## Title

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# Deconstruction of Woody Biomass via Protic and Aprotic Ionic Liquid Pretreatment for Ethanol Production

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**ABSTRACT**

Ionic liquids (ILs) have emerged as important solvents for conversion of lignocellulosic feedstocks to fuels and chemicals due to their ability to enable efficient biomass deconstruction and fractionation. Woody biomass derived from forest and agricultural residues has the potential to be used for production of biofuels and it's removal from forests can help mitigate disastrous wildfires in fire-prone states like California. This study evaluated woody biomass types (pine, almond, walnut, fir) from California as potential biofuel feedstocks. The feedstocks were pretreated with the ILs cholinium lysinate ([Ch][Lys]) and ethanolamine acetate ([EOA][OAc]), followed by enzymatic hydrolysis and fermentation of lignocellulosic sugars to produce ethanol. Under optimal conditions, [EOA][OAc] pretreatment and enzymatic hydrolysis generated glucose and xylose yields in the range of 24-82% and 14-80%, respectively, while glucose and xylose yield for the [Ch][Lys] ranged between 28-83% and 23-80%, respectively. Maximum fermentable sugar was released from almond wood and the lowest amount was from pine and fir. Blends of feedstocks were also explored and a blend with a mass ratio of 2/2/1 (almond:walnut:pine) resulted in maximum glucose and xylose (> 90%) yields using [Ch][Lys]. Fermentation of this hydrolysate using a C5-utilizing strain of *Saccharomyces cerevisiae* resulted in a maximum ethanol concentration of 17.9 g/L for mixture biomass hydrolysate, corresponding to 60.8 % fermentation efficiency. This study represents the first demonstration of the use of these ILs for pretreatment of woody biomass blends that resulted in a high overall conversion efficiency for ethanol production.

**Keywords:** Ionic liquid, pretreatment, woody biomass, fermentation, ethanol

## 1. INTRODUCTION

Deconstruction of lignocellulosic biomass and conversion into biofuels and bioproducts is key route towards a sustainable bioeconomy, paving the path for economic growth, mitigating climate change, ensuring future energy security, and improving human and ecological health.[1] Lignocellulose deconstruction is a critical step to enable efficient downstream processing, and requires a pretreatment step to reduce the recalcitrance to enzymatic hydrolysis of lignocellulosic polysaccharides.[2, 3] Over the last two decades, an array of pretreatment technologies have been developed, facilitating cellulose accessibility to hydrolytic enzymes by increasing surface area, disrupting the rigid cell wall structure, and/or removing lignin and hemicelluloses.[4-6] Major challenges to attaining scalable biomass conversion include water and power consumption, consolidation of unit operations, utilizing mixed biomass, maximization of hexose and pentose sugar yields, solvent recycling, and lignin upgradation.[7, 8] Among all the pretreatment technologies, ionic liquids (ILs), a group of molten salts (melting point ≤ 100 ℃) with unique physicochemical properties, have shown significant promise.[9] ILs are often referred as designer or task-specific solvents due to their ability to combine different cations and anions to tune their functional properties, and have therefore garnered significant interest for researchers in the biofuel industry.[10, 11]

Since the discovery of certain imidazolium ILs' ability to dissolve cellulose, application of imidazolium ILs on lignocellulosic biomass increased exponentially.[12-14] Typically, these

classes of ILs can dissolve lignin and cellulose, and the cellulose-rich fraction can be regenerated from the IL by addition of an antisolvent. In addition to other interactions, strong hydrogen-bonding basicity is known to be a critical parameter for ILs in achieving biomass dissolution.[15-17] Although precipitation of the dissolved material was effective, IL cost, recycling and biocompatibility for downstream processing remains a challenge.[14] To overcome cost and biocompatibility issues, certain choline-based aprotic ILs were investigated and have been demonstrated for compatibility with both enzymes and microbes, allowing the transition towards a one-pot reaction system where all the steps of the process are carried out in a single vessel without any separations.[17] [Ch][Lys] has been effectively used and achieves high cellulose to glucose conversion yields in grasses and more recently in hardwoods and softwoods.[18] To date, we have demonstrated that [Ch][Lys] is effective at promoting high glucose yields in switchgrass (96.5%) and corn stover (>80%).[19, 20] Another category of ILs, called protic ILs (PIL), has also been investigated. The development of protic ILs formed by reversible proton (ion species) transfer precedes the development of aprotic ILs where ion formation results from covalent bond formation and breaking.[21] The susceptible proton inherent to protic ILs is a barrier in lignocellulosic biomass dissolution because of weaker hydrogen-bonding basicity interaction. Hence, whole lignocellulosic biomass dissolution has not been widely demonstrated in a protic IL. However, certain protic ILs can dissolve lignin from biomass and this appears to enable efficient enzymatic deconstruction.[22] In addition, certain protic ILs have been labeled as low cost largely due to more simple synthesis. In our prior findings we reported that a one-pot integrated bioconversion with a low-cost IL (~$1 per kg): ([EOA][OAc]) resulted in a glucose yield of 85% from switchgrass.[22]

Apart from pretreatment, biomass selection and logistics plays a vital role in the effectiveness of a biorefinery. Woody biomass feedstocks are promising raw materials due to their availability, fast growth, and adaptability.[23] Unlike herbaceous biomass, woody biomass has diverse sources including forest residues, timber and milling residues, agricultural residues, and furniture waste, which are available throughout the year to reduce the long-term storage cost.[24, 25] In California, woody biomass has the potential to be a feedstock for production of biofuel and bioproducts, with an estimated projection of there being 38 million bone dry tonnes available annually by 2050.[26] Utilization of woody biomass from forest residues can provide a beneficial end use to mitigate wildfire occurrences while providing employment in the local community. Compositionally, woody biomass has higher carbohydrate content compared to herbaceous biomass, making it economically attractive for biofuel conversion. However, woody biomass is more recalcitrant than herbaceous biomass due to higher lignin content and provides challenges in biomass pretreatment owing to high cellulose crystallinity and the presence of toxic extractives.[23] Therefore, to exploit the value of softwood and hardwood to the maximum extent, effective fractionation of its constituents through green and sustainable processes is essential.[27, 28]

In this work, various California-based woody biomass feedstocks were studied and optimized to efficiently release fermentable sugars via IL pretreatment and enzymatic hydrolysis. Two IL pretreatment methods were studied during process optimization and scale up, 1) IL pretreatment with 100 wt% [EOA][OAc, called “neat” IL pretreatment and 2) an aqueous IL method using 10 wt% [Ch][Lys] in water. This latter process can be implemented in a bioprocess where all the steps of pretreatment, hydrolysis, and fermentation are combined into a single vessel, called “one-pot”. The effects of temperature, solid loading, enzyme loading, reaction time, and particle size were then investigated to maximize glucose and xylose yields. Next, the

one-pot process was scaled-up to 10 L using both pine alone and a 2/2/1 mixture of almond, walnut, and pine, then fermentation was performed on the resulting hydrolysates using C5-utilizing strain of *Saccharomyces cerevisiae* to produce ethanol.

