20.04 ± 0.18 <sup>a</sup>	18.03 ± 0.13 <sup>a</sup>	35.95 ± 0.18 <sup>b</sup>
Xylan	18.86 ± 0.02 <sup>a</sup>	23.70 ± 0.00 <sup>b</sup>	9.06 ± 1.24 <sup>c</sup>
Arabinan	5.56 ± 0.08 <sup>a</sup>	7.28 ± 0.17 <sup>b</sup>	0.00 ± 0.00 <sup>c</sup>
Total polysaccharides	44.46 ± 0.28 <sup>a,b</sup>	49.00 ± 0.31 <sup>b</sup>	43.88 ± 1.44 <sup>a</sup>
TPC (g GAE/L)	–	2.13 ± 0.55 <sup>a</sup>	1.71 ± 0.10 <sup>a</sup>

Different letters between the columns show statistically significant differences ( $P < 0.05$ ).

GAE: gallic acid equivalents.

### 3. Results and discussion

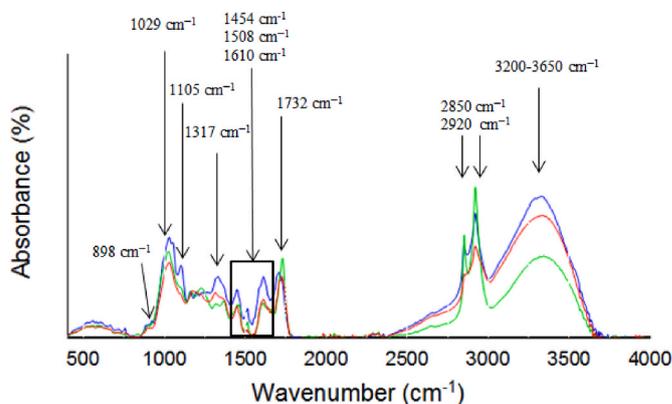
#### 3.1. CB processing

CB are lignocellulosic materials characterized by their rigidity, hardness, and impermeability. The hydrophobicity of CB is mainly attributed to wax, resin and fat [19]. In this sense, the wax layer slow down liquid water penetration [19], what could influence DES pretreatment. For this reason, in order to make easier the accessibility of DES to structural compounds (polysaccharides and lignin) and improve the biomass fractionation, prior to the application of DES, the possibility of partial removal of this layer through washing with distilled water at mild temperature or acid prehydrolysis was evaluated. Thus, both processes were selected according to the most widespread methods used in the literature. Indeed, the water extraction has demonstrated its efficiency in extracting phenols, proteins, free sugars, and pectin [20–22]; meanwhile diluted acid pretreatment with H<sub>2</sub>SO<sub>4</sub> is still used today [23, 24] due to low cost of acids, energy and short operational times needed [25].

Table 1 summarizes the composition of untreated CB used as a control, the washed solid (W-CB) and the residue obtained after a prehydrolysis stage (PreH).

It was observed that the polysaccharide composition was near 45% in untreated CB biomass, mainly due to glucan and xylan content. It was also important to highlight the high lignin content (31.31 ± 0.04%), comprised by acid soluble lignin (ASL) with 11.26 ± 0.26% and acid insoluble lignin (AIL) with 20.04 ± 0.30%, similar to the value of 24.2% of AIL in CB reported by Moure et al., [26]. Besides, ethanol extractives represented 12.86 ± 0.25%, in concordance with Liang et al. [19], who detailed 10.90% of ethanol-benzene extractives. Additionally, they also quantified hot water extractives representing 29.30%.

CB and W-CB exhibited differences in their lignocellulosic composition in ASL, AIL, xylan, arabinan and extractives (Table 1). Thus, it can be observed that water was able to remove 50% of the extractive fraction of CB, from 12.86 ± 0.25 to 6.82 ± 1.21%, being the most remarkable decrease observed in the characterization of W-CB. On the other hand, PreH showed more compositional changes than W-CB and untreated CB, since prehydrolysis combines the concentration of mineral or organic acids and temperature, which improves the biomass deconstruction. As it was reported in our previous work [27], glucan-lignin rich material (PreH) was obtained through prehydrolysis with diluted sulfuric acid due to the solubilization of pentoses decreasing total polysaccharides content until near 44%. During prehydrolysis, hemicelluloses are hydrolyzed into soluble monomers, whereas this pretreatment does not show significant influence in lignin and cellulose [28]. However, other authors confirmed recondensation of lignin after prehydrolysis, as well as a decrease of crystallinity, swelling and depolymerization of cellulose [29].



