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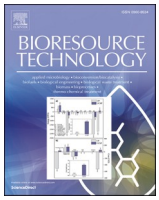

Assessment of the interaction mechanisms for a novel ternary deep eutectic solvent developed to enhance the enzymatic hydrolysis of bamboo

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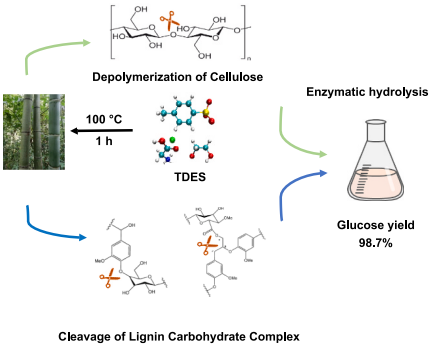
HIGHLIGHTS

- A highly efficient ternary DES pretreatment was developed.
- TDES pretreatment effectively removed lignin (89.0%) and xylan (95.1%) from bamboo residue.
- Decrease in pH value of TDES plays a crucial role in lignin removal.
- Hydrogen bonds formed between TDES and lignin leads to the delignification.
- The enzymatic hydrolysis yield of TDES-treated bamboo residue was 98.7%.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel ternary deep eutectic solvent (TDES) composed of glycine hydrochloride, ethylene glycol, and *p*-toluenesulfonic acid was synthesized. The prepared TDES exhibited highly efficient capacity for the pretreatment of bamboo biomass (*Phyllostachys edulis*). About 95 % of xylan and 89 % of lignin could be removed from the bamboo feedstock after being pretreated with the TDES at 100 °C for 1 h. In addition, a nearly complete glucose yield of 98.7 % could be achieved, which was 9.4 times higher than that of the untreated bamboo. The pH value of the TDES is an important factor affecting the bond cleavage between lignin and carbohydrates. Additionally, the polarity facilitates selective dissolution of lignin via hydrogen bonding. Molecular dynamics simulations reveal an increase in hydrogen bond formation between TDES and lignin model compounds. The findings establish the TDES as a green, efficient method for bamboo biomass pretreatment, facilitating the conversion of this biomass resource into glucose for bioenergy applications.

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1. Introduction

Bamboo represents a renewable biomass resource that is well positioned for bioenergy production and as a sustainable biomaterial because of its rapid growth rate (Dlamini et al., 2021; Rezania et al., 2020). Nonetheless, the full industrial potential of bamboo, like that of many other biomass sources, remains largely untapped due to the complex chemistry, comprised of three primary components: cellulose, hemicellulose, and lignin. These biopolymers are bonded through a complex network of covalent bonds, hydrogen bonds, and other chemical interactions, forming a recalcitrant barrier to conventional digestion techniques (Ying et al., 2022). Addressing this challenge necessitates innovative pretreatment strategies aimed at effectively deconstructing the physical and chemical structure of bamboo biomass (Sai Bharadwaj et al., 2023; Zhang et al., 2023b).

Physical pretreatment methods such as ball milling, ultrasound, and microwave energy can minimize the environmental impact by avoiding the use of chemical reagents. However, these methods are energy-intensive, leading to higher production costs (Verma et al., 2021; Zhao et al., 2024). Chemical pretreatment methods often involve acids, bases, or organic solvents. While acid and alkali pretreatments are highly efficient, they require stringent conditions and generate hazardous waste (Bansod et al., 2024). Among the alternative pretreatment options, those based on deep eutectic solvent (DES) stand out as promising green solvent systems, alleviating the harsh conditions dictated by traditional procedures and demonstrating remarkable qualities in dissolving a wide range of biomass components (Marchel et al., 2022).

Ternary DES systems consisting of choline chloride, alcohols, Brønsted acids, or Lewis acids have been widely used. Fernandes et al. (2021) demonstrated that a DES composed of choline chloride, lactic acid, and tartaric acid removed 95 % of lignin from coastal pine at 175 °C in 1 h. Similarly, Ji et al. (2020) reported that a DES composed of choline chloride, glycerol, and chromium chloride removed 65 % of lignin from sugarcane bagasse at 110 °C in 3 h. These systems often require high pretreatment temperatures or achieve relatively low delignification. Glycine hydrochloride (GH), with three hydrogen bond acceptors compared to two in choline chloride, potentially offers greater stability when forming a DES with hydrogen bond donors. Additionally, the low toxicity of GH makes it an environmentally friendly choice for enhancing biomass separations.

In the field of DES pretreatment, binary systems have shown limited efficiency in separating biomass components. Wu et al. (2024) observed a relatively low removal percentage in the pretreatment of soybean straw, with 22.9 % for lignin and 3.8 % for hemicellulose, using benzyltriethylammonium chloride/glycerol as the DES at 100 °C for 2 h. Introducing *p*-toluenesulfonic acid (PTA) as a third DES component significantly increased removal efficiency, achieving 92.0 % for lignin and 88.2 % for hemicellulose. Similarly, Shen et al. (2022) reported that the addition of $FeCl_3$ to a choline chloride/glycerol acid-based DES markedly improved the delignification percentage from 4.2 % to 70.8 % and hemicellulose removal percentage from 13.6 % to 76.3 %. These findings indicate the potential of employing a selected mixture of several hydrogen bond donors and acceptors to synergistically improve the efficiency of biomass separation (Chourasia et al., 2022). However, it remains to be elucidated how the non-neutral components in DES systems impact the chemical compositions and physicochemical properties of the biomass feedstock.

Recent advancements in biomass pretreatment research have increasingly relied on quantum chemistry and molecular dynamics simulations to model the interactions between DES and lignocellulosic biomass. These methods allow for a deeper understanding of electrostatic potential distributions, hydrogen bonding, and molecular orbital energies (Naseem et al., 2023). Studies have shown molecular dynamics simulations can predict DES performance, especially in predicting lignin solubilization and hemicellulose removal (Zhu et al., 2023).

This study developed a neutral binary deep eutectic solvent (BDES) by utilizing GH as the hydrogen bond acceptor and ethylene glycol (EG) as the hydrogen bond donor. The impacts of introducing *p*-toluenesulfonic acid (PTA) to synthesize a ternary deep eutectic solvent (TDES) were then investigated. Specifically, this included an investigation of the effect of PTA on the solvent properties of the BDES, and for both DES systems, the effects of pretreatment conditions on chemical composition, surface characteristics, and enzymatic hydrolysis efficiency of the pretreated bamboo. Quantum chemistry and molecular dynamics simulations were employed to elucidate the molecular mechanisms of bamboo decomposition with these DES systems. This study aims to establish a theoretical foundation for the effective separation of the main components of bamboo and the subsequent efficient enzymatic hydrolysis the resultant polysaccharide fractions.

2. Materials and methods

2.1. Biomass feedstock and chemicals

Residual bamboo (*Phyllostachys edulis*) biomass, provided by a bamboo furniture factory (Nanjing, Jiangsu province, China), was washed with tap water, dried in an oven (40 °C), and ground by a knife mill to a particle size of 0.30–0.45 mm. Glycine hydrochloride (GH), ethylene glycol (EG), and *p*-toluenesulfonic acid monohydrate (PTA) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. Microcrystalline cellulose (MC) powder was purchased from Sigma Life Science Company (Shanghai, China). Cellulase (Cellic CTec3, Novozymes), with a filter paper activity of 220 FPU/mL and a protein concentration of 122.9 g/L, was employed for enzymatic hydrolysis of bamboo samples before and after DES pretreatment.

2.2. Preparation of binary and ternary DES systems

The neutral binary deep eutectic solvent (BDES) was synthesized by heating GH and EG at a 1:4 M ratio in a conical flask at 80 °C for 30 min, accompanied by magnetic stirring at 500 rpm. The ternary deep eutectic solvent (TDES) was synthesized by heating a mixture of GH, EG and PTA in a molar ratio of 1:4:0.5 in the same manner. Both the BDES and TDES exhibited flowability and stability, remaining in a non-solidified state at room temperature.

