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Understanding the effect of Deep Eutectic Solvent (DES) pretreatment on the utilization of corn stover

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Abstract

Pretreatment is an important step to reduce the recalcitrance factors in biomass for effective biomass utilization. In particular, the choice of processing solvents in the pretreatment influences the quantity and quality of the final products. Although conventional organosolv pretreatments are effective, they are typically performed under harsh conditions. Compared to those approaches, recent studies have shown that the use of Deep Eutectic Solvents (DES) made up of a hydrogen bond donor and acceptor at the eutectic point can be a promising alternative as biomass processing solvents because of their good thermal stability and compatibility with natural components. In this study, DES pretreatment was applied to corn stover, which is the largest agricultural residue in the United States. The performance of the pretreatments was assessed by measuring the removal of xylan and lignin from the corn stover, as well as the production of glucose and xylose by subsequent enzymatic hydrolysis. The results indicated that the DES pretreatment resulted in significantly higher delignification rates (75%) than an organosolv pretreatment (35%) at the same processing temperature. The DES pretreatment also resulted in a more effective conversion of glucan (81%) and xylan (56%) than the organosolv pretreatment. The results indicated that DES pretreatment is a promising processing strategy for biomass utilization.

Keywords

Biorefinery, Pretreatment processing solvent, Enzymatic hydrolysis, Deep Eutectic Solvent, Corn stover, Biomass utilization, Biomass conversion, Fermentation, Fermentable sugars

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Introduction

Lignocellulosic biomass has proven to be a sustainable and renewable source as an alternative for fossil fuels by its production of various platform chemicals and fuels. Corn stover, including cobs, leaves, and stalks, left in corn fields after harvesting, is a promising feedstock due to its advantageous price point and lack of competition with food and feed production, especially as there is a greater emphasis on utilizing renewable energy sources (1,2). The United States is the largest producer of corn in the world, hence the effective utilization of this agricultural waste can be a solution not only for energy security and environmental concerns but also for local farmers. Lignocellulosic biomass is composed of approximately 35–45% cellulose, 25–35% hemicellulose, 15–30% lignin, and other components such as extractables and ash (3). Cellulose, a polysaccharide of glucose, is the largest component in biomass. It has been used for paper production in the traditional biomass industry. It has been recently a subject of research as a source of biofuels and biochemicals. Once it is hydrolyzed into fermentable sugar (i.e., glucose), it is readily converted into useful chemicals and biofuels through biological methods (4).

Enzymes are biocatalysts that facilitate chemical transformations of organic compounds. Cellulase is an enzyme responsible for breaking down cellulose into glucose. However, due to the recalcitrance of intrinsic biomass, this biological degradation of cellulose in biomass is challenging. For instance, cellulose is surrounded by other components, such as hemicellulose and lignin, and has limited enzyme accessibility. In addition, the crystalline structure of cellulose makes its decomposition challenging. Moreover, to maximize the use of biomass, utilization of other components beyond cellulose is beneficial. Lignin, especially, is the largest aromatic resource in nature, hence it can be a source for many alternative petroleum-based chemicals and fuel additives (5). Therefore, it is beneficial to consider the fates of both cellulose and lignin in biomass pretreatment.

Pretreatment is designed to address these challenges because it can change the composition and structural properties of the lignocellulosic biomass. It can overcome the aforementioned challenges and allow better cellulase access to cellulose, improving the production of fermentable sugars like glucose (6). For achieving this objective, diverse pretreatment approaches, such as physical, thermochemical, biological, and others, have been investigated. For instance, steam explosion pretreatment using saturated steam at high pressure with biomass effectively improved biomass conversion (7). Similarly, hot water pretreatment and dilute acid pretreatment also enhanced biomass utilization (8,9). However, these methods mainly focus on cellulose conversion only. To maximize the utilization of biomass, recent pretreatments consider both carbohydrates and lignin fractions together. Deep eutectic solvents (DESs) have been introduced as an effective biomass fractionation solvent for both cellulose and lignin in biomass. These solvents are green and cheaper alternatives to ionic liquids because of their biodegradability, low toxicity, and low cost (10). They are composed of a hydrogen bonding donor (HBD) and acceptor

(HBA) at their eutectic points, allowing for high reactivity and solubility of biomass components under mild conditions, which are comparable to those of ionic liquids. DES are also recyclable. The 'green extraction' solvent properties of DES (11,12) have been studied extensively with regard to its Life Cycle Assessment (LCA) and environmental impact.

