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Enhancing the Biorefinery of Chestnut Burrs, Part II: Influence of Pretreatment with Choline Chloride–Urea-Diluted Deep Eutectic Solvent on Enzymatic Hydrolysis

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Abstract

Agro-industrial chestnut waste derived from chestnut processing is usually discharged without further use. However, these residues are attractive due to their high-value composition, rich in sugars and lignin. Among these residues, chestnut burrs (CB) represent a promising feedstock for biorefinery applications aimed at maximizing the valorization of their main constituents. In this study, we propose an environmentally friendly approach based on deep eutectic solvents (DES) formed by choline chloride and urea (ChCl/U) (1:2, mol/mol) for the selective deconstruction of lignocellulosic architecture, followed by enzymatic hydrolysis to release second-generation (2G) fermentable sugars. Pretreatments were applied to raw CB, washed CB (W-CB), and the obtained solid fraction after prehydrolysis (PreH). Structural and morphological modifications, as well as crystallinity induced by DES pretreatment, were characterized using attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR), field emission scanning electron microscopy (FE-SEM), and X-ray diffraction (XRD). Remarkable results in terms of effectiveness and environmental friendliness on saccharification yields were achieved for PreH subjected to DES treatment for 8 h, reaching approximately 60% glucan and 74% xylan conversion under the lower enzyme loading (23 FPU/g) and liquid-to-solid ratio (LSR) of 20:1 studied. This performance significantly reduces DES pretreatment time from 16 h to 8 h at mild conditions (100 °C), lowers the LSR for enzymatic hydrolysis from 30:1 to 20:1, and decreases enzyme loading from 63.5 FPU/g to 23 FPU/g, therefore improving process efficiency and sustainability.

Keywords: chestnut burrs; diluted ChCl/urea; crystallinity; sequential pretreatments; saccharification



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1. Introduction

Chestnut processing is an emerging sector in the European Union with an increasing production of more than 60% in recent years [1]. Particularly, Spain remains the largest EU producer, with around 182,000 tons of harvested production in 2024 [2]. Consequently, managing the waste produced in this activity is of great interest, following the new guidelines and legislation around zero-waste processes and circular bioeconomy, which has received much attention in recent years as a solution to surmount the present consumption and production demands [3].

Waste derived from chestnut management, including shells, burrs, leaves, and pruning, shows a relevant presence of carbohydrates in its composition [4]. Specifically, burrs made up around 50% on a dry basis, making them a good candidate to obtain monomeric sugars [5]. Nevertheless, the presence of lignin in these materials hinders the recovery of carbohydrates since this polymer acts like a barrier due to the interaction with cellulase enzymes [6] and consequently limits the hydrolysis of cellulose and hemicellulose polymers to produce second-generation (2G) monomeric sugars [7]. Additionally, cellulose crystallinity is another parameter associated with biomass recalcitrance and restriction to the action of enzymes [8,9]. Consequently, it plays a significant role as a predictor of enzymatic hydrolysis as well as the effectiveness of the pretreatment [10]. In this sense, pretreatments are indispensable to deconstructing the lignocellulosic complex, improving the accessibility of the hydrolytic enzymes to the carbohydrate fraction, and consequently improving both the hydrolysis rate and amount of 2G sugars released after enzymatic hydrolysis [11,12].

The use of conventional pretreatments, namely diluted alkali using NaOH as a reagent, is extensively used on biomass due to the great removal of lignin and partial elimination of hemicellulose. Although some disadvantages have been cited as a result of its use, such as costs for NaOH recovery, its environmental impact, or possible condensation of lignin and changes in cellulose towards a more stable structure, which could make enzymatic hydrolysis difficult [13–15]. Furthermore, it is estimated that around 30% lower energy consumption is achieved by using a deep eutectic solvent (DESs) pretreatment compared to the pretreatment with NaOH under the same pretreatment conditions [16]. Thus, the use of mild alkaline-neutral DES, such as choline chloride/urea (ChCl/U), seems to be more feasible from an environmentally friendly point of view than conventional routes.

Therefore, the use of DES as eco-friendly solvents in biorefinery processing has gained special attention in recent years [17–19], since DES show interesting properties as low cost, low vapor pressure, biodegradability, and non-flammability, whereby they are commonly distinguished as green solvents [20]. Particularly, in our previous work, DES pretreatment using diluted ChCl/U showed relevant physicochemical changes on chestnut burrs (CB) [5].

For these reasons, this work aims to develop a novel and efficient biorefinery process to improve the conversion of carbohydrates present in CB into fermentable 2G sugars via DES and subsequent enzymatic hydrolysis. Unlike previous works, our contribution lies in integrating diluted DES pretreatment with systematic evaluation of raw CB, washed CB, and solid residues after prehydrolysis, using ChCl as a hydrogen bond acceptor (HBA) and urea as a hydrogen bond donor (HBD). We hypothesize that DES pretreatment significantly modifies the crystalline structure of CB, therefore improving enzymatic digestibility and reducing enzyme requirements. To test this, we combined carbohydrate-rich materials (CRMs), including those obtained in our previous study (Part I) [5], and assessed their hydrolysis under varying solid loads. Structural characterization (ATR-FTIR, XRD, SEM) was performed to correlate crystallinity changes with hydrolysis efficiency. Finally, enzyme loading was optimized to minimize costs while maintaining high sugar yields, reinforcing the potential of this integrated process for cost-effective biorefinery applications.

2. Materials and Methods

2.1. Synthesis of DES

ChCl (>99%) and urea (>99.5%) were provided by Sigma-Aldrich (Merk Life Science S.L., Tres Cantos, Madrid, Spain). The moisture of the ChCl was maintained in a gel desiccator. DES was prepared by mixing ChCl and urea with a molar ratio of 1:2 (mol/mol) and placed in a water bath at 60 °C with stirring until a colorless liquid was obtained [21]. Then, the mixture ChCl/U was homogenized by adding water at a ratio of 80:20 DES/water (*v/v*) under magnetic stirring.

2.2. Substrates of DES Pretreatment

CBs were provided by local cultivars (Vilardevós, Ourense, Spain) in 2019. Then, they were dried at room temperature and homogenized by milling (SOGO mill, SS-5430 models, Sanysan Appliances SL, Valencia, Spain). The substrates assayed for DES treatments were

- Untreated CB
- Washed CB (W-CB) obtained by mixing untreated CB with distilled water in a glass bottle with an SLR of 1:8 (*w/v*), followed by agitation in an orbital shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 50 °C, 150 rpm for 4 h. The solid fraction was recovered by filtration and dried at 50 °C (Celsius 2007, Memmert, Schwabach, Germany).
- CB from prehydrolysis (PreH). Untreated CB was pretreated with diluted H₂SO₄ under the conditions reported by Costa-Trigo et al. [4]. The solid fraction was recovered, washed with distilled water until neutral pH, and dried at 50 °C (Celsius 2007, Memmert, Schwabach, Germany).

