Green Chemistry

A biomass pretreatment using cellulose-derived solvent Cyrene

Journal:	*Green Chemistry*
Manuscript ID	GC-COM-02-2020-000661.R1
Article Type:	Paper
Date Submitted by the Author:	25-Mar-2020
Complete List of Authors:	Meng, Xianzhi; University of Tennessee, Pu, Yunqiao; Oak Ridge National Laboratory, Joint Institute of Biological Science, Biosciences Division Li, Mi; University of Tennessee Ragauskas, Arthur; University of Tennessee,

SCHOLARONE™
Manuscripts

A biomass pretreatment using cellulose-derived solvent Cyrene

Xianzhi Meng[1], Yunqiao Pu[2], Mi Li[3], Arthur J. Ragauskas[1,2,3,*]

[1] Department of Chemical & Biomolecular Engineering, University of Tennessee Knoxville, Knoxville, TN 37996, USA
[2] Joint Institute for Biological Sciences, Biosciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA
[3] Department of Forestry, Wildlife, and Fisheries; Center for Renewable Carbon, The University of Tennessee Knoxville, Institute of Agriculture, Knoxville, TN 37996, USA

Corresponding Author
Arthur J. Ragauskas. Fax: 865-974-7076; Tel: 865-974-2042; E-mail: argauskas@utk.edu.

Abstract

Despite only recently becoming available in the quantities required for solvent usage, the cellulose-derived solvent, named Cyrene, has gained significant attention in green chemistry in recent years. To fulfill the sustainability criteria of future biorefineries, a novel renewable biomass pretreatment using Cyrene and water was developed for the first time. Results showed that Cyrene has high potential as a green pretreatment solvent in terms of lignin fractionation/recovery and sugar release in the follow-up enzymatic hydrolysis. The mechanism of this pretreatment was revealed by investigating different phenotypes of biomass, and the recovered lignin was also fully characterized to assess its valorization potential. Results indicated that Cyrene pretreatment could be performed at a mild condition (120 °C) to reduce the lignin condensation and the cleavage of β-O-4 linkages without compromising lignin removal and the following sugar platform. The successful utilization of this cellulose-derived solvent in pretreatment chemistry will further contribute to the realization of a "closed-loop" biorefinery process.

1. Introduction

Conversion of biomass to fuels, chemicals, and other beneficial byproducts, known as biorefinery, has attracted significant attention due to the increasing environmental concerns over traditional petroleum-based fuels.[1] In current biorefineries, biomass pretreatment remains an essential step, and the high cost associated with this process still represents a significant limitation. The techno-economic analysis of the lignocellulosic ethanol biorefinery with different individual pretreatments (e.g. hot water,[2] dilute acid,[3] ionic liquid,[4] organosolv,[5] ammonia fiber expansion[6]) has been summarized and compared in several studies.[7-9] It has been estimated that, on a per-gallon basis, the biomass pretreatment step remains the single most expensive processing step, representing an appropriately 20% cost in biofuel production.[10] One way to reduce the biorefinery cost or increase the revenue from lignocellulosic biomass is to maximize the utilization of the feedstock as well as its derivatives.[11] Advances in biotechnology, synthetic chemistry, and chemical engineering are leading to a new concept for converting biomass to liquid fuels and valuable products using biomass-derived solvents, achieving a "closed-loop" biorefinery as summarized in **Fig. 1**. Cellulose, hemicellulose, and lignin are three major components in the plant cell wall of the lignocellulosic biomass. Solvent derived from these components, especially hemicellulose and lignin, including γ-valerolactone (GVL),[12]

tetrahydrofuran (THF),[13] 2-methyltetrahydrofuran,[14] ionic liquid (IL),[15] and deep eutectic solvent (DES)[16] have been all successfully applied on different types of biomass. For example, lignin and hemicellulose derived ILs have been successfully used to replace the expensive imidazolium-based solvent in IL pretreatment of switchgrass.[15] Kim *et al.* reported that lignin-derived phenolic compound such as catechol, vanillin, *p*-coumaric acid, and 4-hydroxyphenyl alcohol could be used as hydrogen bond donors to form DES with choline chloride to pretreat switchgrass, and their results suggested that up to 85.7% of glucose could be released during the subsequent enzymatic hydrolysis process which is comparable with that from the traditional IL pretreatments.[16] Their group further improved this technique by using genetic engineered biomass to produce lignin with aldehyde-rich units, which in turn facilitate the production of phenolic aldehyde-derived DES that could be used in the pretreatment step.[17] Although these solvents make the closed-loop technology highly cost-effective, most of these ILs and DES are not readily available, and some of them require certain functionality; thus, it may require multiple steps to synthesize those solvents.
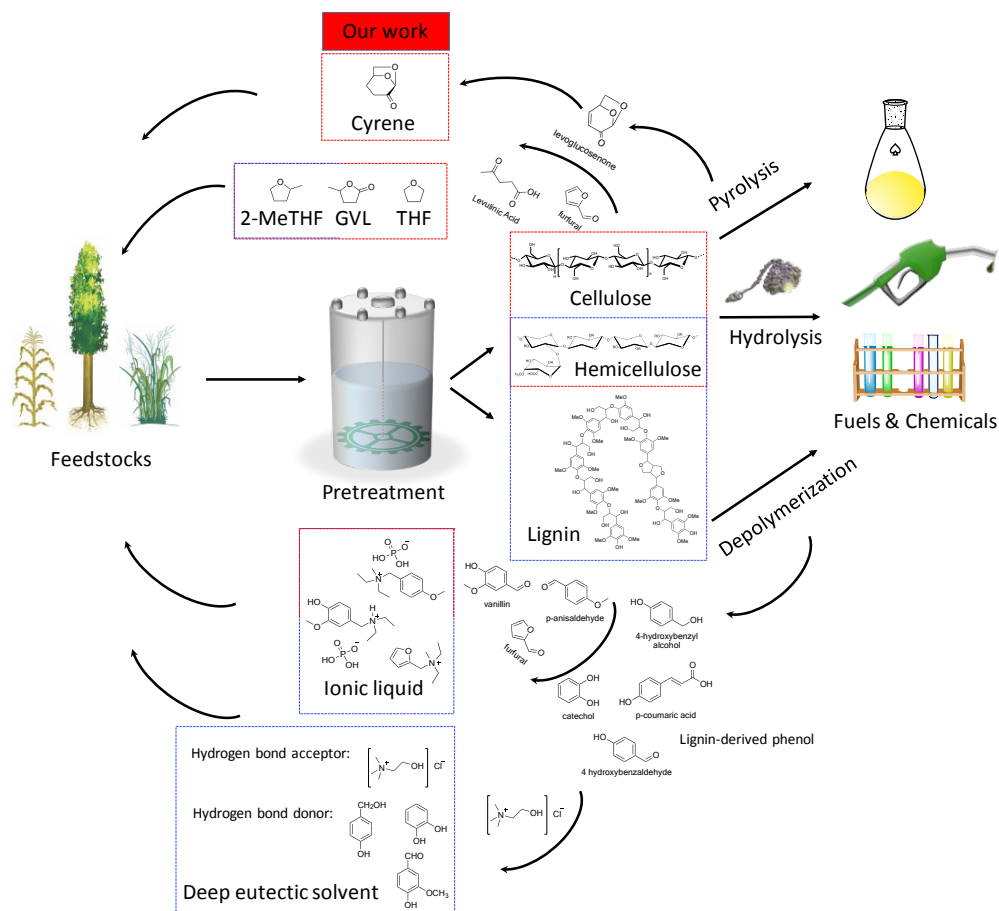
Figure 1. A summary of the recently developed closed-loop biorefinery concept using biomass-derived solvents.