2.3. Binary and ternary DES pretreatment

The pretreatment of bamboo residue (BR) with each DES were performed in a 100-mL conical flask. In short, 5 g of ground BR and 50 g of DES were heated at 60–120 °C for 1 h, with stirring at 500 rpm. For comparison, a BR pretreatment with BDES was performed at 100 °C for 1 h, and two pretreatments with TDES were conducted at 100 °C, one for 0.5 h and the other for 2 h. The pretreated residues were filtered and washed with 1:1 mixture of ethanol and water. Based on the pretreatment temperature and time, the samples were labeled as BDES/BR, TDES-60/BR, TDES-80/BR, TDES-100/BR, TDES-120/BR, TDES-100–0.5/BR and TDES-100–2/BR.

The chemical compositions of BR and pretreated residues were analyzed in accordance with the standard methods from the National Renewable Energy Laboratory (NREL) (Sluiter et al., 2008); the polysaccharide sugars were quantified using a high-performance liquid chromatography (HPLC) system (Agilent 1200, USA), equipped with a Bio-Rad HPX-87H column, using 5 mM sulfuric acid as the eluent at a flow rate of 0.6 mL/min and a temperature of 50 °C. The solid yield, recovery of glucan, and delignification after pretreatment were evaluated using the following equations:

$$\textit{Solid residue yield}\ (\%) = \frac{\textit{weight of bamboo after pretreatment}}{\textit{weight of bamboo before pretreatment}} \times 100 \quad (1)$$

$$Glucan\ recovery\ yield\ (\%) = \frac{weight\ of\ glucan\ in\ bamboo\ after\ pretreatment}{weight\ of\ glucan\ in\ bamboo\ before\ pretreatment} \times 100 \quad (2)$$

$$Delignification\ (\%) = \left(1 - \frac{weight\ of\ lignin\ in\ bamboo\ after\ pretreatment}{weight\ of\ lignin\ in\ bamboo\ before\ pretreatment}\right) \times 100 \quad (3)$$

2.4. Enzymatic hydrolysis

Cellic CTec3 was employed for the enzymatic hydrolysis at a dosage of 10 FPU/g substrate (5.6 mg protein/g substrate). Briefly, 2 g BR (or pretreated residue) and 40 mL sodium citrate buffer solution (pH 4.8) were mixed in a 100 mL conical flask, and incubated in a shaker at 50 °C with stirring at 150 rpm for 72 h. The aliquots were collected regularly, centrifuged at 10,000 rpm for 5 min, and then used to measure the sugar concentration.

$$Glucose\ yield\ (\%) = \frac{released\ glucose\ weight\ content \times 0.9}{initial\ weight\ content\ of\ glucan\ in\ pretreated\ solids} \times 100 \quad (4)$$

2.5. General analysis methods

To determine the affinity between BR and cellulase before and after pretreatment, an enzyme adsorption experiment was performed. Cellulase solutions with 8 different protein concentrations (0.02, 0.04, 0.08, 0.16, 0.5, 1, 2, 3 mg/mL) for the enzyme adsorption experiment were prepared by pipetting different amounts of cellulase, or diluted cellulase solution, into a 100 mL conical flask that contained 39.7 mL sodium citrate buffer (pH 4.8) (Yang et al., 2019). The above solutions were stirred with a magnetic stirrer bar for 5 min to ensure thorough mixing. Then, an aliquot of 3 mL was withdrawn and subjected to UV analysis to determine the actual protein concentration. The 3, 2, and 1 mg/mL cellulase solutions were prepared directly using the original cellulase (protein concentration 122.9 mg/mL). The other 5 cellulase solutions, with protein concentrations of 0.02, 0.04, 0.08, 0.16, 0.5 mg/mL were prepared from diluted cellulase solutions. The adsorption experiments were carried out under 4 °C to minimized the possible hydrolysis of the substrate. In brief, 0.1 g untreated BR and BR residues from DES pretreatment was mixed with 5 mL cellulase solution at various protein concentrations (0.02, 0.04, 0.08, 0.16, 0.5, 1, 2, 3 mg/mL) in a 20 mL conical flask and agitated at 150 rpm for 3 h. Upon the end of desired adsorption time, the supernatant was collected for protein concentration determination by UV–vis analysis using the Bradford method (Bradford, 1976). The adsorbed enzyme protein content was calculated based on the difference between the initial protein content before adsorption and the free enzyme protein content in the supernatant after adsorption.

The maximum adsorption capacity (Γ_m, mg/g substrate) and the Langmuir constant (K, mL/mg) were determined by nonlinear regression of the free enzyme concentration (C, mg/mL) in solution and the corresponding adsorbed enzyme (Γ, mg/g substrate) on the substrate, based on the classical Langmuir adsorption isotherm. The distribution coefficient (R, L/g) was calculated by the formulas:

$$\Gamma = \frac{\Gamma m \times K \times C}{1 + K \times C} \quad (5)$$

$$R = \Gamma m \times K \quad (6)$$

The characteristic functional groups of BR before and after pretreatment were explored by Fourier transform infrared spectroscopy (FT-IR, Nicolet iS5, Thermo Scientific, USA). The distribution of carbon and oxygen elements was determined through X-ray photoelectron spectroscopy (XPS) with a K-Alpha instrument (AXIS UltraDLD, Shimadzu, England). The surface coverage of carbohydrate and lignin was analyzed as described by Yang et al. (2020). The crystallinity index (CrI) of BR before and after pretreatment was analyzed by X-ray diffraction (XRD Ultima IV, Rigaku, Japan). Lignins were isolated from bamboo and DES pretreatment residues using the milled wood lignin (MWL) procedure of Björkman (1954). Lignins were dissolved in dimethyl sulfoxide for structural analysis by 2D-HSQC NMR using a Bruker instrument (AVIII 400 MHz, Germany). Lignins were dissolved in tetrahydrofuran after acetylation with pyridine/acetic anhydride, and then analyzed on a gel permeation chromatograph (Agilent GPC/HPLC 1200), equipped with two Jordi Gel DVB Mixed Bed HPLC columns, to evaluate the number average molecular weight (Mn) and weight average molecular weight (Mw) of lignin (Zhang et al., 2020). Additionally, microcrystalline cellulose (MC) powder was subjected to TDES pretreatment at 100 °C for 1 h; the CrI of cellulose and its enzymatic hydrolysis efficiency before and after pretreatment were measured to evaluate the impact of TDES pretreatment on cellulose alone.

2.6. Quantum chemical calculations

GH, EG and PA were optimized structurally at the B3LYP-D3(BJ)/def2-TZVP(-f) level utilizing ORCA 5.0 software (Neese, 2022). The Multiwfn 3.8 software was employed to ascertain the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO). Additionally, the distribution of surface electrostatic potentials of BDES and TDES was analyzed (Lu & Chen, 2011). Visualization was achieved through the use of VMD 1.9.3 software (Humphrey et al., 1996).