2.3. DES Pretreatment

DES pretreatments were carried out according to the best conditions found in our previous work (Part I) [5]. Briefly, 0.5 g of substrate was mixed with diluted ChCl/U in an SLR of 1:20 (*w/w*) at 100 °C for 4, 8, and 16 h. Then, 50 mL of antisolvent (water) was added and stirred for 30 min. CRMs recovered were washed with 40 mL of water and centrifuged (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) at 3700 rpm for 15 min. The washing process was repeated three times. Finally, CRMs were dried at 50 °C to remove excess water and stored until physicochemical analysis was performed (Section 2.5).

2.4. Enzymatic Hydrolysis

Sigma-Aldrich (Spain) provided the multienzyme complex CellicCTec2 with the following activities: cellulase 254.50 ± 4.53 (FPU/mL), cellobiose 89.53 ± 0.43 (U/mL), and xylanase 12084.88 ± 169.53 (U/mL) [22].

Samples of untreated CB, W-CB, PreH, and all CRMs obtained after DES pretreatments were enzymatically hydrolyzed. A certain volume of citrate buffer (pH 5) was added to achieve an LSR of 30:1 (*v/w*), and commercial enzyme Cellic CTec2 (23, 40, 63, 127, 190, and 254 FPU/g) was added under sterile conditions. Afterwards, glass tubes were placed in an orbital shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) and incubated at 50 °C and 250 rpm for 72 h.

At the end of the enzymatic hydrolysis, samples were boiled for 5 min to inactivate the enzymes. Then, the samples were centrifuged at 3700 rpm for 10 min (Ortoalresa, Consul 21, EBA 20, Hettich Zentrifugen, Germany) and filtered through 0.22 µm pore membranes (Sartorius, Goettingen, Germany) to be analyzed by HPLC (Agilent, model 1200, Palo Alto, CA, USA) following the methodology described by Costa-Trigo et al. [23] and used to calculate the percentage of saccharification as it was described by Costa-Trigo et al. [24].

2.5. Analytical Methods

2.5.1. Lignocellulosic Composition

The characterization of the lignocellulosic substrates and CRMs was performed by quantitative acid hydrolysis according to the official method NREL/TP-510-42618 [25]. Total lignin was calculated as the sum of acid-soluble lignin (ASL) and acid-insoluble lignin (AIL).

2.5.2. Attenuated Total Reflectance Fourier-Transform Infrared (ATR-FTIR) Spectroscopy

The spectra of PreH and CRMs after DES pretreatments on PreH were obtained by attenuated total reflection (ATR) equipped with a diamond crystal (Smart Orbit Diamond ATR, Thermo Fisher, USA) and coupled to infrared spectroscopy equipment (Thermo Nicolet 6700 FTIR Spectrometer, Thermo Fisher Scientific Inc., Madison, WI, USA). A deuterated triglycine sulfate (DTGS) KBr detector was employed to analyze dry samples with a resolution of 4 cm^{-1} and 20 scans, in the range of $4000\text{--}400\text{ cm}^{-1}$.

From FTIR spectra, the Lateral order index (LOI) was calculated as the ratio between 1430 and 898 cm^{-1} absorbances (Equation (1)) [26]; total crystalline index (TCI) was defined as the quotient between the values of absorbances at 1372 and 2900 cm^{-1} [27] (Equation (2)); and the hydrogen bond intensity (HBI) was calculated as the ratio between 3400 cm^{-1} and 1320 cm^{-1} absorbances [26].

$$\text{LOI} = A_{1430} / A_{898} \quad (1)$$

$$\text{TCI} = A_{1372} / A_{2900} \quad (2)$$

$$\text{HBI} = A_{3400} / A_{1320} \quad (3)$$

LOI, TCI, and HBI were calculated for all samples tested, the initial and DES pretreated samples.

2.5.3. X-Ray Diffraction (XRD)

Crystallinity index (*CrI*) was obtained by X-ray spectroscopy (Siemens D5000 Diffractometer) through the diffraction angles spanning from $2\theta = 2\text{--}45^\circ$, with a step size of 0.02° and a step time of 0.5 s. The values obtained for the intensity of the crystalline region at $2\theta = 22.35$ (I_{cry}) and intensity of the amorphous region at $2\theta = 16.17$ (I_{am}) were used following Equation (4) [28]:

$$\text{CrI} = \frac{I_{\text{cry}} - I_{\text{am}}}{I_{\text{cry}}} \times 100 \quad (4)$$

2.5.4. Field Emission Scanning Electron Microscopy (FE-SEM)

The morphological changes in the lignocellulosic substrates and CRMs were performed by an FE-SEM system (Model JSM-6700 F, Jeol, Japan). The dry material was mounted onto aluminum stubs and coated with gold, and the sputter coater (Sputtering Emitech K550X, Quorum Technologies, Kent, UK) was used to analyze for 3 min.

2.6. Statistical Analysis

All data were submitted to one-way ANOVA of the Statgraphics statistical package (Statgraphics Centurion XVI version 16.1.11). Statistically significant differences between means, with a confidence level of 95%, were determined through a least significant difference (LSD) test.

3. Results and Discussion

3.1. Crystallinity of Lignocellulosic Starting Materials

The lignocellulosic composition of substrates and CRMs was reported in our previous work [5]. However, it was added in Section 3 to join the crystallinity of materials to try to acquire enough information about biomasses before and after the sample preparation, with the objective of searching for relationships between the fractions that form biomasses and their crystallinity parameters. Washing of biomass influenced crystallinity, which could have an impact on further enzymatic hydrolysis of materials, due to the removal of free sugars and partial extractives present in CB, as was previously observed in our previous work [5].

Thus, in addition to the lignocellulosic composition of substrates, a deeper knowledge about the changes experienced by the materials before DES treatments was acquired in this work through the study of the crystallinity of the samples. It is worth mentioning that although the method of Segal through XRD is still popular, it usually overestimates the value of the crystallinity index (*CrI*), exceeding the cellulose content [29]. For that reason, other indices calculated from ATR-FTIR analysis were employed to evaluate the crystallinity value. First, the lateral order index (LOI) allows monitoring changes linked to crystalline and amorphous structures in the biomass, being related to the organization of cellulose and directly proportional to the crystallinity [27,30]. Secondly, the total crystallinity index (TCI) is linked to planes assigned to C-H and CH₂ and their behaviors [26]. Finally, hydrogen bond intensity (HBI) is related to an increase in hydroxyl groups in the cellulose hydrogen bonding, promoting high values of HBI (less crystallinity), which occurs in processes where cellulose I converts to cellulose II, although a decrease in total % crystallinity is noted [30].