Cyrene, also known as dihydrolevoglucosenone, is a heterocyclic cycloalkanone which could be prepared via the hydrogenation[18] or enzymatic reduction of levoglucosenone (LGO) (**Fig. S1**).[19] LGO, at the same time, is a major product of acid-catalyzed pyrolysis of carbohydrates, mainly cellulose. Thus, Cyrene is bio-renewable and has been considered as a green alternative to toxic aprotic dipolar organic solvents such as dimethylformamide (DMF) and N-methyl-2-pyrrolidone (NMP).[20] Despite only lately becoming commercially available, Cyrene has been utilized as a sustainable solvent in several applications, including synthetic chemistry,[21, 22] pharmaceutical industry,[23] and polymer chemistry.[24] Nevertheless, using Cyrene as a renewable solvent in biorefinery, especially the pretreatment step, has not been reported to the best of our knowledge. Lignin is known to have excellent solubility in DMF due to their low Hansen relative energy difference (RED) (0.77). Due to its similar aprotic dipolar nature to DMF, the RED between lignin and Cyrene is 0.89 (Table S1), suggesting that Cyrene should be an excellent solvent for lignin as well. In addition, a high hydrogen bond acceptor (HBA) capacity is typically required in order for the solvent system to effectively solubilize lignin, and Cyrene has a relatively high HBA capacity (0.61) based on the Kamlet-Abboud-Taft parameter.[20, 25] A quick solubility test confirmed that organosolv (EtOH) lignin and Co-solvent enhanced lignocellulosic fractionation (CELF) lignin all have excellent solubility in Cyrene despite its high viscosity which might slow down the lignin transportation in the solvent (**Fig. 2A and 2B**). Moreover, Cyrene is miscible with water, and the Cyrene/water ratio was further found to play a critical role in lignin solubility due to the presence of strong hydrogen bond between Cyrene and water which is beneficial for the cleavage of lignin-carbohydrate linkages (**Fig. 2C**). De Bruyn et al. showed that Cyrene could react with water to form a germinal diol type of structure, which further introduces additional polarity from the two OH groups.[26] Futher addition of water decreased the HBA capacity of the solvent system, therefore decreased the lignin solubility in the co-solvent.[25] Cyrene is also a mild Lewis base, thus the presence of a Brønsted acid such as H_2SO_4 could lower the pH of the solution even in very dilute concentrations. The high boiling point of Cyrene (~200 °C) and the fact that it does not form an azeotrope with water makes it possible to distill the latter from the Cyrene/water mixture to recycle pure Cyrene. All these facts

support the hypothesis that Cyrene, together with water, could be a promising green pretreatment solvent for lignocellulosics in which water and Cyrene could be used to dissolve hemicellulose and lignin, respectively.
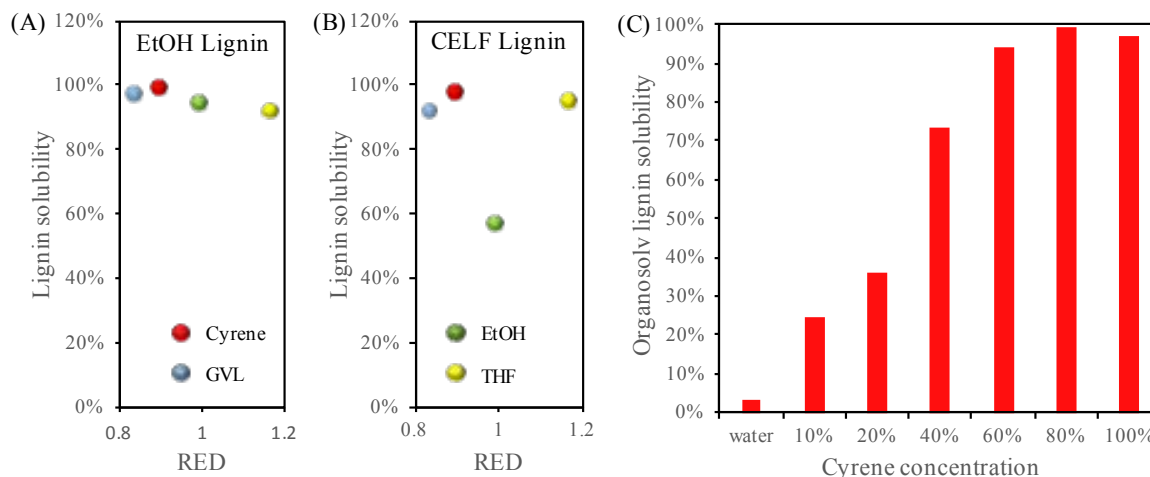

Figure 2. Lignin solubility test in an organic solvent. (A) Solubility of organosolv EtOH lignin in EtOH, THF, GVL, and Cyrene, (B) solubility of CELF lignin in EtOH, THF, GVL, and Cyrene, (C) solubility of EtOH lignin in Cyrene/water co-solvent system at different Cyrene concentrations.

Lignin limits cellulose accessibility and binds to cellulase enzymes unproductively during enzymatic hydrolysis.[27, 28] Acknowledging the negative role of lignin in biomass recalcitrance, high pretreatment severities such as high acid loading, high pretreatment temperature, or long process time are typically required to obtain cellulose-rich residue that favors the sugar production in traditional carbohydrate-first pretreatments. However, lignin under those conditions usually suffers from issues associated with condensation or significant cleavage of ether linkages that affect its subsequent valorization strategies. For example, it has been reported that the hydrogenolysis of native lignin could produce aromatic monomers with a theoretical yield of 20 and 45% from softwood and hardwood, respectively.[29, 30] On the other hand, the limited amounts of reactive aryl ether (β-O-4) linkages and unnecessarily large amounts of C-C linkages (β-5, β-β, and β-1) within the technical lignins significantly affect the yield of aromatic monomers, causing less than 20% of the theoretical yield from native lignin.[31, 32] Therefore, producing a high yield of technical lignins while preserving their ether linkages during the pretreatment step without endangering the lignin removal is essential to achieve an integrated conversion of carbohydrate and lignin to sugars and aromatic monomers, respectively. From this

perspective, the development of advanced pretreatment technology is becoming a primary research topic. Here, we propose that due to Cyrene's excellent ability to dissolve lignin, Cyrene pretreatment could be performed at a mild condition to reduce the lignin condensation and the cleavage of β-O-4 linkages without endangering lignin removal and solubility during pretreatment. The objectives of this study are to i) evaluate the performance of Cyrene pretreatment on woody biomass by measuring sugar release; ii) explore the mechanism of pretreatment by investigating different phenotypes of biomass, and iii) assess the potential valorization of obtained lignin by characterizing its physicochemical structures.

2. Experimental

2.1. Materials and chemicals

Populus trichocarpa x deltoids were air-dried, debarked, and Willey milled to ≤0.4 mm (40 mesh). The extractives were removed by ethanol/toluene (1:2, v/v) using a Soxhlet extraction apparatus for 8 h and then over dried at 45 °C. Compositional analysis was performed using the NREL standard procedure.[33] Chemicals were all purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification except when mentioned specifically.