## 2. MATERIALS AND METHODS

### *2.1. Materials*

Pine, fir, walnut, almond, were donated by Ametis, Inc. (Cupertino, CA, USA) and used as received. They are all California woody biomass obtained from Paddock In. in Oakdale, CA. The almond and walnut wood waste were procured from local orchards, while the pine wood was obtained from forest thinning. The biomass was dried for 24-48 h in a 40 ºC oven. Subsequently, it was a knife-milled with a 2 mm screen (Thomas-Wiley Model 4, Swedesboro, NJ). The resulting biomass was then placed in leak-proof bags and stored in a cool dry place. Additionally, a small portion of the pine and fir were further sieved (mesh #270 with ~ 50 micron opening) to obtain a separate fraction with smaller particle sizes. The following chemicals were purchased from Sigma Aldrich (St. Louis, MO) and used as received: hydroxyethylamine, (ACS reagent, 99.0% purity), acetic acid (ACS reagent, 99.7% purity), Choline hydroxide (46% in $H_2O$). L-lysine monohydrate was purchased from VWR and hydrochloric acid (36-37.5%) was purchased from J.T. Baker (Phillipsburg, NJ) and used without further purification. The enzymes (Cellic® CTec3 and HTec3) were procured from Novozymes North America (Franklinton, NC).

### *2.2. Methods*

*2.2.1 Chemical Synthesis*

*2.2.1.1 Aprotic Ionic Liquid ([Ch][Lys])*

Lysine monohydrate (0.4 mol, 65.68 g) was weighed into a 500 mL round bottom flask and dissolved in 100 mL deionized water at room temperature to obtain a clear solution (light lime-yellow). Then the flask was mounted on an ice-bath (3-5 ℃) and $N_2$ was purged for 20-30 mins. Next 46 wt% of choline hydroxide in water (0.4 mol, 105.15 g) was added dropwise to lysine solution while maintaining the temperature of the ice-bath (3-5 ℃). The mixture was stirred for 48 h at room temperature. Excess water was removed under reduced pressure and the mixture was added to acetonitrile/methanol (9:1, v/v) to remove the excess starting materials. Finally, the solvents were removed under reduced pressure and the mixture was freeze-dried to get the final product (Yield~ 95%, light orange). The product, thus obtained, was characterized by 1H-NMR (Fig. S1) using MeOD as an external lock solvent.

*2.2.1.2. Protic Ionic Liquid (Ethanolammonium Acetate)*

[EOA][OAc] was synthesized by the equimolar addition of the acid and bases as neat reagents (based on the stoichiometric requirements) to eliminate both the need for solvent and the introduction of incidental water. The [EOA][OAc] was synthesized using a round-bottom flask equipped with two addition funnels; one for the acid and one for the base. The reagents (ethanolamine and acetic acid) were slowly added into the flasks and homogenized with a magnetic stirring bar. The flask was mounted in an ice/water bath (~4 ℃) to prevent heat buildup during the reaction. After complete addition, the reaction was continued for 24 h. The

final product was characterized with 1H-NMR (Fig. S2) using DMSO-d6 as an external lock solvent.

*2.2.2 Biomass pretreatment*

*2.2.2.1. One pot pretreatment and enzymatic hydrolysis*

Biomass pretreatment was carried out at a solid loading of 15 wt% in a one-pot configuration. Typically, 0.75 g of biomass was mixed thoroughly with 4.25 g of IL in a pressure tube (15 mL, Ace Glass Inc., Vineland, NJ), followed by heating in an oil bath at 140-160 °C for 3-6 hours. Post pretreatment, 10 M HCl was added to adjust the pH of the biomass slurry to 5. Subsequently, 20-30 mg protein/ g of biomass of commercial enzyme mixtures, Cellic CTec3 and HTec3 (9:1 v/v) was added to the biomass slurry to carry out saccharification at 50 °C for 72 hours at 48 rpm in a rotary incubator (Enviro-Genie, Scientific Industries, Inc.). After hydrolysis, liquid samples were collected and centrifuged at 12,000 rpm for 2 minutes and the supernatant was filtered using 0.45 μm centrifuge filters before performing sugar analysis, as described below.

*2.2.2.2. Conventional pretreatment and enzymatic hydrolysis*

For the PIL, the pretreatment was carried out using the conventional method that involves washing to remove the IL after pretreatment. In a typical experiment, biomass and ionic liquid were loaded into an ace pressure tube (50 mL, Ace Glass Inc., Vineland, NJ) and homogenized. The solid loading was set at 15 wt % solids (based on a 1 g biomass scale) and heated in an oil bath set to 140 ºC for 3 h. After cooling for 30 min, the mixture was washed with 200 mL deionized water. The solid fraction was recovered via centrifugation, then lyophilized for further

use. Enzymatic hydrolysis of the pretreated biomass was carried out in 0.1 M citrate buffer (pH 5), 1 V/V% $NaN_3$ and 30 mg protein/g biomass using a 9:1 mixture of the CTec3/Htec3. The mixture was subsequently incubated at 50 ºC for 72 h. at 50 rpm in a rotary incubator (Enviro-Genie, Scientific Industries, Inc.) The amount of sugars released from the supernatant were quantified using HPLC after the incubation was completed.

The selection of different pretreatment time for both the pretreatment corresponds to the severity factor ($\log R_0$) calculated as described by equation 1.[29]

$$\log R_0 = \log\left[t \times \exp\left((T-100)/14.75\right)\right] \quad (1)$$

Where, $t$ is the treatment time (min), $T$ is the reaction temperature (℃). All the pretreatment experiments were performed in duplicates and standard deviation was estimated to represent errors.

*2.2.3 Compositional Analysis and Analytical techniques*

The dried biomass samples were extracted sequentially using solvents (water (W), 80% ethanol/ water (E), and acetone, (A)).[30] Typically, 1 g of biomass was combined to a tube containing 40 mL of the solvent of choice. The mixture was then homogenized, sonicated for 20 minutes, and then centrifuged (10 min, 4000 RPM) to separate the extracts/solvents from the residual biomass. This extraction cycle was carried out 5 times for each biomass/solvent. Finally, the residual biomass was dried overnight at 40 °C and utilized for the compositional analyses. Compositional analysis of the untreated sorghum was performed to determine the glucan, xylan, and klason lignin following the two-step acid hydrolysis procedure described by NREL.[31, 32] In summary, 300 mg of the dry extractive-free biomass was exposed to 3 mL of 72% w/w $H_2SO_4$, at 30 ºC for

1 hour, followed by secondary hydrolysis at 4% w/w $H_2SO_4$ at 121 ºC for 1 hour. After the two-step acid hydrolysis, acid-insoluble lignin was obtained by filtering the hydrolysates through filter crucibles. Klason lignin was determined by subtracting the weight of oven-dried residual solids (105 ºC) and the ash content (575 ºC). Monomeric sugars (glucose and xylose) were determined by HPLC using an Agilent 1200 series instrument equipped with a refractive index detector and Bio-Rad Aminex HPX-87H column, coupled with a guard column assembly. Product separation was obtained at 60 °C with 4 mM $H_2SO_4$ as a mobile phase at a flow rate of 0.6 mL/min.

Phenolics were analyzed using an HPLC-ESI-TOF-MS as previously described.[33, 34] A 6-point calibration curve of pure chemical standard was used for the absolute quantification of each corresponding analyte. The theoretical m/z of the deprotonated analytes were used to quantify the phenolics of interest; ferulic acid (193.050632), 4-hydroxybenzoic acid (137.024418), protocatechuic acid (153.019332), vanillic acid (167.034982), salicylic acid (137.024418), syringic acid (197.045547), p-coumaric acid (163.040068), vanillin (151.040068), sinapinaldehyde (207.066282), 4-hydroxycinnamyl aldehyde (147.045153), and coniferyl aldehyde (177.055718).

