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## RESEARCH ARTICLE

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## Investigation of the effects of ternary deep eutectic solvent composition on pretreatment of sorghum stover

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## Abstract

Biomass-derived deep eutectic solvents (DESs) have been introduced as promising pretreatment and fractionation solvents because of their mild processing conditions, easy synthesis, and green solvent components from biomass. In recent DES studies, solvent-based third constituents like water, ethanol, and others improve the processibility of typical binary DESs. However, the impacts of these components are not well understood. Here, two solvent-based constituents, including water and ethylene glycol, were applied to 3,4-dihydroxybenzoic acid (DHBA)-based DES system for improving the conversion efficiency of cellulose-rich fraction and the properties of lignin fraction. Chemical composition, enzymatic digestibility, degree of polymerization of cellulose and physicochemical properties of lignin were used to evaluate the impact of each third constituent on biomass processing. Ternary ChCl-DHBA DESs exhibited better performances in delignification, fermentable sugar production, and preservation of  $\beta$ -O-4 ether linkage in lignin compared with binary ChCl-DHBA DES.

## KEYWORDS

3,4-dihydroxybenzoic acid, engineered plant, pretreatment, sorghum stover, ternary deep eutectic solvent

Yunxuan Wang and Jiae Ryu contributed equally to this study.

## 1 | INTRODUCTION

Finding a renewable alternative to petroleum fuels is a significant issue for mankind's sustainability. Lignocellulosic biomass, a promising renewable and abundant material, can produce currently petroleum-derived fuels and platform chemicals by the conversion of its three major components: cellulose, hemicellulose, and lignin.<sup>1</sup> Cellulose, the most abundant carbohydrate component in biomass, can be chemically or biologically converted into biofuels and platform chemicals.<sup>2,3</sup> Hemicellulose also finds value-added applications, as it can be converted to value-added chemicals like furfural and xylitol or applied in bioplastics.<sup>4–6</sup> Lignin is a biopolymer of aromatic compounds and has great potential for future industry as a source of bio-based chemicals.<sup>7–9</sup>

As a green solvent, deep eutectic solvent (DES) has been investigated to overcome the challenges of conventional pretreatment solvents and has great potential to achieve high productivity of fermentable sugars as well as minimal condensation of lignin, which are crucial factors in a successful biorefinery process. DESs can be prepared via the simple mixing and heating of hydrogen bond acceptors (HBA) and donors (HBD). Various compounds including amines, carboxylic acids, polyols, and aromatics have been applied for the formation of DESs.<sup>10–12</sup> Recently, DESs prepared from biomass metabolites such as lactic acid, oxalic acid, and lignin-derived phenolic compounds like *p*-coumaric acid, *p*-hydroxybenzoic acid, and guaiacol have been reported to effectively pretreat the biomass.<sup>13–16</sup> The functional groups in carboxylic acid-based DESs like the  $-COOH$  groups could provide active protons, enhancing the hydrogen-bonding strength and facilitate delignification via the cleavage of lignin bonds.<sup>17</sup> Enhanced fermentable sugar yield and lignin quality can be achieved by DES pretreatment compared with hydrothermal, dilute acid, or conventional organosolv pretreatment due to its mild processing conditions.<sup>12,13,18</sup> Additionally, biomass-derived DESs possess the potential to facilitate a circular process, as fractionated biomass components can be reused as the processing solvent, decreasing the overall cost.<sup>19</sup> However, carboxylic acid groups in the HBD could also lead to excessive bonding and increase the viscosity of the DES.<sup>20</sup> Recent DES studies showed that introducing a third constituent like water, acids, or organic solvents into the DES improved the removal of lignin and hemicellulose from the biomass, further enhancing fermentable sugar yield.<sup>21,22</sup> However, studies on lignin-based three-component DESs are scarce.

3,4-Dihydroxybenzoic acid (DHBA), a metabolite found in several plant species, is a promising platform chemical that can be used as a precursor to industrially important chemicals including muconate, beta-ketoadipate, 2-pyrone-4,6-dicarboxylate, gallates, and vanillin.<sup>23–25</sup> It has also seen applications in the pharmaceutical and food packaging industries. In a recent study, Tian et al. reported the accumulation of DHBA in engineered sorghum by expressing a bacterial dehydroshikimate dehydratase (QsuB) gene, converting 3-dehydroshikimate to protocatechuate.<sup>26</sup> A study by Unda et al. showed increased saccharification yield from poplar expressing QsuB, which led to the reduction of lignin and the incorporation of DHBA into lignin.<sup>27</sup>

Additionally, our study finds that DHBA can act as an HBD to form DESs that can be directly applied in biomass pretreatment.

Herein, we report the influence of the third component in the DHBA-based DES system on the fractionation of the DHBA-enriched engineered sorghum stover in a biorefinery approach. Biomass from the DHBA-enriched engineered sorghum was pretreated by binary DES, composed of choline chloride (ChCl) and DHBA, and ternary DESs with additional third constituents like water and ethylene glycol. Chemical compositions and enzymatic digestibility of the processed sorghum stover were investigated. The structural properties of lignins recovered from each processing method were also analyzed to evaluate the quality of lignin for accomplishing a sustainable biorefinery strategy.

## 2 | EXPERIMENTAL SECTION

### 2.1 | Materials

The sorghum wildtype and transgenic line *pSbUbi:chl-qsuB* #48 (variety Wheatland) used in this study have been described previously.<sup>26</sup> The plants were grown at the University of California (UC) greenhouse Oxford facility and harvested at full maturity without the panicles.<sup>26</sup> Each sample consisted of stem and leaf biomass from five plants, which was dried in an oven at 323.15 K for 5 days and ground using a Model 4 Wiley Mill equipped with a 1-mm mesh (Thomas Scientific, Swedesboro, NJ). Chemicals for DES synthesis, including choline chloride and 3,4-dihydroxybenzoic acid, were purchased from JT Baker and BeanTown Chemical, respectively. The chemical used as the third constituent of the DES (ethylene glycol) was purchased from VWR. Cellulase, enzyme blend Cellic CTec2, sodium acetate trihydrate, and sodium azide were purchased from Sigma-Aldrich. The enzyme was stored at 275.15–281.15 K.

### 2.2 | DES formation

ChCl and DHBA were first mixed at molar ratios of 3:2, 2:1, 1:1, 1:2, and 2:3 at 383.15 K to determine the optimal composition of binary DES prior to the formation of two types of ternary DESs (Figure S1). Among the five tested molar ratios, 3:2 and 2:1 resulted in a clear and homogeneous liquid. Two solvents, distilled water and ethylene glycol, were added as the third constituent in the DES system with a 3:2:5 molar ratio of ChCl, DHBA, and the solvent component. The molar ratio of the third constituent was chosen based on the previous study.<sup>28</sup> The DES solvent formation temperature and time were 363.15–393.15 K for 0.5–3.0 h in a Pyrex bottle.