2.2. Biomass pretreatment

Cyrene pretreatment was performed using a Cyrene/water mixture at different ratios (4:1, 1:1, and 1:2) at 120 °C for 10 and 60 min. ~10 g of the extractive free *Populus* samples were transferred to a 300 mL Parr reactor with H_2SO_4 (75mM) at a 6.6% dry solids loading. The Parr-reactor was heated to 120 °C over ~15 min, held at this temperature (±2 °C) for 10 and 60 min, and then quenched in a water bath at room temperature for ~5 min. The pretreated biomass was filtered to remove the solid material and washed with the same Cyrene/water mixture followed by an excess amount of DI water. Dilute acid pretreatment was done at a very similar procedure without the addition of Cyrene at 120 °C for 60 min. Pretreated samples were never-dried and stored in refrigerator at 4 °C prior to enzyatmic hydrolysis and further analysis. Cyrene pretreatment hydrolysate was combined and diluted with DI water to precipitate the lignin. For the 60min_4:1 Cyrene pretreatment, because of the high dynamic viscosity nature of the mixture, water was directly added to the mixture to decrease the viscosity of the solution mixture. The solid mixture (containing the precipitated lignin and pretreated biomass) was then separated from

the liquid using vacuum filtration through a glass fiber filter paper. Dioxane was used to separate lignin from the solid mixture, and lignin was finally isolated from the dioxane by using a rotary evaporator. The obtained crude lignin was dissolved in 90% acetic acid followed by precipitated into three volumes of DI water. After filtration, lignin samples were dried at 45 °C overnight, and the dry weight was measured to calculate the lignin yield.

2.3.   *Enzyamtic hydrolysis*

Enzymatic hydrolysis of raw and pretreated samples was performed in 50.0 mM citrate buffer at pH 4.8 with commercial Cellic CTec2 loadings of 20 FPU/g and 60 FPU/g cellulose. The mixture was incubated at 50 °C under continuous agitation at 150 rpm for 96 h with antibiotic antimycotic solution (Sigma A5955) 1% (v/v) added to avoid microbiological contamination. Hydrolysis liquid (1.00 mL) was withdrawn from the hydrolysis, quenched by submersion in a boiling water bath for 10 min, and then immediately frozen to -20 °C prior to analysis. In the case of solvent incubation, pretreated substrates were extracted with dioxane, acetone, or dilute alkaline (1 wt%) overnight at room temperature with constant stirring to facilitate the defibrillation. After that, the solid residue was filtrated and washed with an excess amount of DI water, and the never-dried incubated solid materials were subsequently subjected to the enzymatic hydrolysis. The sugar content was measured by a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., USA) with a conductivity detector, a guard CarboPac PA1 column (2 × 50 mm, Dionex), a CarboPac PA1 column (2 × 250 mm, Dionex), a AS40 automated sampler and a PC 10 pneumatic controller at room temperature. Calibration was performed with standard solutions of glucose, and fucose was used as an internal standard.

2.4.   *Lignin solubility test*

Organosolv (EtOH) and Co-Solvent Enhanced Lignocellulosic Fractionation (CELF) lignin (~100mg) obtained from previous study[34] were added into 10 mL organic solvents (Ethanol, GVL, THF, and Cyrene). The mixture was stirred at room temperature for 2 h. The undissolved solid was then recovered by filtration, and dried in a vacuum oven at 45 °C. Finally, the dry weight of the lignin solid was measured to calculate the solubility percentage. EtOH lignin was also mixed with pure Cyrene as well as mixtures of Cyrene/water, ranging from 10 to 80% Cyrene. The lignin solution was again stirred for 2 h at room temperature and then centrifuged at 8000 rpm to separate the undissolved fraction from the solubilized fraction. The dry weight of

the undissolved fraction was obtained to calculate the lignin solubility in Cyrene/water co-solvent.

2.5.   *Isolation of cellulolytic enzyme lignin*

Cellulolytic enzyme lignin (CEL) was isolated from the untreated *Populus* according to the literature.[35] In brief, the extractives-free *Populus* was ball-milled in a porcelain jar with ceramic balls via Retsch PM 100 (Newton, PA) at 600 rpm for 2.5 h followed by enzymatic hydrolysis in acetate buffer (pH 4.8, 50 °C) using Cellic CTec 2 cellulase and HTec 2 hemicellulase (1:1, mass ratio), as the enzymes (150 mg of protein loading/g of biomass) for 48 h. The solid residue was isolated by centrifugation and hydrolyzed again with freshly added buffer and enzymes for another 48 h. After filtration, the solid residue was extracted twice with 96% (v/v) 1,4-dioxane/water mixture at room temperature for 48 h. The extracts were combined, rotary evaporated, and freeze-dried to recover CEL. CEL was then dried overnight in a vacuum oven at 40 °C prior to NMR and GPC analysis.

2.6.   *Celluose and lignin characterization*

The detailed procedures of the cellulose isolation and characterization, lignin molecular weight distribution analysis, HSQC and ^{31}P NMR analysis are listed in the ESI section.

3. Results and discussion

To test our hypothesis, a Cyrene/water mixture at different ratios (4:1, 1:1, and 1:2) was used to pretreat woody biomass *Populus* at 120 °C for different times (10 and 60 min) under acidic conditions. Dilute acid pretreatment (DAP) without the addition of Cyrene was also performed as a control at the same conditions without the addition of organic solvent. A preliminary visual screening suggested that for 30 min pretreatment, no apparent biomass deconstruction was observed for ptreatment performed under 100 °C, and higher temperatures were not tested for safety concerns as the safety data sheet of Cyrene advises temperature above 140 °C should be avoided.[23] Due to its high dynamic viscosity nature, the higher ratio between Cyrene and water (>4:1) was also avoided.

3.1.   *Compositonal analysis*

As shown in **Fig. 3A**, all the Cyrene pretreatment decreased the content of hemicellulose and lignin to a different extent, depending on the pretreatment conditions. Both Cyrene concentration and pretreatment time played a positive role in the solubilization of lignin and hemicellulose. As

shown in **Fig. S2**, up to 74 and 68% of xylan and lignin were removed during the 60 min pretreatment using a 4:1 mixture of Cyrene and water. In contrast, no apparent delignification was observed during the dilute acid pretreatment, although ~40% of xylan was solubilized during the process. From the pretreatment yield and composition analysis based on initial raw biomass (Table S2), it was also found that a significantly higher degree of biomass solubilization in the presence of Cyrene was observed than that in the DAP control.

3.2. *Enzyamtic hydrolysis*

The raw and pretreated *Populus* samples were then subjected to a 96 h enzymatic hydrolysis using the commercial Cellic CTec2 at two different enzyme loadings (20 and 60 FPU/g of cellulose). As shown in **Fig. 3B**, the cellulose conversion of almost all Cyrene pretreated samples was higher than that of the dilute acid pretreated control and the untreated *Populus*. Surprisingly at low cellulase loadings (20 FPU/g), the 60 min Cyrene pretreated *Populus* using a 4:1 mixture of Cyrene and water (60min_4:1) had the lowest cellulose conversion (~17.7%) among all the substrates including the raw untreated one (~21.5%). At the higher enzyme loading, its cellulose conversion increased to 48.7%, which is higher than the untreated sample (~42.6%) but is still significantly lower than other pretreated samples at lower severities. This unexpected phenomenon is also observed for the low cellulase loading enzymatic hydrolysis of Cyrene pretreated *Populus* using a 1:1 mixture of Cyrene and water. Cellulose conversion was found to decrease from 72.2% (10min_1:1) to 56.9% (60min_1:1) as the pretreatment time extended from 10 to 60 min, although higher cellulase loading (60 FPU/g) could overcome this difference (88.4 and 91.5% for 10min_1:1 and 60min_1:1, respectively). At these two ratios (4:1 and 1:1), considering the fact that 60 min pretreatment leads much lower lignin and xylan content than the 10 min pretreatment, it seems to indicate that some inhibitors were formed during the more extended pretreatment period and it is somehow related to the high concentration of Cyrene in the co-solvent mixture. On one hand, longer pretreatment time (60min) is known to increase the efficiency of lignin and xylan removal (**Fig. 3A**), which generally facilitate the enzymatic hydrolysis. On the other hand, it is also believed that the formation of inhibitors is associated with the extended pretreatment period. Once a factor is no longer limiting; the other factor will become dominant. For high Cyrene concentration pretreatment (4:1 and 1:1 ratios), the negative effect caused by inhibitors is probably the dominant factor responsible for the low sugar release of the 60 min pretreated substrate. For the low Cyrene concentration pretreatment (1:2 ratio),