**Fig. 1.** (A) ATR-FTIR spectra of untreated CB (red line), W-CB (green line) and PreH (dark blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

In addition, ATR-FTIR spectra showed the influence of CB processing (Fig. 1). The superposition of the spectra of untreated CB, W-CB, and PreH supported the changes in chemical composition explained above. Thus, the characteristic peak of hemicellulose (1732 cm<sup>-1</sup>) [30] increased slightly in W-CB regarding the untreated CB sample, which was in concordance with the increment in xylan percentage quantified during the characterization of the samples (see Table 1). The presence of free sugars like glucose (2.43 ± 0.23 g/L) and fructose (5.64 ± 0.07 g/L) detected in the liquid stream derived from W-CB, demonstrated their presence in CB. These free sugars could cause interference in the characterization of CB, overestimating the percentage of glucan, since they are quantified as glucose monomers and underestimating the other fractions what would explain the differences in polysaccharide characterization (xylan and arabinan) between CB and W-CB (Table 1). Besides, bands corresponding to three biopolymers of lignocellulosic biomass bonds vibration, 2850 cm<sup>-1</sup> (C–H stretch O–CH<sub>3</sub> group) and 2920 cm<sup>-1</sup> (C–H stretch methyl and methylene groups) [30–32] achieved the highest absorbances what is in concordance with the increase of xylan and arabinan fractions in W-CB (Table 1). The band between 3200 and 3650 cm<sup>-1</sup> attributed to O–H stretch and H-bonded typical of lignin, hemicelluloses, and cellulose [30] and the peak at 1317 cm<sup>-1</sup> related to C–O stretching of substituted aromatic units decreased their absorbance in W-CB regarding untreated CB. An explanation for this behavior could be linked to the high presence of phenolic compounds in CB [19,26], being water reported as an appropriate solvent to recover them [19,20]. In this regard, this was corroborated since a high concentration of phenolic compounds was solubilized during washing in water achieving a value around 2 g GAE/L (Table 1). Therefore, the abovementioned bands in W-CB could be reduced because of phenolic compounds removal by water what also justify the reduction in ASL content in W-CB compared to untreated CB.

Regarding, the spectrum of PreH showed higher absorbance in cellulose and lignin bands demonstrating its cellulose-lignin-rich composition. Characteristic peaks of lignin appeared at the following wavenumbers: 1105 cm<sup>-1</sup> (plane aromatic deformation C–H), 1454 (methyl and methylene C–H deformation), 1508 (stretching and vibration C–C aromatic ring) and 1610 cm<sup>-1</sup> (aromatic skeleton vibration, C=O stretching in conjugated carboxyl and O–H absorption) [31–33]. Furthermore, bands linked to three biopolymers also increased their intensities such as 1317 cm<sup>-1</sup> (CH<sub>2</sub> wagging and C–O stretching of substituted aromatic units), 2850 cm<sup>-1</sup> and 2920 cm<sup>-1</sup>, between 3200 and 3650 cm<sup>-1</sup> band (O–H stretch, H-bonded typical of the three main lignocellulosic polymers: lignin, hemicelluloses, and cellulose) [30]. Bands corresponding to pretreated biomass in 898 cm<sup>-1</sup> (Stretching C–O–C at β-(1,4) glycosidic linkage in cellulose component) can be observed in PreH spectrum. Finally, a slight increase was observed in 1029 cm<sup>-1</sup> peak which was associated with C–H plane deformation and