In this sense, LOI, HBI, and *CrI* showed the same tendency, increasing after sample preparation (W-CB and PreH), as can be seen in Table 1. The higher crystallinity observed in W-CB compared with untreated CB could be a consequence of the solubilization in water of other amorphous compounds such as free sugars, soluble lignin, or extracts [24], increasing the glucan fraction [5] and therefore the *CrI* from 19.90 to 44.90%. Likewise, Haykiri-Acma and Yaman [31] observed a reduction in *CrI* after water treatment; however, they commented on the unclear information about water effects on *CrI*. In addition, the same authors obtained an increment in LOI values in three studied biomasses after water treatment, following the same trend as this work, where the LOI value of CB increased after washing from 1.96 to 2.15, respectively.

Table 1. Moisture (%) and crystallinity of the starting materials: raw CB, washed CB, and solid residue after prehydrolysis (PreH).

	CB	W-CB	PreH
Moisture	10.97 ± 0.11 ^a	2.66 ± 0.02 ^b	4.07 ± 0.05 ^c
<i>CrI</i> (%)	19.90	44.90	56.14
LOI (A1430/A898)	1.96	2.15	3.08
TCI (A1372/A2900)	0.51	0.40	0.50
HBI (A3400/A1320)**	2.60	2.30	2.11

A: absorbance. ** Inversely proportional to crystallinity. Different letters within the columns show statistically significant differences ($p < 0.05$).

On the other hand, PreH achieved the highest *CrI* values (56.14%) due to the strong removal of the amorphous fractions corresponding to pentose carbohydrates like xylan and arabinan, as was mentioned in our previous work, obtaining a cellulose-rich material (39.56 ± 0.20% of glucans) [5]. Additionally, diluted acid pretreatment induces the formation of more stable allomorph cellulose I β from I α cellulose (susceptible to hydrolysis under acidic conditions), diminishing the degree of polymerization (DP) and increasing

crystallinity [32]. In this sense, Huong et al. [33] obtained an increase of around 12% in *CrI* after acid pretreatment of vetiver grass at 0.5% (*v/v*) H₂SO₄ for 60 min. In addition, Santos et al. [34] observed the same behavior in elephant grass plant leaves and stems after pretreatment at all concentrations of H₂SO₄ studied (5, 10, and 15%).

3.2. Influence of DES Pretreatment on Different Biomasses

To the best of our knowledge, no reports have been published so far on processing CB by washing or hydrolyzing with diluted acid prior to diluted ChCl/U pretreatment to increase the saccharification efficiency. According to our previous work [5], DES pretreatment using diluted ChCl/U was a suitable strategy to recover glucan and xylan fractions from CB, achieving percentages of delignification close to 40%. Therefore, this work evaluates the saccharification of different CRMs after the pretreatment with diluted ChCl/U (mild-neutral alkali DES). Additionally, CB substrates non-pretreated by DES were also enzymatically hydrolyzed to compare the different strategies.

Even though the time of the DES pretreatment was set to 16 h in part I [5], the lower hemicellulose content and higher crystallinity presented by the PreH sample (Table 1) because of previous diluted acid pretreatment led to shorter treatment times with DES (4 and 8 h). In addition, DES acts mainly on hemicellulose and lignin [35], while cellulose only rearranges its structure due to DES action into intermolecular H-bonds, promoting the creation of new H-bonds between DES and hydroxyl groups of cellulose [36]. Particularly, this kind of DES can preserve the hemicellulose fraction of CB to a great extent (70–85%) as demonstrated in our previous work [5]. In this sense, as can be seen in Table 2, DES pretreatments carried out on PreH material offered delignification values around 27–28%, which were considerably lower than using DES in untreated CB (39.74 ± 2.40%). DES pretreatment on CB or only washed with water (W-CB) poorly removed the hemicellulose, retaining in great measure the total polysaccharide content up to 70%. While glucan content after DES pretreatment in PreH material achieved values between 47 and 50%, it was higher at longer pretreatment times.

Table 2. Characterization of PreH solids before and after DES pretreatments and CB and W-CB after DES pretreatments at 16 h. All DES pretreatments were carried out at 100 °C and an LSR of 20:1 (*w/w*).

(%)	CB+DES (16 h) •	W-CB+DES (16 h) •	PreH+DES (4 h)	PreH+DES (8 h)	PreH+DES (16 h) •
AIL	17.92 ± 0.68 ^a	22.61 ± 0.95 ^b	35.25 ± 0.30 ^c	33.25 ± 1.02 ^{c,d}	34.42 ± 0.70 ^{c,d}
ASL	2.73 ± 0.14 ^c	4.04 ± 0.12 ^d	1.92 ± 0.15 ^b	1.02 ± 0.00 ^a	2.43 ± 0.11 ^c
Total Lignin	20.65 ± 0.82 ^a	26.86 ± 0.98 ^b	37.17 ± 0.15 ^c	37.21 ± 2.64 ^c	36.86 ± 0.60 ^c
Delignification	39.74 ± 2.40 ^d	19.19 ± 2.95 ^a	27.31 ± 0.30 ^b	27.63 ± 5.15 ^b	28.31 ± 1.16 ^b
Glucan	38.87 ± 0.35 ^b	34.67 ± 1.78 ^a	46.98 ± 2.04 ^c	52.43 ± 2.25 ^d	50.63 ± 2.52 ^{c,d}
Xylan	31.74 ± 0.47 ^c	33.55 ± 2.32 ^c	3.41 ± 0.12 ^a	4.51 ± 0.08 ^b	3.68 ± 1.00 ^{a,b}
Arabinan	4.78 ± 0.11 ^a	3.55 ± 1.09 ^a	n.d.	n.d.	n.d.
Total polysaccharides	75.38 ± 0.71 ^d	70.77 ± 3.01 ^c	50.39 ± 2.17 ^a	56.75 ± 2.45 ^b	55.79 ± 2.04 ^{a,b}
<i>CrI</i> (%)	48.40	53.75	37.04	54.65	38.67
LOI (A1430/A898)	1.79	2.62	2.02	3.43	2.57
TCI (A1372/A2900)	0.66	0.58	0.61	0.68	0.58
HBI (A3400/A1320) [°]	3.54	4.07	3.21	2.76	3.04

AIL: acid-insoluble lignin, ASL: acid-soluble lignin, and A: absorbance. Different letters within the columns show statistically significant differences ($p < 0.05$). [°] Inversely proportional to crystallinity • Results of lignocellulosic composition from Part I [5]. n.d.: non-detected.

Table 2 also shows the crystallinity parameters obtained after DES pretreatment of samples. It can be seen that CB and W-CB after DES pretreatment increased their crystallinity, as indicated by *CrI* and TCI values compared with the starting materials.