### 2.3 | DES pretreatment

Sorghum stover was loaded to a reactor containing DES at a 1:10 weight ratio of biomass-to-solvent. A vortex mixer was used to

rigorously mix the biomass and DES before the reaction. The mixtures were processed at 393.15 K for 3 h in an oil bath with continuous stirring at  $8.3\text{ s}^{-1}$ . Upon the end of the pretreatment, the reaction was quenched by adding ethanol/water 1:1 volume ratio of solution and acetone. The solid fraction was washed with 1:1 volume ratio of ethanol/water solution. Further solvent washing was conducted to completely recover the fractionated lignin from the pretreated biomass.

## 2.4 | Lignin recovery

Lignin was recovered from the liquid fraction using an Amicon stirred ultrafiltration cell (UFSC20001, Amicon Corporation) with a 1000 Da membrane disc. Nitrogen gas was connected to the stirred cell at 207 kPa with the magnetic stirrer set at  $5.0\text{ s}^{-1}$ . The liquid fraction was mixed with 2.3 times of ethanol/water 1:1 (v/v) solution, loaded to the stirred cell, and filtered. Additional ethanol/water 1:1 (v/v) solution was added and filtered to completely remove the free choline chloride, DHBA, and the third constituent. The retained lignin on the membrane disc was air-dried for further characterization.

## 2.5 | Chemical composition analysis of untreated and pretreated biomass

The chemical composition of untreated and pretreated biomass was analyzed in accordance with the NREL procedure.<sup>29</sup> In brief,  $3\text{ cm}^3$  of 72% sulfuric acid and 0.3 g of biomass were mixed in a glass tube, and the mixture was stirred for 1 h at 303.15 K in a water bath. After the reaction, the mixture was diluted with  $84\text{ cm}^3$  of distilled water and autoclaved at 394.15 K for 1 h. The mixture was filtered through medium pore-size crucible glass filters, and the filtrate was collected to determine the content of acid-soluble lignin and sugar. Acid-soluble lignin content was measured by a UV-vis spectrometer at 320 nm wavelength, while the sugar composition was analyzed by a high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity) equipped with a Biorad Aminx HPX-87H column. The crucible was dried, weighed, heated to 848.15 K in a muffle furnace, and weighed again to determine the content of acid-insoluble lignin. All untreated and pretreated biomass were analyzed in duplicates.

## 2.6 | Enzymatic hydrolysis

The productivity of fermentable sugars from wild-type (WT) and transgenic (TG) sorghum stover before and after pretreatments were evaluated by enzymatic digestibility assays. Enzymatic hydrolysis and enzyme activity analysis were conducted in accordance with the NREL procedure.<sup>30,31</sup> The enzyme activity of Cellic CTec2 was measured to be 137 FPU/ $\text{cm}^3$ . Enzymatic hydrolysis of untreated and pretreated biomass was operated in an incubator at 323.15 K with  $3.0\text{ s}^{-1}$  for 72 h. Wet samples equivalent to 0.2 g of dry weight were

loaded into an Erlenmeyer flask with a rubber cap at a solid loading of 1 wt% in  $50\text{ mol/m}^3$  sodium acetate buffer at pH 5.0 with 0.02% sodium azide. The Cellic CTec2 was loaded at 20 FPU/g-biomass. The hydrolysis reaction was quenched by heating at 368.15 K for 360 s in a heating block. The hydrolysate was centrifuged followed by filtering through a  $0.22\text{ }\mu\text{m}$  nylon filter prior to the HPLC analysis. All enzymatic hydrolysis was conducted in duplicates.

## 2.7 | Characterization of recovered lignin structure

Two-dimensional heteronuclear single quantum coherence nuclear magnetic resonance (2D HSQC NMR) spectra of the fractionated lignin were obtained on a Bruker AVANCE III HD 800 MHz NMR equipped with a TCI cryoprobe.  $^1\text{H}$ - $^{13}\text{C}$  HSQC spectra were processed from the hsqcctgsp pulse program with the following acquisition parameters: 32 scans, 1 s of relaxation delay, with F1 ( $^{13}\text{C}$ ) and F2 ( $^1\text{H}$ ) dimensions at 160 and 12 ppm spectral, with 512 and 1024 data points, respectively. Dimethylsulfoxide- $d_6$  (DMSO- $d_6$ ) was used as the solvent for lignin.

## 2.8 | Molecular weight of recovered lignin

The weight-average molecular weight ( $M_w$ ), number-average molecular weight ( $M_n$ ), and dispersity ( $D$ ) of the isolated lignin were measured by an Agilent gel permeation chromatography (GPC) SECurity 1200 system equipped with Waters Styragel columns (Waters Corporation, Milford, MA) and a UV detector at 270 nm. The isolated lignin was acetylated in a pyridine/acetic anhydride 1:1 (v/v) mixture for 48 h, rotary evaporated, dissolved in THF, and filtered through  $45\text{ }\mu\text{m}$  PTFE filters before injection.

## 2.9 | Cellulose degree of polymerization

Dried sorghum stover samples ( $\sim 0.3\text{ g}$ ) were reacted in 32% peracetic acid aqueous solution at 298.15 K for 24 h to isolate holocellulose. The obtained holocellulose samples were washed with distilled water via centrifugation until neutral pH was achieved. The holocellulose samples were further treated in two steps with 17.5% and 8.75% sodium hydroxide aqueous solution, respectively, to obtain  $\alpha$ -cellulose. Prior to molecular weight measurement by GPC,  $\alpha$ -cellulose was converted to cellulose tricarbaniolate as described in a previous study.<sup>15</sup> In brief, 15 mg of oven-dried  $\alpha$ -cellulose was mixed with 4 mL anhydrous pyridine in a glass bottle, followed by the injection of 0.5 mL phenyl isocyanate. The mixture was reacted at 343.15 K for 48 h. Another  $0.5\text{ cm}^3$  of phenyl isocyanate was injected after 24 h. The reaction was quenched by adding methanol. Cellulose tricarbaniolate was precipitated in a methanol/water 7:3 solution, washed via centrifugation, and air-dried. An Agilent GPC SECurity 1200 system equipped with Waters Styragel columns (Waters Corporation, Milford, MA), a refractive index (RI) detector, and a UV detector was used to

measure the  $M_w$  and  $M_n$ . Cellulose tricarbanilate was dissolved in tetrahydrofuran (THF) and filtered through 0.45  $\mu\text{m}$  PTFE filters before injection.

### 3 | RESULTS AND DISCUSSION

#### 3.1 | Determination of the molar ratio between ChCl and DHBA

Among the tested molar ratios of ChCl and DHBA, the molar ratio of 2:1 and 3:2 with ChCl and DHBA formed a homogenous and transparent liquid, as shown in Figure S1. A previous study reported that the DESs with the same components at different molar ratios showed different properties and performances.<sup>32</sup> In this study, prior to investigating ternary DESs, two binary ChCl-DHBA DESs with different molar ratios (3:2 and 2:1) were evaluated by applying them to pretreat TG sorghum stover. The pretreatment was carried out at 393.15 K for 3 h, adapted from our previous study of DES pretreatment with sorghum stover.<sup>28</sup> The pretreatment efficiency was determined based on xylan removal, delignification, and fermentable sugar production, as shown in Table 1. ChCl-DHBA 3:2 DES showed higher xylan removal and delignification than its 2:1 counterpart. Clearly, the composition of DES is an important factor affecting the pretreatment performance. Since the removal of both xylan and lignin could enhance the enzymatic digestibility of the biomass, both ChCl-DHBA DES-pretreated biomass samples were tested for the release of fermentable sugars using a cellulase mixture. The sorghum stover pretreated with ChCl-DHBA-3:2 DES had 87% of glucan digestibility, while that pretreated with ChCl-DHBA 2:1 DES showed a lower amount of glucose (81% of glucan digestibility); therefore, the 3:2 molar ratio of ChCl and DHBA was selected for subsequent ternary DES testing.