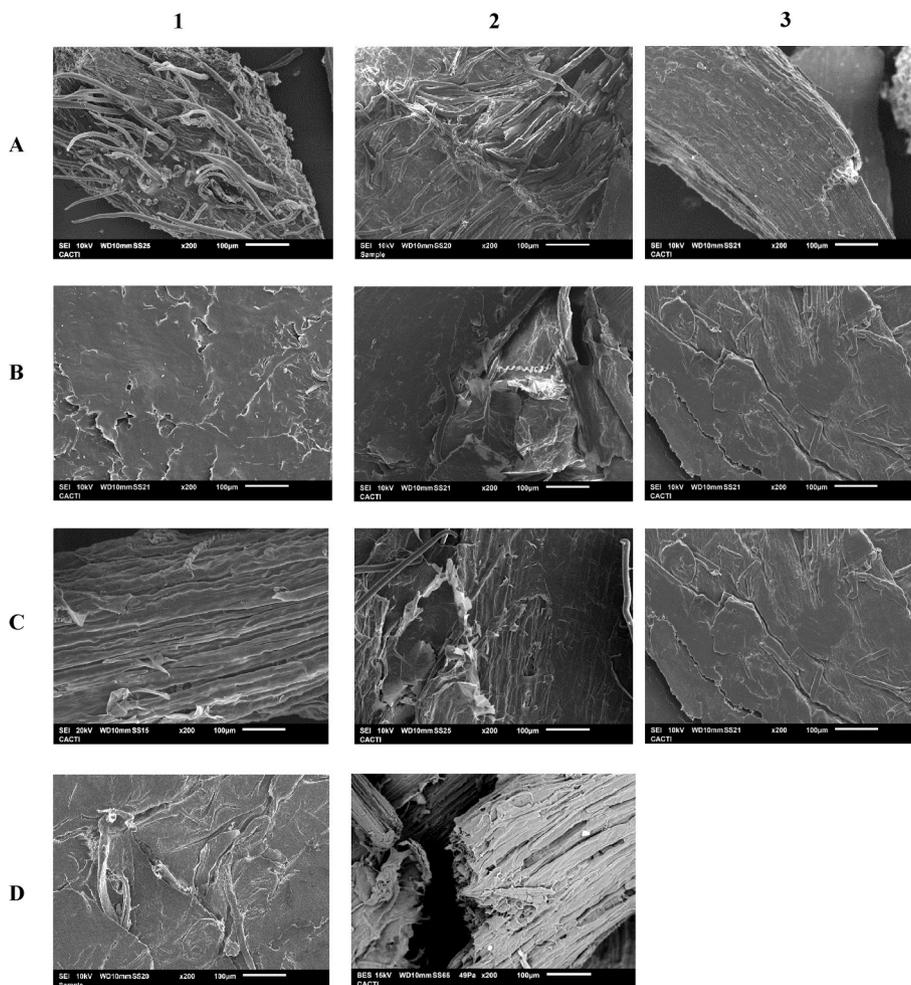


Fig. 2. SEM-photos of samples. First row (A) consists of untreated CB (1), W-CB (2) and PreH (3). Second row (B) shows the influence of time after DES pretreatment (at 120 °C): 4 h (1), 8 h (2), and 16 h (3). Third row (C) disposes the influence of temperature (all for 16 h): 80 °C (1), 100 °C (2), and 120 °C (3). Final row (D) shows SEM photos of W-CB + DES (1) and PreH + DES at 16 h (2).

C–O stretching in cellulose and lignin.

Scanning electron microscopy (SEM) was used to observe differences in morphology of untreated and processed CB (Fig. 2A). Thus, qualitative information related to physicochemical changes could be extracted from SEM images, such as the particle size, detection of microstructures or surface characteristics [34]. In this sense, CB appearance revealed a compact texture, smooth spiny and rigid structure. Similarly, a well-defined spiny surface without breaks nor remarkable alterations could be observed in W-CB, whereas fissures were noted in the PreH material.

3.2. Influence of temperature, reaction time and liquid-solid ratio (LSR) on DES pretreatment

Time, temperature and LSR are key factors for the suitable fractionation of lignocellulosic biomass. Consequently, the influence of these parameters on CB deconstruction by DES was studied. The effectiveness of the treatment could be assessed through the compositional analysis of the lignocellulose, being delignification one of the most important parameters studied. First of all, it was evaluated the effect of time and temperature of DES pretreatment on delignification. For this purpose, the delignification data obtained for each condition were analyzed using a factorial ANOVA to determine the main effects and interactions between parameters.

Initially, the pretreatment with DES was carried out at 60 °C and different times (4, 8 and 16 h). However, the results showed that only

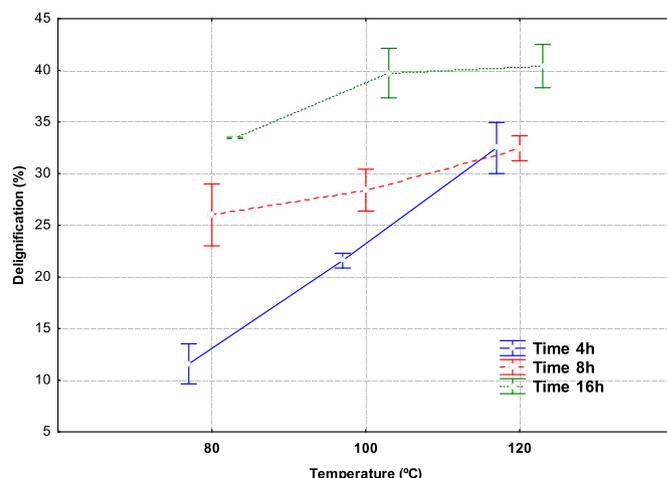


Fig. 3. Main effect of temperature and time in DES pretreatment of CB. Wilks lambda = 0.00137, F(20, 17.533) = 5.5211, P = 0.00035. Vertical bars denote +/- standard errors.

ASL was partially removed at that temperature, without modifying the AIL content, and offering very low delignification results. For this reason, the application of DES at 60 °C was not considered in the subsequent analyses. Moreover, SEM-photos corroborated this observation

**Table 2**

Composition of untreated and pretreated chestnut burrs with ChCl:U under different conditions of time and temperature and percentage of cellulose, xylan and arabinan recovery (PCR, PXnR, PARr). All results are expressed as percentage and experiments were carried out using a LSR 20:1 (w/w).