*2.2.4 Scale-up of ionic liquid pretreatment and enzymatic saccharification*

After the optimization of the pretreatment and saccharification conditions at bench-scale, one-pot ionic liquid pretreatment and saccharification process was scaled-up to 10 L in a Hastelloy C276 Parr vessel (Parr Instrument Company, model: 4555-58, Moline, IL, USA). Two biomass solid loading conditions were evaluated (15% and 25% solid loading) with a 3 Kg final

working weight. Pine and a mixture of pine, almond and walnut (1:2:2) were used as substrate. Pretreatment conditions for all experiments were: 10% wt. [Ch][Lys], 160°C, 50 rpm for 3 h. After pretreatment, the reaction was cooled down to room temperature and 50% (w/w) $H_2SO_4$ was used to adjust the pH to 5. Saccharification step was conducted at 50°C with agitation at 50 rpm for 72 h. Enzyme loading for each process was 30 mg/g biomass with CTec3:HTec3 ratio of 9:1.

*2.2.5 Yeast strain and seed cultivation*

The xylose utilizing yeast strain used for this study, *Saccharomyces cerevisiae* NZ 22202, was engineered by Novozymes and maintained in a 25% (w/v) glycerol stock solution at -80°C. Cells were cultured in a two-tiered seed train. First, cells were grown in 250 mL baffled flasks containing 50 mL YPD media (10 $g.L^{-1}$ yeast extract, 20 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose and 10 $g.L^{-1}$ xylose) inoculated with cell suspension from glycerol stock. Seed 2 was inoculated using a 10% (v/v) inoculum size with cells grown in the YPD media in 250 mL baffled shake flasks with 50 mL of 50:50 mixture of YPD and filtered hydrolysate. In both steps, the cells were incubated at 30°C at 220 rpm for 24 h. The YPD media and hydrolysate were filtered sterilized with 0.2 mm pore filters and 100,000 U/L Penicillin and 100 mg/L Streptomycin were added prior inoculation. Microbial growth was measured by optical density at 600 nm in a spectrophotometer (Thermo Scientific™ GENESYS™ 10S UV-Vis Spectrophotometer.

*2.2.6 Fermentation*

Fermentation experiments were carried out in 100 mL sealed glass bottles with 80 mL working volume. Each bottle was aseptically batched with 72 mL filtered/unfiltered hydrolysate

and 8 mL inoculum from Seed 2. All experiments were performed at 100 rpm for 6 days and 50,000 U/L Penicillin and 50 mg/L Streptomycin were added prior fermentation. Filtered hydrolysate was prepared by centrifuging the hydrolysate at 4000 xg for 20 min to remove the solids and filtered-sterilized (0.2 mm) prior to use.

## 3. RESULTS AND DISCUSSION

### *3.1 Biomass composition and extractives*

Compositional analysis (Table 1) of the biomass was determined, first examining the extractives, then the polysaccharide and lignin content. The total extractives for almond, pine, fir, and walnut were 38.4, 17.5, 34.3, 34.0 % of biomass, respectively. The extractives in woody biomass (softwood and hardwood) are typically composed of free sugars, terpenoids, fatty acids, and phenolics.[35, 36] Free phenolics tend to be major constituent of the extractives and are attractive due to their potential utility as bioactives, such as antioxidant and anti-UV agents.[37]

Figure 1 shows the composition of the phenolic extractives recovered from the biomass using ethanol and water. The ethanol extract from softwoods contained predominantly protocatechuic acid, vanillic acid, salicylic acid and p-coumaric acid with trace amounts of syringic acid, p-coumaric acid, sinapine aldehyde, and coniferyl aldehyde. On the other hand, the hardwoods generated a larger amount of phenolics with both water and ethanol. The water extracts were predominantly 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, and syringic acid for almond, while walnut comprised of ferulic acid, protocatechuic acid, vanillic acid, and syringic acid. Similarly, the ethanol extract consisted mainly of protocatechuic acid and salicylic acid for almond, whereas walnut extractives composed of ferulic acid, protocatechuic acid, vanillic acid,

salicylic acid, and coniferyl aldehyde. For additional extractive characterization, different solvents and different analytical techniques could be used to determine the complete composition of components, such as free sugars, terpenoids, fatty acids etc., that are present within the raw biomass.

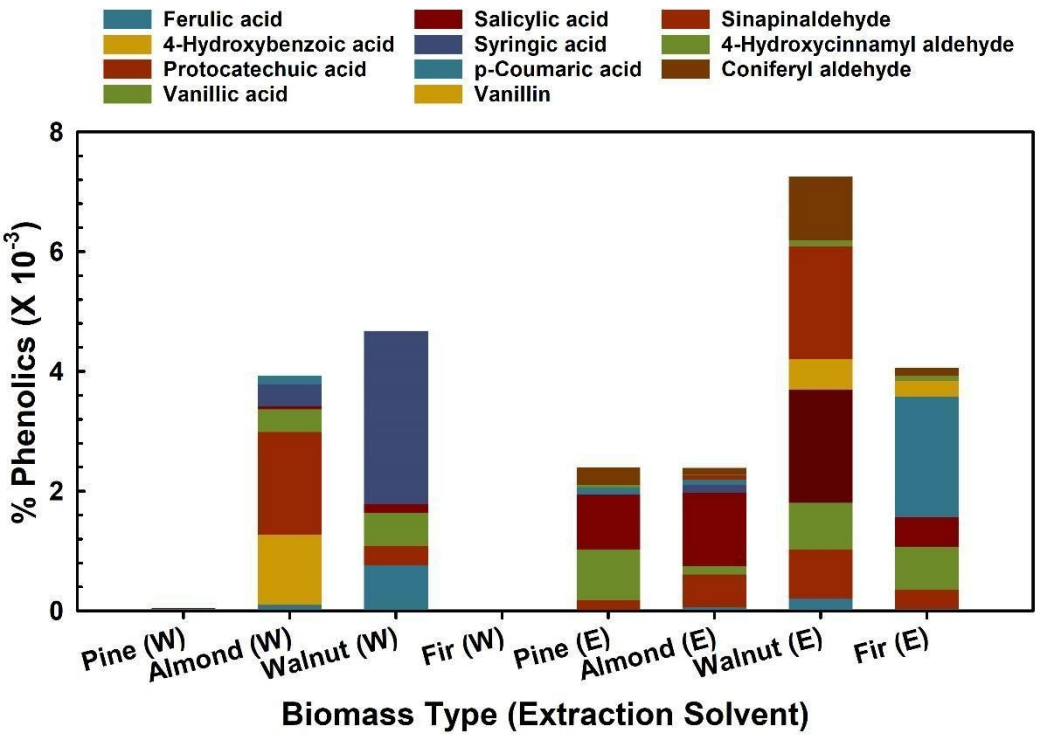


**Figure 1.** Extractive composition (phenolic content) for four woody biomasses using water (W) and 80% (v/v) ethanol (E).

Examining the polysaccharide content, the highest glucan content of 33.0% was observed in pine, while almond had lowest glucan content of 22.3%. The xylan content of almond (11.1% of biomass) and walnut (10.6 % of biomass) were very similar, while pine (14.0% of biomass) and fir (12.0 % of biomass) were significantly different. Combining both glucan and xylan, the total fermentable sugars for the four biomasses ranged from 33.3-47.0 % of dry biomass. In addition to cellulose and hemicellulose, lignin is another vital building block of the plant cell wall that

accounts for approximately 10–30% of the biomass. Lignin content for almond, pine, fir, and walnut were 20.0, 27.4, 23.4, 20.0 % of biomass, respectively. The presence of high lignin content has been associated with biomass recalcitrance to deconstruction into fermentable sugars, so these feedstocks are expected to be challenging material for conversion to biofuel.[38] One positive aspect of a higher lignin content is that provides the opportunity for upgrading it into value added products such as phenols, activated carbons, composites, energy storage materials, and antimicrobial agents, to name a few.[39-41] Overall, the results illustrate that compositionally woody biomass is a promising feedstock for the production of biofuels and platform chemicals; however, differences in composition could lead to variability in the process design and performance, and in the product streams that are generated.