#### 3.2 | Impact of the third constituent in ChCl-DHBA DES system on the chemical compositions of sorghum stover

One of the challenges in the application of several binary DESs is its relatively high viscosity, limiting mass transfer. Hou et al. reported that lowering the viscosity of the binary ChCl-malic acid DES by using water as a third constituent improved solubility of lignin over three-

**TABLE 1** Xylan removal, delignification, and glucan digestibility of the pretreated transgenic (TG) sorghum stover with ChCl-DHBA at different molar ratios at 393.15 K for 3 h. Enzymatic hydrolysis was conducted for 72 h with enzyme loading at 15 FPU/g-biomass.

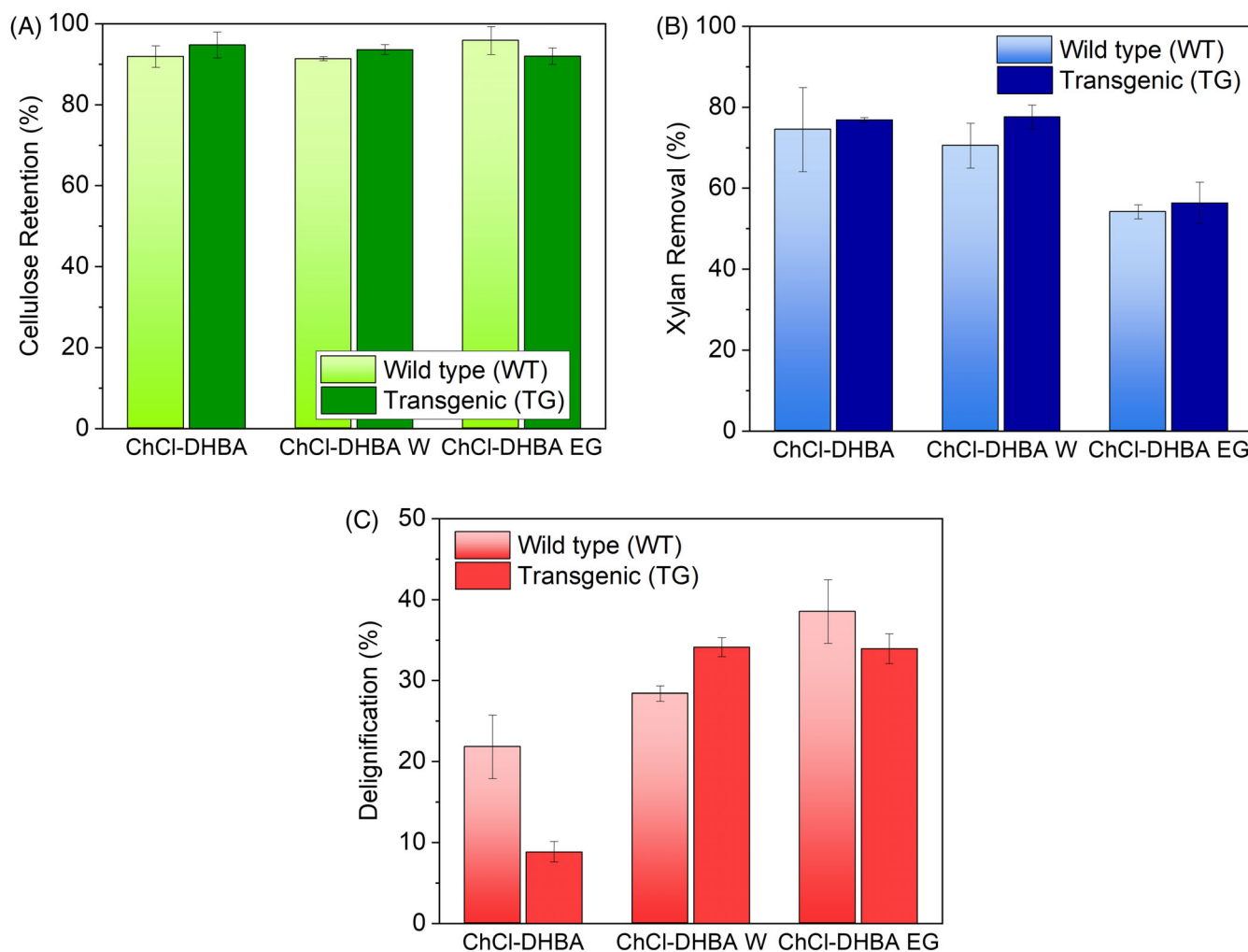
| DES composition | Xylan removal (%) | Delignification (%) | Glucan digestibility (%) |
|-----------------|-------------------|---------------------|--------------------------|
| ChCl-DHBA 2:1   | 22.6 $\pm$ 1.3    | 28.4 $\pm$ 1.7      | 81                       |
| ChCl-DHBA 3:2   | 60.6 $\pm$ 3.7    | 37.6 $\pm$ 7.3      | 87                       |

fold.<sup>33</sup> Here, ternary DESs were synthesized with the selected 3:2 molar ratio of ChCl and DHBA mixture and an additional third constituent, either water (W) or ethylene glycol (EG). The molar ratio between ChCl, DHBA, and the third constituent was 3:2:5, according to our previous study.<sup>28</sup> The prepared ternary DESs were applied to the pretreatment of two different biomass, WT and TG sorghum stover at 413.15 K for 3 h. The contents of glucan and xylan in intrinsic WT and TG sorghum stover were similar. Although TG sorghum stover showed slightly lower lignin content (12.8%) than WT biomass did (14.4%), the values are comparable (Table 2), which is in accordance with the previous study.<sup>26</sup> All the applied DES pretreatments removed over 50% of the initial mass of biomass regardless of the DES composition, resulting in solid recovery yields ranging from 43% to 46%. For the further evaluation of the performance of DHBA-based ternary DESs, the changes in the chemical composition of the pretreated biomass were measured with cellulose retention, xylan removal, and delignification, as shown in Figure 1. The preservation of cellulose in the solid is a critical factor affecting the yield of glucose in the following enzymatic hydrolysis step. As depicted in Figure 1A, after pretreatment, over 90% of cellulose remained in the solid fraction, regardless of the biomass type and DES composition. A previous study indicated that ChCl in the DESs could actively bind and stabilize the cellulose fractions, while the HBD facilitates the removal of hemicellulose and lignin,<sup>34</sup> which aligns well with the current results. On the other hand, the DESs having different compositions resulted in varied xylan removal and delignification, as the xylan removal ranged from 54% to 77% and delignification ranged from 9% to 40% (Figure 1B,C). Detailed information on solid recovery after different pretreatment and chemical compositions of untreated and pretreated biomass are in Table 2.

