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Effects of polyol-based deep eutectic solvents on the efficiency of rice straw enzymatic hydrolysis

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ABSTRACT

Deep eutectic solvent treatment to overcome lignocellulose resistance to deconstruction has great potential for the production of biofuels and bioproducts. Most investigations have been focused on optimizing the sugar yield from specific feedstocks. However, little is known about the interaction between deep eutectic solvent and cellulose during pretreatment, post-treatment cellulose accessibility to enzymes, and lignin fractionation. We evaluated four deep eutectic solvents that consisted of choline chloride and biomass-derived polyols (ethylene glycol, glycerol, xylitol, and sorbitol). Choline chloride-glycerol (ChCl-Gly) pretreatment retained >90 % glucan from rice straw, even at high pretreatment severity (>4.2 severity factor). In addition, ChCl-Gly pretreatment fractionated 74 wt.% lignin and completely removed acetyl groups from xylan, which enhanced glucan digestibility from 21 % to 87 %. However, the ChCl-Gly deep eutectic solvent became deposited on the solids and limited cellulose accessibility to cellulase enzymes. A sodium carbonate wash was highly effective in removing the deposited ChCl-Gly from the pretreated solids and enhancing cellulase accessibility to cellulose. The simple production and biodegradability without interfering with glucan make ChCl-Gly a promising lignocellulose pretreatment agent. In addition, the ability to fractionate both lignin and sugars enables biorefineries to use the lignin as a co-product.

1. Introduction

Petroleum processing for fuels and chemicals produces a large amount of carbon dioxide, which negatively affects the ecosystem (Lynd et al., 2008; Naik et al., 2010; Zhang et al., 2007; Zhang, 2008). The production of fuels and chemicals from renewable lignocellulose, such as corn stover, wheat straw, and rice straw, has the potential to mitigate carbon dioxide emissions. Among lignocellulose sources, rice straw, a byproduct of the rice grain harvest, is one of the most abundant agricultural residues globally (Binod et al., 2010). The growing global population will increase rice demand, which will significantly increase the amount of available rice straw. The conversion of rice straw into biofuels and bioproducts will improve global energy and economic security and facilitate a sustainable society. However, upgrading rice straw is difficult because cellulose, hemicellulose, and lignin of rice straw are woven together into a recalcitrant structure that prevents efficient release of sugars by enzymatic degradation. Hence, a pretreatment step is typically required to increase cellulose accessibility to enzymes and boost sugar yield (Alvira et al., 2010; Mosier et al., 2005).

Numerous pretreatment technologies have been developed to

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overcome lignocellulose's recalcitrance, including physical (Chang et al., 1981; Cybulska et al., 2013; Ewanick and Bura, 2010; Fang et al., 2015; Ryu et al., 1982), chemical (Sathitsuksanoh et al., 2010, 2012), biological (Ma et al., 2010), and their combinations. A major challenge in pretreatment is to fractionate lignin without touching glucan, so the glucose can be used for biofuels and the fractionated lignin can be used as a co-product in biorefineries (Scheme 1). Pretreatment with deep eutectic solvents (DESs), a physico-chemical process, has garnered interest in lignocellulose pretreatment because of the cost-effectiveness, ease of synthesis, and biodegradability of DES (Smith et al., 2014; Xu et al., 2018; Zhang et al., 2012a, b). Moreover, numerous DESs are nontoxic to microbes in downstream biofuels fermentation (Huang et al., 2017); thus, a costly detoxification step is eliminated.

Deep eutectic solvents are formed by the interaction between a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) such that the mixture forms a eutectic solvent with a melting point lower than that of each component (Abbott et al., 2003, 2006). Polyols, such as ethylene glycol (EG) (Chen et al., 2018a, 2020; Zhang et al., 2016a) and glycerol (Gly) (Wang et al., 2020b; Zhang et al., 2016a), have been used as HBDs. These polyol-based DESs have free hydroxyl groups that interact with the free and etherified hydroxyl groups of lignin (Zhang et al., 2016a); this interaction enables the high lignin fractionation efficiency and enhanced cellulose accessibility to cellulase enzymes (Alvarez-Vasco et al., 2016; Kim et al., 2018; Wang et al., 2020a). As indicated earlier, these DESs are biodegradable and not toxic to microbes in downstream fermentation (Huang et al., 2017). Despite these advantages of DESs, we know little about their dynamic interaction with carbohydrates and lignin and their effect on glucan digestibility. The lack of this information slows the design of DESs for efficient lignocellulose conversion in biorefineries.

Our goal in this study was to determine the extent to which the pretreatment severity by polyol-based DESs affected sugar release and to establish a relationship between pretreatment severity and changes in the physical and chemical properties of pretreated biomass. We screened four polyol-based DESs, ChCl-ethylene glycol (EG), ChCl-glycerol (Gly), ChCl-xylitol (Xyl), and ChCl-sorbitol (Sor), for pretreatment of rice straw at 80−150 °C for 3−24 h. We chose these temperature and time ranges to best establish the relationship between pretreatment efficiency and pretreatment severity factor. We found that ChCl-Gly fractionated 74 wt. % lignin and completely deacetylated rice straw, leaving >90 % glucan-rich treated solids even at >4 severity factor. We also found that washing the treated solids with sodium carbonate increased glucan digestibility to 87 %, a 28 % increase compared with a water wash. Lastly, we characterized the pretreated and sodium carbonate washed solids to identify the factor(s) responsible for increased glucan digestibility. These characterizations serve as a reference for the effects of ChCl-based eutectic solvents on interaction with lignin and glucan digestibility. Our findings can guide biorefineries in the processing of other types of lignocellulose.

2. Material and methods

2.1. Materials

Rice straw was provided by Industrial Technology Research Institute (Taiwan). The straw was dried overnight at 80 °C, knife-milled, and sieved to the size of ~0.7−1.2 mm (16–25 mesh). All chemicals and reagents were purchased from VWR (USA) as analytical grade and used as received unless otherwise noted.

2.2. Synthesis of deep eutectic solvents (DESs)

Twelve lignocellulose-derived polyol-based DESs were synthesized using choline chloride (ChCl) as a hydrogen bond acceptor (HBA) and four lignocellulose-derived polyols as hydrogen bond donors (HBDs), i.e., ethylene glycol (EG), glycerol (GLY), xylitol (XYL), and sorbitol (SOR). In a typical synthesis, ChCl and the HBD partner were mixed in three molar ratios: 2:1, 1:1, and 1:2. The mixtures were heated at 80 °C until they formed a colorless transparent liquid; stirring was continued for 2 h, and the mixtures were cooled to room temperature. DESs were kept for 72 h at room temperature.