only a limited amount of inhibitors was formed, therefore the positive effect of lignin and xylan removal becomes the dominant factor responsible for the high sugar release of the 60 min pretreated substrate.
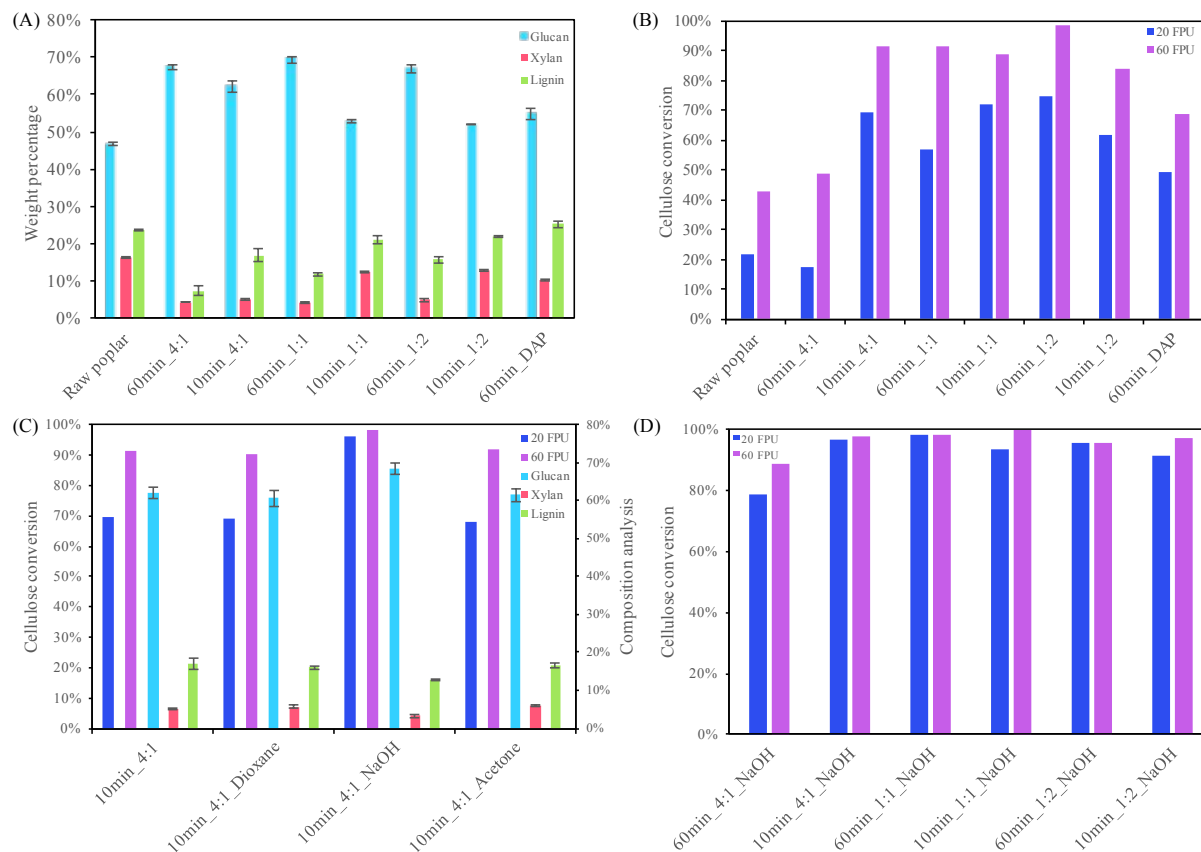

Figure 3. Cyrene pretreatment of *Populus* at 120 °C with different Cyrene concentrations (4:1, 1:1, and 1:2) and pretreatment times (10 and 60 min). (A) Composition analysis of untreated and pretreated *Populus*, (B) enzymatic hydrolysis of untreated and pretreated *Populus* at different enzyme loadings, (C) effect of organic solvent and alkali extraction on enzymatic digestibilities of 10min_4:1 pretreated *Populus*, (D) enzymatic hydrolysis of alkali incubated Cyrene pretreated *Populus* at different enzyme loadings.

There could be three possible reasons, which are all related to the physicochemical properties of the new solvent Cyrene. The first one could be due to its high dynamic viscosity nature, and it might be difficult to completely remove the leftover Cyrene from the pretreated substrate, especially at higher concentrations, which deactivated the enzymes during the enzymatic hydrolysis process. To test this hypothesis, the 60min_4:1 pretreated *Populus* were

soaked and stirred in fresh hydrolysis acetate buffer overnight. After filtration, the "contaminated" buffer was then directly applied to enzymatic hydrolysis of Avicel, and the sugar release was measured and compared to the enzymatic hydrolysis of Avicel using "clean" fresh buffer. Results showed that they have almost identical sugar release (**Fig. S3**), suggesting Cyrene itself is probably not the reason causing the unexpected low cellulose conversion of these 60 min pretreated samples in light of the high delignification that was achieved. The second possible reason is also related to the high viscosity of Cyrene. During the DI water washing process, dissolved lignin in the leftover Cyrene could be precipitated and deposited right back on the surface of pretreated biomass, causing significant lignin inhibition during the enzymatic hydrolysis process. The last possible reason could be due to the grafting of the acetate type of structure on the cellulose surface, ultimately blocking the access of enzymes to cellulose. Shuai et al. showed that incubation of GVL pretreated biomass could significantly improve the conversion of cellulose to glucose.[12] A subsequent study revealed that this increase of sugar release efficiency is attributed to both the depletion of the ester groups on cellulose surface as well as further partial removal of lignin and hemicellulose components from the pretreated biomass.[36] To test these hypotheses, the 10min_4:1 pretreated *Populus* was selected and extracted with 1,4-dioxane, acetone, and dilute alkali solution (1 wt% NaOH) at room temperature in an incubator overnight before the enzymatic hydrolysis experiment. The purpose of this incubation is to hydrolyze any possible ester groups on the surface of cellulose or solubilize any possible lignin residues on the surface of biomass substrates. As shown in **Fig. 3C**, the cellulose conversion after dioxane and acetone extraction remain relatively unchanged, possibly due to the very limited hemicellulose and lignin removal. In contrast, alkali incubation dramatically increased the cellulose conversion to 96.4% even at low cellulase loading, suggesting chemical grafting on the surface of cellulose is probably the primary reason causing the unexpected low glucose yield. This result agrees with the fact that despite having much lower lignin and xylan content, pretreated *Populus* at higher Cyrene concentration and more prolonged pretreatment had lower enzymatic digestibilities. Also, the slight decrease of hemicellulose and lignin content after dilute alkali incubation is likely to contribute to the increase of glucose yield as well. In conclusion, dilute alkali incubation was proved to be an excellent way to overcome the negative effect of high concentration of Cyrene or long pretreatment time. Ongoing studies are underway to confirm the exact chemical grafting on the surface of cellulose. All the Cyrene

pretreated substrates were subjected to dilute alkali incubation, followed by enzymatic hydrolysis, and their sugar release was shown in **Fig. 3D**. In conclusion, the 10min_4:1 and 60min_1:1 Cyrene pretreated *Populus* had the highest biomass saccharification after alkaline incubation at low enzyme loadings. The enzymatic hydrolysis was enhanced by up to 4.5-fold for the sample treated with Cyrene/water system as compared to the raw sample.