The primary goal of biomass pre-processing, including pretreatment and fractionation, is reducing and removing the recalcitrance factor from biomass. Hemicellulose and lignin physically hinder the enzyme accessibility to cellulose and decrease the enzyme efficiency by productive and non-productive bindings. Therefore, the removals of hemicellulose and lignin are important parameters to measure processing effectiveness. The numerical data for the cellulose retention, xylan removal, and delignification (Figure 1) are tabulated in Table S1. Cellulose retention was over 90% after DES pretreatments with both WT and TG sorghum stover (Figure 1A). There was no significant difference in cellulose retention between WT and TG as well as among three DES pretreatments. As Figure 1B shows, xylan removals from WT and TG sorghum stover showed similar trends. Binary DES (ChCl-DHBA) and ChCl-DHBA-water (ChCl-DHBA-W) had approximately 20% higher than the ternary DESs with EG (ChCl-DHBA-EG). Also, binary DES and ChCl-DHBA-W resulted in similar xylan removal with WT sorghum stover (75% and 71%, respectively) as well as with TG biomass (77% and 78%, respectively). However, ChCl-DHBA-EG was not as efficient as the former two DESs in terms of xylan removal (54%–56%). Li et al. reported that the content of EG in a ternary DES showed contradictory effects on xylan removal.<sup>35</sup> EG content in ChCl-oxalic acid-EG DES showed positive effects on xylan removal when the molar ratio between EG and ChCl was below 3:1, while xylan

**TABLE 2** Solid recovery and chemical compositions of WT and TG sorghum stover before and after binary and ternary DES pretreatments with different compositions.

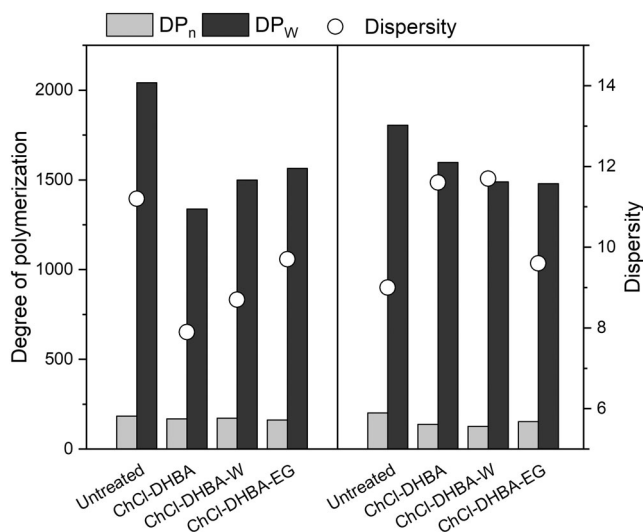
| Biomass         | Pretreatment     | Solid recovery (%) | Chemical composition (%) |             |             |             |            |
|-----------------|------------------|--------------------|--------------------------|-------------|-------------|-------------|------------|
|                 |                  |                    | Glucan                   | Xylan       | Lignin      | Extractives | Ash        |
| Wild type (WT)  | Untreated        | —                  | 26.5 (±0.1)              | 17.6 (±0.1) | 14.4 (±0.3) | 26.4 (±1.8) | 8.7 (±0.2) |
|                 | Binary ChCl-DHBA | 44.1 (±0.9)        | 55.3 (±2.7)              | 10.1 (±3.9) | 25.6 (±1.8) | —           | —          |
|                 | ChCl-DHBA-W      | 45.1 (±1.0)        | 53.7 (±0.9)              | 11.5 (±2.4) | 22.9 (±0.2) | —           | —          |
|                 | ChCl-DHBA-EG     | 46.4 (±0.5)        | 54.7 (±1.4)              | 17.3 (±0.5) | 19.1 (±1.4) | —           | —          |
| Transgenic (TG) | Untreated        | —                  | 25.7 (±0.7)              | 17.4 (±0.6) | 12.8 (±0.1) | 29.0 (±2.2) | 9.4 (±0.1) |
|                 | Binary ChCl-DHBA | 43.4 (±3.2)        | 60.0 (±0.7)              | 9.7 (±0.1)  | 25.9 (±1.8) | —           | —          |
|                 | ChCl-DHBA-W      | 43.3 (±2.1)        | 57.2 (±3.3)              | 9.3 (±1.6)  | 21.8 (±1.4) | —           | —          |
|                 | ChCl-DHBA-EG     | 44.0 (±0.4)        | 53.7 (±1.6)              | 17.2 (±1.9) | 19.3 (±0.4) | —           | —          |

**FIGURE 1** (A) Cellulose retention, (B) xylan removal, and (C) delignification of WT and TG sorghum stover before and after binary and ternary DES pretreatments with different compositions. Pretreatment conditions: 413.15 K for 3 h.

removal was reduced with increasing EG content when the molar ratio of EG and ChCl was above 3:1.

The third constituent in the ChCl-DHBA DES influenced more significantly the delignification of biomass. The additional solvents as the third constituent enhanced the delignification. Binary ChCl-DHBA

DES showed 22% of delignification with WT biomass while resulting in only 9% with TG biomass. Water-incorporated ternary DES (ChCl-DHBA-W) increased delignification of WT biomass to 28%, and the one with TG biomass was improved to 34%. A recent study by Wang et al. showed that the addition of a co-solvent could promote



**FIGURE 2** Number-averaged degree of polymerization ( $DP_n$ ), weight-average degree of polymerization ( $DP_w$ ), and dispersity ( $D$ ) of cellulose isolated from untreated and pretreated WT and TG sorghum stover.

the aggregation of ions and hydrogen bonding in an ionic liquid (IL) system, resulting in the ternary systems having better biomass dissolution than the neat ILs.<sup>36</sup> They calculated the ionicity of a ternary ionic liquid system with co-solvents and a superbase by analyzing the relationship between viscosity and ionic conductivity. The addition of the co-solvent with a certain range of molar ratios between the co-solvent and IL decreased the fraction of free ions in the system rather than promoting the dissociation of the IL. In another previous study, ethanol-incorporated ChCl-polyol DES systems showed higher solubility of aromatics compared with the binary DES.<sup>37</sup> Since ethylene glycol leads to higher lignin solubility than water, higher delignification with ChCl-DHBA-EG was expected. Interestingly, delignification yields of WT and TG sorghum stover by ethylene glycol-incorporated ternary DES (ChCl-DHBA-EG) were 39% and 34%, respectively, which were higher than binary DES. However, compared with the delignification with ChCl-DHBA-W, the yield of ChCl-DHBA-EG was not notably improved, especially with TG biomass. It is possible that other factors like the viscosity of the DES could be more significant than lignin solubility in this ternary DES system; therefore, the enhancement of pretreatment effects could be explained by the improved mass and heat transfer during the pretreatment process. Nonetheless, the third constituent clearly altered the fractionation performance of the DES, as evidenced by the changed delignification and xylan removal with the tested three DESs.