In this study, DES pretreatment was applied to corn stover to understand its effects on biomass transformation. Choline chloride (ChCl) and urea were used as an HBA and HBD, respectively, to understand their impact on corn stover utilization. It has been noted that several alkaline solutions such as ammonium hydroxide and urea were effective on biomass pretreatment (13,14). However, these alkaline pretreatments were not as effective when applied to woody biomass. In this study, urea was used as HBD in DES to enhance the pretreatment effect. Also, choline chloride (ChCl), commonly used for biochemical processes, was selected as HBA. The changes in the chemical composition of corn stover and the production of fermentable sugars from the fractionated cellulose were used to elucidate the effects of DES. The performance of the applied DES pretreatment was also compared with that of organosolv pretreatment, which was successfully applied to woody biomass in a previous study (15).

Materials and Methods

Materials

Corn stover was supplied by the U.S. Department of Agriculture Research Service Eastern Regional Research Center. The corn stover received was Wiley-milled and screened to a uniform size (20–100 mesh). Choline chloride and sulfuric acid were purchased from JT Baker Chemical Company. The urea and ethanol were supplied by Fisher Scientific Inc. and Decon Laboratories Ltd, respectively. The enzyme used in this study was cellulase enzyme blend Cellic Ctec 2, purchased from Sigma-Aldrich with a hydrolysis (Units/g) > 1000. All these and other chemicals used in this study were used without further purification.

Biomass pretreatment

The overall utilization process of corn stover using ChCl/Urea DES is shown in Figure 1. In brief, the DES was synthesized at a 1:2 molar ratio of choline chloride (ChCl) and urea as a hydrogen bond acceptor and donor, respectively. The DES was synthesized with 11.0 g of ChCl (98% purity) and 9.4 g of urea (98% purity) in a 150 mL glass reaction vial equipped with pressure relief caps and stirred at 80 ℃ until a clear solution was formed. Corn stover (2 g dry weight) was pretreated with 20 g of the DES at different temperatures, 140 ℃ (T1) and 160 ℃ (T2). The reaction was performed for 120 min with magnetic stirring at 500 rpm. After the pretreatment, the solid fraction was washed using 100-150 mL of 50 v/v% ethanol solution per reaction and was soaked overnight in the solution. This was repeated until the filtrate became colorless. The washed solid was stored in a 4 - 8 ℃ refrigerator until chemical composition and enzymatic hydrolysis analyses were performed. The organosolv pretreatment with 60 v/v% ethanol solution with sulfuric acid (1.25% based on biomass weight) was applied to the same corn stover as a control; however, this experiment was performed in a Parr reactor because of the generated pressure resulting from the procedure. Since ethanol organosolv

pretreatment has been applied to biomass in many studies, this comparison served to measure of how much more effective the ChCl/Urea DES pretreatment was. The organosolv pretreatment condition (160 ℃ for 60 min) was modified from the reference (15). Corn stover (3.0 g) was loaded with aqueous ethanol solution (27 ml) into a glass lined cylindrical reaction vessel. 16.2 mL of ethanol, 10.8 mL DI water, and 0.05 mL sulfuric acid were mixed in advance to form an aqueous ethanol solution. After organosolv pretreatment, the pretreated biomass solid was washed with deionized water until the pH of the solid reached 6 - 7, as determined by measuring the pH of the filtrate. The washed solid residue was used for chemical composition analysis and enzymatic hydrolysis.