However, this trend was only followed for LOI values in the case of W-CB+DES (from 2.15 to 2.62). Meanwhile, HBI showed the opposite tendency, decreasing crystallinity in three materials (CB, W-CB, and PreH) after DES pretreatment. Furthermore, it can be highlighted that PreH biomass subjected to different time pretreatments with DES showed similar behavior for all crystallinity indexes, increasing from 4 to 8 h and then decreasing again at 16 h. Excessively harsh conditions could promote the destruction of some cellulose crystalline areas [37], and cleavage of hydrogen bonds of biomass [38], which probably explains the reduction in crystallinity from 8 to 16 h. Additionally, the differences found in terms of crystallinity could be supported by the changes observed in ATR-FTIR and SEM analyses further explained.

In order to understand the influence of time in DES pretreatment on PreH material, since their chemical composition was very similar (see above in Table 2), ATR-FTIR spectra are presented in Figure 1a. The spectra showed some similarities between PreH and PreH+DES at 8 h. Accordingly, the spectra remained quite similar in the 3125–3600 cm^{-1} band corresponding to OH free and hydrogen-bonded stretching of cellulose and cellulose derivatives [39], at 1029 cm^{-1} corresponding to C–H plane deformation and C–O stretching in cellulose and lignin [5], supporting cellulose retention caused by DES pretreatment. Conversely, characteristic peaks of lignin were decreased after DES pretreatment in PreH in bands 1610 cm^{-1} (C=O corresponding to stretching of aromatic ring in lignin and in carboxylic groups, carboxylic acid, and ester compounds) [40], 1108 cm^{-1} (aromatic deformation of lignin C–H), 1160 cm^{-1} (C–O–C stretching of cellulose and hemicellulose [26], and 2850 cm^{-1} and 2920 cm^{-1} (related to aromatic methoxyl groups stretching C–H and in methyl and methylene groups of side chains) [41], what is in concordance with delignification reported in Table 2. Moreover, it was also demonstrated that the effect of DES pretreatment at 8 h, observing lower intensities than PreH in feature peak at 1317 cm^{-1} , linked to stretching C–O of substituted aromatic units [26] and 1732 cm^{-1} which can be attributed to lignin (off-plane C–H vibration in p-hydroxyphenyl units) [42], indicating the bonds of lignin-carbohydrate complexes and proving partial lignin removal of the PreH biomass as was previously reported. DES pretreatment at 4 and 16 h showed spectra with lower intensities than PreH, suggesting the remarkable impact of DES on this biomass. However, SEM-photos in PreH+DES at 4 h (Figure 1b) exhibited minor alterations in its structure than PreH+DES at 8 and 16 h, in which great morphology changes were noticed, highlighting the great number of pores and rupture of fibrils across the biomass.

3.3. Enzymatic Hydrolysis

Enzymatic hydrolysis of CB, W-CB, and all CRMs obtained after pretreatment with ChCl/U was carried out for 72 h, assaying different enzyme loads because of the high total polysaccharide content. Released sugars (glucose, xylose, and total sugars) (Figure 2A) and glucose and xylose saccharifications (Figure 2B) were notably enhanced when ChCl/U pretreatment was carried out. In this sense, the enzymatic hydrolysis of CB and W-CB without DES pretreatment yielded the worst percentages of saccharification and, therefore, low concentrations of released monomeric sugars. Under the highest concentrations of enzyme load, only 4.85 and 7.83 g/L were released from CB and W-CB, respectively. However, after DES pretreatment, CB+DES and W-CB+DES showed the best percentages of total released sugars at maximum enzyme load, reaching more than 12 g/L. The same trend was followed by the attending glucose and xylose saccharifications, achieving around 60% and 40%, respectively, in both residues.

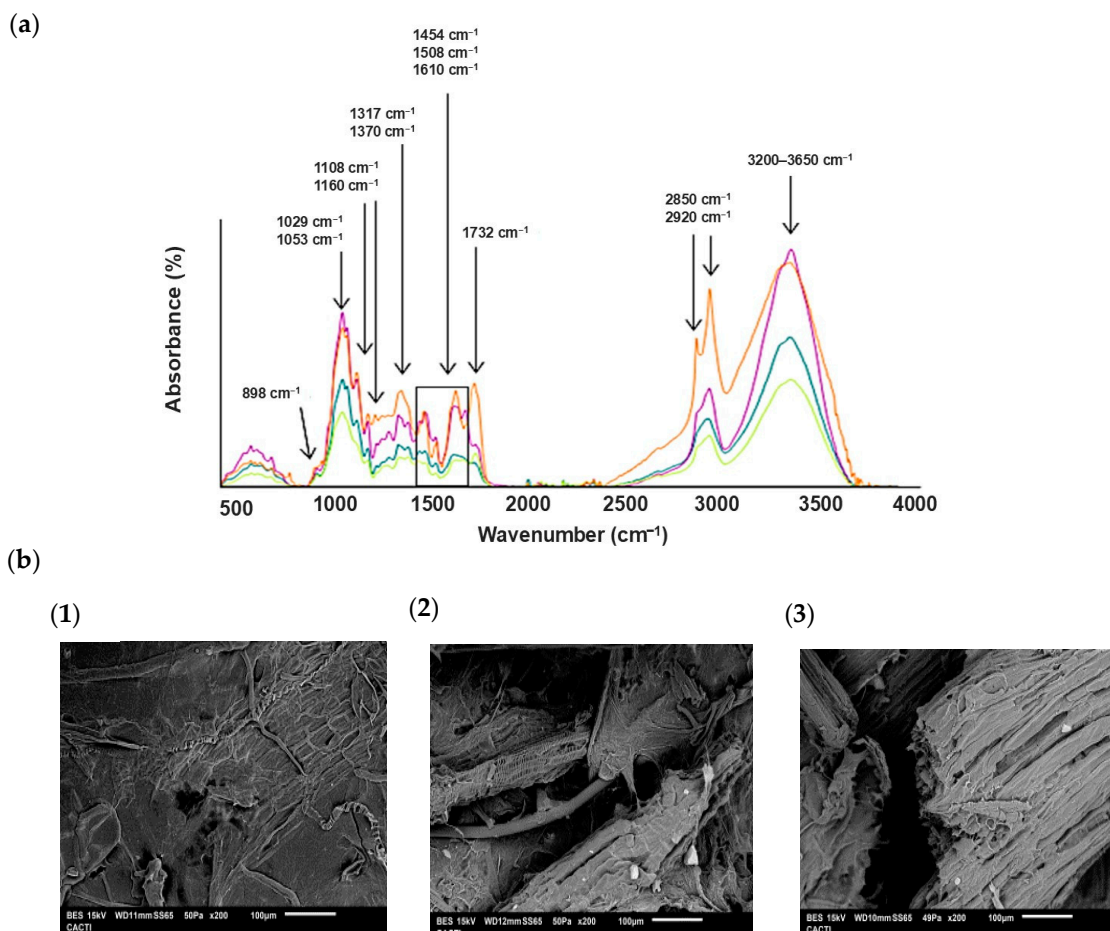


Figure 1. (a) ATR-FTIR spectra of PreH (orange line) *, PreH+DES 4 h (blue line), PreH+DES 8 h (purple line), and PreH+DES 16 h (light green line), and (b) SEM images of PreH DES 4 h (1), PreH+DES 8 h (2), and PreH+DES 16 h (3) *. * Results from part I [5].