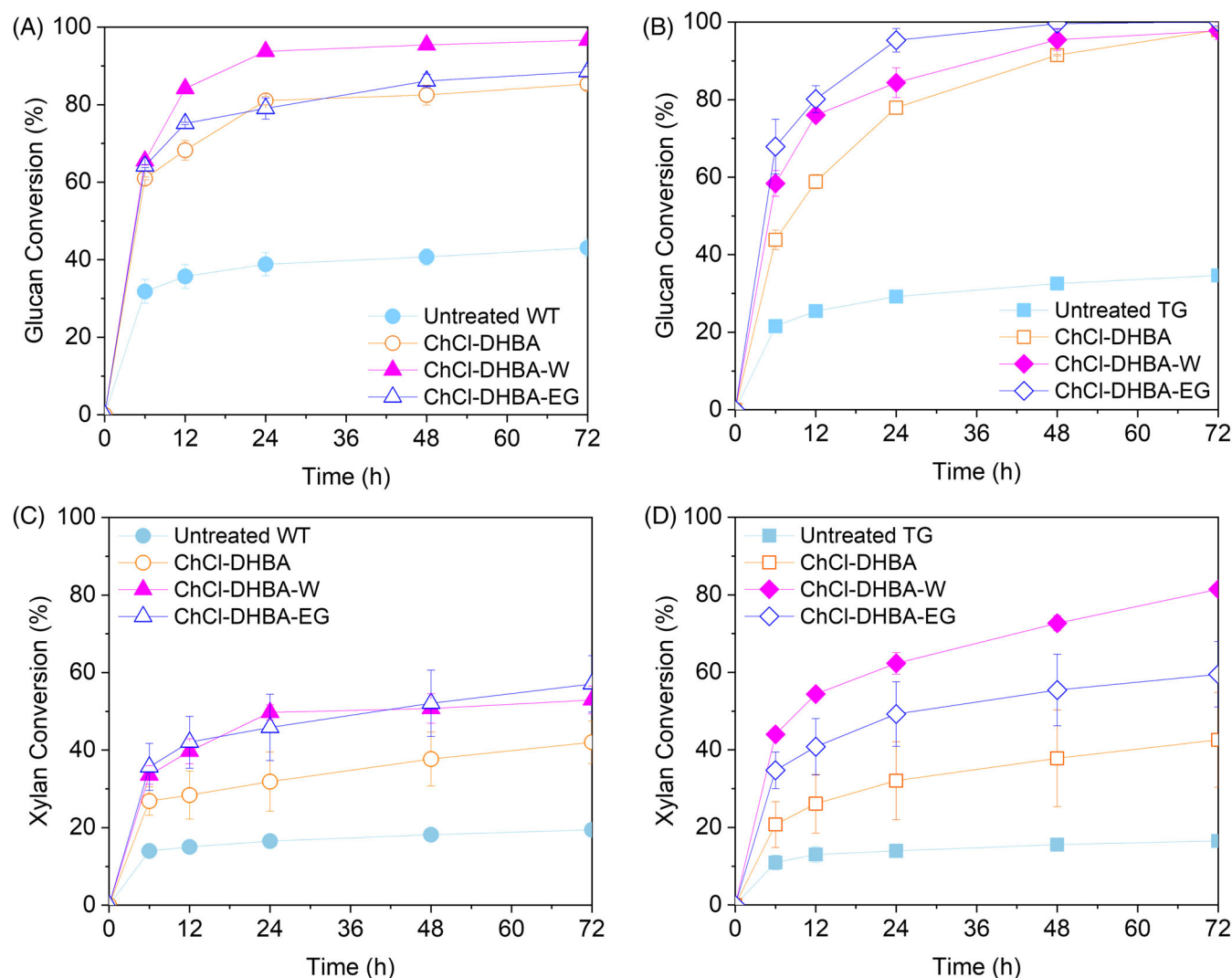
### 3.3 | Impact of the third constituent in ChCl-DHBA DES system on cellulose degree of polymerization

According to the previous study, the degree of polymerization of cellulose is a crucial factor in biomass conversion.<sup>38</sup> The cleavage of cellulose generates more reducing ends which is beneficial in many

biomass conversion processes. The degree of polymerization (DP) of cellulose isolated from untreated and pretreated WT and TG sorghum stover is presented in Figure 2 and tabulated in Table S2. The weight-average degree of polymerization ( $DP_w$ ) of cellulose in untreated WT and TG sorghum stover were 2042 and 1805, respectively. The dispersity ( $D$ ) of cellulose in untreated WT and TG biomass were 11 and 9, respectively. All three pretreatments substantially reduced cellulose  $DP_w$  in this study. The cellulose of WT biomass was more significantly depolymerized than the one in TG biomass. The highest reduction of cellulose  $DP_w$  of WT biomass was 34% by the binary ChCl-DHBA DES pretreatment. Binary DES cleaved more cellulose chains in WT biomass (1338) than in TG one (1598), while both ternary DES pretreatments were more effective in reducing the cellulose  $DP_w$  in TG biomass (1479–1489) than in WT one (1499–1599). Overall, the impacts of binary and ternary DESs on cellulose DP were not significantly different, indicating that the third component did not significantly modify glycosidic bond cleavage. Both binary and ternary ChCl-DHBA DESs showed much less impact on cellulose degree of polymerization compared with dilute acid pretreatment, where up to 90% of cellulose DP reduction was reported.<sup>39</sup> The observation on cellulose DP is consistent with results of cellulose retention, indicating that neither binary nor ternary ChCl-DHBA DES significantly modified the cellulose fraction of the biomass.

### 3.4 | Impact of the third constituent in ChCl-DHBA DES system on enzymatic hydrolysis of sorghum stover

The pretreatment efficiency of each DES was evaluated by measuring fermentable sugar production via enzymatic hydrolysis. The average value and standard deviation are in Figure 3 and Table S2 of the Supplementary Materials for glucan conversion and Table S4 for xylan conversion, respectively. Overall, both binary and ternary ChCl-DHBA DESs improved the glucan and xylan conversion of sorghum stover. Over 80% of glucan conversion and over 40% of xylan conversion were obtained with the DES pretreated WT and TG biomass. The glucan conversion yields of TG biomass pretreated by both binary and ternary DESs after 72 h-enzymatic hydrolysis ranged 98%–100%, which were higher than pretreated WT biomass had. It is well-known that xylan removal and delignification are well correlated with glucan conversion in previous studies.<sup>40,41</sup> However, in this study, maximum sugar yields were not well correlated with delignification and xylan removal, especially for the TG biomass. The binary ChCl-DHBA DES pretreatment, having lower delignification, resulted in a similar 72 h-glucan conversion compared with ChCl-DHBA-W DES. However, the hydrolysis rate of the ChCl-DHBA-W pretreated biomass was faster than the binary DES pretreated one, which can be attributed to the difference in lignin content of each pretreated TG biomass (ChCl-DHBA: 26%, ChCl-DHBA-W: 22%). The non-productive binding of lignin to cellulose subsequently decreases the rate of enzymatic hydrolysis, as reported in the previous study.<sup>42</sup> Other factors, such as potential