Table 1. Chemical composition of four woody biomass

| **Biomass** | **Extractives (%)** | **Glucan (%)** | **Xylan (%)** | **Klason Lignin (%)** | **Ash (%)** |
|---|---|---|---|---|---|
| Almond | 38.4 ± 4.0 | 22.3 ± 2.3 | 11.1 ± 1.2 | 20.0 ± 2.4 | 0.0 ± 0.0 |
| Pine | 17.5 ± 0.5 | 33.0 ± 1.0 | 14.0 ± 0.4 | 27.4 ± 4.1 | 0.1 ± 0.0 |
| Fir | 34.3 ± 0.3 | 28.5 ± 0.3 | 12.0 ± 0.2 | 23.4 ± 1.9 | 0.1 ± 0.0 |
| Walnut | 34.0 ± 0.3 | 26.5 ± 0.3 | 10.6 ± 0.3 | 20.0 ± 1.5 | 0.1 ± 0.0 |

*3.2 Fermentable sugar yields from woody biomass using ionic liquids*

To establish a baseline to compare the performance of different pretreatment methods, glucose and xylose yields from the four biomasses was determined by performing enzymatic hydrolysis at 20 and 30 mg protein/g biomass of enzyme without any pretreatment (Fig. S3). Glucose yields (Fig. S3 A) for the biomasses at 20 mg enzyme protein/ g biomass were between 11.5-15.6% of

dry biomass. Under similar enzymes loading xylose yield ranged between 4.5-6.9% of dry biomass. The total sugar released for all the biomass varied between 16.0-21.8 % of dry biomass, with lowest and highest total sugar yields obtained from walnut and pine, respectively. Similarly, glucose yield (Fig. S3 B) for almond, pine, fir, and walnut at 30 mg enzyme protein/ g biomass ranged between 16.5-21.5% of dry biomass, while xylose yield fell between 6.5-13.1% of dry biomass. There is a slight increase in xylose release for almonds as the enzyme loading is increased. The total sugar released for all the biomass varied between 23.0-34.6 % of dry biomass, with lowest and highest total sugar yields obtained from walnut and almond, respectively.

To improve the fermentable sugar released, all the biomasses were pretreated with [Ch][Lys] and [EOA][OAc] with three different pretreatment severity factors (SF) of 3.4, 4.0, and 4.3 corresponding to different reaction time and temperature. The selection of different pretreatment time for both the pretreatment corresponds to the severity factor Log $R_0$ calculated as described by equation 1. The pretreatment severity range was chosen from past experience that suggested it would give the highest total glucose plus xylose yields from the combined operations of pretreatment and enzymatic saccharification.

*3.2.1 Impact of [Ch][Lys]*

Unlike traditional biomass conversion technologies, “one-pot” conversion of lignocellulosic biomass to fuels can reduce the operating cost by consolidating three (pretreatment, saccharification, and fermentation) unit operations and reduces the energy input for the mass transfer between reactors.[20] Previous studies have shown that the IL [Ch][Lys] diluted into water

can be as effective as the pure IL for pretreating biomass and extracting lignin.[42, 43] In addition to its ability to solubilize lignin when diluted in water, it also has low enzyme inhibition and low toxicity to microbes, making it an ideal candidate for one-pot approach.[20] Figure 2A lists the glucose and xylose yields from all the four biomasses at 140 °C, 3 h, (SF=3.4) and 20 mg protein/g biomass of enzyme. Results show that under the SF of 3.4, glucose yield for the four biomasses ranged between 22.5 (pine)-76.5 (almond & walnut) % of dry biomass. Similarly, xylose yield fell between 17.6 (pine)-94.4 (walnut)% of dry biomass. Further increasing the pretreatment severity to 4.0 (Fig. 2 B) and keeping 20 mg protein/g biomass of enzyme loading constant lead to an increase in the fermentable sugar release for almond and pine, while a decrease was noticed for fir and walnut. The average sugar released from all the four biomasses varied between 20.4-81.5% of dry biomass, with pine showing the highest 17% increase in total sugar released, whereas fir showed a 25% decrease in the average sugar released. Keeping the pretreatment severity (4.0) constant, an increased enzyme loading of 30 mg protein/g biomass led to a significant increase in glucose yield for almond and pine (Fig. 2 C). Glucose yield for almond, pine, fir, and walnut were 83.0, 28.2, 29.3, 58.2% of dry biomass, respectively, while xylose yield for almond, pine, fir, and walnut were 80.0, 22.5, 28.1, 77.0 % of dry biomass, respectively. Taken together, pretreatment at 160 ℃, 3 h, (SF= 4) and 30 mg protein/g biomass of enzyme was selected as the best condition to maximize fermentable sugars from almond, pine, and fir, which was used as a benchmark for further optimization experiments.