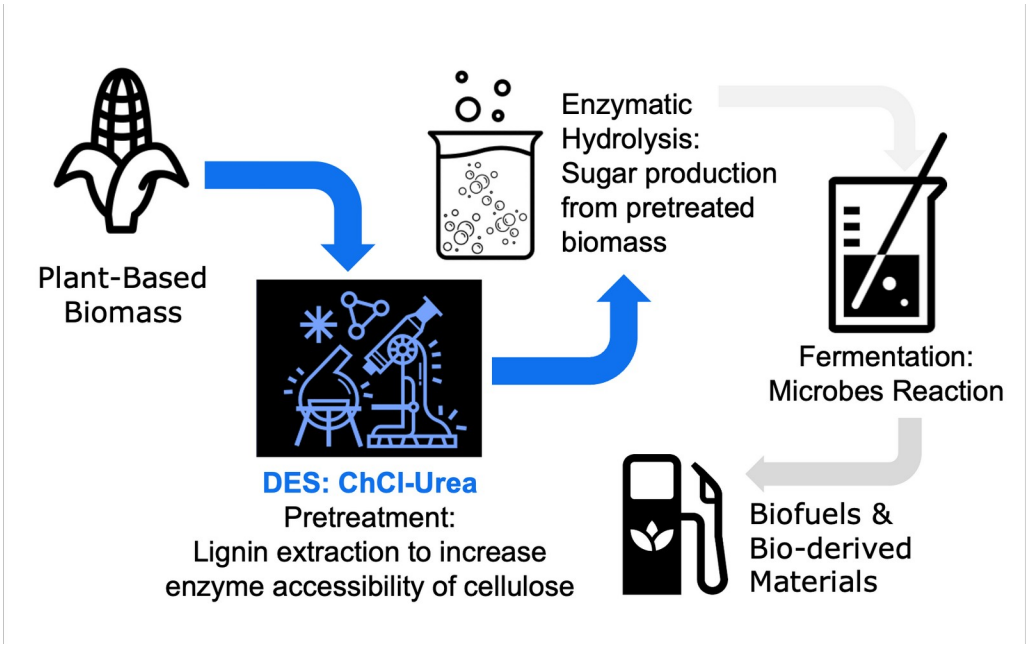

Figure 1. Overall biomass utilization process using DES.

Thermal properties of DES

The melting temperature of ChCl/Urea DES was measured using differential scanning calorimetry (DSC, TA Instruments Q200). The heat flow of the sample was compared with an empty pan as a reference, executing a controlled heating/cooling rate of 10 ºC/min while scanning from -70 ºC to 60 ºC with a nitrogen purge at 50 ml/min. A calibration curve was prepared with indium following standard procedure from TA Instruments. A secondary heating scan was used for the analysis. The thermal stability inherent in the ChCl/Urea DES was also determined by thermogravimetric analysis (TGA). The prepared ChCl/Urea DES was tested using a Q500 TGA (TA Instruments) from 30 to 400 ºC, with a heating rate of 10 ºC/min.

Chemical composition analysis of biomass

To measure the effect of each pretreatment, a chemical composition analysis was performed with untreated and ChCl/Urea DES and ethanol organosolv pretreated corn stover samples. Before the analysis, the biomass was air-dried to reduce the moisture content (as measured by

gravimetry) below 10%. The contents of cellulose, hemicellulose, and lignin in the biomass samples were measured using a two-step acid hydrolysis method following the NREL procedure (16). Firstly, acid hydrolysis was performed using 0.3 g of biomass and 3 ml of 72% sulfuric acid. Both sulfuric acid and biomass were loaded into test tubes and stirred using a glass rod. The test tubes were kept in a water bath and periodically stirred using a glass rod every 5 min at 30 °C for 60 min. After the primary hydrolysis, the entire sample mixture was transferred to a 200 ml Pyrex bottle and diluted to a 4% sulfuric acid solution by adding 84 mL of deionized water. The Pyrex bottle was placed in an autoclave (Tuttnauer 2340M-B/L, Heidolph) at 121 °C for 60 min. The two-step hydrolysis process hydrolyzed all the carbohydrates in the solution. Vacuum filtration was then performed with the mixture after the two-step hydrolysis using filtering crucibles to separate residual solids from the mixture. The filtrate was used to measure the acid-insoluble lignin content (%AIL). The %AIL was determined by measuring the weight difference between the dried weight of the solid fraction on the crucible and the weight of ash that remained after the calcination of the solid. The ash content was measured with a muffle furnace, heated up to 575 °C for 24 hours. The filtrated solids were washed with deionized water until these solids were neutralized. The acid-soluble lignin content (%ASL) was measured using ultraviolet-visible (UV) spectroscopy at 320 nm, and the concentrations of carbohydrates, including glucose and xylose from cellulose and hemicellulose, were analyzed using high-performance liquid chromatography (HPLC, Agilent Technologies 1260 Infinity) equipped with a Bio-Rad Aminx HPX-87H column with a RefractoMax 520 refractive index (RI) detector. All liquid samples for HPLC analysis were filtered with a nylon syringe (0.2 μm) filter before analysis and filled in 1.5 ml HPLC vials. To confirm the reproducibility of the reported results, all the experiments except for the control (organosolv pretreated biomass) were performed at least twice, with a maximum Relative Standard Deviation (RSD) of < 5%. Quantification of sugar was performed with 1.25–20 g/L of pure glucose and xylose as external standard. Calibration curves for both glucose and xylose were made with an R^2 >0.99. All the analyzed sample concentrations were within the ranges of the external standards. The total lignin of the biomass sample was calculated by summing the AIL and ASL. Based on the chemical composition, the xylan removal and lignin removal (delignification) were calculated as follows.