3.3.   *Cellulose characterization*

To better understand how Cyrene pretreatment overcomes biomass recalcitrance. Molecular weight, crystallinity, and accessible surface area of cellulose, which are known to play essential roles in biomass conversion, were analyzed (**Fig. 4**). The substrates pretreated with a 4:1 mixture of Cyrene and water (10 and 60 min) along with untreated and dilute acid pretreated samples were selected for these investigations. Simons Stain (SS) represents an effective semi-quantitative technique to assess the accessible surface area of cellulose to cellulases using a direct orange dye to imitate the generic cellulase enzyme because of their similar sizes.[37] Accessibility test suggested that all the pretreated samples, especially the organosolv pretreated *Populus* using Cyrene, significantly increased cellulose accessibility compared to the untreated one (**Fig. 4A and 4B**). Even the 10 min Cyrene pretreatment could outperform the 60 min DAP in terms of the cellulose accessibility increase due to its exceptional ability to solubilize hemicellulose and lignin. Cellulose degree of polymerization (DP) was also decreased after all the pretreatments as a result of cleavage of glycosidic bond, and the most reduction of cellulose DP was found in the 10min_4:1 pretreated *Populus* (**Fig. 4C**). It has been proposed in several studies that shorter cellulose chains could expose more reducing ends, which is beneficial to the subsequent enzymatic saccharification process.[38, 39] However, the lower cellulose DP was not observed to dramatically improve the cellulose digestibility of the 60min_4:1 Cyrene pretreated Populus. This is likely due to the reason that that biomass recalcitrance is a multi-variant phenomenon, and it cannot be simply judged on one solely substrate factor such as cellulose DP.[40] The cellulose crystallinity (CrI) was slightly increased from 50.2% to 51.9, 52.8, and 51.8% for 10min_4:1, 60min_4:1, and dilute acid pretreated *Populus*, respectively, which are attributed to the removal of amorphous regions of cellulose (**Fig. 4D**). Foston and Sannigrahi et al. all reported that cellulose CrI significantly increased after acid-catalyzed pretreatment.[41, 42] It should be noted that the cellulose CrI increase reported here is smaller than those reported in the literature, which might due to the low pretreatment severity applied in this study.
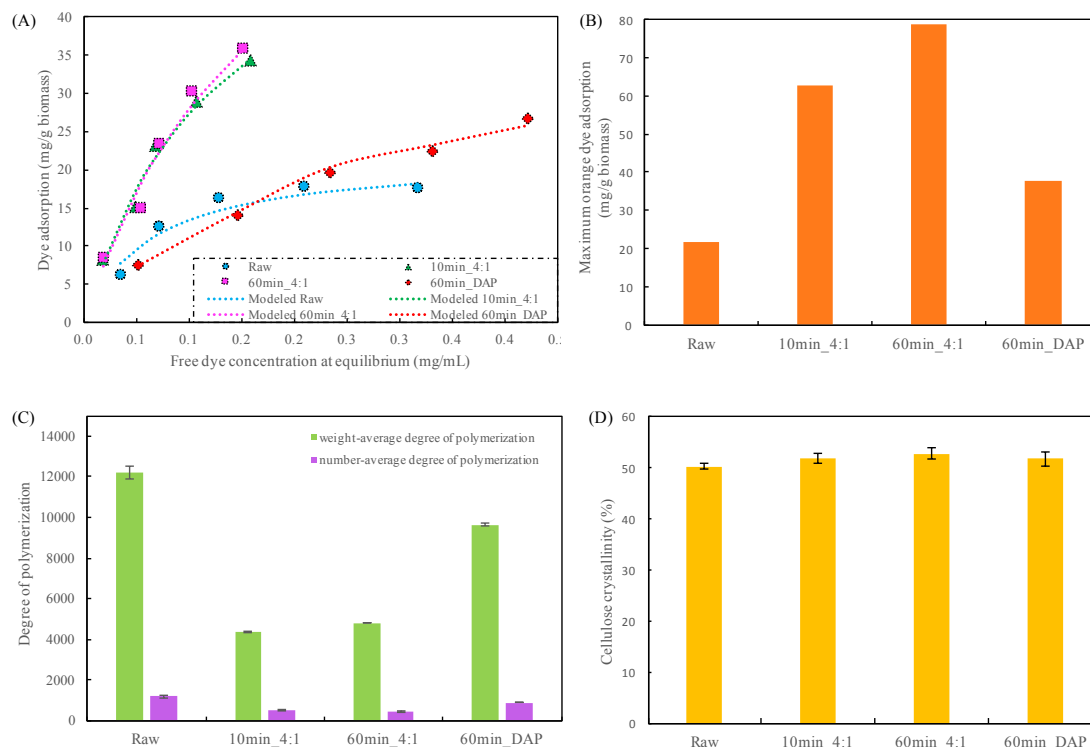
Figure 4. Characterization of cellulose isolated from untreated and pretreated *Populus*. (A) Simons staining orange dye isotherm adsorption curves, (B) maximum amount of dye adsorbed, (C) cellulose degree of polymerization, (D), cellulose crystallinity.

3.4.   *Lignin characterization*

Lignin, as the most recalcitrant components of biomass, plays an important role in the economic viability and sustainability of biorefining strategy. It has been treated as a waste product in the current pulp and paper industry. The future integrated biorefinery highly depends on the efficient valorization of lignin, which requires a deep understanding of the chemical nature of the isolated lignin. Because the content of reactive ether linkages in lignin is a crucial factor for the downstream conversion of lignin to aromatic chemicals, biomass pretreatment or lignin isolation technologies that could prevent severe lignin degradation have attracted considerable interest in recent years. Bhagia and Ragauskas recently reported higher aryl ether linkages could be preserved in cellulolytic enzyme lignin (CEL) by using ultra-friction grinding instead of planetary ball milling for size reduction of Populus.[43] Zhou et al. developed a rapid flow-through fractionation method using formic acid to separate the dissolved lignin from the reaction system, preserving up to 75 to 85% of the labile ether linkages in the solubilized lignin.[44] Costa Sousa et

al. reported a new liquid ammonia pretreatment that could produce lignin with an almost intact structure. Besides, the trade-off between lignin yield and structure quality was often demonstrated in the literature. During our Cyrene pretreatment, up to 60% of the original lignin could be recovered from the co-solvent system, and the impact of pretreatment conditions on the physicochemical properties of lignin was investigated and compared to the raw CEL.