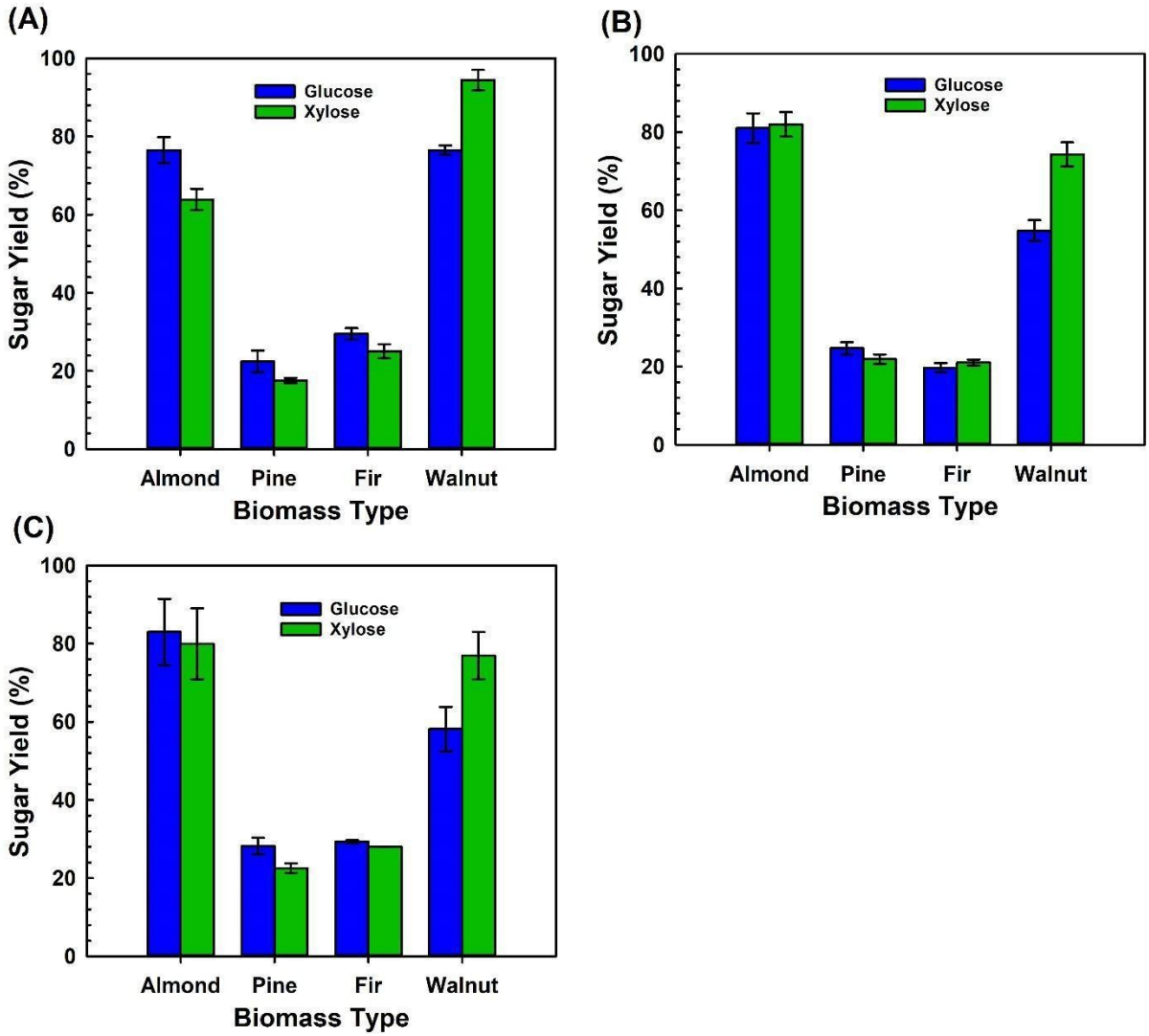


**Figure 2**. Glucose and xylose yield from enzymatic hydrolysis of [Ch][Lys] pretreated samples: A) 140 ℃, 3 h, and 20 mg protein/g biomass of enzyme; B) 160 ℃, 3 h, and 20 mg protein/g biomass of enzyme; C) 160 ℃, 3 h, and 30 mg protein/g biomass of enzyme.

*3.2.2 Impact of [EOA[OAc]*

The use of PIL for biomass pretreatment is a cost competitive option that has been reported to reduce ethanol selling process by up to 40% as compared to more conventional ILs.[22] In

particular, the biocompatible PIL ([EOA][OAc]) is associated with a low cost (~$1 per kg) and has been used for the integrated biofuel production (without pH adjustments, water-wash and solid–liquid separations). Figures 3 A-C show the glucose and xylose yields from the enzymatic hydrolysis of [EOA][OAc] pretreated biomass at 140 and 160 ℃, using 20-30 mg of enzyme protein/ g of starting biomass. Figure 3 A illustrates the glucose and xylose yields from all the four biomasses at 140 °C, 3 h, (SF=3.4) and 20 mg protein/g biomass of enzyme. The results show that under the SF of 3.4, glucose yield for almond, pine, fir, and walnut were 35.0, 18.7, 21.0, 38.3 % of dry biomass, respectively. Similarly, xylose yield for almond, pine, fir, and walnut were 27.9, 11.3, 15.5, 49.2 % of dry biomass, respectively. Despite the improvement in sugar release, neither of the processes employed could fully release the sugars present within the biomass (see Table 1). Therefore, additional optimization was carried out by further increasing the pretreatment severity to 4.0 and keeping 20 mg protein/g biomass of enzyme loading constant. This led to an increase in the fermentable sugar release for almond and walnut, while there was a negligible change for pine and fir. This represents an average improvement of 29% and 32% for glucose and xylose yields respectively (compared to SF=3.4). Keeping the pretreatment severity (4.0) constant, an increased enzyme loading of 30 mg protein/g biomass was employed and led to a significant increase in glucose yield for almond and walnut (Fig. 3 C). The glucose yield for the four-biomass ranged between 24.2 (pine)-82.4 (almond)% of dry biomass, while xylose yield ranged between 14.3 (pine)-79.8 (walnut)%. This indicates an average of 31% total sugar increase (amongst all four biomasses), because of the increased enzyme loading. Based on these results, pretreatment at 160 ℃, 3 h, (SF= 4) and 30 mg protein/

g biomass of enzyme was selected as the best pretreatment condition to maximize fermentable sugars and was used as a benchmark for further optimization experiments.

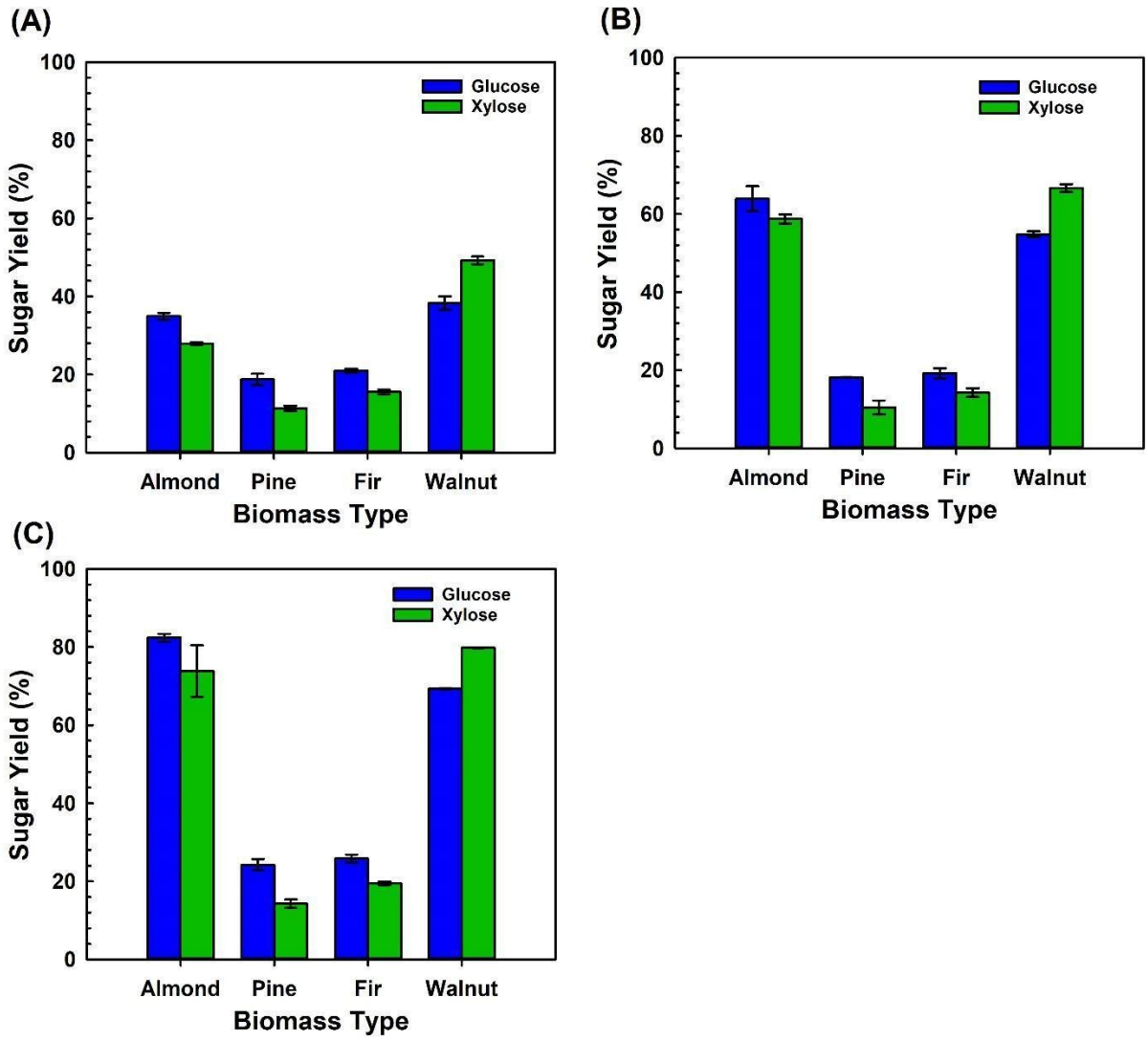


**Figure 3**. Glucose and xylose yields after enzymatic hydrolysis of [EOA][OAc] pretreated samples: A) 140 ℃, 3 h, and 20 mg protein/g biomass of enzyme; B) 160 ℃, 3 h, and 20 mg protein/g biomass of enzyme; C) 160 ℃, 3 h, and 30 mg protein/g biomass of enzyme.