	Glucan (%)	PCR (%)	Xylan (%)	PXnR (%)	Arabinan (%)	PARr (%)	Acetyl groups (%)
<b>Untreated</b>	23.00 ± 0.20	–	21.64 ± 0.02	–	6.38 ± 0.09	–	3.33 ± 0.48
<b>DES 4 h</b>							
80 °C	30.32 ± 0.54	82.15 ± 2.29	29.88 ± 0.07	86.68 ± 4.13	5.75 ± 0.44	55.67 ± 6.73	6.89 ± 1.33
100 °C	30.37 ± 1.98	73.88 ± 4.99	29.18 ± 3.36	71.95 ± 4.93	5.40 ± 0.63	46.47 ± 5.27	5.35 ± 0.76
120 °C	35.29 ± 0.48	83.22 ± 1.31	30.55 ± 0.32	76.13 ± 0.96	5.58 ± 0.57	46.31 ± 4.79	5.51 ± 0.67
<b>DES 8 h</b>							
80 °C	28.54 ± 1.54	80.20 ± 0.04	26.50 ± 1.43	78.32 ± 2.03	4.92 ± 0.29	43.37 ± 1.61	5.48 ± 0.39
100 °C	32.87 ± 0.04	83.75 ± 3.43	30.00 ± 0.03	81.75 ± 3.32	4.75 ± 0.20	43.18 ± 3.56	4.79 ± 0.36
120 °C	36.71 ± 1.55	83.39 ± 4.81	30.88 ± 0.71	75.01 ± 2.90 <sup>a</sup>	4.88 ± 0.15	39.44 ± 0.56	5.51 ± 0.45
<b>DES 16 h</b>							
80 °C	33.04 ± 1.94	82.69 ± 0.88	31.19 ± 2.30	83.51 ± 0.89	6.37 ± 0.42	56.78 ± 0.61	5.33 ± 0.80
100 °C	38.87 ± 0.35	93.22 ± 1.86	31.74 ± 0.47	79.12 ± 1.58	4.78 ± 0.11	41.42 ± 0.83	3.24 ± 0.25
120 °C	41.39 ± 0.03	97.36 ± 0.80	32.10 ± 0.44	80.46 ± 0.66	3.62 ± 0.46	30.90 ± 0.25	n.d.

PCR: percentage of cellulose recovery, PXnR: percentage of xylan recovery, PARr: percentage of arabinan recovery, n.d.: non detected.

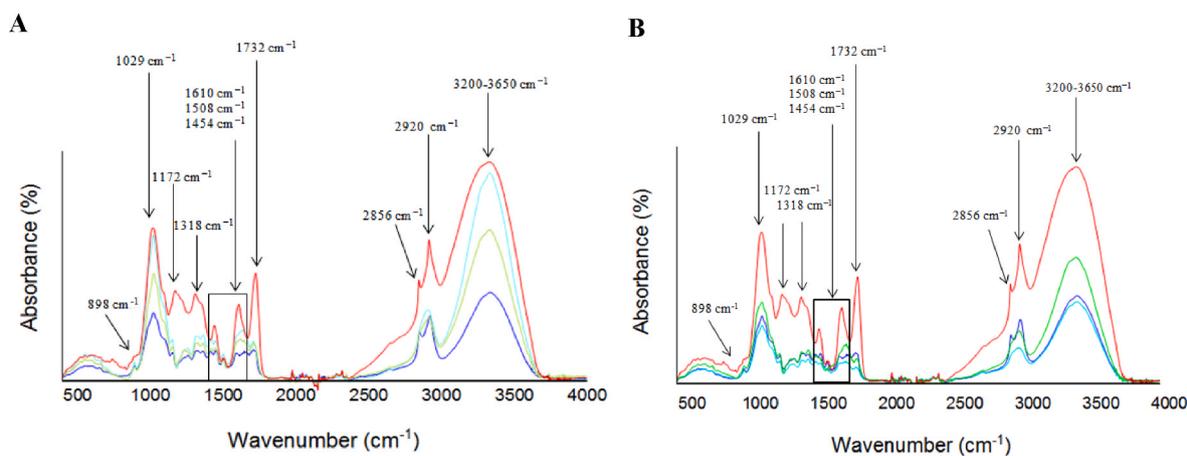
(data non-shown), since the appearance and shape of biomass after 60 °C was very similar to that observed with untreated CB. It must be said that temperatures above 120 °C were not contemplated in this work because the boiling point of diluted ChCl:U with 20% of water is between 130 and 140 °C. Experiments carried out near boiling point could break linkages between Cl<sup>-</sup> and lignin due to vibrations of molecules experimented at these temperatures, resulting in a decrease in delignification [11].

Fig. 3 summarizes the main effects of time and temperature pretreatment attending on the best delignification. This type of DES (diluted ChCl:U) can join to lignin complexes through hydrogen bonds when DES impregnates the biomass [11], promoting the delignification process. Moreover, the addition of water led to improve the mass transfer between solvent and CB, causing better delignification ratios by increasing the formation of hydrogen bonds [11]. As can be seen, the slope of the treatments at 4 h was more pronounced than for 8 h and 16 h, indicating that temperature has a greater effect at shorter treatments than at longer treatment times. Besides, it was highlighted the interaction between DES pretreatments at 4 and 8 h at 120 °C, this means that at the latter temperature there were no significant differences ( $P > 0.05$ ) in the delignification between pretreating the material for 4 or 8 h ( $32.49 \pm 2.47$  and  $32.46 \pm 1.20\%$  respectively). However, if the time was doubled until 16 h, delignification reached its maximum ( $40.41 \pm 2.10\%$ ). According to Yu et al. [35], amorphous compounds, including lignin, are fractionated to a greater extent, breaking the lignin-carbohydrate bonds, when the operating conditions of the DES pretreatment are more severe. Nevertheless, this percentage could also

be reached at the same time (16 h) at lower temperatures (100 °C), founding significant differences ( $P < 0.05$ ) with the DES pretreatment at 80 °C ( $33.46 \pm 0.01\%$ ). On the contrary, the low slope of the 8 h timeline can also be observed in Fig. 3, indicating that there are no significant differences ( $P > 0.05$ ) between temperatures. Therefore, in terms of CB delignification, the most suitable treatment with DES would be the condition of 16 h and 100 °C.