2.3. Rice straw pretreatment

The rice straw pretreatment was performed at a solid loading of 5 wt. %. In short, ~0.5 g rice straw was added to 9.5 g DES in a 15 mL glass pressure tube. The solution was vortexed vigorously. The pressure tube was heated in an oil bath at 80−150 °C for 3−24 h. After pretreatment, the pressure tube was cooled in an ice bath. Water (~25 mL) was added to regenerate the pretreated solids. The pretreated solids were washed twice with 25 mL water or sodium carbonate solution (5 wt.%). The washed samples were further washed with water until the pH dropped to between 5 and 6; the samples were freeze-dried for compositional analysis, enzymatic hydrolysis, and chemical structure characterization. All experiments were performed in triplicate, and data represented the mean with the standard deviation < 9%. Solid recovery, glucan retention, xylan removal, and lignin removal were calculated by Eq. 1–4:

$$\text{Solid recovery (wt.\%)} = \frac{\text{solid}_{\text{before}} - \text{solid}_{\text{after}}}{\text{solid}_{\text{before}}} \text{x100} \quad (1)$$

$$\text{Glucan retention (wt.\%)} = \frac{\text{glucan}_{\text{before}} - \text{glucan}_{\text{after}}}{\text{glucan}_{\text{before}}} \text{x100} \quad (2)$$

$$\text{Xylan removal (wt.\%)} = \frac{\text{xylan}_{\text{before}} - \text{xylan}_{\text{after}}}{\text{xylan}_{\text{before}}} \text{x100} \quad (3)$$

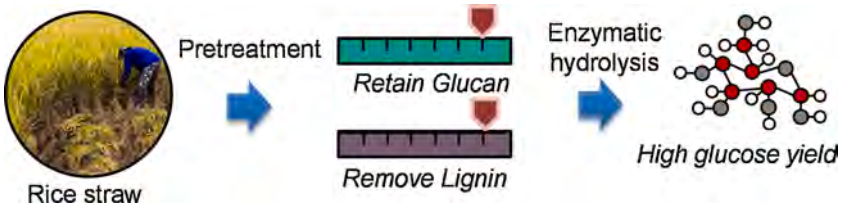
$$\text{Lignin removal (wt.\%)} = \frac{\text{lignin}_{\text{before}} - \text{lignin}_{\text{after}}}{\text{lignin}_{\text{before}}} \text{x100} \quad (4)$$

where $\text{solid}_{\text{before}}$, $\text{gluan}_{\text{before}}$, $\text{xylan}_{\text{before}}$, and $\text{lignin}_{\text{before}}$ represent the mass of solid, glucan, xylan, and lignin before pretreatment. $\text{Solid}_{\text{after}}$, $\text{glucan}_{\text{after}}$, $\text{xylan}_{\text{after}}$, and $\text{lignin}_{\text{after}}$ indicate mass of solid, glucan, xylan, and lignin after pretreatment.

The severity factor (R_0) of pretreatment was calculated by Eq. 5:

$$R_0 = t \cdot \exp\left(\frac{T\text{-}100}{14.75}\right) \quad (5)$$

where t is pretreatment time (min), and T is pretreatment temperature (^{o}C).



Scheme 1. The challenge in biorefinery is to fractionate lignin in high yield with high glucan retention.

2.4. Enzymatic hydrolysis

Enzymatic hydrolysis of the pretreated solids was conducted either in a single pot to measure the toxicity of the DES on enzymatic hydrolysis or in a conventional process in which the solid fraction was washed and separated for the enzymatic saccharification. Enzymatic hydrolysis was conducted in 50 mM citric acid buffer (pH 4.5) with a solid loading of 10 g glucan/L (23.2 g biomass/L). Sodium azide (NaN_3, 0.01 % (w/v)) was included as an antimicrobial agent. All hydrolysis experiments were performed in a rotary shaker at 50 °C at 250 rpm. The enzyme loading was 15 mg protein/g glucan and a Novozyme® cellulase (Ctec 2, protein concentration: 188 mg protein/mL) to hemicellulose (Htec 2, protein concentration: 190 mg protein/mL) ratio of 9/1 (v/v) or otherwise described. Enzymatic glucose and xylose yields were calculated by Eq. 6–7:

$$\text{Enzymatic glucose yield}\,(\text{wt.}\%) = \frac{\text{glucose in the enzymatic hydrolysate}}{\text{glucose equivalent in the pretreated solid}} \text{x}100 \quad (6)$$

$$\text{Enzymatic xylose yield}\,(\text{wt.}\%) = \frac{\text{xylose in the enzymatic hydrolysate}}{\text{xylose equivalent in the pretreated solid}} \text{x}100 \quad (7)$$

2.5. Compositional analysis of pretreated rice straw

The compositions of untreated and pretreated rice straw samples were determined by National Renewable Energy Laboratory analytical procedures (Sluiter et al., 2012). All sugars and organic acid concentrations in the acid-digested solutions were analyzed by HPLC equipped with a refractive index detector (RID) and a diode array detector (DAD). The Aminex HPX-87H column (300 × 7.8 mm, Bio-Rad®, Hercules, CA, USA) was used to separate sugars at 60 °C with 0.6 mL/min of 4 mM H_2SO_4 as a mobile phase. The concentrations of sugars were determined by the RID signals' peak area (HMF and furfural were determined by DAD signals at 280 nm). All sugars were calibrated against certified standards (Absolute Standards Inc., Hamden, CT, USA). The rice straw used in this study was composed of 30.3 wt.% glucan, 15.6 wt.% xylan, 1.1 wt.% arabinan, 21.5 wt.% lignin, 13.2 wt.% ash (~67 wt.% SiO_2), 1.4 wt.% acetyl content, and 16.9 wt.% others (proteins and extractives).

2.6. Characterization of DESs and pretreated rice straw samples

To determine the characteristics of rice straw after pretreatment in DESs, the pretreated solids were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FTIR), and two-dimensional (2D) ^{13}C-^{1}H Heteronuclear Single-Quantum Correlation (HSQC) Nuclear Magnetic Resonance (NMR) spectroscopy. Untreated rice straw was used as a control.

2.6.1. Scanning electron microscopy (SEM)

SEM was performed on treated solids to determine the changes in morphology. We used a TESCAN Vega3 SEM (Warrendale, PA, USA) with an energy dispersive x-ray spectrometry (EDS) detector for elemental mapping. Before performing SEM, pretreated solids were sputter-coated by an SPI-Module™ Sputter Coater (West Chester, PA, USA) using a gold target for 90 s under Argon gas.