2.7. Molecular dynamics simulations

Molecular dynamics simulations were performed using molecular structures optimized with the ORCA software. The advanced restrained electrostatic potential (RESP2) atomic charges were employed, calculated with Multiwfn software based on the electrostatic potential charges. Molecular topology files were generated using Sobtop 1.0 software, based on the GAFF force field (Lu, 2023). Molecular dynamics simulations were performed using GROMACS 2020 with the Amber99sb-ildn force field (Abraham et al., 2015). Two distinct systems, BDES and TDES, were constructed using Packmol 18 software within a system box (10 nm × 10 nm × 10 nm) (Martinez et al., 2009). The BDES system was composed of 10 GH^+, 10 Cl^-, 40 EG, and 10 lignin model compounds. Similarly, the TDES system consisted of 10 GH^+, 10 Cl^-, 40 EG, 5 PTA and 10 lignin model compounds. The Berendsen method was employed to maintain system pressure at 1 atm during the equilibrium phase, while the Parrinello-Rahman method was applied during the production phase to keep the pressure constant at 1 atm. An annealing process occurred in the equilibrium phase, with the temperature increasing from 0 to 373.15 K over 450 ps and then being maintained thereafter. Temperature control was achieved using the V-rescale method in both the equilibrium and production phases. The production phase was conducted for 30 ns, with a step size of 1 fs, with periodic boundary conditions applied in all directions throughout the simulations.

2.8. Reuse of DES

After the separation of the solid residue and liquid from a TDES pretreatment cycle, a rotary evaporator was utilized to remove the water and ethanol from the spent TDES. Subsequently, 4-times volume of deionized water than that of the recovered TDES was added to precipitate the lignin, which was then removed by centrifugation at 5000 rpm.

The supernatant was then subjected to rotary evaporation to remove water. Subsequently, the recovered TDES was dried in a vacuum oven at 70 °C until a constant weight was achieved. About 1.0 g fresh TDES was added to the vacuum-dried TDES, which was then used for another pretreatment cycle (Chen et al., 2018).

2.9. Statistical analysis

Statistical analyses were evaluated by ANOVA with Tukey's post hoc test using Python 3.10, and statistical significance was determined at $p \leq 0.05$.

3. Result and discussion

3.1. Characteristics of binary and ternary DES

The pH, and solvation parameters (hydrogen bond donor acidity, hydrogen bond acceptor basicity, and polarization value) of DES with different molar ratios are provided in the supplementary materials. A stable DES system was formed using a molar ratio of 1:4:0.5 for GH, EG, and PTA whereas a DES with a molar ratio of 1:3:0.5 could not be synthesized.

Three different molar ratios of GH: EG: PTA (1:4:0.1, 1:4:0.5, 1:4:1) were used in the preparation of the TDES and their performances in the bamboo biomass pretreatment were compared. It was found that increasing the PTA content caused the pH of the TDES to drop from 2.59 to 1.43 while the polarization value and hydrogen bond donor acidity increased from 0.99 to 1.06 and from 1.81 to 1.84, respectively. These findings suggest that introducing PTA into the DES could increase the hydrogen bond donor acidity of the solvent, which is generally positively correlated with lignin removal efficiency. In addition, lower pH values would enhance the lignin dissolution in acidic DES pretreatments (Lobato-Rodríguez et al., 2023). Similar observations were found in previous research (Bai et al., 2024), where a decrease in pH values, and an increase in polarization values and hydrogen bond donor acidity, led to more removal of xylan and lignin.

The physical and chemical properties of the BDES and TDES were characterized and the results are provided in the supplementary materials, including DSC, FTIR, viscosity, and moisture stability analysis. The glass transition temperatures of the BDES and TDES are −96.2 °C and −93.3 °C, respectively. The addition of PTA increased the glass transition temperature, likely due to the formation of hydrogen bonds with other components (Ee et al., 2023). Morrison et al. (2009) prepared a DES using choline chloride and malonic acid, but they did not observe any thermal events. They explained that the high viscosity of the liquid hinders nucleation and crystal growth during heating.

Viscosity is a key factor influencing biomass-DES interactions. The TDES possesses a slightly higher viscosity (17.1 cP) than that of the BDES (13.1 cP) at 100 °C. However, the viscosities of both BDES and TDES decreased at higher temperatures as expected and the differences between the viscosities of the two DES systems gradually decreased with the increasing temperatures. Previous research has reported that the viscosity of the DES showed less significant impact on the biomass pretreatment if below 20 cP (Hou et al., 2024).

The moisture contents of the synthesized DES systems were determined over 5 days at room temperature (28 °C) and 65 % humidity. The maximum moisture contents of BDES and TDES were 21.3 % and 19.1 %, respectively, indicating that PTA reduces the water absorption of TDES. To maintain performance, DES should be stored in a dry, moisture-proof environment to prevent absorbed moisture from interfering with experimental results.

The FTIR of the individual components, BDES, and TDES are provided in the supplementary materials. The broadening of the hydroxyl peak is more pronounced in TDES than that of BDES, indicating the formation of more hydrogen bonds.

3.2. Electrostatic potential distribution and band gap of DES

Quantum chemical calculations were performed to analyze the electrostatic potential distribution of the BDES and TDES and the results are displayed in Fig. 1 (a). The electrostatic potential is comprised of negative (blue area) and positive (red area) regions. Within the DES systems, chloride ion, sulfonic acid groups, and hydroxyl oxygen atoms exhibited negative potentials, while amino groups and hydroxyl hydrogen atoms exhibited positive potentials. The electrostatic potential range for BDES extended from −38.6 kcal/mol to 62.9 kcal/mol, whereas it ranged from −38.0 kcal/mol to 49.1 kcal/mol for TDES. The lower maximum positive electrostatic potential observed in TDES compared to BDES should be due to the introducing of PTA in the TDES that was prone to form additional hydrogen bonds with GH and EG and therefore reduced the overall positive charge of the TDES as well as stabilizing the TDES system (Zhang et al., 2024).

Fig. 1(b) and (c) depict the energy band gaps between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) of the BDES and TDES, which were 5.5 eV and 4.9 eV, respectively. The energy band gap is considered as an indicator of the energy associated with molecular excitation of a compound. A lower band gap value implies a lower requisite energy for molecular excitation and therefore the reactions are more likely to occur (Naseem et al., 2023). The lower band gap of the TDES suggests that it should possess higher chemical reactivity compared to the BDES, which would enhance the ability of TDES to interact with the main components of bamboo biomass during the pretreatment process (Haroon et al., 2023; Rocha et al., 2015).

3.3. Chemical compositions of BR before and after DES pretreatment

The selectivity of DES for bamboo residues was investigated using BDES and TDES for the pretreatment of the BR under various conditions. The efficiency of each DES pretreatment was determined by analyzing the solid residue yield, changes in chemical composition, and the recovery or removal percentages of individual components. As shown in Table 1, untreated BR contained 39.1 ± 0.5 % glucan, 18.7 ± 1.0 % xylan, and 30.1 ± 0.4 % lignin. After BDES pretreatment, the solid residue yield decreased to 76.1 ± 0.5 %, with a glucan recovery percentage of 92.1 ± 0.7 %, 51.9 ± 0.4 % reduction in xylan, and 43.9 ± 0.5 % delignification. The analyses showed that in BDES-treated BR, the glucan content increased to 47.3 ± 0.3 %, while the xylan and lignin contents decreased to 11.8 ± 0.8 % and 22.2 ± 0.4 %, respectively. Prior research demonstrated that a tetramethylammonium chloride/lactic acid pretreatment only removed 44.5 % of hemicellulose and 31.0 % of lignin from a different grass (*Pennisetum giganteum*) at 110 °C for 3 h (Sun et al., 2023). It was reported that the neutral DES lacked sufficient polarity and Lewis acidity, which were critical for breaking the bonds in the lignin structure and between lignin and carbohydrates (Xie et al., 2021).