3.4.1. *Molecular weight analysis*

Biomass pretreatment performed under acidic conditions could cause lignin depolymerization, as shown in the molecular weight analysis (**Fig. 5B and 5C**). For example, the weight-average molecular weight (M_w) of Cyrene pretreated lignin substrates was decreased from 13133 g/mol (for CEL) to much lower values (4028 to 6311 g/mol). A high ratio of Cyrene in the co-solvent mixture seems to have a positive effect on the molecular weight values, as the two pretreated lignin samples using 1:2 mixtures of Cyrene and water have the lowest molecular weight among all the pretreated lignin substrates. This result could be ascribed to the increased solubility of large lignin fragments in concentrated Cyrene aqueous solutions. Meanwhile, acid pretreatment also systematically suffers from the irreversible repolymerization issue, normally leading to a low yield of low molecular lignin fragments that can be ultimately upgraded to value-added aromatic chemicals at high yields.[45] One of the interesting findings in the molecular weight study is that despite lignin continued to be solubilized in the co-solvent as pretreatment time is extended from 10 to 60 min (**Fig. 3A**), it does not have a prominent effect on lignin molecular weight (**Fig. 5C**). This observation is promising as the molecular weight is typically positively related to the content of breakable β-O-4 linkages, suggesting that moderate ether linkages were probably preserved at the end of batch pretreatment. ^{31}P and 2D HSQC NMR analyses were subsequently performed to test if this is true.[46, 47]
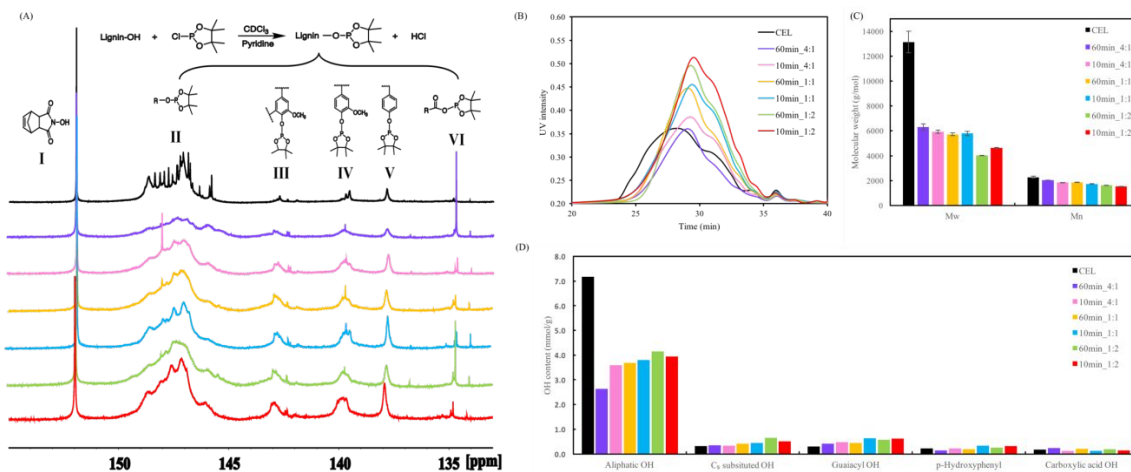
Figure 5. ^{31}P NMR and GPC analysis of CEL and Cyrene lignin. (A) Quantitative ^{31}P NMR spectra, (B) molecular weight distributions curves, (C) weight-average and number-average molecular weight of lignin, (D) contents of various aliphatic, phenolic, and carboxylic hydroxyl groups.
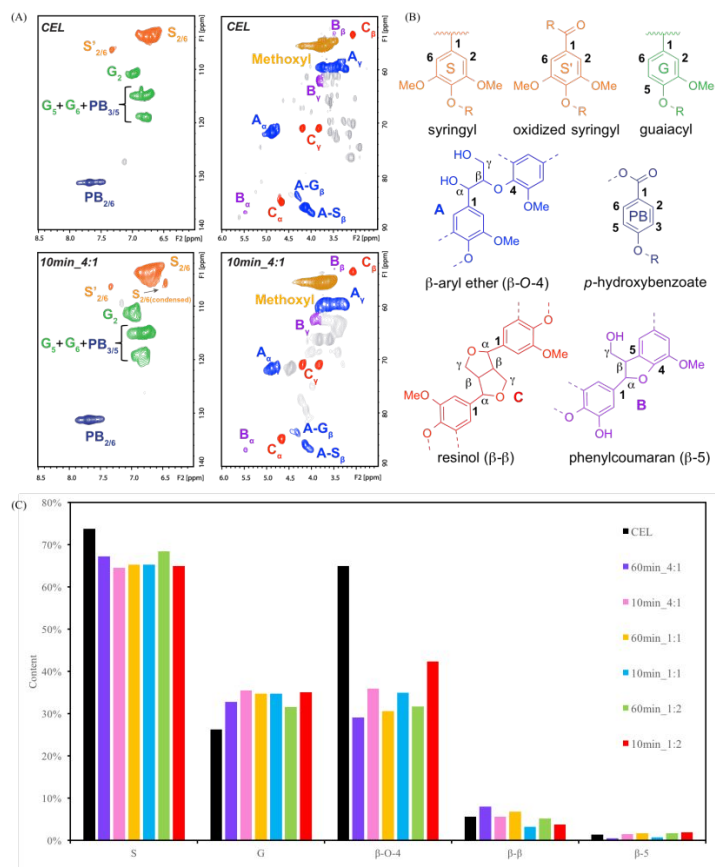
Figure 6. 2D HSQC NMR characterization of lignin. (A). Aromatic and aliphatic regions of NMR spectra from CEL and 10min_4:1 pretreated Cyrene lignin, (B). common lignin sub-units and inter-linkages, (C). semi-quantitative information for lignin subunits and inter-unit linkages

3.4.2. ^{31}P and HSQC NMR analysis

^{31}P NMR was first used to calculate the content of various hydroxyl (OH) groups including aliphatic, guaiacyl, C_5 substituted, p-hydroxy OH, and carboxylic acid present in lignin samples. Syringyl and condensed guaiacyl were combined into C_5 substituted phenolic units to prevent a possible overestimation of syringyl units or underestimation of condensed guaiacyl units according to a recent study.[48] Results show that the content of the aliphatic OH group decreased from 7.18 to ~2.16-4.15 mmol/g (**Fig. 5D**). On the other hand, the content of the phenolic OH group increased to different extents after the pretreatment, confirming the cleavage of lignin inter-units (mainly ether) linkages. Unlike common technical lignins such as Alcell and Indulin AT kraft lignin which typically have a large amount of C_5-substituted OH groups due to the recondensation reactions, Cyrene lignin has much lower C_5-substituted OH content, further suggesting that lignin condensation is quite limited as a result of the mild pretreatment conditions applied.[47] The contents of carboxylic and *p*-hydroxyphenyl OH were not dramatically affected by the pretreatment. **Fig. 6A** shows a detailed comparison of HSQC spectra of the 10min_4:1 Cyrene lignin and the control CEL, which is used as a representative of native lignin. The cross-peaks are identified according to the literature (Table S4).[40] As shown in the HSQC figure (**Fig. 6A and 6B**), the structure of solubilized lignin in Cyrene is very similar to the native lignin which contains various lignin substructures (S: syringyl, G: guaiacyl, PB: p-hydroxybenzoate) and inter-unit linkages (A: β-O-4, B: β-5, C: β-β). In particular, β-O-4 linkages remain as the predominant units (84% of total linkages), possibly due to the mild nature of pretreatment conditions (Table S5). A comparable amount of β-O-4 linkages was also preserved in GVL lignin at similar pretreatment conditions.[34] Shen et al. fractionated *Eucalyptus* by a renewable deep eutectic solvent derived from biomass (lactic acid) and analyzed the structure of lignin isolated from pretreated biomass at different conditions.[49] Results showed that the content of breakable aryl ether linkage significantly decreased from 69.5 (native) to 5.36 per 100 aromatic rings at the same pretreatment temperature (120 °C), and this number was further decreased to trace as the temperature slightly increased to 130 °C. Their study, along with several other

studies also reported that recondensation of S and G units could occur at elevated pretreatment temperatures (>110 °C), while only a small amount of condensed S units was detected at a noise level in our study.[49] Semi-quantitative analysis of lignin subunits and inter-unit linkages suggested that all the Cyrene lignin have high contents of syringyl units and β-O-4 linkages which favor the downstream valorization, especially the thermochemical depolymerization process (**Fig. 6C**). Moreover, the slight decrease of the S/G ratio after pretreatment indicated that S units are preferentially degraded over G units (Table S5).