2.6.2. X-ray diffraction (XRD)

To determine the degree of crystallinity, X-ray diffraction was performed on pretreated samples using the Bruker D8 (Billerica, MA, USA) with CuK_α radiation (λ =0.15418 Å). The scanning rate was 0.5 s/step (0.02 step increment), ranging from 10° to 45° unless otherwise noted. The change in the degree of crystallinity of biomass was expressed in terms of the crystallinity index (CrI). The CrI value was calculated based on the Segal method (Segal et al., 1959) using the relationship between the height of the crystalline peak corresponding to (002) lattice plane (I_{002}) and the amorphous region (I_{am}), which was the minimum (~18°) between (110) and (002) lattice planes as shown in Eq. 8:

$$\text{CrI} = \frac{I_{(002)} - I_{am}}{I_{(002)}} \text{x}100 \quad (8)$$

2.6.3. Fourier-transform infrared spectroscopy (FTIR) spectroscopy

The changes in the chemical structures of pretreated rice straw (bond strength between sugar monomers and lignin-carbohydrates) relative to untreated samples were characterized by the JASCO 4700 FT-IR Spectrometer (Akron, OH, USA) equipped with Attenuated Total Reflection (ATR, Pike Technologies, Madison, WI, USA). The samples were scanned in the spectral range between 400 and 4000 cm^{-1} for 256 scans at 4 cm^{-1} resolution. The lateral order index (LOI) and total crystallinity index (TCI) were calculated by the intensity ratio of 1423/897 (A_{1423}/A_{897})(Nelson and O'Connor, 1964) and 1372/2900 (A_{1372}/A_{2900}) (Åkerholm et al., 2004; Kataoka and Kondo, 1998; Nelson and O'Connor, 1964), respectively. The crystalline cellulose bands can be observed at 1423 and 1372 cm^{-1}, and the band at 897 cm^{-1} is associated with amorphous cellulose. The band at 2900 cm^{-1} represents the C–H and CH_2 stretching of the cellulose. Thus, TCI represents the proportion of crystalline cellulose, and LOI indicates the overall degree of crystallinity of cellulose. The syringyl (*S*)- and guaiacyl (G)-like lignin unit (S/G) ratio was determined using the ratio of A_{1260}/A_{1330} (Pal et al., 2016).

2.6.4. Thermogravimetric analysis (TGA) of the DES

The TGA of the DESs was conducted to determine their thermal stability, using High-Res TGA 2950 (TA instruments, New Castle, DE, USA) under N_2 flow of 100 cc/min from room temperature to 350 °C with a ramping rate of 10 °C/min. The derivative thermogravimetric (DTG) curves were plotted to determine the points at which the weight loss was apparent. The decomposition temperature was identified when the weight loss of the DES was >10 wt%.

2.6.5. 2D ^{13}C-^{1}H Heteronuclear Single-Quantum Correlation (HSQC) nuclear magnetic resonance (NMR) spectroscopy

Raw rice straw and six pretreated rice straw samples were dried and ground to pass an 80-mesh sieve. The ground samples were ball-milled using a Retsch PM-400 planetary ball-mill (Newtown, PA). For each sample, approximately 200−300 mg of ground material was added to a 50 mL ZrO_2 jar with ten 10-mm ZrO_2 balls and milled at 300 rpm for a total of 10 h (20 min milling, 10 min pause) to give 400 min actual milling time. Afterward, approximately 30 mg of each milled sample was dissolved directly in a 5 mm NMR tube (7 in. in length) using 500 µL of DMSO-d_6 and sonicated at 35 °C for 1 h. The dissolved samples were homogeneous and clear solutions. NMR spectra were acquired on a Bruker-Biospin (Rheinstetten, Germany) AVANCE III HD™ 500 MHz spectrometer fitted with a nitrogen-cooled 5 mm Prodigy™ TCI gradient cryo-probe with inverse geometry. One-bond ^{13}C–^{1}Hcorrelation (HSQC) spectra were acquired using adiabatic Bruker pulse program hsqcetgpsisp2.2 and processed as described (Yelle et al., 2008) using Bruker TopSpin 3.6.2 software. The relative contour intensities in this study were expressed relative to the methoxyl contour (OMe) as an internal standard.

3. Results

We synthesized 12 choline chloride (ChCl)-based DESs as hydrogen bond acceptors (HBA) and four lignocellulose-derived polyols as hydrogen bond donor (HBD) partners, i.e., ethylene glycol (EG), glycerol (Gly), xylitol (Xyl), and sorbitol (Sor), with HBA:HBD molar ratios of 2:1, 1:1, and 1:2. ChCl was solid at room temperature. ChCl-EG (1:1), ChCl-Gly (1:2), ChCl-Xyl (1:1), and ChCl-Sor (1:1) were homogeneous

liquids at room temperature (**Fig. S1A**). Hence, we selected those four DESs for further studies (*see Supporting Information*). First, we examined their physical and thermal properties (**Table S1**). TGA profiles showed that ChCl-Gly, ChCl-Xyl, and ChCl-Sor were stable at temperature < 150 °C (**Fig. S1B**), whereas the ChCl-EG was not stable at 150 °C and decomposed at ~99 °C (**Table S1**). All DESs were viscous compared with water (0.8 cP at 25 °C). ChCl-EG and ChCl-Gly had lower viscosity compared with ChCl-Sor and ChCl-Xyl because of the formation of strong hydrogen bonds (Garcia et al., 2019). In turn, their high viscosity made them difficult to handle at room temperature and use in lignocellulose pretreatment (*See supporting information*). Second, we used the four DESs to treat rice straw at 150 °C for 15 h. Then, we characterized changes in the composition of pretreated solids and their enzymatic glucan digestibility (**Fig. S2**). ChCl-EG-pretreated solid had the highest glucan digestibility of 80 % (**Fig S2A**). However, as already indicated, ChCl-EG had low thermal stability and decomposed at < 150 °C. Interestingly, ChCl-Gly had the highest glucan retention (96.5 %) and lignin fractionation (52.2 %), which was the most promising pretreatment (**Fig. S2B**). As detailed next, we characterized the DESs-pretreated solids to understand how DES-mediated changes in chemical structure affected hydrolysis efficiency.