The introduction of PTA as a third constituent to formulate TDES for the pretreatment of BR led to significant changes in the chemical compositions of the pretreatment residues. As shown in Table 1, an increase in pretreatment temperature from 60 °C to 120 °C resulted in significant reduction in the solid residue yield from 68.9 ± 1.1 % to 43.7 ± 0.8 %. Glucan proved challenging to depolymerize during DES pretreatment, remaining as the major component within the pretreatment residue with a retention of over 89 %. Conversely, TDES pretreatment selectively facilitated a more pronounced removal of xylan and lignin. With the increase in temperature, the xylan content significantly decreased from 10.2 ± 0.5 % at 60 °C to 1.9 ± 0.5 % at 100 °C, culminating in the near-complete removal (99.9 %) of xylan during TDES pretreatment at 120 °C. After TDES pretreatment, the lignin content decreased to 17.2 ± 0.2 % of TDES-60/BR, 12.5 ± 0.8 % of TDES-80/BR, 6.9 ± 0.4 % of TDES-100/BR, and 2.6 % of TDES-120/BR, corresponding to delignifications of 60.6 ± 0.5 %, 76.1 ± 0.4 %, 89.0 ± 1.0 %, and 96.1 ± 0.7 %,

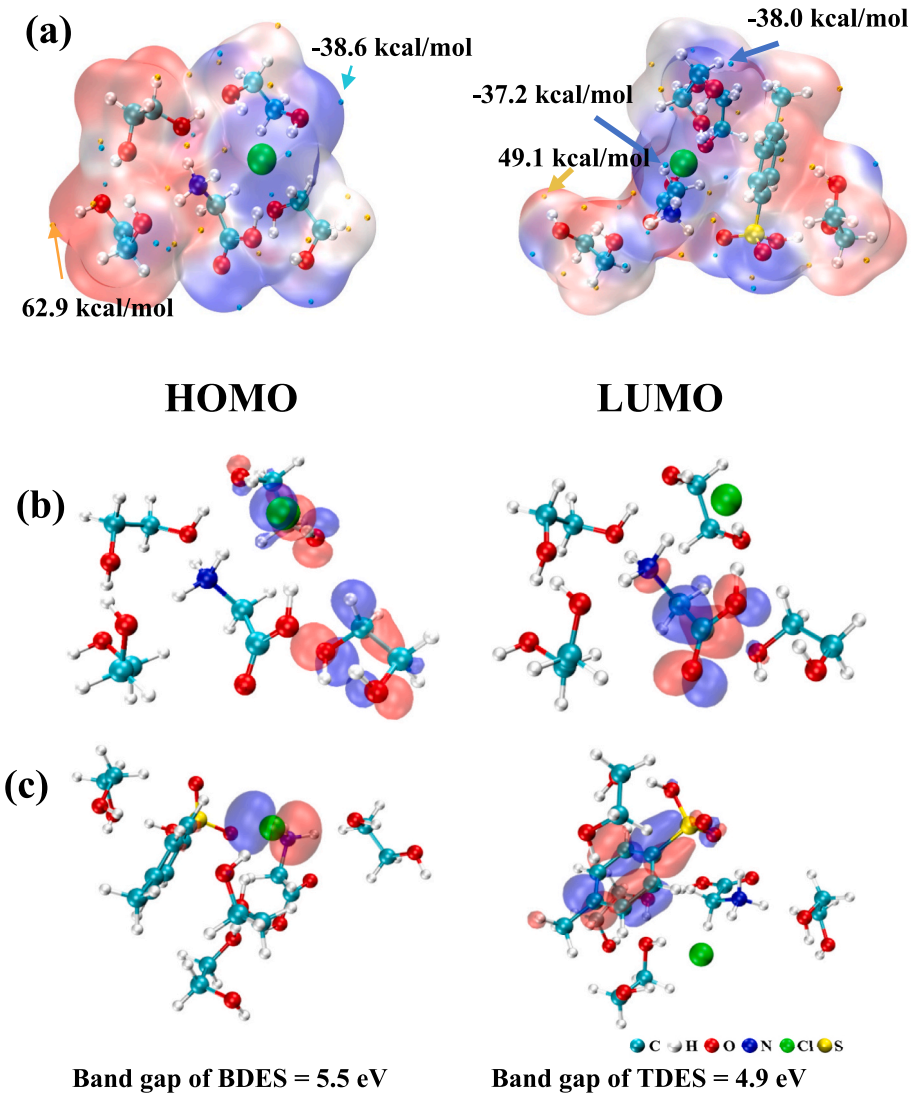

Fig. 1. (a) Electrostatic potential distribution of BDES and TDES, (b) HOMO-LUMO band gap of BDES, (c) HOMO-LUMO band gap of TDES. (HOMO: Highest Occupied Molecular Orbital, LUMO: Lowest Unoccupied Molecular Orbital).

Table 1
Solid residue yields and chemical components determinations for BR before and after DES pretreatment varying DES system, temperature, and time.

Pretreatment residue	Solid residue yield[1] (%)	Glucan (%)	Xylan (%)	Lignin (%)	Glucan recovery (%)	Xylan removal (%)	Delignification (%)
BR	na[2]	39.1 ± 0.5^{A}	18.7 ± 1.0^{A}	30.1 ± 0.4^{A}	na	na	na
BDES/BR	76.1 ± 0.5^{A}	47.3 ± 0.3^{A}	11.8 ± 0.8^{B}	22.2 ± 0.4^{B}	92.1 ± 0.7^{A}	51.9 ± 0.4^{A}	43.9 ± 0.5^{A}
TDES-60/BR	68.9 ± 1.1^{A}	53.2 ± 0.6^{A}	10.2 ± 0.5^{B}	17.2 ± 0.2^{C}	93.7 ± 0.6^{A}	62.4 ± 0.7^{A}	60.6 ± 0.5^{B}
TDES-80/BR	57.7 ± 1.0^{B}	63.1 ± 0.8^{B}	5.3 ± 0.6^{C}	12.5 ± 0.8^{D}	93.2 ± 1.2^{A}	83.6 ± 0.7^{B}	76.1 ± 0.4^{C}
TDES-100/BR	47.7 ± 1.0^{C}	73.6 ± 0.8^{B}	1.9 ± 0.5^{D}	6.9 ± 0.4^{E}	89.3 ± 0.7^{A}	95.1 ± 0.4^{C}	89.0 ± 1.0^{D}
TDES-120/BR	43.7 ± 0.8^{C}	81.4 ± 0.6^{C}	0.1 ± 0.1^{D}	2.6 ± 0.3^{F}	90.9 ± 0.5^{A}	99.9 ± 0.1^{C}	96.1 ± 0.7^{D}
TDES-100–0.5/BR	59.6 ± 1.0^{B}	61.0 ± 0.5^{B}	7.3 ± 0.8^{C}	14.5 ± 0.4^{D}	93.0 ± 0.5^{A}	76.7 ± 0.3^{B}	71.3 ± 0.4^{C}
TDES-100–2/BR	43.2 ± 0.5^{C}	79.3 ± 0.3^{C}	0.8 ± 0.8^{D}	3.2 ± 0.3^{F}	89.1 ± 0.3^{A}	97.9 ± 0.4^{C}	93.9 ± 0.5^{D}

[1]Value in the same column with different superscript letters indicate a significant difference at $p \leq 0.05$.
[2]not applicable.

respectively. Delignification and xylan removal of TDES-100/BR were 1.19 and 0.92 times higher than those of BDES/BR, indicating that a higher propensity of TDES to disrupt the chemical and hydrogen bonds within bamboo constituents compared with BDES. The addition of PTA to BDES significantly increased the affinity of the DES for the amorphous components of biomass. That is, the TDES effectively disrupted the weaker intermolecular forces within the amorphous regions, thereby facilitating the separation and dissolution of hemicellulose and lignin. This selectivity is attributed to both the disruption of existing hydrogen bonds and the formation of new hydrogen bonds in the TDES (Wu et al., 2024).

