Peer review

I liked reading the manuscript even though there is similar work already in the public domain. I have some concerns that need to be addressed.

1.Need operating conditions of the HPLC such as solvents (gradient elution or isocratic), flow rates, injection volume (split ratio if applicable), detector (FID, UV-VIS?) if UV-Vis, wavelength, elution time (for the 3 analytes), standard curve R^2, internal standard if any. I am assuming that linearity, reproducibility, peak purity, specificity, LOQ, LOD etc. was checked beforehand. Please provide sufficient information so that your work can be replicated in other labs.

2.Need autoclave make, manufacturer and model. Need what inert purge flow gas was used in the DSC and its flow rate. I am assuming DSC was calibrated using Indium? Also, need calibration procedure for DTA.

3.What was the weight percent of corn stover for the organosolv treatment? Was the DES treatment performed in a Parr reactor as well? If not then describe or present reaction vessel for DES treatment.

4.You state "…until the pH of the solid reached 6-7….." How do you check the pH of a solid?

5.you mention that the solvent is green. Present approximate calculations for carbon neutrality in the manufacture of choline chloride, urea, sulfuric acid, ethanol for the amounts used in your experiments.

6.This paper suggests that the chloride ion is responsible for the lignocellulosic breakdown; finding that NaCl was as effective as choline chloride. see: https://doi.org/10.1021/acs.iecr.9b03588 , if that is the case, will a urea-NaCl solution be as effective as a urea-choline chloride solution in pretreatment ? This paper suggests that using only urea is effective for delignification of corn stover (https://doi.org/10.1016/j.biortech.2020.123097), please comment and discuss in the manuscript.

7.You show a purported eutectic at 11.8 Celsius with cch:urea mixture. If this is indeed a eutectic, then the same sample heated to 350 celsius should not show melting endotherms for urea (mp:133 C) or for choline chloride (mp:300 C). I don't see that this is the case because your run stops at 60 C. Please present thermograms that show that no endotherms are visible at 133 or 300 C when the DSC for the mixture is run past 300 C. Present and discuss in the manscript. Be sure to also present individual thermograms of CCh and Urea. References can be used/cited instead of generating actual data or performing these experiments.

8.How does steam explosion pretreatment compare with DES ? See: https://doi.org/10.3389/fchem.2021.705358 , discuss in the manuscript.

9.you state "…..The pretreated biomass was soaked in the same concentration of ethanol solution to eliminate the possibility of the residual DES components trapped in the pretreated biomass…." what volume of etOH was used, for how long ?

10.you mention "….The content of acid-insoluble lignin (%AIL) was calculated with the dried weight of the solid fraction and the weight of ash obtained after calcination of the solid using a furnace….." please present time and temperature of calcination and furnace type.

11.You mention "….72-hour reaction by a certain concentration of enzyme (15 FPU/g of biomass) was used to measure their efficiency…." present what enzyme was used, potency and vendor. I am assuming the glucan and xylan percent conversion were determined by aliquots of fermentation liquor at those times using HPLC ? I am also assuming the sample was filtered before being injected into HPLC ? what filter? what pore size? how do you know that the filter membrane did not adsorb glucose or xylose? Please present all this information and analysis in the manuscript.

Response to Reviewers' Comments

Title: Understanding of the impacts of deep eutectic solvent (DES) pretreatment on utilization of corn stover

We appreciate the reviewers' valuable comments. We carefully reviewed and addressed each comment.

Comments to the Author:
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Response: We appreciate the reviewer's comment. HPLC operating conditions are included as suggested. Sugar concentrations were analyzed using HPLC with RefractoMax 520 refractive index (RI) detector. To confirm the reproducibility of the reported results, all the experiments were conducted at least twice, except enzymatic hydrolysate due to the limited test sample. Also, quantification of sugar was conducted with 1.25–20 g/L of pure glucose and xylose as external standard. Calibration curves for both glucose and xylose were made with over 0.99 of R^2 value. All the analyzed sample concentrations were within the ranges of external standards, indicating that a proper range of external standards was used in this study.

"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) installed 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 located in 1.5 ml HPLC vial. To confirm the reproducibility of the reported results, all the experiments were conducted at least twice. Also, quantification of sugar was conducted with 1.25–20 g/L of pure glucose and xylose as external standard. Calibration curves for both glucose and xylose were made with >0.99 of R^2 value. All the analyzed sample concentrations were within the ranges of 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."