Solid recovery yield (S, %) = $(m_{pretreated}/m_{initial})\times 100$
where, $m_{pretreated}$ represents the dry weight of pretreated biomass (g), and $m_{initial}$ is the dry weight of untreated initial biomass (g)

Xylan removal (%) = $(X_1-(X_2\times S/100))/X_1\times 100$
where, X_1 is xylan content of untreated initial biomass (%), X_2 is xylan content of pretreated biomass (%)

Delignification (%) = $(L_1-(L_2\times S/100))/L_1\times 100$
where, L_1 is lignin content of untreated initial biomass (%), L_2 is lignin content of pretreated biomass (%)

Enzymatic hydrolysis

To evaluate the performance of each biomass pretreatment, enzymatic hydrolysis was performed with the pretreated biomass (organosolv and DES separately) and untreated corn stover. Due to the limited biomass, the enzymatic hydrolysis of the DES treated biomass was performed only once. The enzymatic hydrolysis was performed with 1 wt % of biomass loading. The concentration of sodium acetate buffer and sodium azide were 50 mM and 0.02%, respectively. The pH was adjusted to 5.0 using acetic acid, and enzyme loading was 15 Filter Paper Units, FPU/g of biomass. The reaction was performed in a shaking incubator at 50 °C for 72 hours. The hydrolyzed liquid sample (1 ml) was collected at 6, 12, 24, 48, and 72 hours for HPLC analysis. To deactivate the enzyme in the collected liquid samples, the liquid samples were placed in a heating block at 95 °C for 6 min, filtered through a 0.22 μm nylon filter and loaded into a 1.5 ml HPLC vial. The glucan and xylan conversions were calculated based on the following equations.

Glucan conversion yield (%) = $(Glucose_{hydrolysate}\times 0.90/Glucan_{biomass})\times 100$
where, $Glucose_{hydrolysate}$ is the weight of glucose in liquid aliquot after enzymatic hydrolysis (g), and $Glucan_{biomass}$ is the glucan content of biomass before enzymatic hydrolysis (g).

Xylan conversion yield (%) = $(Xylose_{hydrolysate}\times 0.88/Xylan_{biomass})\times 100$
where, $Xylose_{hydrolysate}$ is the weight of xylose in liquid aliquot after enzymatic hydrolysis (g), and $Xylan_{biomass}$ is the xylan content of biomass before enzymatic hydrolysis (g).

Results and Discussion

Thermal properties of ChCl/Urea DES

To confirm the formation of DES, its unique thermal behavior at the eutectic point was measured. According to the DSC thermogram in Figure 2a, ChCl/urea DES was successfully formed at the eutectic point. The melting point of ChCl/urea DES was visible at ~ 12 ºC. It was much lower than the melting points of individual choline chloride (302 ºC) and urea (133 ºC), indicating a generation of the deep eutectic point (17). The lowered melting point of DES underscored the unique thermophysical properties compared to the pure chemical constituents, offering insights into its potential applications across other scientific domains.