Overall, [Ch][Lys] and [EOA][OAc] pretreatment on woody biomass were effective at releasing fermentable sugars. Results show that under tested conditions both the IL's were able to unlock

the highest sugar releasing threshold for almond and walnut. However, under those conditions pine and fir showed little or no change in total sugar yield. Hence, further optimization to experimental conditions were performed to improve the sugar yields for pine and fir.

*3.3 Optimization of sugar release from pine and fir*

Due to their recalcitrance to deconstruction, further process optimization via the severity factor, enzyme loading, and particle size was carried out for the softwoods (Pine and Fir) to improve fermentable sugar release. Figures S4 A-B show the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] and [EOA][OAc] pretreated biomass at SF of 4.3 using 30 mg of enzyme protein/ g of starting biomass. The average sugar yields for pine and fir in presence of the ILs at SF of 3.4 and 4.0 showed little or no change. However, further increasing the SF to 4.3 resulted in a decrease in average sugar yields for both pine and fir, which can be attributed to the degradation of sugars and polysaccharides at the more severe pretreatment condition.[8] Enzyme doses and pretreatment conditions need to be optimized to make the conversion process economically viable.[44, 45] Although high yields can be realized by applying high enzyme loadings post biomass pretreatment, from a commercial and economic standpoint, the lowest possible amount of enzyme must be used to maximize fermentable sugar release.[46] Figures S5 A-B show the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] and [EOA][OAc] pretreated biomass at 160 ℃, 3 h, using ~ 27 mg of enzyme protein/ g of starting biomass at a ratio of 7/3 Ctec3/HTec3. Results for both the ILs illustrate that, under the constant cellulase enzyme loadings, a decrease in glucose yield was observed. However, under the high xylanase enzyme loadings, xylose yield for pine and fir with [Ch][Lys] showed little or no change, but

after pretreatment with [EOA][OAc] an increase of 24% and 22% in xylose yields for pine and fir, respectively was observed.

It is commonly known that biomass particle size can substantially affect the efficiency of pretreatment and subsequent enzymatic hydrolysis. Particle size reduction enhances the effective surface area to volume ratio, facilitating enzyme accessibility into their substrates within the feedstocks.[47, 48] Figure S6 A show the glucose and xylose yields from the enzymatic hydrolysis of [Ch][Lys] pretreated biomass at 50 μm, 160 °C, 3 h, using 30 mg of enzyme protein/ g of starting biomass at a ratio of 9/1 Ctec3/HTec3. There was a significant increase in the average sugar yield for pine (43.4% of dry biomass) and fir (44.4% of dry biomass), which accounts for an increase of 62% for pine and 48% for fir compared to results at 2mm particle size. For the [EOA][OAc] pretreated biomass, there was an 85% vs 16% and 89% vs 26% increase in glucose vs xylose yields for pine and fir, respectively, compared to the analogous 2mm particle size pretreatments. The reduction in particle size improves mass transfer and allows the IL's to penetrate the biomass' cell wall to disrupt the lignin carbohydrate complex. Nevertheless, biomass comminution is an energy intensive strategy that is not practical at larger scales. Overall, the decrease in particle size was effective at further deconstructing the biomass, reducing its recalcitrance, thereby, improving the sugar yields. However, modification of the pretreatment severity and the ratios of the biomass-deconstructing enzyme cocktail did not improve the fermentable sugar release.

*3.4 Mixed biomass and intermediate scale up*

In order for a biorefinery to maintain productivity and profitability, it must use feedstocks that are readily available at an affordable price.[49, 50] The available feedstocks in a biorefinery will most likely be a mixture of different plants with variable composition and fluctuating prices that will change over time. Therefore, it is highly desirable for the biorefineries to be able to effectively process mixed biomass feedstocks with minimal adverse impact on overall performance, including sugar and fuel titers. In this study three different mixed biomass were studied by varying the weight fraction of almond (A), walnut (W), pine (P) and fir (F). Figure 4 A shows the effect of mixed biomass on glucose and xylose yields after [Ch][Lys] pretreatment and enzymatic hydrolysis. The glucose and xylose yield for a mixture containing equal fraction (1/1/1/1) for all the four biomasses were 49.1 and 50.1 % of dry biomass, respectively. Interestingly, when fir was eliminated from the mixture while keeping equal fractions (1/1/1/0) for almond, pine, and walnut, a significant increase in glucose (58.6 % of dry biomass) and xylose (61.7 % of dry biomass) yields were obtained. Further modification of the mixture ratios to 20% pine and 40% of both almond and walnut resulted in a slight increase in glucose (62.3 % of dry biomass) and xylose (66.6 % of dry biomass) yields. For the [EOA][OAc] pretreated biomass (Figure 4B), the results show that glucose vs xylose yields for mixed biomass were 54.0 vs 56.6, 45.2 vs 43.3, 39.7 vs 39.6 % of dry biomass for mass ratio of 2/2/1/0, 1/1/1/0, and 1/1/1/1 A/W/P/F, respectively. The overall sugars released were ~12% less than the expected amount (based on the ratios for the pure biomass streams). Nevertheless, once this slight reduction has been accounted for, the response was very close to that of a linear correlation between biomass ratios and sugar yields.