The advantage of Cyrene pretreatment in preserving lignin integrity while maintaining relatively high delignification was demonstrated by comparing Cyrene lignin with lignin from other batch (Ethanol,[34] THF,[34] GVL,[34] DES[49]) and flow-through (formal acid[44, 50]) pretreatments from literature (see **Fig. 7**). Take delignification into consideration, Cyrene pretreatment showed comparative performance to GVL pretreatment. It is noticed that flow through fractionation is a promising method to extract non-condensed and structure-preserved lignin from the plant cell wall. Last but not least, because of the possible formation of germinal diol type of structure in Cyrene/water mixture (**Fig. S4**), a nucleophilic addition reaction between the solvent (i.e., diol) and the reactive benzyl carbocation at the α position of the side chain could occur, suppressing the possibility of subsequent acidolysis cleavage of ether linkages or further condensation reactions (i.e., C-C bond formation). However, this explanation needs to be further confirmed by the model compound study. One of the potential drawbacks of the Cyrene pretreatment is the need to distill larger amounts of water out to regain pure Cyrene, which is often energy-intensive. How to effectively recycle Cyrene from the co-solvent system and reuse it for further pretreatment without significant loss of its pretreatment performance is currently under investigation. The possibilities of running Cyrene pretreatment at a lower temperature (e.g., <100 °C) at the expense of a more prolonged pretreatment or lower Cyrene concentration at the expense of high temperature without the addition of acids are currently under investigation as well.
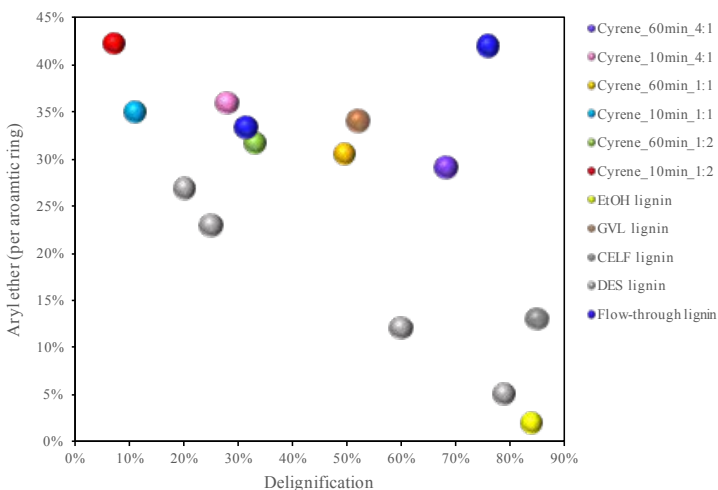
Figure 7. Relationship between delignification and content of preserved aryl ether linkages for different technical lignins.

4. Conclusion

In the modern biorefinery system, how to integrate lignin quality into the established sugar platform is very important but remains quite a challenge. A novel biorefinery concept using a renewable co-solvent system, including Cyrene and water, is introduced in this study to simultaneously achieve a good quality of carbohydrate and lignin. The addition of Cyrene helps improve lignin removal, creates more cellulose reducing end, and significantly increases the accessible surface area of cellulose. A near 100% cellulose conversion could be achieved for the enzymatic hydrolysis of Cyrene pretreated *Populus*. We also demonstrated that Cyrene pretreatment could be performed at a mild temperature (120 °C) to reduce the lignin condensation and avoid significant cleavage of β-O-4 linkages without compromising the lignin removal and solubility in the co-solvent system. The obtained Cyrene lignin could be a promising reactive material for a wide range of applications such as aromatic compounds, carbon fiber, and polyurethane precursors. The versatility of this pretreatment toward various other kinds of feedstock, such as grass, softwood, and agriculture energy crops, will be further investigated.

Acknowledgements

Conflicts of interest

The authors declare no conflict of interest.

Reference

1.   C. Huang, W. Lin, C. Lai, X. Li, Y. Jin and Q. Yong, *Bioresour. Technol.*, 2019, **285**, 121355.
2.   V. Archambault-Léger, Z. Losordo and L. R. Lynd, *Biofuels, Bioproducts and Biorefining*, 2015, **9**, 95-108.
3.   A. Aden and T. Foust, *Cellulose*, 2009, **16**, 535-545.
4.   D. Klein-Marcuschamer, B. A. Simmons and H. W. Blanch, *Biofuels, Bioproducts and Biorefining*, 2011, **5**, 562-569.
5.   A. Rodrigues Gurgel da Silva, M. Errico and B.-G. Rong, *Clean Technologies and Environmental Policy*, 2018, **20**, 1401-1412.
6.   R. J. Stoklosa, A. del Pilar Orjuela, L. da Costa Sousa, N. Uppugundla, D. L. Williams, B. E. Dale, D. B. Hodge and V. Balan, *Bioresour. Technol.*, 2017, **226**, 9-17.
7.   A. R. G. da Silva, C. E. Torres Ortega and B.-G. Rong, *Bioresour. Technol.*, 2016, **218**, 561-570.
8.   H. Olcay, R. Malina, A. A. Upadhye, J. I. Hileman, G. W. Huber and S. R. H. Barrett, *Energy & Environmental Science*, 2018, **11**, 2085-2101.
9.   F. K. Kazi, J. A. Fortman, R. P. Anex, D. D. Hsu, A. Aden, A. Dutta and G. Kothandaraman, *Fuel*, 2010, **89**, S20-S28.
10.   B. Yang and C. E. Wyman, *Biofuels Bioproducts Biorefining*, 2007, **2**.
11.   D. M. Alonso, S. H. Hakim, S. Zhou, W. Won, O. Hosseinaei, J. Tao, V. Garcia-Negron, A. H. Motagamwala, M. A. Mellmer, K. Huang, C. J. Houtman, N. Labbé, D. P. Harper, C. T. Maravelias, T. Runge and J. A. Dumesic, *Science Advances*, 2017, **3**, e1603301.
12.   L. Shuai, Y. M. Questell-Santiago and J. S. Luterbacher, *Green Chemistry*, 2016, **18**, 937-943.
13.   C. M. Cai, T. Zhang, R. Kumar and C. E. Wyman, *Green Chemistry*, 2013, **15**, 3140-3145.
14.   S.-X. Li, M.-F. Li, J. Bian, S.-N. Sun, F. Peng and Z.-M. Xue, *Bioresour. Technol.*, 2017, **243**, 1105-1111.
15.   A. M. Socha, R. Parthasarathi, J. Shi, S. Pattathil, D. Whyte, M. Bergeron, A. George, K. Tran, V. Stavila, S. Venkatachalam, M. G. Hahn, B. A. Simmons and S. Singh, *Proceedings of the National Academy of Sciences*, 2014, **111**, E3587-E3595.
16.   K. H. Kim, T. Dutta, J. Sun, B. Simmons and S. Singh, *Green Chemistry*, 2018, **20**, 809-815.
17.   K. H. Kim, A. Eudes, K. Jeong, C. G. Yoo, C. S. Kim and A. Ragauskas, *Proceedings of the National Academy of Sciences*, 2019, **116**, 13816.

18. S. H. Krishna, R. S. Assary, Q. A. Rashke, Z. R. Schmidt, L. A. Curtiss, J. A. Dumesic and G. W. Huber, *ACS Catalysis*, 2018, **8**, 3743-3753.

19. L. M. M. Mouterde, F. Allais and J. D. Stewart, *Green Chemistry*, 2018, **20**, 5528-5532.

20. J. Sherwood, M. De bruyn, A. Constantinou, L. Moity, C. R. McElroy, T. J. Farmer, T. Duncan, W. Raverty, A. J. Hunt and J. H. Clark, *Chem. Commun.*, 2014, **50**, 9650-9652.

21. L. Mistry, K. Mapesa, T. W. Bousfield and J. E. Camp, *Green Chemistry*, 2017, **19**, 2123-2128.

22. J. Zhang, G. B. White, M. D. Ryan, A. J. Hunt and M. J. Katz, *ACS Sustainable Chemistry & Engineering*, 2016, **4**, 7186-7192.

23. L. Hughes, C. R. McElroy, A. C. Whitwood and A. J. Hunt, *Green Chemistry*, 2018, **20**, 4423-4427.

24. P. Ray, T. Hughes, C. Smith, M. Hibbert, K. Saito and G. P. Simon, *Polymer Chemistry*, 2019, **10**, 3334-3341.

25. G. Wang, X. Liu, B. Yang, C. Si, A. M. Parvez, J. Jang and Y. Ni, *ACS Sustainable Chemistry & Engineering*, 2019, **7**, 10112-10120.