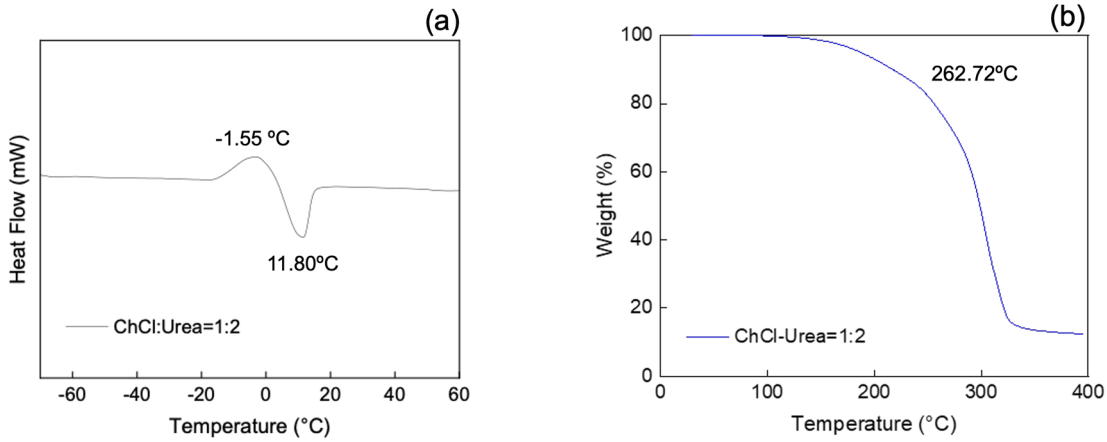

Figure 2. Phase transition temperature (a) and thermal degradation temperature (b) of ChCl/Urea DES (1:2 mol ratio).

The thermal degradation temperature (i.e., onset decomposition temperature) is an important property in determining biomass pretreatment temperature. The thermal degradation temperature is the weight loss of a sample as the temperature increases, providing the DES network behavior between constituents (18). As a result, the onset decomposition temperature of ChCl/urea DES, predicted from the crossing point between baseline and first inflection point of the solvent (18), was determined to be 263 ºC as presented in Figure 2b. The value signified the temperature at which the solvent begins measurable degradation. The results implied that the ChCl/urea DES was thermally stable during the pretreatment at both 140 ˚C and 160 ˚C.

Chemical composition of DES pretreated corn stover

Pretreatment is designed to reduce the recalcitrance factors from biomass and increase the conversion of biomass into target products such as fermentable sugars. Previous studies found that lignin in lignocellulosic biomass inhibits the biological conversion of cellulose by physically limiting the accessibility of enzymes as well as by deactivating them through non-productive binding (19,20). Therefore, the changes in the chemical composition of corn stover before and after the pretreatments were used to determine the effect of the DES pretreatments. After the DES pretreatments, the color of corn stover was notably changed as presented in Figure 3. The changes in biomass color were associated with the extraction of xylan and lignin from corn stover. Those observations were confirmed with the changes of chemical composition of corn stover after the pretreatments (Figures 4 and 5). The DES pretreated corn stover presented with higher glucan and xylan content than the untreated and the organosolv pretreated biomass. The DES T1 glucan and xylan content was 51.2% and 31.0% respectively. The DES T2, treated at 160 ℃, presented with a similar 31.5% xylan content but with a higher glucan content (59.2%) (Figure 4). Compared to the glucan and xylan

contents in untreated corn stover (38.8% glucan and 26.7% xylan), the DES pretreated biomass presented with greater carbohydrate contents. The organosolv pretreated biomass presented with glucan and xylan content of 50.3% and 16.7% respectively. The increases in both glucan and xylan content were caused by the removal of another component, lignin, which is one of the main recalcitrance factors in biomass utilization. The lignin content in the DES T1, DES T2 and the organosolv pretreated biomass was 10.0%, 6.4% and 24.2% respectively.

Figure 3. Morphologies of biomass including corn stover and organosolv and DES pretreated biomass.

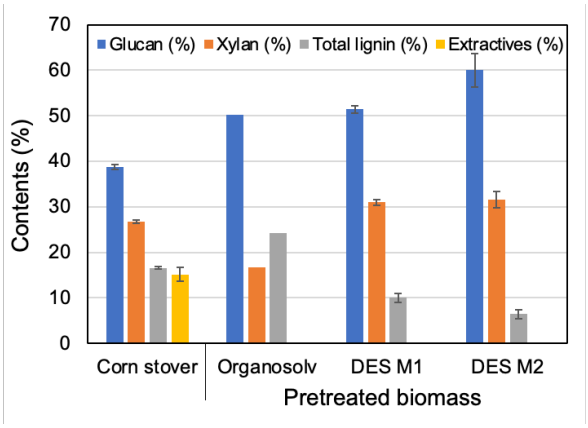

Figure 4. The chemical composition of untreated and pretreated corn stover.