Comment: Need autoclave make, manufacturer and model. Need what inert purge flow gas was used in the DSC and its flow rate. I am assuming DSC was calibrated using Indium? Also, need calibration procedure for DTA.
Response: The information of autoclave and DSC are included in the revised manuscript as below.

"The Pyrex bottle was placed in an autoclave (Tuttnauer 2340M-B/L, Heidolph) at 121 ˚C for 60 min."

"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 purging at 50 ml/min. A calibration curve was prepared with indium followed by standard procedures from TA Instruments."

Comment: What was the weight percent of corn stover for the organosolv treatment? Was the DES treatment performed in a Parr reactor as well? If not then describe or present reaction vessel for DES treatment.
Response: The information on corn stover loading and reactors for DES and organosolv pretreatments are included below.

"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 under different temperatures, 140 ℃ (T1) and 160 ℃ (T2). The reaction was conducted for 120 min in a 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 done thoroughly until the filtrate became colorless. The washed solid was stored at a 4 ℃ refrigerator until its chemical composition analysis and enzymatic hydrolysis. 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 in order to account for safety regarding the generated pressure resulting from the procedure. Since ethanol organosolv pretreatment has been applied to biomass in many studies, this comparison can give a clue as to how effective the ChCl-Urea DES pretreatment is. The organosolv pretreatment condition (160 ℃ for 60 min) was modified from the reference.[8] Corn stover (3.0 g) was loaded with aqueous ethanol solution (27 ml) into a glass cylindrical liner. In aqueous ethanol solution, 16.2 mL of ethanol, 10.8 mL DI water, and 0.05 mL sulfuric acid were blended in advance. After organosolv pretreatment, the pretreated biomass solid was washed with deionized water until the pH of the solid reached 6-7, as determined by using the filtrate. The washed solid residue was used for chemical composition analysis and enzymatic hydrolysis."

Comment: You state "…until the pH of the solid reached 6-7….." How do you check the pH of a solid?
Response: To clarify the pH measurement method, the below sentence is added.

"The pH of solid residue was monitored with the washing filtrate using a pH meter."

Comment: you mention that the solvent is green. Present approximate calculations for carbon neutrality in the manufacture of choline chloride, urea, sulfuric acid, ethanol for the amounts used in your experiments.
Response: We agree that the greenness of the solvent and methods can be explained by carbon neutrality with life cycle assessment. However, it's beyond the scope of this work. Instead, we justified the environmental benefits of DES with the references in the introduction.

"Also, DES is employed as an efficient solvent due to its recyclability. Regarding to the environmental impacts in life cycle theory, the life cycle assessment (LCA) has been studied widely to elucidate the green extraction solvent of DES.[11,12]"

Comment: This paper suggests that the chloride ion is responsible for the lignocellulosic breakdown; finding that NaCl was as effective as choline chloride. see: https://doi.org/10.1021/acs.iecr.9b03588, if that is the case, will a urea-NaCl solution be as effective as a urea-choline chloride solution in pretreatment? This paper suggests that using only urea is effective for delignification of corn stover (https://doi.org/10.1016/j.biortech.2020.123097), please comment and discuss in the manuscript.
Response: Thanks for bringing up interesting discussion points. Formation, properties and performances of DESs vary depending on the selection of HBD and HBA, as well as their molar ratios. Since the cited paper is with lactic acid as HBD, it is hard to answer whether NaCl-Urea will give us comparable results or not. Also, due to the chemical structure difference between lactic acid and urea, the hydrogen bonds between HBD (i.e., urea) and HBAs (e.g., NaCl, ChCl) can be different. We briefly added a discussion about the possibility of other HBAs in the results and discussion section.

For the second question, alkaline pretreatments including urea pretreatment are effective on herbaceous plants like corn stover, but it is also known that many alkaline pretreatments are not effective with woody biomass. Although we studied corn stover, this DES can extensively be applied to different biomass including woody biomass, so it has broader applications compared to urea alone. We also included this discussion in the manuscript.

"It has been noted that several alkaline solutions such as ammonium hydroxide and urea were effective on biomass pretreatment.[13,14] However, many alkaline pretreatments showed limitations on 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."

"Although this study only showed the impacts of ChCl-urea DES on corn stover conversion, various combinations of HBA and HBD are available. For instance, Smink et al. discussed the impacts of diverse salts on lactic acid pulping.[22] Therefore, biomass conversion can be further improved by selecting and optimizing DES components."