3.1. HSQC NMR and FTIR revealed deacetylation and G-like lignin removal by ChCl-Gly

To understand how these DES pretreatments affected the chemical structure of lignocellulose solids and their glucan digestibility, we used HSQC NMR to characterize the chemical groups of the treated solids (Fig. 1). The assignments of HSQC cross-peaks are shown in **Table S2**. ChCl-EG, ChCl-Gly, and ChCl-Xyl were chosen as pretreatment agents because of their low viscosities and ease of handling. When we compared the sample spectra and integration data in the aliphatic region, acetyl groups were almost entirely removed by ChCl-EG and ChCl-Gly pretreatments. It was clear that the ChCl-Gly pretreatment was the most effective at removing the lignin sidechains (β-O-4 and β-5), cinnamyl alcohol end-groups (X_1), and acetyl groups substituted along xylan (2-*O*- and 3-*O*-Ac-β-D-Xyl*p*) units. The ChCl-Xyl pretreatment showed the least removal of lignin sidechains and acetylated xylan. However, ChCl-Xyl exhibited a large amount of β-O-4 removal (87 %) and almost complete β-5 removal. Xylan in nature is highly acetylated, which causes lignocellulose recalcitrance and inhibits binding of cellulase enzymes (Chen et al., 2012; Pan et al., 2006). Therefore, the disappearance of acetyl group cross-peaks mediated by ChCl-Gly

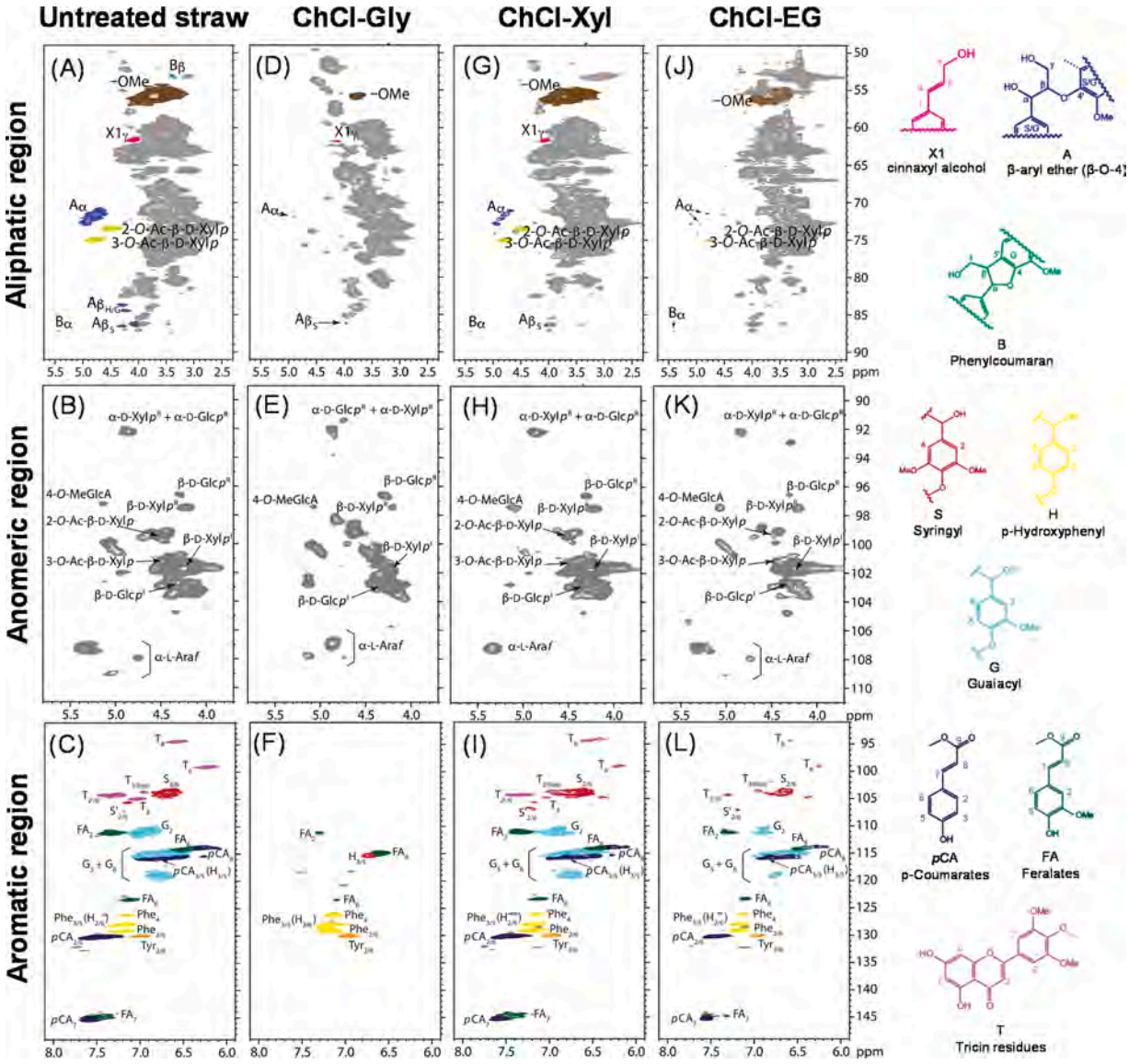


Fig. 1. 2D HSQC NMR of rice straw pretreated with ChCl-Gly (D, E, and F), ChCl-Xyl (G, H, and I), and ChCl-EG (J, K, and L) with untreated rice straw as a control (A, B, C).

indicated improved cellulose enzymatic accessibility (Chen et al., 2012; Pan et al., 2006) and corroborated the high glucan digestibility of pretreated solids.

When we compared the sample spectra and integration data in the aromatic region, the ChCl-Gly pretreatment displayed the most dramatic removal of the syringyl (*S*)- and guaiacyl (G)-like lignin units, along with the removal or depletion of the hydroxycinnamates (*p*CA and FA) and tricin (T), a flavonoid that is covalently incorporated into the lignin polymer in monocots (Lan et al., 2015). This substantial removal of the S, G, *p*CA, and FA units by the ChCl-Gly indicated nearly complete removal of lignin (Fig. 1F). The ChCl-Xyl pretreatment did not affect the aromatic region to any noticeable degree compared to that of untreated rice straw. Then we calculated the S/G ratio of treated solids. The ratio increased from 0.45 (untreated rice straw) to 0.87 in ChCl-Xyl and to 1.05 in ChCl-EG (Table 1). These increases in S/G ratio after pretreatment suggested an increase in G-like lignin unit removal. G-like lignin units are branched and covalently linked with other units, whereas S-like lignin units are linear and can link only to two other units (Boudet et al., 1998). The low S/G ratio of untreated rice straw was indicative of a branched lignin structure with low cellulose accessibility to enzymes. The aromatic region of the ChCl-Gly-treated solid did not have a strong signal because >50 % lignin was removed after pretreatment. Overall, after pretreatment by DES, treated solids had increased S/G ratios that suggested the removal of G-like lignin and greater cellulose accessibility to enzymes compared with untreated rice straw.