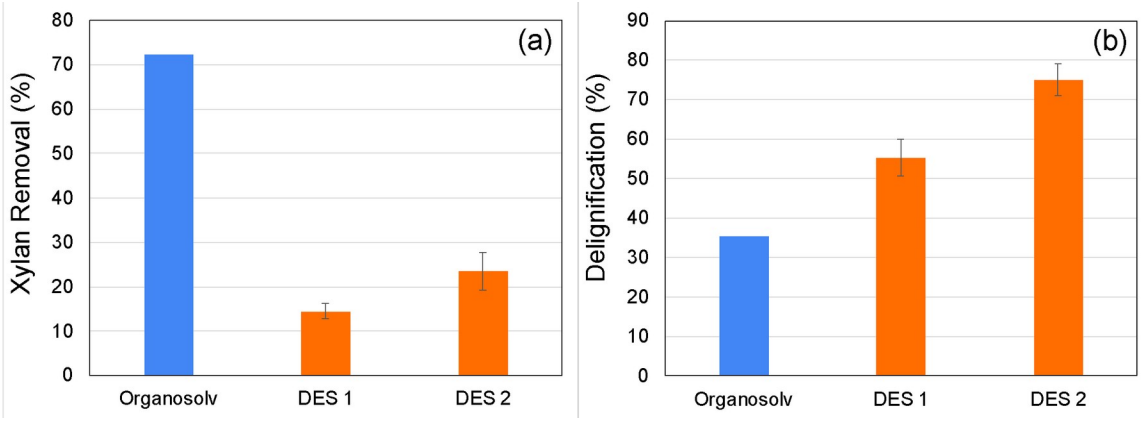

Figure 5. The removal of (a) xylan and (b) lignin (i.e., delignification) from corn stover using organosolv and DES pretreatments.

The results showed that lignin was selectively removed by the DES pretreatments, and the delignification (i.e., lignin removal) when subjected to DES pretreatment was more effective than when pretreated with organosolv. For quantitative comparison of the effect of each pretreatment, delignification and xylan removal were calculated based on the chemical composition changes after the pretreatments. Figure 5 shows that 55.5% (DES T1) and 75.0% (DES T2) of lignin was removed from the corn stover after the DES pretreatments, unlike the organosolv pretreatment, which removed 35.5% of the lignin. Microorganisms such as yeast can only ferment glucose; hence, hemicelluloses like xylan need to be removed in the fermentation process to increase the cellulose conversion in biomass. However, the development of enzymes and engineered microorganisms has allowed the utilization of both hexoses (e.g., glucose) and pentoses (e.g., xylose) together (21). The DES pretreatments exhibited lesser xylan loss (14.5%, DES T1 and 23.5%, DES T2) when compared with the organosolv pretreatment (72.4%). The DES pretreatments hence retained more fermentable sugars in the pretreated biomass for subsequent enzymatic hydrolysis into fermentable sugars.

Enzymatic hydrolysis of DES pretreated corn stover

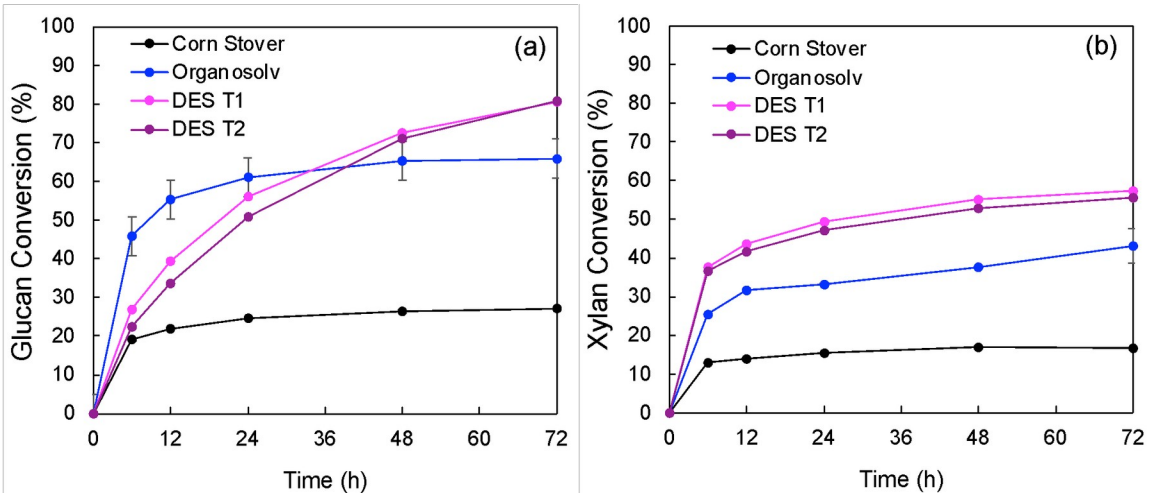

Figure 6. The (a) glucan and (b) xylan conversion of corn stover and its pretreated biomass treated with organosolv and DES.