**FIGURE 3** Glucan and xylan conversion of unpretreated and pretreated WT (A and C) and TG (B and D) sorghum stover after 72 h enzymatic hydrolysis.

pseudo lignin deposition and cellulose crystallinity, could also be possible reasons.<sup>43–45</sup>

The maximum glucan conversion yields of binary DES pretreated biomass were 85% with WT and 98% with TG sorghum stover. The addition of the solvent-based third constituents further improved the glucan conversion of the binary DES pretreated biomass. ChCl-DHBA-W pretreated WT sorghum stover showed a 97% of glucan conversion yield, while ChCl-DHBA-EG pretreated one showed a similar yield (89%) to binary ChCl-DHBA DES. The effect of ternary DES on xylan conversion yield was more pronounced. The ChCl-DHBA-W and ChCl-DHBA-EG DES pretreated WT biomass had 11% and 15% higher xylan conversion yields than the binary DES pretreated one. The ternary DES pretreated TG biomass also had 17%–39% enhancement in xylan conversion compared with binary DES pretreated one.

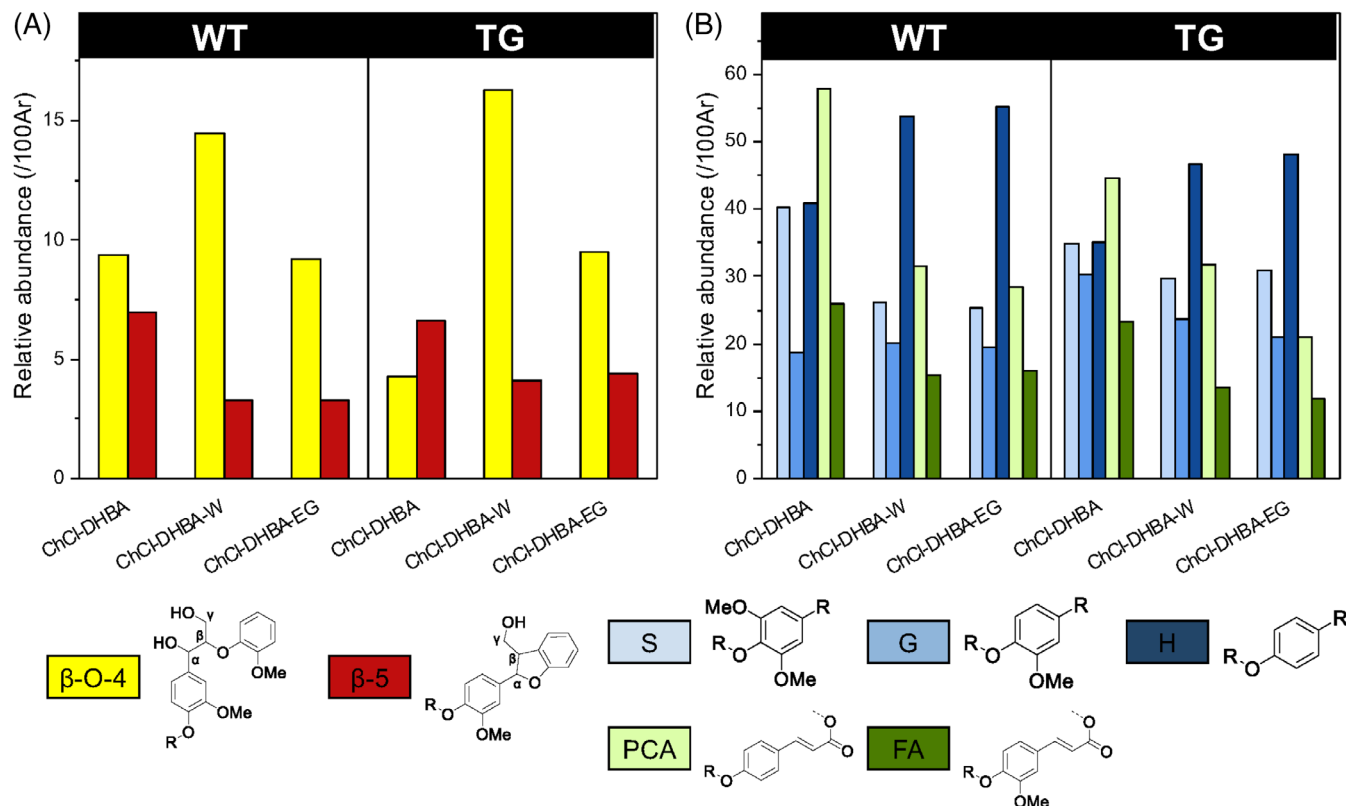
ChCl-DHBA-W DES pretreated biomass showed similar glucan conversion yields to WT (97%) and TG (98%). The ChCl-DHBA-W DES exhibited the highest glucan and xylan conversion yields

with WT sorghum stover. This DES also resulted in a notably higher xylan conversion yield (81%) with TG biomass. Another ternary DES, ChCl-DHBA-EG DES, showed higher glucan and xylan conversion yields (89% and 57%) than ChCl-DHBA DES (85% and 42%) with the pretreated WT. The ChCl-DHBA-EG DES provided superior sugar conversion with the pretreated TG biomass (99%) compared with the WT biomass (88%) even though the chemical composition changes in WT and TG biomass were similar (Figure 1, Tables 1 and S1).

### 3.5 | The impacts of ChCl-DHBA DES pretreatments on the physicochemical properties of lignin

The characteristics of the fractionated lignin from biomass pretreatment are important to achieve a successful biorefinery process. Therefore, the properties of lignin are as important as its recovery.





**FIGURE 4** Semiquantitative 2D HSQC analysis of lignin isolated from WT and TG sorghum stover after pretreatment using DESs with different compositions. (A) Relative abundance of interunit linkages; (B) relative abundance of subunits. The relative abundance expressed as 100/Ar is calculated based on the total integration areas of S, G, and H units.