Additionally, extending the pretreatment time from 0.5 to 2 h at 100 °C led to a higher glucan content in pretreatment residue from 61.0 ± 0.5 % to 79.3 ± 0.3 %, with delignification increasing from 71.3 ± 0.4 % to 93.9 ± 0.5 %. However, the values with TDES-100–2/BR remained below the glucan content (81.4 ± 0.6 %) and delignification

(96.1 ± 0.7 %) of TDES-120/BR, suggesting that further prolonging the pretreatment time would not significantly increase the delignification compared with TDES-120/BR.

3.4. Effect of DES pretreatment on enzymatic hydrolysis and cellulase distribution

Bamboo residues before and after DES pretreatment were subjected to enzymatic hydrolysis with cellulase loading of 10 FPU/g substrate. Fig. 2(a) and (b) show the glucose yield and cellulase distribution, respectively, in BDES and TDES-treated BR during the 72-h enzymatic hydrolysis process. As indicated in Fig. 2(a), the glucose yield of untreated BR remained below 10 % (9.5 %), which increased significantly to 45.5 % after BDES pretreatment. However, TDES pretreatment led to a progressive increase in glucose yields from 33.7 % to 81.1 % as the temperature increased from 60 to 80 °C. Notably, at the pretreatment temperatures of 100 °C and 120 °C, glucose yields were 98.7 % and 98.8 %, respectively, indicating nearly complete glucan conversion to glucose, which was 1.16 to 1.17-times higher than those from BDES/BR under identical cellulase dosages. These observations suggested that xylan and lignin present within the BR act as considerable barriers to glucan hydrolysis. The TDES pretreatment significantly removed xylan and lignin, consequently increasing the number of binding sites available for cellulase on glucan and facilitating near-complete glucan degradation. Additionally, prolonging the TDES pretreatment of BR at 100 °C from 1 h to 2 h resulted in essentially the same glucose yield, from 98.8 % to 98.9 %. This indicated that the TDES pretreatment system attained the maximum conversion percentage of glucan to glucose with 1 h at 100 °C, and any further increases in pretreatment temperature or duration are unlikely to significantly enhance the glucose yield.

The distributions of cellulases in the supernatants during the enzymatic hydrolysis of BR before and after DES pretreatment are shown in Fig. 2 (b). Over the initial 10-h period of enzymatic hydrolysis, the concentration of free cellulase exhibited an initial rise and then decline. This pattern implies that the cellulase was initially bound to the lignocellulosic substrates, and then released back into the solution. For the BR, the concentration of free cellulase decreased from 0.21 g/L at the 4-h interval to 0.09 g/L by 72 h. Similarly, for the BDES/BR, there was a decrease in the concentration of free cellulase from 0.16 g/L at 4 h to 0.08 g/L by 72 h. Result with TDES-100/BR was quite different, showing the concentration of free cellulase only slightly decreased from 0.09 g/L at 4 h to 0.07 g/L by 72 h. The shared downward trends could be related to either cellulase degradation or the possibility of adsorption of the cellulase onto non-carbohydrate components following cellulose digestion. Remarkably, for the TDES-120/BR, the free cellulase concentration increased from 0.12 g/L at the 4-h interval to 0.14 g/L by 72 h. This was associated with the reduced lignin content in the TDES-120/BR substrate, which may be resulted in less adsorption of the cellulase onto non-carbohydrate components (Lan et al., 2020).

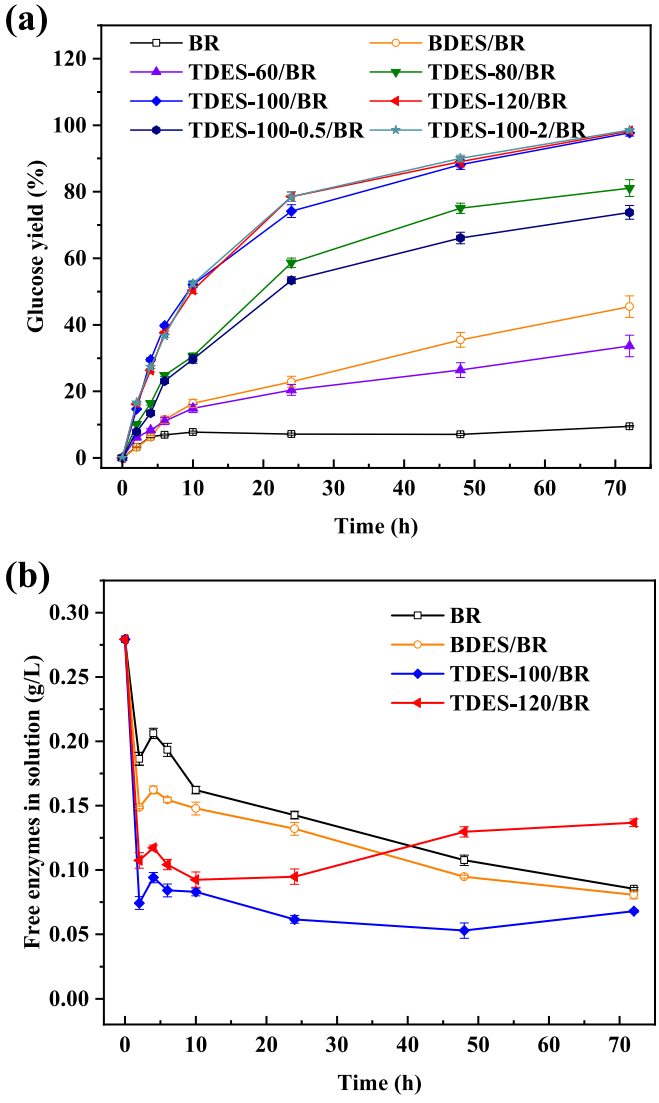

Fig. 2. (a) Glucose yield and (b) cellulases distribution for BR, before and after DES pretreatment, during the 72-h enzymatic hydrolysis process.

3.5. Effect of DES pretreatment on cellulase adsorption on BR

The Langmuir adsorption isotherm was employed to characterize the adsorption capacity of cellulase on BR before and after DES pretreatment (Zhang et al., 2023a). As depicted in Fig. 3 and Table 2, the adsorption capacity (Γ_m) of untreated BR was noted at a relatively low value of 57.2 mg/g, which increased to 67.7 mg/g after the BDES pretreatment. The TDES pretreatment led to an enhancement to 74.8 mg/g for TDE-100/BR and 77.7 mg/g for TDES-120/BR, representing an increase of approximately 11–15 % relative to BDES/BR. Additionally, a slight alteration in the adsorption constant (K) from 0.6 to 0.63 mL/mg was observed for BR before and after DES pretreatment, indicating a weak correlation with cellulase adsorption onto substrates. Furthermore, the distribution coefficient (R) represented the interaction strength between substrates and cellulase. Relative to BR (0.034 L/g), the R-value for

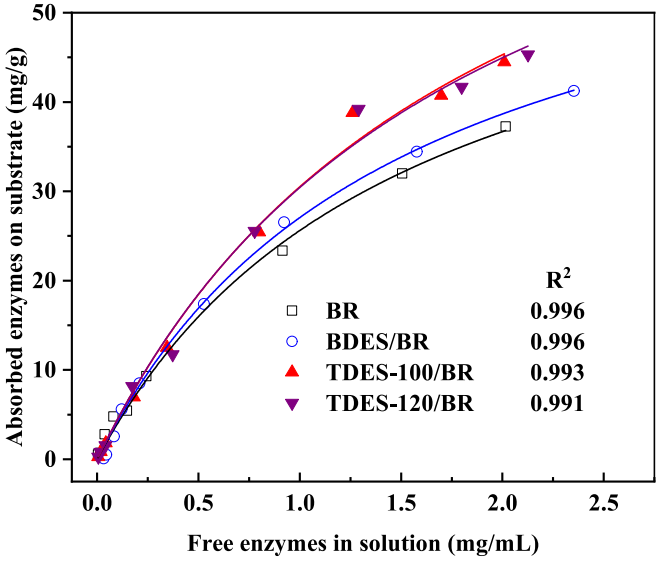

Fig. 3. Enzyme adsorption isotherms on BR before and after DES pretreatment.