Linked to the above mentioned, New et al. [11], reported percentages of delignification of 17% during the pretreatment of oil palm fronds with the same diluted eutectic mixture at 120 °C during 4 h. Besides, similar results of delignification (17%) were obtained by Oh et al. [8], in pine wood pretreated with pure ChCl:U at 130 °C during 6 h. Meanwhile in this work, under similar operational conditions (120 °C and 4 h), delignification raised to 32.5%, almost doubling the results attained by these authors. Otherwise, Ong et al. [36], recently reported a higher value of delignification of oil palm fronds (47%) using a combined pretreatment consisting of ultrasonic-assisted alkaline solution with a DES mixture of water and ChCl:U (20–80%).

To the best of our knowledge, this is the first report using a DES pretreatment to fractionate CB and there are few references that propose new sustainable management strategies. This work postulates the use of ChCl:U as DES to break the structure of CB recovering those fractions rich in polysaccharides. According to Zulkefli et al. [37], the aromatic structure of lignin can join with protons (H<sup>+</sup>), derived from Cl<sup>-</sup> of ChCl, forming hydrogen bond donors causing link breakage between lignin and hemicellulose [9]. Besides, Simeonov and Afonso [38] commented that ammonia formation, due to urea decomposition from ChCl:U



**Fig. 4.** ATR-FTIR spectra to evaluate the effect of time (A) on the pretreatment of CB by DES at 100 °C: CB (red line), 4 h (green line), 8 h (blue line) and 16 h (dark blue line) and temperature (B): CB (red line), 80 °C (green line), 100 °C (light blue line) and 120 °C (dark blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

surpassing 80 °C, disturbs lignin-carbohydrate chains. Furthermore, according to Keshav et al. [39], the addition of water in biomass pretreatment with DES improves lignin removal. For that reason, in this work the ChCl:U was diluted at a ratio of 80:20 DES/water (v/v).

The effectiveness of treating CB with ChCl:U can be also observed in the data summarized in Table 2 through the enrichment of glucan and xylan content in all CRMs. For instance, under the pretreatment carried out at 16 h at 100 °C, there was a 1.80 and 1.48-fold times increment regarding untreated CB. The enrichment of polysaccharide content, especially the increase in xylan, could be highlighted with this DES since xylan removal yields are regularly reported in acid-based DES pretreatments, avoiding the exploitation of high added value compounds that could be used in biorefinery processes [18]. On the contrary, it can be observed a reduction in the arabinan fraction of CRMs after ChCl:U pretreatment (from  $6.38 \pm 0.09$  to  $3.62 \pm 0.46\%$ ) and a decrease in PArR to only  $30.90 \pm 0.25\%$ . This could be related to delignification effect [40] showed in Fig. 3 when temperature increased from 80 to 120 °C at 16 h. In this sense, according to Morán-Aguilar et al. [41], DES usually dissolves lignin, xylan, and arabinan, as a result of breaking the  $\beta$ -O-4 aryl ester bonds between lignin and polysaccharides, which could explain arabinan losses.

The effect of time and temperature of DES pretreatment on CB was also studied by ATR-FTIR. Characteristic bands between 4000 and 400  $\text{cm}^{-1}$  are shown in Fig. 4A, revealing the effect of time (4, 8 and 16 h) on the composition of CB. In this sense, feature peaks of lignocellulosic materials around 2850  $\text{cm}^{-1}$  (C–H stretch O–CH<sub>3</sub> group) corresponding to three biopolymers of lignocellulosic biomass bonds vibration and 2920  $\text{cm}^{-1}$  (C–H stretch methyl and methylene groups) were identified, showing a diminution after DES pretreatments at different reaction times. The same behavior could be observed between 3200 and 3650  $\text{cm}^{-1}$  bands (O–H stretch, H-bonded typical of the three main lignocellulosic polymers: lignin, hemicelluloses, and cellulose). It was noted that these characteristic peaks greatly declined in intensity after pretreatments with ChCl:U under the three reaction times studied, which demonstrates the effect of DES in biomass, changing the composition of initial material and being congruent with the results of characterization detailed above. Furthermore, lignin band at 1610  $\text{cm}^{-1}$  (aromatic skeletal C=C alkene) [31] and near 1454  $\text{cm}^{-1}$  (asymmetric bending in CH<sub>3</sub> of lignin) [32] were reduced in samples pretreated with DES. Nevertheless, the peak at 1508  $\text{cm}^{-1}$ , attributed to aromatic skeleton vibration in lignin [42], remained visible after DES pretreatment.