The sample spectra in the anomeric region revealed that ChCl-Xyl-treated samples did not appear visually different from the untreated rice straw with respect to polysaccharide intensities (Figs. 1**B-H**). Thus, ChCl-Xyl pretreatment did not affect the crystalline cellulose structure to a significant extent. This result was confirmed by comparing the reducing end-groups of β-glucan and β-xylan by calculating the ratios of β-D-Glc*p*^{R} / β-D-Xyl*p*^{R} integrals for all four samples, as seen in Table 1. The β-D-Glc*p*^{R} / β-D-Xyl*p*^{R} ratio for ChCl-Xyl was 0.26, which is similar to the β-D-Glc*p*^{R} / β-D-Xyl*p*^{R} ratio of the untreated (0.23). The ChCl-Gly spectrum visually showed more β-glucan reducing end-groups (i.e., β-D-Glc*p*^{R}) than untreated rice straw, which suggested that ChCl-Gly decreased the degree of polymerization of cellulose, and the pretreated solids had enhanced cellulose accessibility to enzymes. By comparing the β-D-Glc*p*^{R} / β-D-Xyl*p*^{R} ratios of the ChCl-Gly pretreated and untreated rice straw, the ChCl-Gly gave a much higher ratio of 5.22, thus confirming the high cellulose accessibility. The spectra of untreated rice straw and ChCl-EG did not show visible differences in β-glucan and β-xylan reducing end-groups, but the β-D-Glc*p*^{R}/β-D-Xyl*p*^{R} ratios were slightly different, with the ChCl-EG at 0.17 compared to the untreated at 0.23. The high β-glucan digestibility of the ChCl-EG would suggest that this β-D-Glc*p*^{R} / β-D-Xyl*p*^{R} ratio should be much higher than the untreated, but this was not seen here. Is it possible that there is some esterification occurring at the reducing ends with the ChCl-EG pretreatment. Esterification of reducing ends was recently reported by Sapouna and Lawoko (2021) when evaluating lignin-carbohydrate linkages. If that is the case for ChCl-EG, we would see a small contour for β-D-Glc*p*^{R} instead of an increase in size, like for ChCl-Gly, and we suggest this is the case here. Therefore, the likelihood is high that β-D-Glc*p*^{R} end-groups in ChCl-EG are reacting in a way that masks the actual decrease in the degree of polymerization of cellulose. What is more apparent is that the contours of the internal glucan and xylan residues (i.e., β-D-Glc*p*^{I} and β-D-Xyl*p*^{I}) of the ChCl-EG as depicted in Fig. 1**K** are significantly smaller visually than those of the untreated rice straw (Fig. 1B). These anomeric region results corroborated the high glucan digestibility of pretreated rice straw samples promoted by ChCl-EG and ChCl-Gly pretreatments.

We used FTIR to confirm changes in the chemical bonds and surface functional groups of treated solids. **Table S3** shows the observable characteristic FTIR peaks. There were no visible differences in band intensities or band shifts between the FTIR spectra of untreated and treated solids (**Fig. S3**). We calculated S/G ratio using bands at 1260 and 1330 cm^{-1}. The S/G ratios of treated solids were higher than untreated rice straw (**Fig. S3**), similar to the trend observed by HSQC (Table 1). These results confirmed that DES pretreatment removed G-like lignin from rice straw lignin. Overall, characterization results and composition changes demonstrated that ChCl-Gly pretreatment was promising with a high lignin fractionation efficiency, high glucan retention, and a moderate glucan digestibility of 68 %. We next investigated the temperature- and time-dependent aspects of ChCl-Gly pretreatment.

Table 1
Integration of contours for HSQC spectra of treated solids.

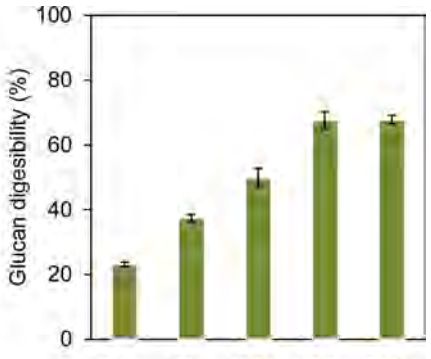
Spectral region	Number of protons	Untreated rice straw	ChCl-Gly	ChCl-Xyl	ChCl-EG
Aliphatic					
β-O-4 (A_α)	1	0.0541	nd[a]	0.0068	0.0013
β-5 (B_α)	1	0.0007	nd[a]	ni[b]	ni[b]
2-O-Ac-β-D-Xylp (2)	1	0.0324	nd[a]	0.0204	ni[b]
3-O-Ac-β-D-Xylp (3)	1	0.0390	nd[a]	0.0241	0.0024
X_1 (γ)	1	0.0076	nd[a]	0.0027	0.0016
Aromatic					
$S_{2/6}$	2	0.0293	nd[a]	0.0266	0.0155
G_2	1	0.0655	nd[a]	0.0305	0.0148
S/G ratio	–	0.45	nd[a]	0.87	1.05
Anomeric					
β-D-Glc*p*^{R} / β-D-Xyl*p*^{R}	–	0.23	5.22	0.26	0.17

Note. [a]nd = not detected; [b]ni = not integrated due to low intensity of contour. All integrals are relative to the methoxyl contour, where applicable.

3.2. ChCl-Gly deposition on treated biomass blocked cellulose accessibility to cellulase enzymes

Pretreatment temperature and time are important factors that affect lignin fractionation efficiency and cellulose accessibility to enzymes. To determine how these factors affected rice straw composition and glucan digestibility, we first varied the pretreatment temperature from 80 to 150 °C. We observed only a slight change in composition and enzymatic hydrolysis of pretreated solids at temperatures below 120 °C (**Fig. S4**). These results suggested that (1) rice straw was recalcitrant; and/or (2) a higher temperature was needed to activate the cleavage of lignin-carbohydrate complex linkages and release lignin from the lignocellulose structure. Procentese et al. (2015) observed that ChCl-Gly pretreatment of corncob at 115 °C failed to exhibit lignin fractionation efficiency (Procentese et al., 2015). However, they fractionated 30 wt.% lignin after increasing the temperature to 150 °C, which corroborated our findings. Zhang et al. (2016) hypothesized that the free hydroxyl groups of polyols interact with the free and etherified hydroxyl groups of lignin (Zhang et al., 2016a), enabling the cleavage of the lignin-carbohydrate complex linkages at 150 °C and releasing lignin.