Enzymatic hydrolysis is a method of decomposing polysaccharides such as cellulose and hemicellulose in the plant cell walls into fermentable sugars for their subsequent biological conversion to biofuels and biochemicals. As discussed earlier, pretreatment is designed to reduce the recalcitrance factors in biomass; therefore, pretreatment efficiency was evaluated by this enzymatic hydrolysis yield. In this study, each biomass was hydrolyzed by cellulase (15 FPU/g of biomass) to convert glucan and xylan into fermentable mono-sugars (i.e., glucose and xylose) (%) over a 72-hour reaction period to assess the pretreatment effects (Figure 6). DES pretreatment at T1 and T2 resulted in 80.4%

and 80.7% glucan and 57.3% and 55.5% of xylan conversions at 72 hours of enzymatic hydrolysis. These yields from DES pretreated biomass were greater than the conversion of glucan (27.2%) and xylan (16.7%) with the untreated corn stover and greater than the organosolv pretreated biomass that presented with a 65.9% of glucan and 43.1% of xylan conversion. However, despite a notable difference in delignification between DES T1 pretreated biomass and DES T2 pretreated biomass in Figure 5b, the enzymatic conversions of those biomass samples did not parallel this decrease in lignin.

Mass balance of DES pretreated corn stover

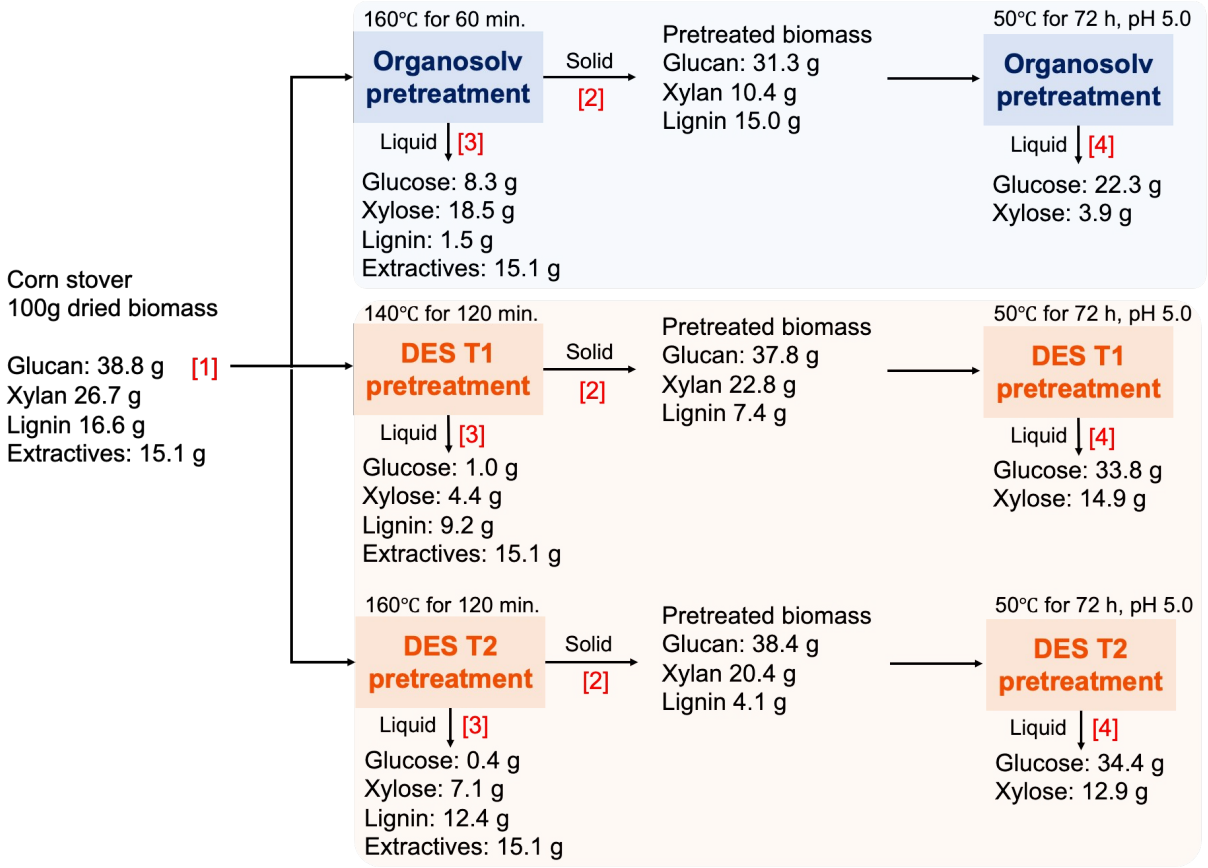

Figure 7. The mass balance of corn stover pretreatments. The [number] represents specific processes, [1] Pretreatment, [2] Solid obtained after pretreatment, [3] Filtrate after pretreatment, [4] Fermentable sugars after treatment

The mass balance of the system provided information on how to design the overall process, including reactor size, and post-utilization strategies such as fermentation. It also provided critical insights into the selectivity and efficiency of the applied methods. In this study, the overall mass balance of corn stover with each pretreatment was

calculated based on 100 g of dried corn stover as the starting material (Figure 7). The mass balance measurements began with the mass of chemical components in the untreated corn stover; the continued all the way to the fermentable sugars. To maximize the fermentable sugar yield, a high content of glucan and xylan in pretreated biomass (19) as well as effective enzymatic conversion are desired. The DES pretreatments resulted in carbohydrate-rich solid fractions containing glucan (37–39 g) and xylan (20–23 g) from the 100g of raw corn stover. Through the enzymatic hydrolysis, the highest sugar production was achieved with the DES T1 pretreated biomass, resulting in 48.7 g of glucose and xylose, while 47.3 g of total fermentable sugars were produced with the DES T2 pretreated biomass. This was because DES T1 exhibited lesser xylan loss while presenting with similar enzymatic hydrolysis yield as DES T2 (Figure 6), even though DES T2 pretreatment resulted in greater delignification (Figure 5). This showed that even though lignin is the