Procentese et al. [43] achieved similar results of saccharification (58% of glucan and 31% of xylan) in corncob after ChCl/U pretreatment at 115 °C for 15 h of adding a high enzyme dosage (166 FPU/mL). Meanwhile, Pan et al. [44] reported similar saccharifications after all pretreatments with ChCl/U at conditions between 110 and 130 °C for 4 to 8 h, compared with the untreated rice straw using a low enzyme dosage of 89 µL of cellulase per gram. Therefore, an elevated amount of enzyme is necessary to obtain a reasonable sugar yield and release total sugars from CRMs after preparing CB with ChCl/U as it was reported in the literature and corroborated in this work. Nevertheless, recent works proved the suitability of using combined or sequential methods with this type of DES, observing better efficiencies in pretreatments [14,45]. Particularly, Ong et al. [46] reported higher sugar yields combining ultrasounds with NaOH-diluted ChCl/U in oil palm fronds. For that reason, an acid-diluted hydrolysis was postulated before DES pretreatment.

As other pretreatments were added to the process, lower amounts of enzyme (23, 40, and 63 FPU/g) were assayed in materials after sequential pretreatments (W-CB+DES, PreH+DES 4 h, PreH+DES 8 h, and PreH+DES 16 h) compared to prior enzymatic hydrolysis. In this sense, the initial three materials (CB, W-CB, and CB+DES) were also submitted to enzymatic hydrolysis under lower enzyme amounts to obtain comparative data (Figures 3 and 4).

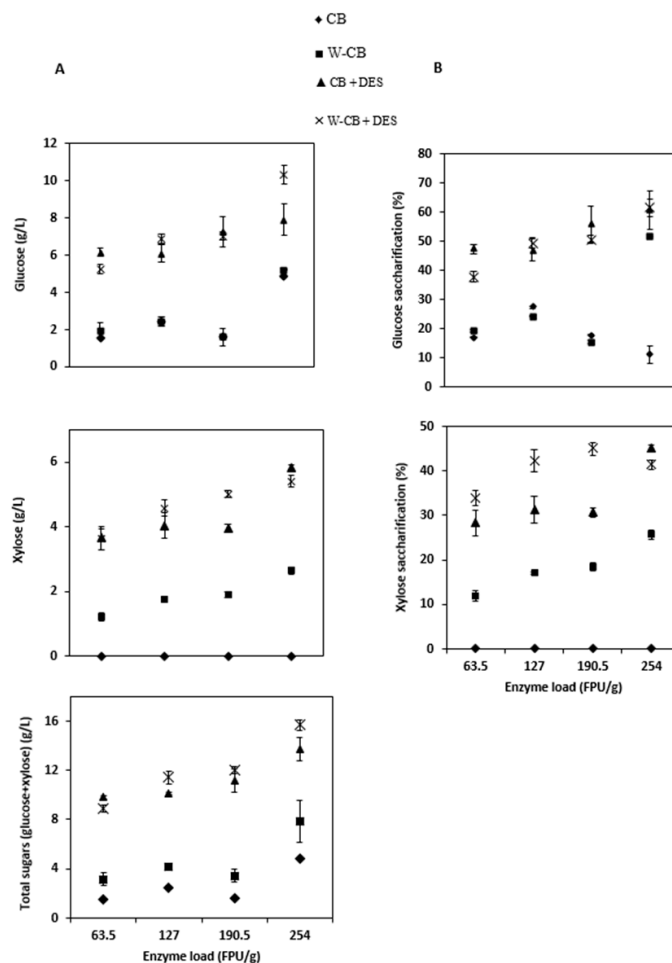


Figure 2. Study of enzyme dosage in enzymatic hydrolysis of CB, W-CB, and all CRMs after DES pretreatment, (A) released glucose, xylose, and total sugars (g/L) and (B) glucose and xylose saccharification (%). Error bars correspond to $n = 2$.

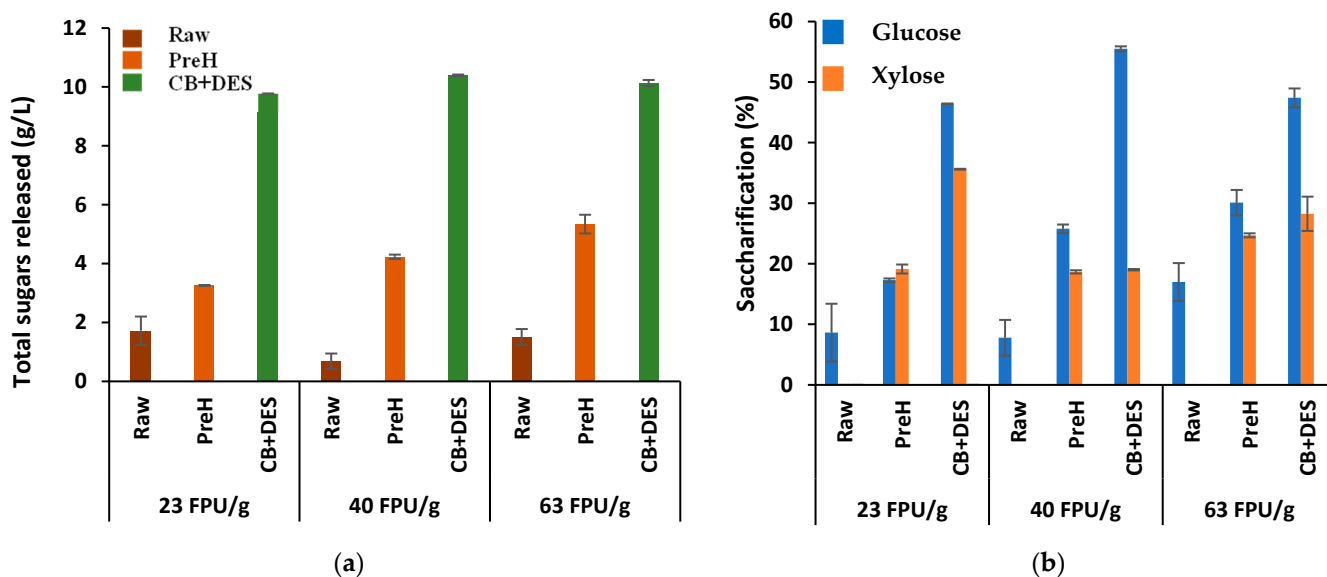


Figure 3. (a) Total sugar released during enzymatic hydrolysis and (b) saccharification percentage with lower dosage of enzyme (23, 40, and 63 FPU/g) of CB, PreH, and CRMs after DES pretreatments. DES pretreatments were conducted at 100 °C, 16 h, and LSR of 20:1 (w/w). Error bars correspond to $n = 2$.