Next, we varied pretreatment time from 3 to 24 h. Pretreatment for 3 h did not increase glucan digestibility compared to untreated rice straw (**Fig. S5A**). Increasing pretreatment time beyond 3 h progressively increased the lignin fractionation efficiency, which reached 52 % at 15 h. Pretreatment for 24 h did not increase lignin fractionation any further (**Fig. S5C**). Interestingly, there were no visible differences between pretreated solid compositions at 15 h and 24 h. Also, the degree of deacetylation progressively increased with increasing pretreatment time (**Fig. S5C**). Importantly, the glucan retention on the treated solids remained >90 wt.% regardless of pretreatment time. These results demonstrated the high selectivity of ChCl-Gly at 150 °C toward lignin fractionation. To understand the foregoing observation, we enzymatically hydrolyzed these pretreated solids. The glucan digestibility of pretreated solids progressively increased with increasing pretreatment time and reached 68 % at 15 h. Beyond 15 h, we did not observe a significant improvement in glucan digestibility (Fig. 2). Thus, increased



	3 h	6 h	9 h	15 h	24 h
Solid retention (%)	77.7	76.1	76.1	69.8	66.9
Glucan retention (%)	93.7	97.7	97.7	96.5	91.7
Lignin removal (%)	7.8	14.3	14.3	52.2	53.3
Xylan removal (%)	6.7	5.6	5.6	29.6	38.5

Fig. 2. Fractionation efficiency of ChCl-Gly and the glucan digestibility of pretreated solids at 150 °C with various pretreatment times.

lignin fractionation and deacetylation were responsible for enhanced glucan digestibility of pretreated solids (**Fig. S5B and S5C**).

To identify changes in the chemical structure after varying pretreatment time, we used XRD to characterize the ChCl-Gly treated solids (**Fig. S6A**). On the basis of the XRD spectra of pretreated solids, we calculated the crystallinity index (CrI). The rice straw sample had a CrI of 36.7 % (**Table S4**), similar to reported CrI values (Baramee et al., 2020; Hou et al., 2017, 2018a; Hou et al., 2012). The CrI of treated solids at 3 h was higher than that of untreated rice straw because of the removal of amorphous xylan (7%) and lignin (8%). Pretreatment longer than 3 h removed more amorphous xylan and lignin. Hence, we anticipated that the CrI of treated solids would increase. However, we observed a decrease in CrI, which suggested that treated solids eventually became more amorphous. We hypothesized that a decrease in CrI with higher lignin and xylan removal was due to the breaking of highly ordered hydrogen bonds of crystalline cellulose.

To test this hypothesis, we evaluated the treated solids by FTIR (**Fig. S6B**). The FTIR spectra of ChCl-Gly treated solids at varying pretreatment times were not visibly different. The lateral order index (LOI) and total crystallinity index (TCI) of untreated rice straw was 1.36 and 0.45, respectively. The TCI of the treated solids at 3 h increased to 0.53 (**Table S4**). Beyond 3 h, the TCI of pretreated solids progressively decreased to 0.41 at 24 h. The LOI and TCI trends were similar to the CrI trend. These results suggested that pretreatment time longer than 3 h was needed to break the highly ordered hydrogen bonds of crystalline cellulose.

To establish the effect of pretreatment condition on delignification, deacetylation and glucan digestibility, we calculated the severity factor (R_0) from all pretreatment conditions (80−150 °C and 3−24 h). We found that there was a threshold in the severity factor that affected glucan digestibility. A severity factor below 4.2 gave a glucan digestibility <50 % and did not improve delignification, deacetylation, or glucan digestibility (Fig. 3). A severity factor higher than 4.2 considerably enhanced glucan digestibility to ~67 %. This increase in glucan digestibility was related to an increase in delignification and deacetylation. Our results correspond with previous findings that a severity factor of hydrothermal pretreatment higher than ~3.5 greatly removed lignin and hemicellulose, resulting in increased glucose release (Dogaris et al., 2009; Li et al., 2014; Simangunsong et al., 2020). On the basis of similar glucan digestibility, lignin fractionation efficiency of pretreated solids at 15 h and 24 h, and high viscosity of ChCl-Gly, we hypothesized that residual DES was bound on the surface of the pretreated solids during regeneration and washing, which blocked cellulase accessibility to cellulose.

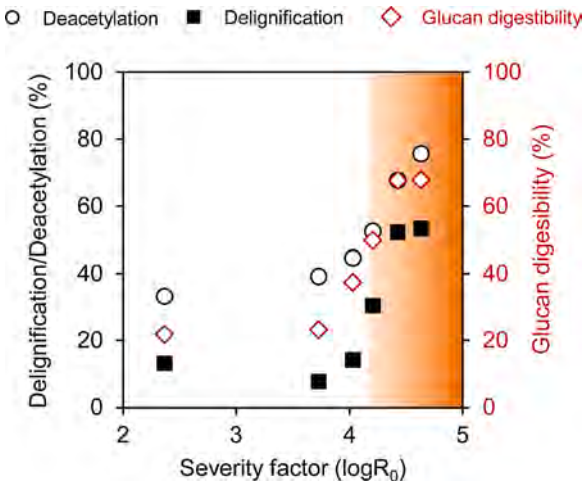


Fig. 3. Correlation of delignification, deacetylation, and glucan digestibility with severity factor ($logR_0$).

3.3. Alkaline wash of the treated solids efficiently removed residual ChCl-Gly and enhanced glucan digestibility

The high viscosity of DES makes it stick to the surface of pretreated biomass. We summarize the current ChCl-Gly pretreatment in various feedstocks (Table 2). Previous work showed low glucan digestibility (13–30 %) by washing pretreated solids with organic solvent and water. Zhang et al. (2016) used dilute NaOH solution to wash the ChCl-Gly treated solids to yield a glucan digestibility of 96.4 % (Zhang et al., 2016a). However, the rationale for dilute NaOH was not assessed. Tenhunen et al. (2018) used ChCl-urea DES and reported that residual DES remained on the treated solids even after extensive washing by organic solvents (Tenhunen et al., 2018). We hypothesized that the residual DES on pretreated biomass surface caused low surface accessibility to enzymes and resulted in a low glucan digestibility.