Table 2
Langmuir adsorption isotherm parameters of cellulase adsorption on BR before and after DES pretreatment.

Substrate	Γ_m (mg/g)	K (mL/mg)	R (L/g)
BR	57.17 ± 4.93	0.60 ± 0.09	0.034 ± 0.011
BDES/BR	67.67 ± 3.91	0.61 ± 0.08	0.041 ± 0.012
TDES-100/BR	74.83 ± 7.20	0.63 ± 0.11	0.047 ± 0.011
TDES-120/BR	77.72 ± 9.43	0.62 ± 0.13	0.048 ± 0.009

BDES/BR increased to 0.041 L/g, while TDES-100/BR and TDES-120/BR exhibited enhanced *R*-values of 0.047 L/g and 0.048 L/g, respectively. Substrates pretreated with TDES at 100 and 120 °C displayed comparable *R*-values, coinciding with the glucose yields.

3.6. Surface and structural characterization of BR after DES pretreatment

3.6.1. FT-IR spectroscopy

The FT-IR spectra were utilized to discern changes in the functional groups and chemical structure of the BR before and after DES pretreatment (see supplementary material). The spectral peaks characteristic of hemicellulose at 1732 cm^{-1} (C═O stretching) and 1250 cm^{-1} (C—O stretching vibration) exhibited a diminishing trend or vanished entirely as the intensity of the pretreatment increased, indicating the degradation of hemicellulose. Furthermore, peaks discernible at 1602 cm^{-1} (aromatic skeleton vibrations), 1514 cm^{-1} (C═C stretching vibrations in the aromatic rings), and 1462 cm^{-1} (C—H deformations), assigned to lignin, exhibited a progressive reduction in intensity, suggesting effective lignin removal by the TDES system. Finally, the peak at 830 cm^{-1}, attributable to the phenolic hydroxyl groups in lignin, weakened after pretreatment, further suggesting delignification.

3.6.2. XPS

XPS analysis was utilized to evaluate changes in the chemical elements of the BR before and after DES pretreatment, as shown in Table 3. The untreated BR exhibited an oxygen to carbon (O/C) ratio of 0.42, with lignin covering 82 % of the surface, suggesting that the surface of the BR was primarily covered with lignin. Pretreatment exerted a significant influence on the distribution of oxygen and carbon elements on the surface of the BR. After BDES pretreatment, the O/C ratio increased to 0.63, and the surface coverage of lignin decreased to 41 %, indicating that carbohydrates were exposed on the surface. More substantial modifications were observed with TDES pretreatment at 100 °C and 120 °C, where the O/C ratio increased to 0.79 and 0.80, respectively, corresponding to the decrease in lignin content as shown in the chemical composition of the pretreated BR (Table 1). Furthermore, the surface analysis revealed that the lignin coverage decreased to 8 % in TDES-100/BR and 6 % in TDES-120/BR, accompanied by increases in carbohydrate coverages to 92 % and 94 %, respectively. These results indicate that TDES pretreatment facilitated greater lignin removal from the surface of the BR and thus greater exposure of carbohydrates compared to BDES pretreatment.

XPS analysis was conducted to assess changes in chemical bonds, with the findings shown in Table 3. Three carbon peaks, identified as C1, C2, and C3, were situated at 284.6 eV, 286.1 eV, and 287.4 eV, respectively. These peaks corresponded to various linkages, C—C/C—H derived from lignin, C—O typical of carbohydrates, and the carbonyl (C═O) and esters (O—C═O) associated with hemicellulose (Gao et al., 2020). As shown in Table 3, C1 and C2 accounted for 80 % of the total carbon present in the BR. Compared with raw BR (41.2 %), BDES/BR (32.2 %), TDES-100/BR (33.3 %), and TDES-120/BR (34.0 %) all exhibited a lesser amount of C1, suggesting that DES pretreatment removed lignin from the surface of the BR thereby improving the accessibility of cellulose to cellulase. An increase in C2 content was also observed in DES-treated BR, particularly with TDES-100/BR (46.3 %) and TDES-120/BR (45.1 %), compared with BR (36.0 %), indicating an enrichment in carbohydrate content. Moreover, the C3 proportion within TDES-100/BR and TDES-120/BR decreased to 20.3 % and 20.9 %, respectively, indicating that the extraction of xylan contributed to the variations in C3 content of the BR.

Table 3
Element compositions, surface chemical bonds, and crystallinity index (CrI) of BR before and after DES pretreatment.

Samples	Element composition			Surface chemical bonds			CrI (%)
	O/C ratio	S_L (%)	S_C (%)	C1 (%)	C2 (%)	C3 (%)	
BR	0.42	82	18	41.2	36.0	22.8	50.6
BDES/BR	0.63	41	59	32.2	42.7	25.0	54.6
TDES-100/BR	0.79	8	92	33.3	46.3	20.3	60.0
TDES-120/BR	0.80	6	94	34.0	45.1	20.9	62.8

3.6.3. XRD

The crystallinity of cellulose, as measured by XRD spectroscopy, indicated the proportion of crystalline cellulose in the pretreated BR, accounting for the amorphous regions, including amorphous cellulose, hemicellulose, and lignin. As shown in Table 3, the crystallinity index (CrI) of BR was 50.6 %, which increased to 54.6 % following the BDES pretreatment. When BR underwent TDES pretreatment, the CrI for bamboo residues rose to 60.0 % for TDES-100/BR and 62.8 % for TDES-120/BR. The enhancement in CrI was primarily attributable to the removal of non-crystalline components resulting in an increase in the proportion of crystalline cellulose.

3.7. Characterization of isolated lignins by NMR

The structural alterations on the side-chain region (δ_C/δ_H 50–90/2.5–6 ppm) and the aromatic region (δ_C/δ_H 100–135/5.5–8 ppm) of the isolated lignin from the TDES pretreatment compared to that in untreated bamboo (i.e., in the form of milled wood lignin) were revealed by the 2D-HSQC NMR analysis. As illustrated in the 2D-HSQC NMR spectra and the main ^{13}C–^{1}H cross-signals (see supplementary materials), the side-chain of the milled wood lignin (MWL) isolated from BR displayed signals of β-O-4 alkyl ethers (A), resinols (B), phenylcoumaran (C), and *p*-hydroxycinnamyl alcohol (I), in which the A unit was the predominant structure (Hu et al., 2024). In the aromatic region, signals were detected from syringyl unit (S), guaiacyl unit (G), *p*-hydroxyphenyl unit (H), ferulates (FA), and *p*-coumarates (PCE). Compared to the BR lignin, only signals from G and PCE units are observed while the signals from S, H, and FA units almost completely disappear in the aromatic region for the lignin isolated from the TDES pretreatment. This result indicated that the primary mechanism of lignin isolation with TDES involved the cleavage of β-O-4 alkyl ethers. Furthermore, the absence of signals from S, H, and FA units implied severe degradation of these lignin structural subunits compared to G and PCE units.

The molecular weights (Mn and Mw) of the extracted lignin were determined using GPC (see supplementary materials). The Mn and Mw of BR lignin were 3935 and 11312 g/mol, respectively. After pretreatment with BDES, the Mn and Mw of extracted lignin decreased to 1487 and 3123 g/mol, respectively. The reduced molecular weight indicated the occurrence of lignin depolymerization during the BDES pretreatment process. Additionally, after TDES pretreatment, the Mn and Mw of extracted lignin further decreased to 1253 and 2572 g/mol, respectively. This indicated that TDES caused more significant degradation of lignin compared to BDES pretreatment as also confirmed by the NMR analysis. This result should be ascribed to the lower pH of TDES than BDES, which would promote the degradation of lignin. The polydispersity (PDI) of extracted lignin with TDES decreased from 2.87 in BR lignin to 2.05, suggested a more evenly distributed molecular weight and is generally assumed being advantageous for the higher-value applications of lignin

(Zhai et al., 2020).