Moreover, the changes in lignin reveal how the pretreatment chemicals and solvents transformed biomass components. Lignin was recovered from the DESs after the pretreatment of WT and TG sorghum stover. Molecular weight distribution, the composition of aromatic units, and abundance of interunit linkages were characterized by GPC and 2D HSQC NMR analyses. Detailed NMR spectra and corresponding structures are shown in Figure S2. By assigning peaks based on previous studies,<sup>46,47</sup> cross-signals corresponding to syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H), *p*-coumaric acid (PCA), ferulic acid (FA) and triclin (T) could be observed in the aromatic regions of the spectra (Figure S2b). Signals correlated to condensed S units were also detected but only at a low level. In the aliphatic regions, peaks of  $\beta$ -O-4 ether (A) and methoxy groups (OMe) were identified, as well as a low level of  $\beta$ -5 linkages (Figure S2a). Semiquantitative analysis results based on S, G, and H units of the NMR spectra are shown in Figure 4. Overall, the lignins fractionated from WT and TG sorghum stover by the same ternary DES, either ChCl-DHBA-EG or ChCl-DHBA-W, were structurally similar, while binary DES resulted in a different abundance of  $\beta$ -O-4 linkage with two biomass. As shown in Figure 4A, lignin isolated from ChCl-DHBA DES pretreated WT biomass had 9/100Ar  $\beta$ -O-4 linkage, which was similar to lignin fractionated by ChCl-DHBA-EG DES pretreatment. However, for the TG biomass,

$\beta$ -O-4 linkage only accounted for 4/100Ar after ChCl-DHBA DES pretreatment. It is worth noting that ChCl-DHBA DES pretreatment removed much less amount of lignin from TG biomass (9%) than from WT biomass (22%). Examining the abundances of subunits (Figure 4B) shows that lignin isolated from binary ChCl-DHBA DES pretreatment had higher hydroxycinnamates (PCA and FA) than those fractionated by the ternary ChCl-DHBA DES pretreatments. Combining with the lower delignification of the binary DES than the ternary DESs, it can be inferred that binary ChCl-DHBA DES mostly extracted the aromatic units incorporated in lignin via ester linkages which are susceptible to cleavage,<sup>48,49</sup> rather than extracting the bulk lignin. The higher amounts of hydroxycinnamates in WT lignin compared to TG lignin also indicate that binary DES was less effective on the engineered sorghum stover.

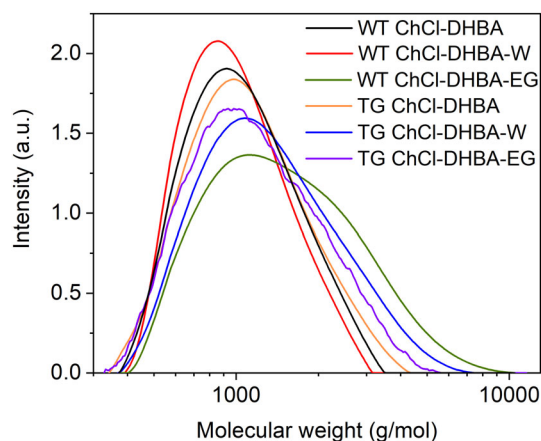
Different DES compositions resulted in varied alterations of lignin interunit linkages and aromatic subunits. In both WT and TG sorghum, lignin fractionated by ChCl-DHBA-W pretreatment had the highest  $\beta$ -O-4 ether content, being approximately 15/100Ar. The impact of ternary DESs on lignin did not show significance between the WT and TG biomass, unlike the binary DES, for both interunit linkages and aromatic subunits (Figure 4). Another important observation from the 2D HSQC NMR spectra is cross-signals corresponding to DHBA, identified with the

**TABLE 3** Number-average molecular weight ( $M_n$ ), weight-average molecular weight ( $M_w$ ), and dispersity ( $\mathcal{D}$ ) of lignin isolated from WT and TG sorghum stover after pretreatment using DESs with different compositions.

| Biomass | DES type     | $M_n$ | $M_w$ | $\mathcal{D}$ |
|---------|--------------|-------|-------|---------------|
| WT      | ChCl-DHBA    | 948   | 1155  | 1.2           |
|         | ChCl-DHBA-W  | 990   | 1078  | 1.2           |
|         | ChCl-DHBA-EG | 1240  | 1776  | 1.4           |
| TG      | ChCl-DHBA    | 969   | 1218  | 1.3           |
|         | ChCl-DHBA-W  | 1122  | 1508  | 1.3           |
|         | ChCl-DHBA-EG | 1011  | 1330  | 1.3           |

peaks at  $\delta_C/\delta_H = 116.3/7.3$  ppm,  $121.6/7.3$  ppm,  $121.6/6.8$  ppm, and  $114.8/6.8$  ppm (overlapping with G5), as indicated in Figure S2. The appearance of DHBA in the recovered lignin could be caused by the accumulation of DHBA *in planta* due to genetic modification and/or arose from residue DES. Even with the addition of a third constitute like EG, the complete separation of the ternary DESs from the biomass after pretreatment is still challenging. A certain portion of DES components are possibly grafted to the lignin. Nonetheless, our previous study has already demonstrated the feasibility of selectively isolating the genetically accumulated and residue aromatic compound from the recovered lignin via hydrothermal processing.<sup>28</sup> Therefore, a sustainable biorefinery process via the selective recovery of DHBA from the lignin is achievable.