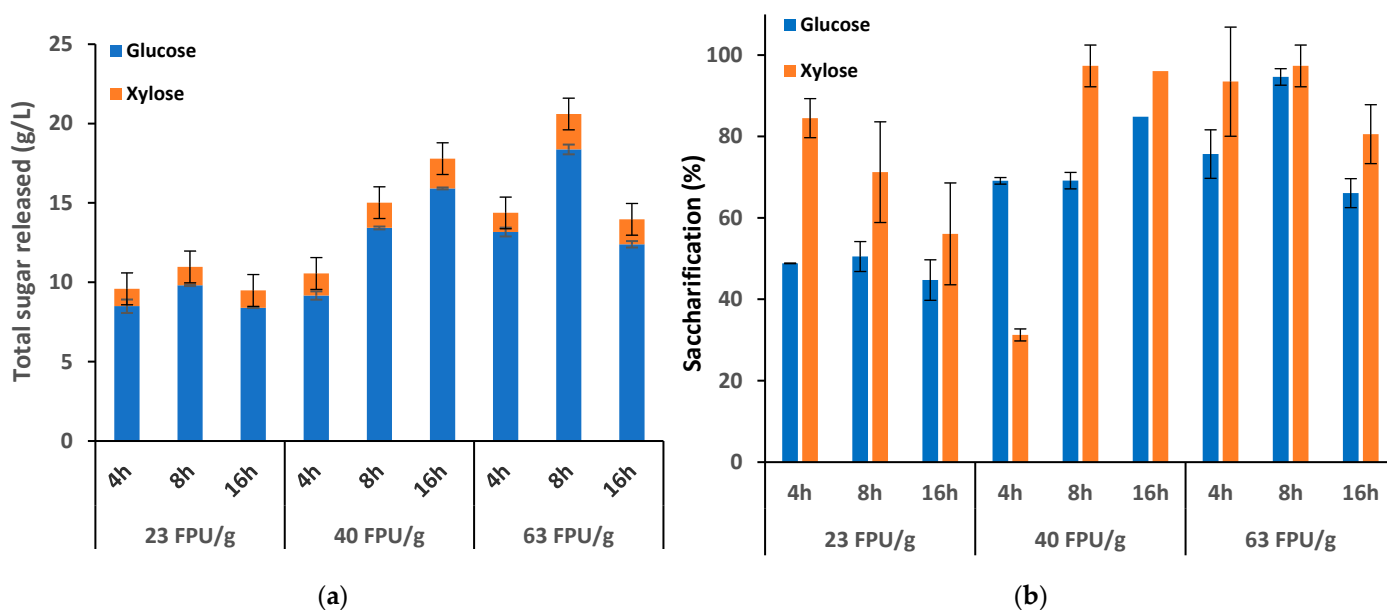


Figure 4. (a) Total sugar released during enzymatic hydrolysis and (b) saccharification percentage with lower dosages of enzyme (23, 40, and 63 FPU/g) of PreH + DES pretreatments at different times. All DES pretreatments were conducted at 100 °C and LSR of 30:1 (*w/w*). Error bars correspond to $n = 2$.

The results of total sugar released in CB+DES showed the same trend independently of the quantity of enzyme, with around 10 g/L (Figure 3a). However, PreH achieved higher concentrations of sugars as the quantity of enzyme added increased, reaching half (5 g/L) of that of DES pretreatment at 63 FPU/g. This fact corroborated the effectiveness of DES pretreatment to enhance enzymatic hydrolysis compared to conventional pretreatments such as diluted acid. Additionally, this advantage was confirmed in Figure 3b, which showed enzymatic saccharification percentages in the same three materials, achieving similar results to those previously described. Thus, DES pretreatment raised higher saccharifications than other materials at any enzyme charge studied. Particularly, glucose saccharification in CB+DES surpassed 50%, more than double that of PreH; meanwhile, xylose saccharification was around 18% for both materials, PreH and CB+DES, at 40 FPU/g. Thus, it was demonstrated the effectiveness and suitability of DES pretreatment compared to diluted acid hydrolysis in terms of saccharification. Zhang et al. [47] also confirmed the scarce performance in terms of sugars released after diluted acid hydrolysis on sunflower stalk bark using 1% of H_2SO_4 at 150 °C for 60 min, raising only 26% of glucose saccharification.

Sole DES pretreatment did not overstep 46% and 35% saccharification yields of glucose and xylose, respectively. However, sequential prehydrolysis and DES pretreatments showed the best saccharification yields at any time of DES pretreatment studied (Figure 4a). In this sense, PreH+DES 8 h achieved the best performance under any enzyme load studied. For instance, at intermediate enzyme load (40 FPU/g), release around 15 g/L of total sugars, corresponding with 70% of glucose and more than 95% of xylose saccharifications (Figure 4b). In this sense, Auxenfans et al. [48] also noted that sequential diluted acid and ionic liquid pretreatment improved saccharification yields compared to single pretreatments.

Figure 5 shows the sugar release (Figure 5a) and enzymatic saccharification (Figure 5b) of the best-performing material (PreH+DES 100 °C, 8 h) at different LSRs (10:1, 20:1, and 30:1).