26. M. De bruyn, V. L. Budarin, A. Misefari, S. Shimizu, H. Fish, M. Cockett, A. J. Hunt, H. Hofstetter, B. M. Weckhuysen, J. H. Clark and D. J. Macquarrie, *ACS Sustainable Chemistry & Engineering*, 2019, **7**, 7878-7883.

27. C. G. Yoo, X. Meng, Y. Pu and A. J. Ragauskas, *Bioresour. Technol.*, 2020, **301**, 122784.

28. H. Dong, L. Zheng, P. Yu, Q. Jiang, Y. Wu, C. Huang and B. Yin, *ACS Sustainable Chemistry & Engineering*, 2020, **8**, 256-266.

29. S. Van den Bosch, W. Schutyser, R. Vanholme, T. Driessen, S. F. Koelewijn, T. Renders, B. De Meester, W. J. J. Huijgen, W. Dehaen, C. M. Courtin, B. Lagrain, W. Boerjan and B. F. Sels, *Energy & Environmental Science*, 2015, **8**, 1748-1763.

30. L. Shuai, M. T. Amiri, Y. M. Questell-Santiago, F. Heroguel, Y. Li, H. Kim, R. Meilan, C. Chapple, J. Ralph and J. S. Luterbacher, *Science (Washington, DC, U. S.)*, 2016, **354**, 329-333.

31. W. Lan and J. S. Luterbacher, *CHIMIA International Journal for Chemistry*, 2019, **73**, 591-598.

32. H. Wang, M. Tucker and Y. Ji, *J. Appl. Chem. (N. Y., NY, U. S.)*, 2013, DOI: 10.1155/2013/838645, 1-10.

33. A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker, *Determination of structural carbohydrates and lignin in biomass*, Laboratory Analytical Procedure, National Renewable Energy Laboratory, Golden, 2008.

34. X. Meng, S. Bhagia, Y. Wang, Y. Zhou, Y. Pu, J. R. Dunlap, L. Shuai, A. J. Ragauskas and C. G. Yoo, *Industrial Crops and Products*, 2020, **146**, 112144.

35. X. Meng, A. Parikh, B. Seemala, R. Kumar, Y. Pu, P. Christopher, C. E. Wyman, C. M. Cai and A. J. Ragauskas, *ACS Sustainable Chemistry & Engineering*, 2018, **6**, 8711-8718.

36. L. Jia, Y. Qin, P. Wen, T. Zhang and J. Zhang, *Bioresour. Technol.*, 2019, **278**, 440-443.

37. X. Meng, M. Foston, J. Leisen, J. DeMartini, C. E. Wyman and A. J. Ragauskas, *Bioresour. Technol.*, 2013, **144**, 467-476.

38. B. B. Hallac, P. Sannigrahi, Y. Pu, M. Ray, R. J. Murphy and A. J. Ragauskas, *Ind. Eng. Chem. Res.*, 2010, **49**.

39. Y. Pu, F. Hu, F. Huang, B. H. Davison and A. J. Ragauskas, *Biotechnology for Biofuels*, 2013, **6**, 1-13.

40. X. Meng, Y. Pu, C. G. Yoo, M. Li, G. Bali, D.-Y. Park, E. Gjersing, M. F. Davis, W. Muchero, G. A. Tuskan, T. J. Tschaplinski and A. J. Ragauskas, *ChemSusChem*, 2017, **10**, 139-150.
41. R. Samuel, Y. Pu, M. Foston and A. J. Ragauskas, *Biofuels*, 2010, **1**.
42. M. Foston and A. J. Ragauskas, *Biomass Bioenergy*, 2010, **34**, 1885-1895.
43. S. Bhagia and A. J. Ragauskas, *ACS Sustainable Chemistry & Engineering*, 2020, **8**, 34-37.
44. H. Zhou, J. Y. Xu, Y. Fu, H. Zhang, Z. Yuan, M. Qin and Z. Wang, *Green Chemistry*, 2019, **21**, 4625-4632.
45. L. Shuai and B. Saha, *Green Chemistry*, 2017, **19**, 3752-3758.
46. X. Meng, C. Crestini, H. Ben, N. Hao, Y. Pu, A. J. Ragauskas and D. S. Argyropoulos, *Nature Protocols*, 2019, **14**, 2627-2647.
47. S. Constant, H. L. J. Wienk, A. E. Frissen, P. d. Peinder, R. Boelens, D. S. van Es, R. J. H. Grisel, B. M. Weckhuysen, W. J. J. Huijgen, R. J. A. Gosselink and P. C. A. Bruijnincx, *Green Chemistry*, 2016, **18**, 2651-2665.
48. M. Balakshin and E. Capanema, *J. Wood Chem. Technol.*, 2015, **35**, 220-237.
49. X.-J. Shen, J.-L. Wen, Q.-Q. Mei, X. Chen, D. Sun, T.-Q. Yuan and R.-C. Sun, *Green Chemistry*, 2019, **21**, 275-283.
50. H. Zhou, L. Tan, Y. Fu, H. Zhang, N. Liu, M. Qin and Z. Wang, *ChemSusChem*, 2019, **12**, 1213-1221.

Table of Content
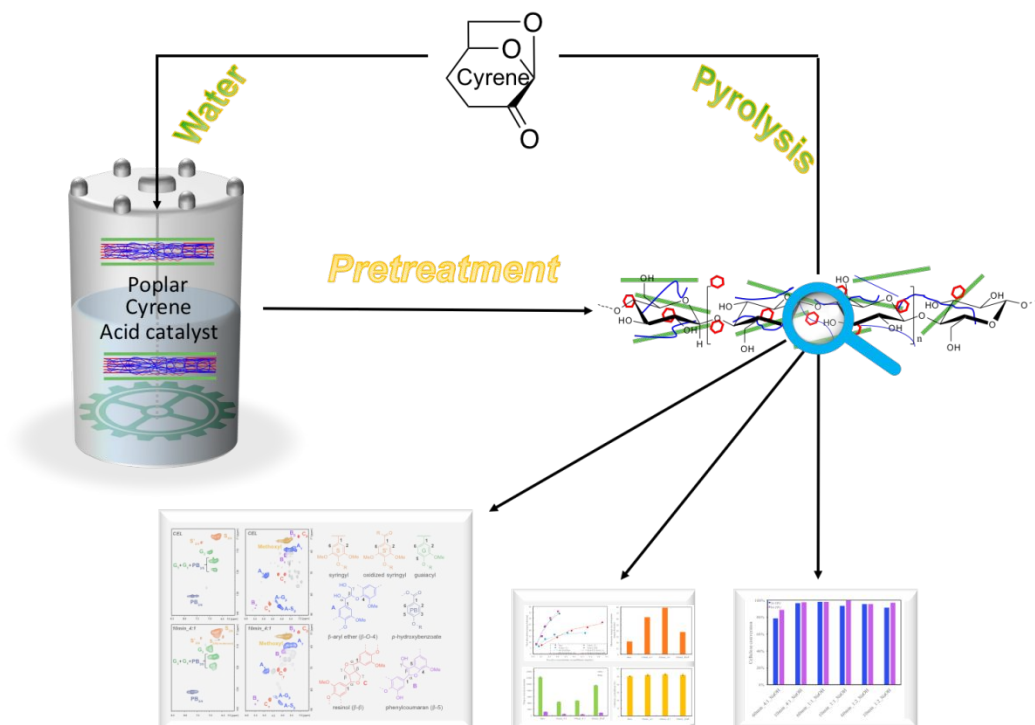
To fulfill the sustainability criteria of future biorefineries, a novel renewable biomass pretreatment using Cyrene was developed for the first time.