Comment: You show a purported eutectic at 11.8 Celsius with cch:urea mixture. If this is indeed a eutectic, then the same sample heated to 350 celsius should not show melting endotherms for urea (mp:133 C) or for choline chloride (mp:300 C). I don't see that this is the case because your run stops at 60 C. Please present thermograms that show that no endotherms are visible at 133 or 300 C when the DSC for the mixture is run past 300 C. Present and discuss in the manscript. Be sure to also present individual thermograms of CCh and Urea. References can be used/cited instead of generating actual data or performing these experiments.
Response: Prior to operating DSC, TGA results are necessary. In general, we run DSC to a temperature within 0.5% thermal degradation based on TGA results. As the TGA result shows, the weight loss of DES was already over 80% at 300 ℃. For this reason, the operation of DSC over 300 ℃ is not

available with our current facility. Instead of the requested DSC test, we included the related references for the T_m of ChCl and urea.

"The melting point of ChCl/urea DES showed clearly at 12 ºC. It was much lower than the points of individual choline chloride (302 ºC) and urea (133 ºC), indicating its generation of deep eutectic point.[17] The lowered melting point of DES underscored the unique thermophysical properties compared to its pure chemical, offering insights into its potential applications across other scientific domains."

Comment: How does steam explosion pretreatment compare with DES? see: https://doi.org/10.3389/fchem.2021.705358, discuss in the manuscript.
Response: While the studied organosolv and DES pretreatments are thermochemical methods, steam explosion is physical pretreatment by exposing the biomass to high pressure steam. This pretreatment is also effective for corn stover, but this is not a method to selectively separate lignin from biomass. In this study, we consider lignin as a potential co-product of the process; therefore, it is hard to compare those two methods directly.

"Pretreatment is designed to address these challenges as it can change the composition and structural properties of lignocellulosic biomass. It can reduce the aforementioned barriers and allow better cellulase access to cellulose, improving the production of fermentable sugars like glucose.[6] 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) under eutectic points, allowing their high reactivity and solubility of biomass components under mild conditions, which are comparable to ionic liquids."

Comment: you state "…..The pretreated biomass was soaked in the same concentration of ethanol solution to eliminate the possibility of the residual DES components trapped in the pretreated biomass…." what volume of etOH was used, for how long?
Response: The requested information is added to the manuscript as below.

"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 under different temperatures, 140 ℃ (T1) and 160 ℃ (T2). The reaction was conducted for 120 min in a 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 done thoroughly until the filtrate became colorless. The washed solid was stored at a 4 ℃ refrigerator until its chemical composition analysis and enzymatic hydrolysis. 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 in order to account for safety regarding the generated pressure resulting from the procedure. Since ethanol organosolv

pretreatment has been applied to biomass in many studies, this comparison can give a clue as to how effective the ChCl-Urea DES pretreatment is. 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 cylindrical liner. In aqueous ethanol solution, 16.2 mL of ethanol, 10.8 mL DI water, and 0.05 mL sulfuric acid were blended in advance. After organosolv pretreatment, the pretreated biomass solid was washed with deionized water until the pH of the solid reached 6-7, as determined by using the filtrate. The washed solid residue was used for chemical composition analysis and enzymatic hydrolysis."

Comment: you mention "….The content of acid-insoluble lignin (%AIL) was calculated with the dried weight of the solid fraction and the weight of ash obtained after calcination of the solid using a furnace….." please present time and temperature of calcination and furnace type.
Response: The requested information is added to the manuscript.

"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 of biomass was measured using a muffle furnace at 575 ˚C for 24 hours."

Comment: You mention "….72-hour reaction by a certain concentration of enzyme (15 FPU/g of biomass) was used to measure their efficiency…." present what enzyme was used, potency and vendor. I am assuming the glucan and xylan percent conversion were determined by aliquots of fermentation liquor at those times using HPLC? I am also assuming the sample was filtered before being injected into HPLC? what filter? what pore size? how do you know that the filter membrane did not adsorb glucose or xylose? Please present all this information and analysis in the manuscript.
Response: Detail information about enzymatic hydrolysis experiment is added as below. Enzyme was cellulase enzyme blend Cellic Ctec 2, purchased from Sigma-Aldrich. Also, all liquid samples for HPLC analysis were filtered with a nylon syringe (0.2 μm) filter before analysis and located in 1.5 ml HPLC vial.

"The urea and ethanol were supplied by Fisher Scientific Inc. and Decon Laboratories Ltd., respectively. Cellulase (enzyme blend Cellic Ctec 2) was obtained from Sigma-Aldrich. All other chemicals used in this study were used without further purification.

Thank you for addressing my comments. Accept