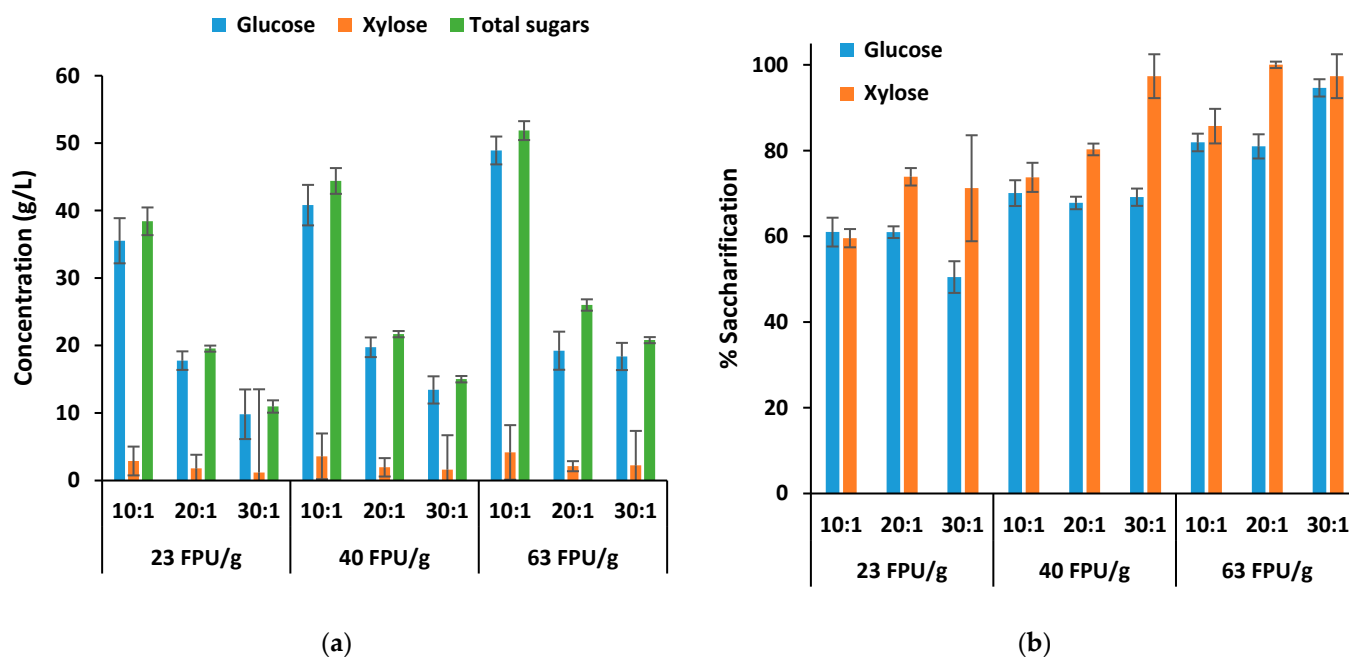


Figure 5. (a) Total sugar released during enzymatic hydrolysis and (b) saccharification percentages with lower dosages of enzymes (23, 40, and 63 FPU/g) at different LSR (10:1, 20:1, and 30:1) during enzymatic hydrolysis of PreH+DES pretreatments at 8 h, 100 °C, and LSR 20:1 (*w/w*). Error bars correspond to $n = 2$.

The results illustrated that when the enzyme load increases, maintaining the same LSR, the total sugars released also increase. This trend was observed for all LSRs assayed. For instance, at LSR 20:1, the total sugars increased from 19.55 g/L < 21.69 g/L < 26.01 g/L in ascending order of enzyme load (23 < 40 < 63 FPU/g). As expected, enzymatic saccharification also followed the same tendency previously mentioned; therefore, the best results were achieved at higher enzyme load using 63 FPU/g at LSR 30:1 (*v/w*) with 95% and 97% of glucose and xylose saccharifications, respectively. However, remarkable results were also attained at lower enzyme load (23 FPU/g) using LSR 20:1 (*v/w*) with 60.97% and 73.89% saccharification percentages of glucose and xylose.

These results clearly outperform those reported in previous works using ChCl/urea-based pretreatments for lignocellulosic biomass (see Table 3). While earlier studies on rice straw, wheat straw, and oil palm empty fruit bunches (OPEFB) achieved relatively modest saccharification yields—typically below 40% glucose and 20% xylose—even under severe pretreatment conditions (120–130 °C, 3–8 h) [44,49,50], the approach developed in this work reached up to 60% glucose and 74% xylose using mild temperatures (100 °C) during DES pretreatment and low enzyme load in enzymatic hydrolysis (23 FPU/g). This remarkable improvement can be attributed to the synergistic effect of the acid prehydrolysis step combined with diluted DES treatment, which significantly promotes hemicellulose solubilization in the first step and reduces biomass recalcitrance and structural disruption by the combination of both. Compared to alternative strategies such as ultrasound-assisted pretreatment (yielding only 35% glucose) [51], our approach demonstrates higher efficiency in releasing monomeric sugars. These findings highlight the potential of subsequent DES pretreatment to prehydrolysis solid residue as a promising route for enhancing enzymatic hydrolysis and biorefinery processes.

Table 3. Comparative analysis on ChCl/urea as pretreatment in different biomasses and subsequent enzymatic hydrolysis to release monomeric sugars.

Biomass	DES	Pretreatment Conditions	Enzymatic Parameters	Enzyme Load	Saccharification	Reference
Rice straw	ChCl/urea (1:2)	110 °C, 8 h, LSR 20:1 <i>w/w</i>	T: 50 °C, t: 48 h, LSR: 10/1 <i>w/v</i>	100 µL cellulase/g substrate	Glucose: 0.78% Xylose: 0.26%	[44]
OPEFB *	ChCl/urea (1:2)	120 °C, 3 h, LSR 10:1 <i>w/w</i>	T: 50 °C, t: 48 h, LSR: 2/1 <i>v/w</i>	10 mg of <i>Trichoderma viride</i> cellulase/mL	Total reducing sugars: 19.60%	[49]
Wheat straw	ChCl/urea (1:2)	120 °C, 3 h, LSR 30:1 <i>w/w</i>	T: 50 °C, t: 72 h, LSR: 50/1 <i>v/w</i>	15 FPU CellicCtec2/g substrate	Glucose: 40% Xylose: 20%	[50]
Napier grass	ChCl/urea (1:2)	130 °C, 5 h, LSR 20:1 <i>w/w</i>	T: 50 °C, t: 72 h, LSR; 40:1 <i>v/w</i>	30 FPU CTech 2/g biomass	Glucose: 120 mg/g Xylose: 10 mg/g	[52]
OPEFB *	Ultrasounds assisted ChCl/urea (1:2)	50 °C, 30', 53 KHz, 210 W, LSR 10:1 <i>w/v</i>	T: 50 °C, t: 72 h, LSR: 40:1 <i>v/w</i>	<i>Trichoderma viride</i> cellulase 30–50 FPU/g	Total reducing sugars: 35%	[51]
Chestnut burrs	Diluted ChCl/urea (1:2)	100 °C, 16 h, LSR 20:1 <i>w/w</i>	T: 50 °C, t: 72 h LSR: 30:1 <i>v/w</i>	23 FPU Cellic Ctec2/g substrate	Glucose: 46% Xylose: 35%	This study
Chestnut burrs are pretreated after prehydrolysis	Diluted acid- ChCl/urea (1:2)	100 °C, 8 h, LSR 20:1 <i>w/w</i>	T: 50 °C, t: 72 h LSR: 20:1 <i>v/w</i>	23 FPU Cellic Ctec2/g substrate	Glucose: 60% Xylose: 74%	This study

* oil palm empty fruit bunch.