3.8. Cleavage mechanisms of lignin carbohydrate complexes

Based on literature-recognized mechanisms and 2D-HSQC NMR analysis, we propose the following degradation mechanism for lignin-carbohydrate complexes and lignin:

(1) The ester bond of xylan breaks under acidic conditions, producing xylose that dissolves in the solvent (Deng et al., 2022). (2) Sulfonic acid groups from PTA attack ester bonds in lignin-carbohydrate complexes, breaking ether bonds. The lignin portion forms protonated fragments and dissolves via hydrogen bonding with sulfonic acid groups (Zhai et al., 2020). (3) Glycine hydrochloride and hydrogen ions from PTA attack β-O-4 linkages in lignin, protonating oxygen atoms into positively charged O^+ ions. Ethylene glycol approaches the positively charged O atom and undergoes nucleophilic reactions, causing lignin to dissociate into small molecule fragments or form hydrogen bonds with ethylene glycol, resulting in dissolution (Ye & Yokoyama, 2020).

3.9. Molecular dynamic simulations

Molecular dynamics simulations were performed using GROMACS 2020 software to investigate the interactions (i.e., electrostatic and van der Waals energy, number of hydrogen bonds, and the lifetime of hydrogen bonds) between DES and lignin using the structural units identified from the 2D NMR analysis of lignin samples as the lignin model compounds (Ee et al., 2023; Zhu et al., 2023). As illustrated in Fig. 4 (a), the interaction energy, including the electrostatic and Van der Waals energy, between TDES and the most lignin model compounds were lower than those of BDES. For instance, the interaction energy of BDES and β-O-4 alkyl ethers (B:A) was −1848.8 kJ/mol, while it was −2084.8 kJ/mol for the TDES and β-O-4 alkyl ethers (T:A), suggesting TDES should exhibit a better lignin dissolution property compared to BDES (Xu et al., 2021).

Fig. 4(b) shows the number and lifetime of hydrogen bonds between the DES systems and lignin model compounds. It can be seen that the number of hydrogen bonds formed by TDES with lignin was higher than that of BDES, indicating that TDES was more likely to form hydrogen bonds and interact with lignin, thereby explaining the observed higher lignin fractionation ability with TDES. Interactions with β-O-4 alkyl ethers by TDES engendered 30.6 hydrogen bonds, outstripping the 28.8 formed by BDES. Additionally, TDES recorded a superior hydrogen bond number with other lignin models in comparison to BDES. The average lifetime of hydrogen bonds demonstrated the stability of bond formation between acceptors and donors (Fu et al., 2022), with the duration attributed to β-O-4 alkyl ethers in TDES averaging 5.4 picoseconds (ps), marginally outperforming the 5.1 ps marked in BDES, indicating enhanced stability of lignin-derived hydrogen bonds within the TDES system over those formed in BDES. This observation further confirmed the superior separation efficiency of bamboo biomass afforded by TDES, particularly noting that the fractionation efficacy of lignin was more pronounced with TDES than with BDES (Fu et al., 2022). In the TDES pretreatment process, the combination of PTA and GH-EG significantly improved the removal percentage of lignin and hemicellulose, which promoted the conversion of cellulose to glucose. In addition, the DES solution contained a significant amount of xylose after pretreatment; Muryanto et al. (2023) found that hemicellulose was converted to furfural through a one-step DES pretreatment at elevated temperatures, which may be a potentially effective method for converting biomass to value-added products.

3.10. Mass balance

The chemical compositions and enzymatic hydrolysis yields of BR pretreated with TDES systems at 100 °C for 1 h are summarized in a flowchart (Fig. 5). The solid–liquid mass changes were monitored before

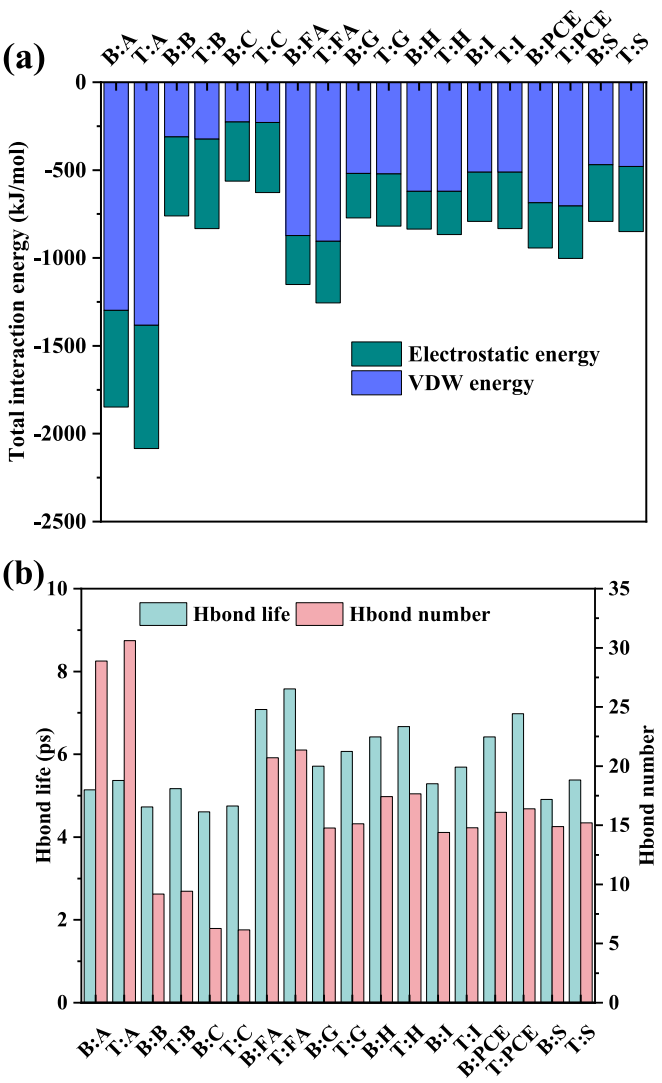

Fig. 4. (a) Electrostatic energy and Van der Waals (VDW) energy of BDES-lignin models and TDES-lignin models. (b) Hydrogen bond life and hydrogen bond number of BDES-lignin models and TDES-lignin models. Lignin models are provided in supplementary materials ("B" represented BDES, "T" represented TDES, for instance, "B: A" represents the interaction between BDES and the β-O-4 alkyl ethers constituents of lignin.).

and after pretreatment, with the mass balance determined by scaling the pretreatment experiment 20-fold. The total mass weight at the beginning of the pretreatment was 1100 g, including 100 g BR and 1000 g TDES. After the pretreatment and the following filtration process, about 988 g liquid fraction was obtained. And the solid residue yield was 47.7 g after washing and drying process. At this stage, it could be calculated that the weight loss from the starting materials was 64.3 g. Given the high TDES/BR ratio during the pretreatment, it could be assumed that the weight loss of 64.3 was mostly the TDES, which was lost during the washing of solid residue after filtration. Therefore, the amount of remaining TDES was 935.7 g, which suggests that the total weight of dissolved lignin, mono-sugars, and other degraded compounds was 52.3 g. The amounts of mono-sugars and some degraded compounds that could be determined by HPLC was 24.5 g in total. Furthermore, the lignin that could be recovered from the liquid fraction was 14.5 g, which left the fraction of other degraded compounds that could not be determined by HPLC was 13.3 g. This fraction could contain degraded compounds from cellulose, hemicellulose, or lignin, or even compounds formed from the degradation fragments of cellulose, hemicellulose, and lignin.