major recalcitrance factor of biomass conversion in enzymatic hydrolysis and should be removed from the raw biomass, the preservation of carbohydrates in the pretreated biomass is also an important factor in achieving successful biological conversion yield before further processing such as fermentation. Along these lines, it could also be speculated that a decreased time of organosolv pretreatment would preserve a larger part of the glucan and xylan that was otherwise lost in this process (8.3 g and 18.5 g respectively), and therefore would yield amounts of fermentable sugars comparable to the DES pretreatments. This seems plausible since not much lignin was removed (~ 9%) in the organosolv pretreatment (Figure 7). It may also be possible to dispense with the organosolv pretreatment entirely.

Although this study only investigated the effect of ChCl/urea DES on corn stover conversion, various combinations of HBA and HBD are available. For instance, Smink et al. investigated the effect of diverse salts on lactic acid pulping (22). Therefore, biomass conversion could potentially be further improved by selecting and optimizing DES components. Also, during biomass pretreatment, energy consumption should be considered, which could be controlled by the biomass processing parameters such as temperature and time (23). With this aspect, the energy cost can be saved by reducing the pretreatment temperature from 160 ℃ to 140 ℃ for the same reaction time. Both DES pretreated biomasses produced a higher total sugar yield (48.7 g and 47.3 g for T1 and T2 respectively) when compared with the organosolv pretreated biomass; which yielded a total of 26.2 g of total fermentable sugars. The results indicated that DES pretreatment resulted in > 1.8 times the fermentable sugar production compared to the organosolv pretreatment. The results indicated that DES pretreatment could produce a higher yield of fermentable sugars under milder conditions when compared to the conventional organosolv pretreatment.

Conclusion

This study investigated the applications of ChCl/Urea DES on biomass pretreatment with corn stover. DES was successfully prepared with ChCl and urea as measured by the thermal properties of the solvent. The prepared DES effectively removed lignin, a major

recalcitrance factor of corn stover, under mild reaction conditions and resulted in > 80% glucan conversion by enzymatic hydrolysis. From 100 g of untreated corn stover, 33.8 g of glucose and 14.9 g of xylose were produced using the DES pretreatment at 140 ˚C, then followed by enzymatic hydrolysis. This yield was > 1.8 times that obtained then using the conventional organosolv pretreatment. The results indicated that the synthesized DES solvent with easily procurable components such as ChCl and urea could be applied as a biomass pretreatment solvent, achieving a sustainable and greener approach for future biofuel and biochemical applications.

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