To test this hypothesis, we used sodium carbonate solution to wash the pretreated biomass at room temperature and the characterized chemical structure of the pretreated solids. We selected sodium carbonate based on a report by Wang et al. (2003) that Na_2CO_3 was effective at lignin removal without damaging the cellulose fibers (Wang et al., 2003). As a control, we washed untreated rice straw with sodium carbonate. We obtained similar composition and enzyme hydrolysis profiles to those of untreated rice straw (**Fig. S7**). These results implied that the sodium carbonate washing at 30 °C was mild and did not have a significant removal effect on any lignocellulose components. The 24 h-treated solids washed by sodium carbonate had a glucan digestibility of 87 % at 72 h, a 20 % increase compared with that of a water wash (Fig. 4B). Moreover, the sodium carbonate filtrate of treated solid was darker compared with the filtrate from untreated rice straw. Composition analysis revealed that washing treated solid with sodium carbonate further fractionated lignin by 21 wt.% compared with the water wash. This increase in lignin removal efficiency occurred because ChCl-Gly fractionated a large amount of lignin. As a result, the deposited ChCl-Gly contained dissolved lignin.

To confirm the presence of residual ChCl-Gly on pretreated solids, we analyzed the pretreated solids by FTIR. The FTIR spectra of pure ChCl-Gly DES showed various characteristic peaks of N—C—C bending (956 cm^{-1}), N—H and C—N bending (1335 cm^{-1}), C–H stretching (2965 cm^{-1}), and N—H stretching (3339 cm^{-1}), in agreement with previous studies (**Fig. S8**)(Ahmadi et al., 2018; Banjare et al., 2018). One of the most pronounced peaks was the N—H and C—N bending at 1335 cm^{-1}. As expected, the ChCl-Gly-treated solids after water washing had similar characteristic peaks to those of ChCl-Gly DES, which suggested the presence of residual DES on the surface of pretreated biomass (Fig. 4A). Conversely, we did not observe DES characteristic peaks after

Table 2
Literature review of ChCl-Gly DES pretreatment of various feedstocks.

Molar ratio	Feed	Pretreatment condition	Washing solvent	Enzymatic hydrolysis		Glucan digestibility (%)	Ref
				Solid loading (g biomass/L)	Enzyme loading (FPU/ g glucan)		
2:1	Rice straw	5% biomass loading, 150 °C, 15 h,	Hot water	23.2	11	67.8	This work
			Na_2CO_3	23.2	11	87.1	
1:1	Corncob	5 % biomass loading 90 °C, 24 h	NaOH	2.5	324[a]	96.4	(Zhang et al., 2016a)
1:1	Rice straw	5% biomass loading, 120 °C, 3 h	Hot water	2.8	16.7[a]	30.2	(Hou et al., 2018b)
1:2	Oil palm trunk	5% biomass loading, 100 °C, 48 h	H_2O:EtOH (1:2)	10	10.2[a]	13.0	(Zulkefli et al., 2017)
1:2	Date Palm Residues	16.7 % biomass loading, 70 °C, 15 h	N.D.	100	43.6	20.0	(Fang et al., 2017)
1:2	Switchgrass	9% biomass loading, 10 % H_2O, 0.9% H_2SO_4, 130 °C, 1h	Acetone:H_2O (1:1)	15	20.0[b]	93.8	(Chen et al., 2018b)

[a]Calculated based on 62 FPU/mL of Celluclast 1.5 L (Cannella et al., 2012).
[a]Calculated based on 0.49 FPU/mg protein of Ctec2 (Cannella et al., 2012).

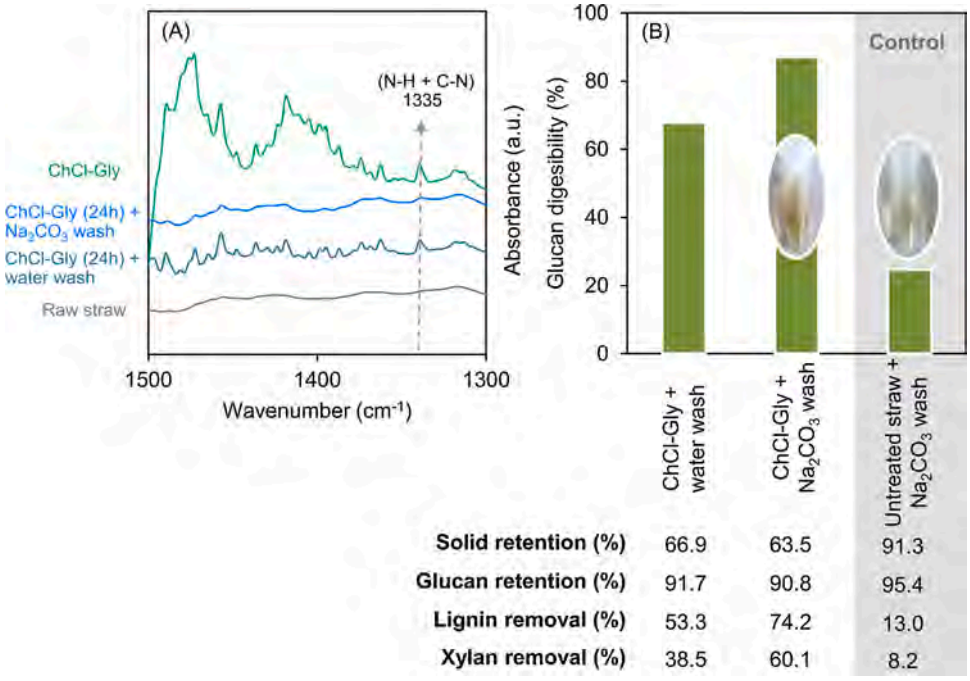


Fig. 4. Fractionation efficiency of ChCl-Gly and the glucan digestibility of pretreated solids washed with sodium carbonate (A) and FTIR spectra of ChCl-Gly treated solid with a water wash and sodium carbonate wash (B).

sodium carbonate wash. We further characterized the treated solids by XRD and SEM (**Figs. S9—10**). Our XRD spectra showed that sodium carbonate-washed treated solids had a higher CrI compared with unwashed solids. We hypothesized that the increase in CrI after sodium carbonate washing was due to the removal of dissolved lignin in the DES on the surface of treated solids. Taken together, these results indicated the importance of alkali wash in the removal of residual DES on the treated solid.

We also hydrolyzed the ChCl-Gly-treated solids after sodium carbonate washing. After sodium carbonate wash, the pretreated solids had 87 % glucan digestibility, a 19 % increase compared with the water wash (Fig. 4B). Our hydrolysis results corresponded with the FTIR data and showed that sodium carbonate wash removed residual DES on the pretreated solid surface, increasing cellulose accessibility to cellulase and glucan digestibility.