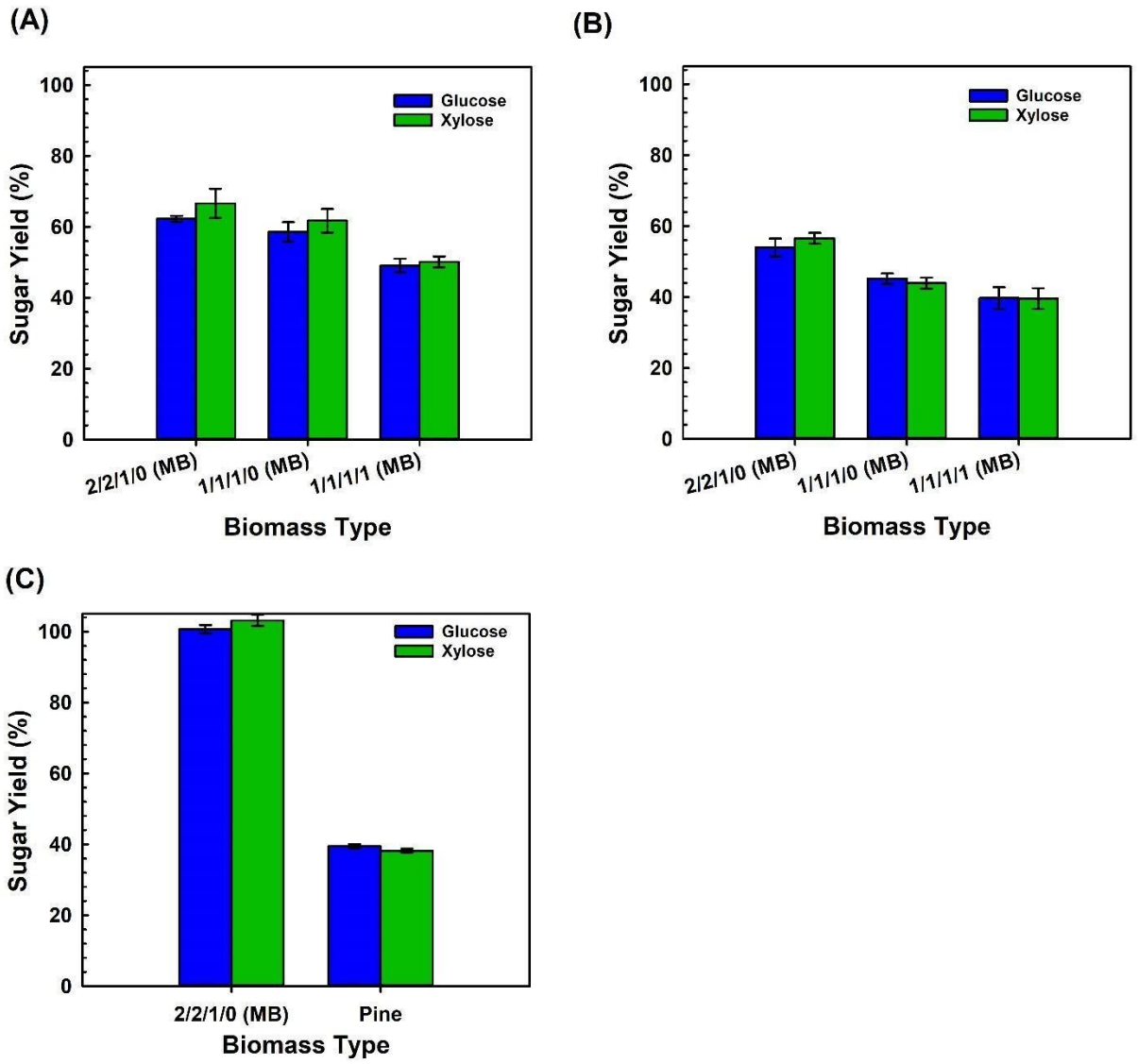


**Figure 4**. Glucose and xylose yield from enzymatic hydrolysis of 2 mm particle sized mixed biomass (MB) (weight fraction of Almond/Walnut/Pine/Fir are listed in the figure as A/W/F/P) of 160 ℃, 3 h, and 30 mg protein/g biomass of enzyme for (A) [Ch][Lys]; (B) [EOA][OAc] pretreated samples; (C) larger scale Parr reactor (30g-scale) via [Ch][Lys].

The combined results show that both the IL's were effective in pretreating biomass mixture of almond, walnut, pine, and fir. Under optimal pretreatment conditions, biomass mixture of 20% pine and 40% of both almond and walnut resulted in highest fermentable sugar release. At this mixture, the average sugar yields for [Ch][Lys] and [EOA][OAc] were 64.4 and 55.3% of dry

biomass, respectively. Despite the effectiveness for sugar release from [EOA][OAc], further process scaleup was carried out using [Ch][Lys] because of its biocompatible nature that enables a simpler process configuration. Figure 4 C shows the results from a 30 g scale process using [Ch][Lys] and pine or 2/2/1 A/W/P. Glucose and xylose yields for pine were 39.5% and 38.2%, respectively, resulting in 19.6 g/L glucose and 8 g/L of xylose in the hydrolysate. The average sugar yield for the mixed biomass resulted in a 62% increase compared to small scale (2 g, Figure 4 A). Glucose and xylose yield for the mixed biomass were near theoretical, resulting in a 41.5 g/L titer of glucose and 19.6 g/L of xylose in the final hydrolysate. The increase in fermentable sugar yields can be attributed to better mixing of the biomass in the reactor vessel leading to improved mass transfer. The decline in hydrolysis efficiency when using pine compared with the feedstock blend is likely due to a higher lignin content and a higher proportion of C-C linkages in pine.[51] It is known that lignin is the most recalcitrant component of the plant cell wall, thus the higher the proportion of lignin the lower the bioavailability of the substrate.[29, 52]

*3.5 Scale Up and Fermentation of mixed biomass*

Using the best parameters determined at the bench-scale, the process was scaled to 10L using both pine and the 2/2/1 A/W/P feedstock blend. At this scale, a rapid glucose and xylose release at the beginning of the saccharification was observed followed by a decrease in the hydrolysis rate over the next 48 hours. As expected, sugar concentrations were higher for the mixed biomass condition, resulting in a maximum of 39.5 g/L glucose and 19 g/L xylose in the final hydrolysate (Fig. 5A). Glucan conversion was 91.7%, while xylan conversion was complete

(Fig. 5B). Glucose and xylose titers from pine were 21.6 g/L and 11.1 g/L, respectively, which corresponds to 39.7% and 48.2% glucan and xylan yields, respectively.

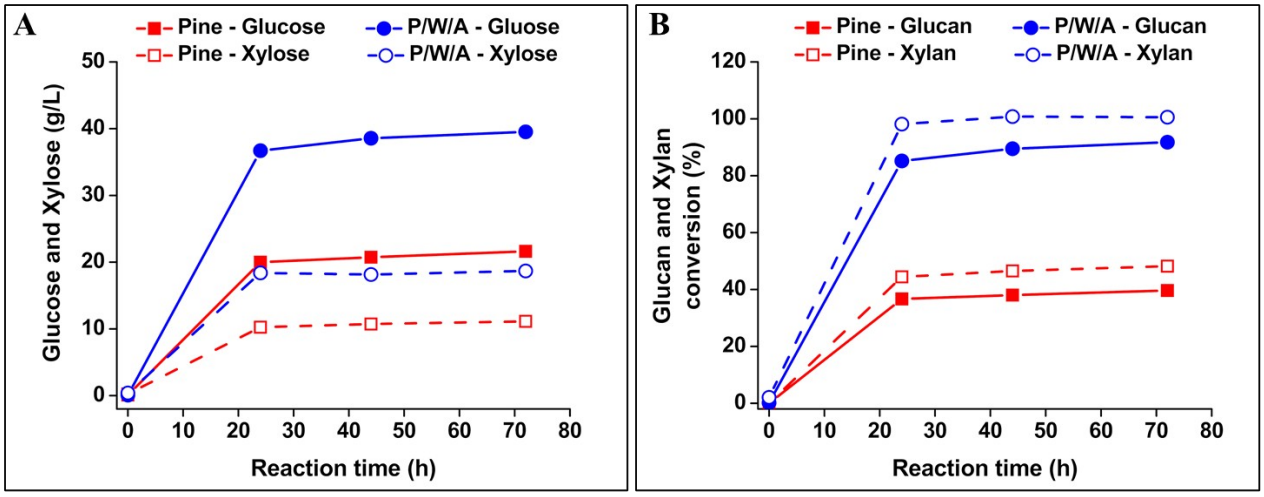


**Figure 5.** Sugars released **(A)** and sugars conversion **(B)** during enzymatic hydrolysis of Pine and Pine/Almond/Walnut with [Ch][Lys] pretreated biomass at 15% solid loading. Enzyme loading: 30 mg/g biomass; temp.: 50°C; pH 5

Since the feedstock blend resulted in higher conversion at scale compared to the single biomass condition, it was used to study the effect of biomass loading on the one-pot ionic liquid pretreatment and saccharification process. Figure 6 shows that an increase of initial solids from 15% to 25% lead to a decrease in glucan conversion from 91.7% to 53% while xylan conversion declined 44.3%. This reduction was likely due to mass transfer limitations, difficulties in mixing and absorption and retention of liquid hydrolysate by residual non-hydrolyzed biomass resulting

in a diminution of the available volume.[53, 54] In addition to reduced hydrolysis yield and lower sugar concentrations at the end of saccharification, the higher solids loading results in an increased concentration of fermentation inhibitors like acetic acid and phenolic lignin degradation products, which can hamper the performance of the conversion host. For this reason, the hydrolysate at 15% solid loading was selected for fermentation testing of both pine and the blended feedstock hydrolysates.