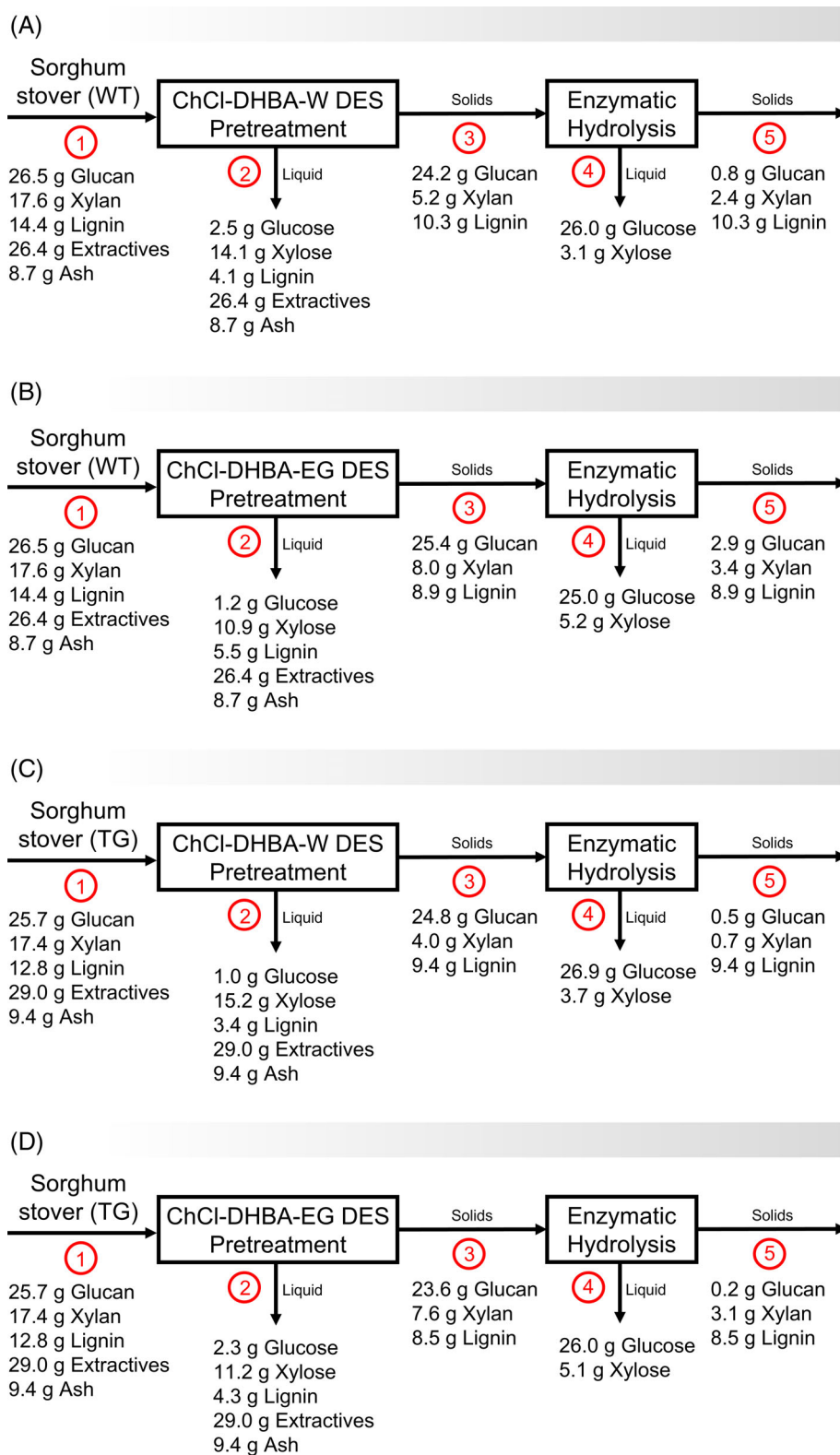
The molecular weight of lignin provides important information on the structural modification during the pretreatment and plays an important role in lignin valorization. For example, lignin with high molecular weight and low PDI exhibit enhanced spinnability and crystallite size, which leads to a boost in the mechanical performance of lignin-based carbon fiber,<sup>50</sup> while lignin with low molecular weights shows better miscibility for the making of lignin-copolymers.<sup>51</sup> The weight-average molecular weight ( $M_w$ ), number-average molecular weight ( $M_n$ ), and dispersity ( $\mathcal{D}$ ) are shown in Table 3, while the molecular weight distributions are displayed in Figure 5. The  $\mathcal{D}$  of the fractionated lignins showed a narrow range, indicating that lignins had relatively uniform size distribution after pretreatment. The molecular weights and  $\mathcal{D}$  of lignin fractionated from the two sorghum stover by the aforementioned ChCl-DHBA DESs were similar, with standard deviations of 240 g/mol and 0.1, respectively. Lignin fractionated from WT sorghum stover by ChCl-DHBA-W showed the lowest molecular weight of 1078 g/mol, while ChCl-DHBA-EG pretreatment of the TG biomass resulted in the highest lignin molecular weight of 1776 g/mol, which could be attributed to the depolymerization and repolymerization of lignin during the DES pretreatments. Lignin fractionated by binary DES pretreatment showed lower molecular weights than those fractionated by ternary DESs, which is consistent to the 2D HSQC NMR results.



**FIGURE 5** GPC molecular weight distribution curves of lignin isolated from WT and TG sorghum stover after pretreatment using DESs with different compositions.

### 3.6 | The mass balance of ternary ChCl-DHBA DES pretreatment processes with WT and TG sorghum stover

Compared with the binary DES, both ternary DESs (ChCl-DHBA-W and ChCl-DHBA-EG) resulted in higher fermentable sugar production. For a better understanding of the overall mass balance of both approaches with WT and TG sorghum stover, process flow diagrams with the mass balance of major biomass components were presented in Figure 6. The mass balance was calculated based on 100 g of sorghum stover as the starting material. The average value of each treatment was used for the mass balance calculation. Stream 1 describes the amount of major biomass components in the untreated feedstock (i.e., sorghum stover), and Stream 4 shows the final products (fermentable sugars after enzymatic hydrolysis). As shown in Figure 6, both WT and TG sorghum stovers have over 26 g of extractives and around 9 g of ash, and the three main components (glucan, xylan, and lignin) accounted for ~60 g per 100 g biomass. WT biomass had slightly higher glucan and lignin content than TG biomass, with the TG biomass having more extractives. Among four scenarios, ChCl-DHBA-W DES exhibited higher glucose production (26.0 and 26.9 g with the pretreated WT and TG biomass) than ChCl-DHBA-EG one (25.0 and 26.0 g with WT and TG biomass). However, ethylene glycol-incorporated DHBA DES resulted in higher xylose production (5.2 and 5.1 g with the pretreated WT and TG biomass) than the water-incorporated one (3.1 and 3.7 g with the pretreated WT and TG biomass) even though its xylan conversion was lower (Figure 3). This is because ChCl-DHBA-EG pretreated biomass retained more xylan during the pretreatment (Table 2). For this reason, ChCl-DHBA-EG pretreated TG sorghum stover produced the highest amount (31.1 g) of the total fermentable sugars (glucose + xylose) with the pretreated TG biomass, shown in Figure 6D. However, a large portion of the hemicellulose was solubilized in the liquid fraction during the pretreatment; therefore, the separation and utilization of these sugars after the recovery of lignin and DES should be considered to complete the biorefinery strategy.



**FIGURE 6** Overall biomass pretreatment mass balance of WT sorghum stover by pretreatment (413.15 K for 3 h) and enzymatic hydrolysis (20 FPU/g of biomass for 72 h) using (A) ChCl-DHBA-W DES and (B) ChCl-DHBA-EG DES, and TG sorghum stover using (C) ChCl-DHBA-W DES and (D) ChCl-DHBA-EG DES.

## 4 | CONCLUSIONS

In this study, the effects of the constituents of ChCl-DHBA DES on WT and TG sorghum stover with *in planta* accumulation of DHBA were investigated. Binary DES and ternary DESs were synthesized

with ChCl, DHBA, and the solvent-based third constituents including water and ethylene glycol. TG biomass had a similar chemical composition to WT biomass and lower enzymatic hydrolysis yields. However, it became more susceptible to enzymatic hydrolysis after the DHBA-based DES pretreatments, releasing higher amounts of fermentable

sugars than WT biomass. The third constituent led to dramatically enhanced pretreatment performances. Binary ChCl-DHBA DES removed over 70% of xylan with less than 20% of delignification. EG-incorporated ternary DES doubled the delignification while having 20% less xylan removal compared with the binary DES. ChCl-DHBA-W DES increased both high xylan removal and delignification. Ternary DESs resulted in a significant enhancement in the conversion of glucan and xylan compared with the binary DES. However, the enhanced fermentable sugar production after the pretreatments with ChCl-DHBA-EG and ChCl-DHBA-W are not easily concluded with a single dominant factor, either xylan removal or delignification, in this study. Investigation of lignin structure after pretreatment indicates that using water as the third constituent for ternary DES preserved the most  $\beta$ -O-4 linkage, while binary ChCl-DHBA DES mostly only removed lignin from the low molecular weight fraction. Taking fermentable sugar conversion and lignin structure together, both water and ethylene glycol showed great potential as the third constituent in developing a sustainable biorefinery process for total biomass utilization. The results demonstrated that synthesizing ternary DESs could enhance the pretreatment performance of binary DESs.

#### AUTHOR CONTRIBUTIONS

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#### DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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