3.4. Mass balance of pretreatment by ChCl-Gly and enzymatic hydrolysis

To evaluate how DES treatment affected the overall sugar yield, we constructed the mass balance. We demonstrated an enzymatic glucose yield of 87 % and 72 % yield of xylose (stream 5, Fig. 5), which corresponded to 26.6 g glucose and 5.1 g xylose based on 100 g rice straw. Moreover, we fractionated 74 % lignin in the liquid stream (stream 2), which provided the potential to use the fractionated lignin as a co-product.

4. Discussion

We ascertained the interaction between ChCl-Gly and lignocellulose components based on the severity factor and explained how this interaction enhanced the glucan digestibility of the pretreated rice straw. Specifically, we demonstrated that the residual DES decreased the cellulase accessibility to cellulose. By removing the residual ChCl-Gly

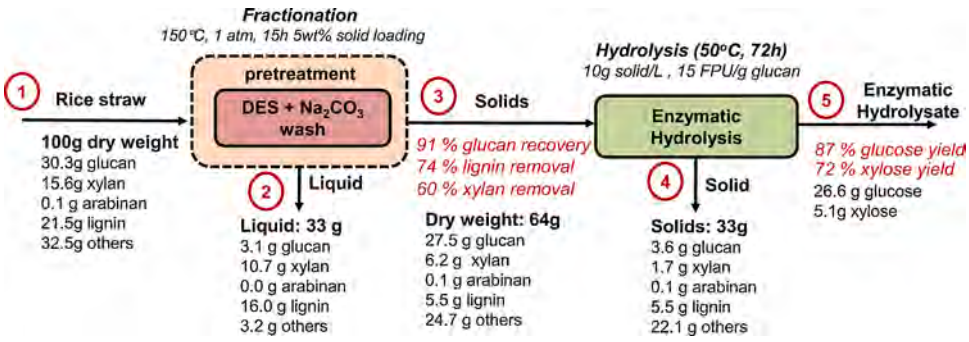


Fig. 5. Mass balance of ChCl-Gly DES pretreatment and enzymatic hydrolysis.

DES from the treated solid's surface, we achieved 87 % glucan digestibility with high glucan retention (91 wt.%).

Our important finding was that the residual DES on the surface of pretreated solids blocks accessibility of cellulase to the cellulose. Our FTIR results showed the characteristic peaks of ChCl-Gly DES on treated solids with ChCl-Gly. The quaternary ammonium groups of ChCl-Gly DES increase the positive charge on ChCl-Gly-treated solids. Cellulase enzymes (CTec2) have a negative charge in buffer at pH 4.8 (Zheng et al., 2020). As a result, the cellulase enzymes are attracted by electrostatic interaction to residual ChCl-Gly on the treated solid surface, thereby decreasing the amount of enzyme available for cellulose hydrolysis. Zheng et al. (2020) observed a similar charge phenomenon after adding lignosulfonate in the enzymatic hydrolysis reaction of pure cellulose (Avicel). The lignosulfonate increased the negative change on Avicel and repulsed cellulases, resulting in a low glucan digestibility (Zheng et al., 2020). Hence, it is important to remove residual ChCl-Gly on the surface of pretreated solids to maximize the available cellulase content for hydrolysis.

Another important finding is that the glycerol in ChCl-Gly DES provides free hydroxyl groups that interact with the free and etherified hydroxyl groups of lignin (Zhang et al., 2016a) and acetyl groups, enabling the 74 % lignin fractionation efficiency and complete deacetylation. Previous studies showed that acetyl groups (Pan et al., 2006) and lignin (Vermaas et al., 2015) in biomass inhibited the cellulose accessibility to cellulase. The ability of ChCl-Gly to fractionate a large amount of lignin and completely deacetylate biomass is beneficial to the high sugar release.

Our findings offered a novel perspective on designing the pretreatment process that maximizes the sugar yield. The high glucan retention, lignin fractionation, and glucan digestibility make this process attractive. Although the combination of DES pretreatment with sodium carbonate wash resulted in a high lignin fractionation, we do not know the chemical characteristics of fractionated lignin. Moreover, this DES treatment used a long treatment time (15 h). Future studies will focus on characterizing the fractionated lignin, residual lignin after hydrolysis, and surface characterization to probe residual DES and ensure the potential use of DESs in biorefineries. In addition, the severity factor is a robust indicator that combines time and temperature. It is possible to achieve the same severity at much shorter times, such as 12 min. (Zhang et al., 2016b), if higher temperatures are used. Further studies will explore the same severity factor strategy at a higher treatment temperature and shorter time (<15 h) and evaluate its effect on sugar yield to ensure the economic feasibility of the process.

5. Conclusion

We investigated the interaction between ChCl-Gly deep eutectic solvent (DES) and lignocellulosic components of rice straw and the effect of this interaction on lignin fractionation and glucan digestibility. The ChCl-Gly pretreatment fractionated 74 wt.% lignin with 91 % glucan retention at 4.1 severity factor. The residual ChCl-Gly remained on the surface of pretreated solids and limited cellulase enzyme activity to 68 %. Sodium carbonate wash at room temperature effectively removed the residual DES and increased the glucan digestibility to 87 %. Our HSQC NMR, XRD, and FTIR analyses revealed that ChCl-Gly pretreatment completely removed the native acetyl units, severed ordered hydrogen bonding among cellulose chains, and selectively removed G-type lignin units. These attributes enhanced cellulose accessibility to cellulase and promoted high glucan digestibility. Our study offers a novel perspective on the dynamic interaction of ChCl-Gly and the deposition of residual ChCl-Gly on pretreated solids. This DES pretreatment strategy can be used profitably with other types of lignocellulose for biorefineries.

CRediT authorship contribution statement

Md Anwar Hossain: Conceptualization, Methodology, Investigation, Writing-original draft. **Mohammad Shahinur Rahaman:** Methodology, Investigation, Writing - original draft. **Daniel Yelle:** Conceptualization, Validation, Formal analysis, Investigation, Writing - review & editing, Visualization. **Hong Shang:** Investigation. **Zhihui Sun:** Supervision, Funding acquisition. **Scott Renneckar:** Conceptualization, Validation, Writing - review & editing. **Jie Dong:** Supervision. **Sarttrawut Tulaphol:** Conceptualization, Validation, Formal analysis, Writing - review & editing, Visualization. **Noppadon Sathitsuksanoh:** Conceptualization, Methodology, Validation, Visualization, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.indcrop.2021.113480.

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