Based on the previously described, Fig. 4B shows characteristic bands of untreated CB and CRMs obtained after 16 h of DES pretreatments carried out at 60, 80, 100 and 120 °C. The same peaks identified in Fig. 4A were also observed in Fig. 4B. Thus, 1508 and 1610  $\text{cm}^{-1}$  bands, both associated with aromatic skeletal of lignin, appeared with similar intensity in untreated CB and the CRM at 60 °C. Further, considering the higher absorbance at 1454  $\text{cm}^{-1}$  in CRM at 60 °C, associated with asymmetric bending in CH<sub>3</sub>, it can be concluded that delignification at minor temperatures may not be effective as reveals ATR-FTIR. However, when pretreatments were carried out at 80, 100, and 120 °C, these characteristic bands of lignin greatly decreased, observing the largest reduction at 100 °C, what sustained the selection of performing the pretreatment at this temperature as it was previously commented. Besides, it was also noticed that absorption corresponding to CH<sub>2</sub> wagging crystalline cellulose (around 1310  $\text{cm}^{-1}$ ) almost disappear under the hardest pretreatments, demonstrating the necessity of using temperatures above 80 °C to strongly modify the structure of cellulose in CB.

CB appearance reveals a compact texture, smooth spiny and rigid structure (Fig. 2A1), whereas CB + DES materials at times under study (see Fig. 2B) showed radical changes onto the surface, being flatter and with several grooves. A similar behavior was also observed when the effect of temperature was studied in CB + DES (Fig. 2C), showing several breaks onto the surface, making messy complexes when spines are blending with the rest of biomass.

**Table 3**

CRMs composition achieved after treatment of CB with DES under different solid-liquid ratios (LSR).

Composition (%)	LSR		
	10:1 (w/w)	15:1 (w/w)	20:1 (w/w)
AIL	20.37 ± 0.46 <sup>a</sup>	17.09 ± 0.19 <sup>b</sup>	17.92 ± 0.68 <sup>b</sup>
ASL	2.74 ± 0.02 <sup>a</sup>	2.75 ± 0.14 <sup>a</sup>	2.73 ± 0.14 <sup>a</sup>
Total lignin	23.11 ± 0.48 <sup>a</sup>	19.84 ± 0.33 <sup>b</sup>	20.65 ± 0.82 <sup>a,b</sup>
Delignification	32.56 ± 1.41 <sup>a</sup>	42.09 ± 0.97 <sup>b</sup>	39.74 ± 2.40 <sup>a,b</sup>
Glucan	34.32 ± 0.34 <sup>a</sup>	35.90 ± 0.76 <sup>a</sup>	38.87 ± 0.35 <sup>b</sup>
Xylan	30.73 ± 0.15 <sup>a</sup>	30.34 ± 0.29 <sup>a</sup>	31.74 ± 0.47 <sup>a</sup>
Arabinan	5.10 ± 0.28 <sup>a</sup>	5.00 ± 0.04 <sup>a</sup>	4.78 ± 0.11 <sup>a</sup>
Total polysaccharides	70.14 ± 0.20 <sup>a</sup>	71.23 ± 1.08 <sup>a</sup>	75.38 ± 0.71 <sup>b</sup>
Acetyl groups	4.06 ± 0.44 <sup>a</sup>	3.74 ± 0.17 <sup>a</sup>	3.24 ± 0.25 <sup>a</sup>
PCR	82.42 ± 0.41 <sup>a</sup>	83.88 ± 2.11 <sup>a</sup>	93.22 ± 1.86 <sup>b</sup>
PXnR	78.93 ± 0.00 <sup>a</sup>	75.84 ± 1.90 <sup>a</sup>	79.12 ± 1.58 <sup>a</sup>
PArR	43.60 ± 2.61 <sup>a</sup>	41.57 ± 1.04 <sup>a</sup>	41.42 ± 0.83 <sup>a</sup>

AIL: acid insoluble lignin, ASL: acid soluble lignin, PCR: percentage of cellulose recovery, PXnR: percentage of xylan recovery, PArR: percentage of arabinan recovery.

\*Different letters between the columns show statistically significant differences ( $P < 0.05$ ).

Finally, in addition to time and temperature, another factor to consider during pretreatment is the LSR, an important parameter in pretreatments that also affects to their effectiveness and pretreatment costs. Low LSR could avoid complete saturation of biomass by solvent [43]. However, the excessive increase in LSR could lead to oversized pretreatment reaction equipment, losses of solvent, excess of energy expense, and negligible amounts of biomass treated per time [43,44]. Furthermore, Lyu et al. [45], reported that an excessive high LSR induces peeling reactions of cellulose and hemicellulose in alkali conditions, what it would not be affordable to further enzymatic hydrolysis after DES pretreatment. For these reasons, three different loadings were assayed in this work (10:1, 15:1 and 20:1, w/w). Table 3 summarizes the CRMs composition at different loadings (biomass:DES) as well as PCR, PXnR and PArR.