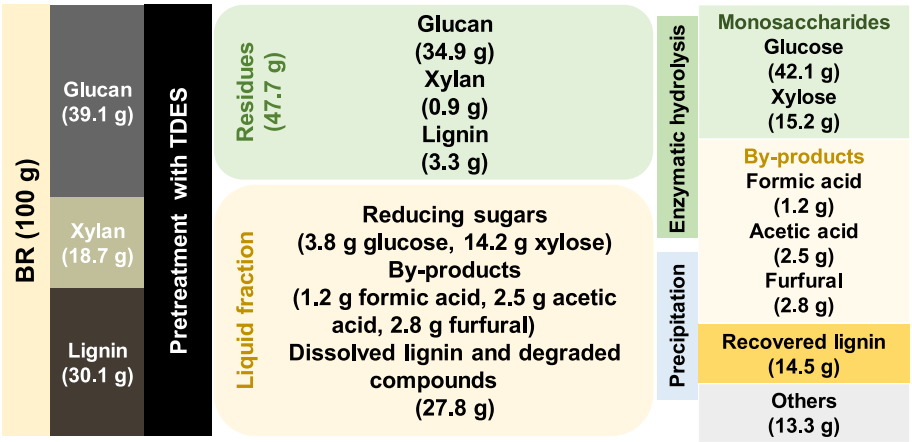

Fig. 5. Mass balance flowchart of enzymatic hydrolysis of TDES pretreated BR.

The comparison of glucose yield of this work and previous studies from DES pretreatment on bamboo were summarized in the supplementary materials. It showed that the TDES pretreatment was at a high level among these DES pretreatments, achieving a glucose yield of 98.7 % at a relatively mild temperature under an enzyme load of 10 FPU/g substrate. Therefore, among these pretreatment methods, TDES pretreatment showed potential in improving the refining efficiency and reducing costs of bamboo.

3.11. *Effect of TDES pretreatment on CrI and enzymatic hydrolysis of microcrystalline cellulose*

Microcrystalline cellulose (MC) with a high CrI was pretreated with TDES at 100 °C and 1 h to investigate its impact on cellulose alone. The untreated cellulose demonstrated a high CrI of 78.5 % (see supplementary materials). After TDES pretreatment, the CrI decreased to 76.4 %. Wang et al. (2023) reported a similar result when bamboo was pretreated with *p*-toluenesulfonic acid at 100 °C and 110 °C for 1 h, the CrI decreased from 63.4 % to 60.7 %. In the present study, after 72 h of enzymatic hydrolysis of MC before and after TDES pretreatment, the glucose yield increased from 67.7 % to 72.1 %. Hence, the increased glucose yield in TDES-pretreated BR can be attributed to the synergistic effects of removing amorphous regions (such as hemicellulose and lignin) and disrupting the cellulose crystalline structure.

3.12. *Reuse of spent DES*

Compared to conventional organic solvents, DES systems generally demonstrate remarkable recyclability. After initial usage, the TDES was separated from dissolved lignin and was subjected to multiple cycles to assess its recyclability based on delignification efficiency and glucose yield. With each pretreatment cycle, approximately 90 % of the TDES was recovered, highlighting its suitability for repeated use. However, as shown in Fig. 6, in the consecutive second, third, and fourth pretreatment cycles, there was a marked reduction in delignification to 56.1 %, 39.1 %, and 30.1 %, respectively. Concurrently, glucose yields also diminished to 43.2 %, 30.1 %, and 25.6 %. These results suggest that the sustained use of the TDES system is likely compromised by residual lignin and hemicellulose degradation products within the DES (Huang et al., 2022). Considering that approximately 90 % of the initial mass fraction of TDES was recovered during each cycle, we found that the performance of the recovered TDES could be revitalized by replenishing it with fresh DES to the initial mass. When BR was pretreated with the refreshed TDES, delignification were observed at 80.1 %, 75.1 %, and 70.3 %, and glucose yields improved to 85.3 %, 77.1 %, and 72.1 %. These findings illustrated the promising sustainability and renewability of our DES systems, particularly for the biorefining of bamboo.

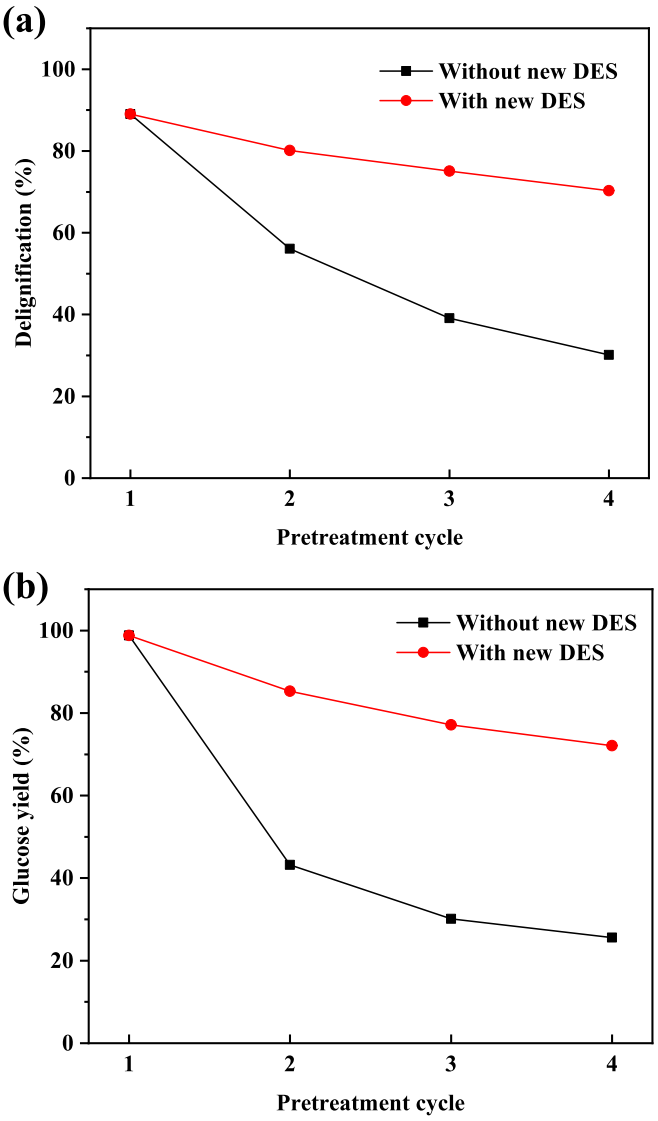

Fig. 6. (a) Delignification and (b) glucose yield with and without the addition of fresh DES over the 4 pretreatment cycles of TDES.

4. Conclusion

This work introduced PTA into a GH-EG BDES, developing a novel TDES (molar ratio if GH: EG: PTA of 1:4:0.5) with high pretreatment efficiency. After the TDES pretreatment, 95.1 % of hemicellulose and 89.0 % of lignin were removed from BR, yielding 98.7 % glucose conversion. The decrease in pH value, increase in polarization value and hydrogen bond donor acidity, coupled with the formation of more hydrogen bonding between the TDES and the lignin are the important factors for TDES to remove more lignin. Delignification from the TDES facilitates cellulase loading onto BR, resulting in an increase in saccharification yield. These findings demonstrated the potential of TDES system for biomass pretreatment. The high recyclability of the TDES underscores its potential for sustainable industrial applications in biorefining.

CRediT authorship contribution statement

Fanyang Meng: Writing – original draft. **Thomas L. Eberhardt:** Writing – review & editing. **Yaru Li:** Visualization, Formal analysis. **Chen Xu:** Supervision. **Hui Pan:** Writing – review & editing, Conceptualization.

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 data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132737.

Data availability

No data was used for the research described in the article.

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