3.4. Relation Between Cellulose Crystallinity and Enzymatic Saccharification

Generally, the best results in saccharification of pretreated biomass were attributed to lower crystallinity values of pretreated biomass than raw biomass [53,54]. For instance, Kumar et al. [55] found that pretreatments commonly reduce crystallinity of biomass due to alterations in cellulose conformation. This fact could be explained by the disruption of cellulose microfibrils that decreases the cellulose polymerization degree, enhancing enzymatic digestibility [56]. However, we found in this study a positive trend between crystallinity and enzymatic saccharification, with saccharification generally enhanced as crystallinity increased. The TCI values, among all crystallinity parameters (LOI, *CrI*, and HBI), were the ones that fit best with saccharification results; they are represented in Figure 6. Thus, it was observed a value of R-square near 0.8 when TCI values of materials (CB, W-CB, CB+DES, and W-CB+DES) were correlated with their enzymatic saccharifications (Figure 6a). Meanwhile, when PreH+DES data was added, the R-square decreased to around 0.63. Several authors reported an increase in crystallinity, specifically after DES pretreatments [57–59]. For instance, Wang et al. [60] assayed four different polyol-based DES in mosso bamboo, noting a crystallinity change from 36.2% to nearly 70% and saccharification up to 80% after 48 h. Ong et al. [46] also observed higher crystallinity values after DES pretreatment with ultrasound-assisted ChCl/U in oil palm fronds, increasing *CrI* from 45.10% to 49.61%, although no improvements were observed in enzymatic saccharification, probably due to the short pretreatment time (30 min). However, Hou et al. [45] reported a two-fold improvement in cellulose digestibility and glucose yield when *CrI* values increased from 43.8% to 54.3% in rice straw compared to pretreated rice straw using ChCl/U for 12 h at 120 °C. This behavior could be explained by the retention of cellulose occurring due to the DES pretreatment and removal of amorphous fractions (lignin, hemicellulose, or amorphous cellulose), obtaining an increase in cellulose content of pretreated materials and, therefore, in crystallinity [61,62]. Furthermore, it was demonstrated that ChCl/U can produce nanofibrillation of bleached birch pulp at room temperature without changes in

crystallinity and DP [63]. In addition, nanofibers are more susceptible to being hydrolyzed than lignocellulose [64] due to the increase in specific surface area and amorphous zones, which allows enzyme action and fast hydrolysis [65]. Additionally, Badillo et al. [66] also noticed slight swelling of cellulose using ChCl/U. For all these reasons, materials after DES pretreatment achieved better results in glucose saccharification than starting materials (CB, W-CB, and PreH), even having higher crystallinity values.

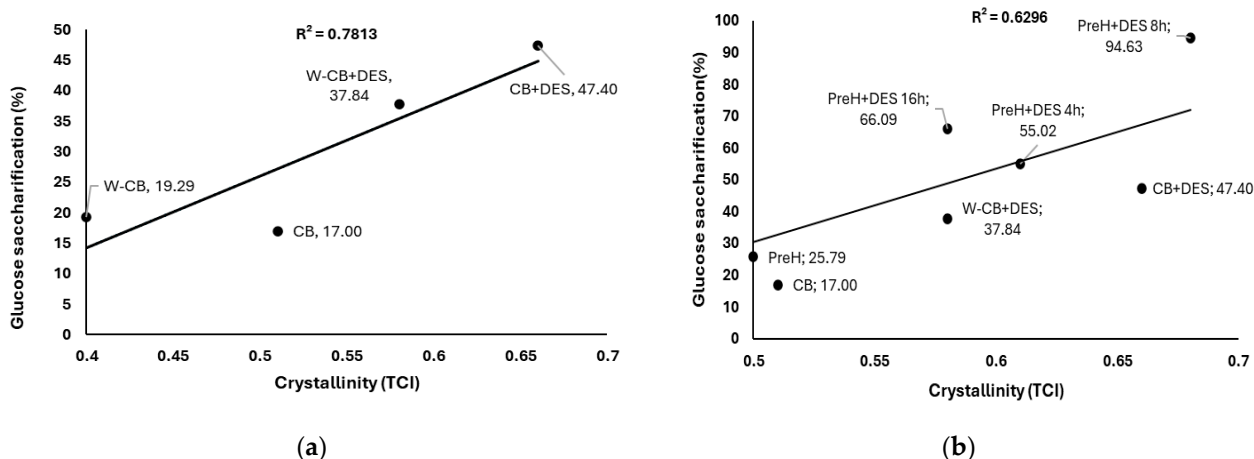


Figure 6. (a) Relationship between glucose saccharification of CB, W-CB, CB+DES, and W-CB+DES at 63.5 FPU/g and their TCI values, and (b) Fit between glucose saccharification of CB, CB+DES, PreH, PreH+DES 4 h, PreH+DES 8 h, and PreH+DES 16 h at 40 FPU/g.

Finally, the effectiveness of ChCl/U as a post-prehydrolysis pretreatment was confirmed, as well as a substantial improvement in glucose saccharification yield while requiring a markedly lower enzyme dosage compared to either PreH alone or CB treated directly with DES. Likewise, a positive correlation was demonstrated between biomass crystallinity and enzymatic saccharification efficiency, suggesting that structural ordering plays a critical role in substrate accessibility and hydrolytic performance. This finding reinforces the importance of pretreatment strategies that modulate crystallinity to optimize sugar release.

4. Conclusions

This study demonstrates the potential of chestnut burrs (CB) as a valuable feedstock for biorefinery applications through an environmentally friendly pretreatment strategy. The use of deep eutectic solvents (DES) composed of choline chloride and urea (ChCl/U, 1:2 mol/mol) effectively disrupted the lignocellulosic structure, enabling high saccharification yields after enzymatic hydrolysis. The best performance achieved up to 60% glucan and 74% xylan conversion under mild conditions (100 °C) of DES pretreatment in PreH material at 8 h, with further reduction in enzymatic hydrolysis parameters such as enzyme loading and LSR. These improvements significantly decrease pretreatment time from 16 h to 8 h, minimize energy consumption, and reduce the expenses of enzyme and buffer in the enzymatic hydrolysis step, enhancing both process efficiency and environmental sustainability. Overall, the proposed approach offers a promising pathway (prehydrolysis–DES–enzymatic hydrolysis) for the valorization of agro-industrial chestnut residues, contributing to the development of greener and more cost-effective biorefinery processes.

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analysis, I.C.-T., M.G.M.-A. and J.M.D.; investigation, I.C.-T., M.G.M.-A. and J.M.D.; resources, N.P.G. and J.M.D.; data curation, I.C.-T. and M.G.M.-A.; writing—original draft preparation, I.C.-T. and M.G.M.-A.; writing—review and editing, N.P.G., R.P.d.S.O. and J.M.D.; visualization, I.C.-T. and M.G.M.-A.; supervision, N.P.G., R.P.d.S.O. and J.M.D.; project administration, J.M.D.; funding acquisition, J.M.D. and R.P.d.S.O. All authors have read and agreed to the published version of the manuscript.

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