Regarding delignification, the best pretreatment (100 °C, 16 h) was attained using the LSR of 15:1 (w/w), achieving  $42.09 \pm 0.97\%$  of delignification and not showing significant differences ( $P > 0.05$ ) with pretreatment using a LSR of 20:1 (w/w). Moreover, attending to glucans, the best PCR results were achieved under the highest LSR (20:1 w/w) what could be due to a greater swelling of the biomass in the pretreatment process [46]. Therefore, these results demonstrated beneficial effects in lignocellulosic materials dissolution at highest LSR. An explanation for that could be a higher interaction between total surface area of lignocellulosic material particles and solvent since the volume of solvent is not saturated and can react more frequently with particles. Besides, minor viscosity was achieved at lower biomass loading, improving the dissolution of biomass [47]. Finally, LSR of 20:1 (w/w) was selected as the best condition between three studied LSR to perform in W-CB and PreH by DES, since it was the best assay attending on PCR, being the best option for subsequent enzymatic hydrolysis.

### 3.2.1. Influence of CB preparation on DES pretreatment

In order to obtain a deeper knowledge on the structural changes occurring during the pretreatment with diluted ChCl:U, the three different substrates (CB, W-CB and PreH) were evaluated. Furthermore, some authors proposed a two-stage pretreatment with diluted acid and alkali to valorize biomass in a complete way [48,49]. Since, diluted acid solubilizes firstly the hemicellulosic fraction, this pretreatment is usually followed by alkali hydrolysis to remove lignin and improve the subsequent enzymatic hydrolysis of cellulose [50]. Thus, one of the most used alkalis in pretreatments is NaOH and its cost per ton is around 440 USD according to Yu et al., [51]. Meanwhile, Peng et al. [52], informed

**Table 4**

Characterization of CRMs of CB, W-CB and PreH. All DES pretreatments were carried out at 100 °C, 16 h and a LSR 20:1 (w/w).

(%)	CB + DES	W-CB + DES	PreH + DES
AIL	17.92 ± 0.68 <sup>a</sup>	22.61 ± 0.95 <sup>b</sup>	34.42 ± 0.70 <sup>a</sup>
ASL	2.73 ± 0.14 <sup>a</sup>	4.04 ± 0.12 <sup>b</sup>	2.43 ± 0.11 <sup>a</sup>
Total lignin	20.65 ± 0.82 <sup>a</sup>	26.86 ± 0.98 <sup>b</sup>	36.86 ± 0.60 <sup>c</sup>
Delignification	39.74 ± 2.40 <sup>a</sup>	19.19 ± 2.95 <sup>b</sup>	28.31 ± 1.16 <sup>c</sup>
Glucan	38.87 ± 0.35 <sup>a</sup>	34.67 ± 1.78 <sup>b</sup>	50.63 ± 2.52 <sup>c</sup>
Xylan	31.74 ± 0.47 <sup>a</sup>	33.55 ± 2.32 <sup>a</sup>	5.16 ± 0.49 <sup>b</sup>
Arabinan	4.78 ± 0.11 <sup>a</sup>	3.55 ± 1.09 <sup>a</sup>	0.00 ± 0.00 <sup>b</sup>
Total polysaccharides	75.38 ± 0.71 <sup>a</sup>	70.77 ± 3.01 <sup>a</sup>	55.79 ± 2.04 <sup>b</sup>

AIL: acid insoluble lignin, ASL: acid soluble lignin.

Different letters between the columns show statistically significant differences ( $P < 0.05$ ).

about the price of choline chloride and urea (200 and 90 \$/ton, respectively).

On the other hand, although in this work the recyclability of DES was not studied, Lee et al. [53], reported recently until 2 cycles of reuse in the pretreatment of oil palm empty fruit bunch with ChCl:U, showing better results in reducing sugars yields after cycles despite on delignification decreased after each cycle.

Moreover, the type of pretreatment influences the cost of subsequent processing such as detoxification, the use of enzymes, as well as the separation of products [54]. In this sense, the detoxification step could be avoided after DES pretreatment due to inhibitors like acetic acid, furfurals and 5-HMF were produced in low amounts because of the dissociation process that occurs between lignin and DES under alkaline conditions [55]. For all the reasons mentioned above, an alkali DES, ChCl:U was set out in this work to study the delignification of the remaining solid after diluted acid pre-pretreatment.

Table 4 shows the lignocellulosic composition of CRMs obtained after DES pretreatment. In all cases, ChCl:U was assayed at 100 °C and a LSR 20:1 (w/w). As observed, the highest percentage of delignification ( $39.74 \pm 2.40\%$ ) was obtained with the CB + DES sample. However, Pre-H was composed mainly by cellulose and lignin, which hinders the ChCl:U efficiency for greater lignin solubilization.

Regarding total polysaccharides, it can be observed that W-CB + DES achieved the same amount as CB + DES with more than 70% content. This fact could be explained according to SEM-photos, which showed that W-CB was a heterogeneous material, with visible spines, similar to untreated CB. Additionally, CRMs of PreH + DES increased carbohydrate content, although did not overcome 60%.

Attending on ATR-FTIR spectra of W-CB + DES and PreH + DES (Fig. 5A and B, respectively), the reduction in the intensity of characteristic peaks of lignin previously cited ( $1454\text{--}1610\text{ cm}^{-1}$ ) corroborated

the delignification of biomass previously discussed due to ChCl:U pretreatment. Furthermore, the almost complete disappearance of hemicellulose peak at  $1724\text{ cm}^{-1}$  in all residues after DES could suggest a deconstruction of biomass structure due to lignin-hemicellulose bonds breakdown.

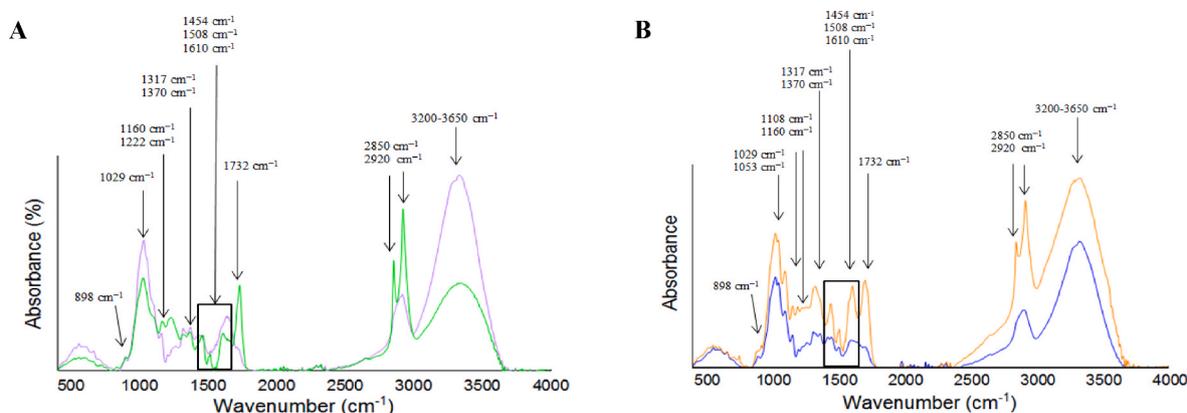
Particularly, new characteristic peaks were detected in W-CB, appearing at  $1160$  and  $1222\text{ cm}^{-1}$  attributed to C–O–C stretching of cellulose and hemicellulose and C–O in guaiacyl unit [30], respectively. These two bands disappeared after DES pretreatment what reinforced that previously discussed. Regarding DES pretreatment in PreH samples, peaks at  $1108\text{ cm}^{-1}$  (aromatic deformation of lignin C–H),  $1160\text{ cm}^{-1}$  (C–O–C stretching of cellulose and hemicellulose) and  $1370\text{ cm}^{-1}$  (C–H bending and  $\text{CH}_3$  stretching corresponding with three biopolymers) [30] were diminished.

Moreover, as can be seen in SEM-photos (Fig. 2D1), W-CB + DES the surface changed in great measure compared to W-CB (Fig. 2A2). Accordingly, similar behavior was observed after DES pretreatment in PreH at 16 h (Fig. 2D2), showing great morphological changes and highlighting the great number of pores and rupture of fibrils, in concordance with the ATR-FTIR study.

In summary, with this work the suitability of ChCl:U as a pretreatment after untreated and processed CB was demonstrated. According to that explained above, this DES is suitable to keep glucan and xylan fractions, what would be convenient to release sugars through enzymatic hydrolysis. Furthermore, alkaline DES are considered not harmful agents to environment, and they can be reused [56]. Besides, other advantages of ChCl:U compared to acidic DES, is the non-corrosion of stainless steel [57], what could be appropriated in a scale up step when tanks are usually made up with this material.

#### 4. Conclusion

Compositional differences in processed biomass compared to untreated CB were observed and further supported by ATR-FTIR and SEM studies. ATR-FTIR and SEM-photos studies helped to understand the influence of temperature and time in the physicochemical composition of CB pretreated with DES. This work shows a more efficient process with DES in terms of delignification on untreated CB (around 40%) after the best conditions; meanwhile it was drastically reduced in prepared biomass (12–21%). Polysaccharides enrichment materials were obtained as a result of DES pretreatments in CB and W-CB, as well as the quantity of carbohydrates in PreH + DES increased compared to PreH. PCR and PXnR showed high values in CB by DES pretreatment postulated as a good pretreatment to improve polysaccharides content and recover them.



**Fig. 5.** (A) ATR-FTIR spectra of W-CB (light green line) and W-CB+DES (light violet line); (B) ATR-FTIR spectra of PreH (orange line) and PreH+DES 16 h (dark blue line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

## Data availability

Data will be made available on request.

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