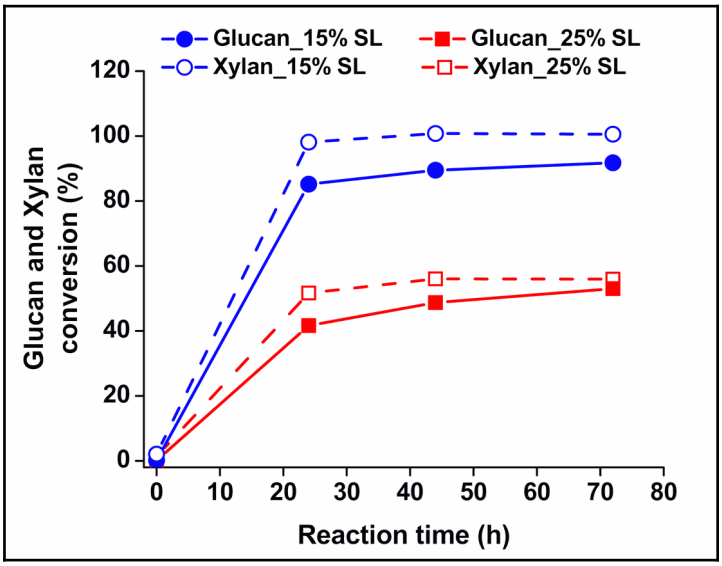


**Figure 6.** Glucan and xylan conversion during enzymatic hydrolysis of mixed biomass (Pine/Almond/Walnut) at 15% and 25% solid loading. Enzyme loading: 30 mg/g biomass; temp.: 50°C; pH 5

To reduce the effect of hydrolysate toxicity on the *S. cerevisiae* strain used for fermentation, a two-stage seed train was employed with yeast cells propagated initially in YPD media and subsequently in a 50% YPD/50% filtered hydrolysate mixture. The 50/50 mixture helps the strain adapt to the hydrolysate prior to the fermentation. Since separation of residual lignin is costly, particularly at large scale, we opted to use unfiltered hydrolysate for the

fermentation process. Glucose utilization was complete for all conditions while approximately 50% xylose was not consumed after 6 days of fermentation when the hydrolysate from the blended feedstock condition was used (Fig. 7). Higher initial sugar concentration improved ethanol titers, with maximum ethanol concentration of 17.9 g/L for the mixture biomass hydrolysate.

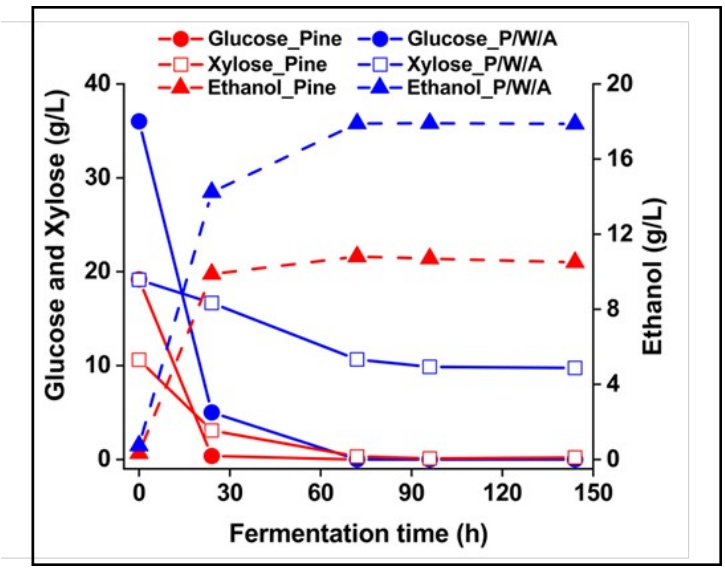


**Figure 7.** Batch ethanol fermentation of Pine and Pine/Almond/Walnut hydrolysates pretreated with [Ch][Lys]. Temp.: 30°C; pH 5, *S. cerevisiae* NS 22202

## 4. CONCLUSIONS

This study is the first to demonstrate that pretreatment of residual woody biomass (pine, fir, walnut, and almond) using the ILs [Ch][Lys] and [EOA][OAc] can enable efficient conversion of these feedstocks and their blends to ethanol. Compositional analysis of these feedstocks demonstrates that they have large variations in glucan (22-33%), xylan (11-14%), lignin (20-27%) and extractives (18-38%), which will result in hydrolysates with a range of maximum potential sugar titers. Pine has the highest sugar content but is the most difficult to deconstruct, so

we explored whether blending it with other feedstocks could enable its efficient deconstruction. To do this, pretreatment process parameters for [Ch][Lys] and [EOA][OAc] were optimized to maximize fermentable sugar release. Under the optimal conditions (temperature 160 ℃, time 3h, and 30 mg protein/g biomass of enzyme), a > 80% yield of glucose was obtained for almond and walnut, but both pine and fir performed poorly. The lower saccharification efficiency of the softwoods can be attributed to its lignin type (abundant G units). To enable conversion of the more recalcitrant feedstocks, various woody biomass blends were pretreated via IL and under optimal conditions and a 2/2/1 (almond: walnut: pine) blend achieved a >90% yield of fermentable sugars. The process was then scale-up to 10 L using [Ch][Lys] pretreatment on either pine or the 2/2/1 (almond: walnut: pine) blend. With better mixing at scale, resulting in a titer of 39.5 g/L glucose (91.7% glucan conversion) and 19 g/L xylose (complete xylan conversion) in the hydrolysate. For pine, glucose and xylose titers were 21.6 g/L (39.7% glucan conversion) and 11.1 g/L (48.2% xylan conversion), respectively. Overall, blending hardwood with softwood synergistically increased the conversion efficiency of the mixed feedstock. Fermentation of the hydrolysate using *Saccharomyces cerevisiae* resulted in an ethanol titer of 17.9 g/L from the blend hydrolysate, corresponding to a 60.8 % fermentation efficiency. These results are promising and with further process optimization, they indicate that this process has potential for commercialization.

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**REFERENCES**


1. report, B. t., https://www.energy.gov/sites/prod/files/2016/08/f33/BillionTon_Report_2016_8.18.2016.pdf. Last accessed 01/10/2021

2. Chundawat, S. P.; Beckham, G. T.; Himmel, M. E.; Dale, B. E., Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annual review of cehmical and biomolecular engineering* **2011,** 2, 121-145. Doi.org/10.1146/annurev-chembioeng-061010-114205

3. Wyman, C. E.; Dale, B. E.; Elander, R. T.; Holtzapple, M.; Ladisch, M. R.; Lee, Y., Coordinated development of leading biomass pretreatment technologies. *Bioresource technology* **2005,** *96* (18), 1959-1966. doi.org/10.1016/j.biortech.2005.01.010

4. Zheng, Y.; Pan, Z.; Zhang, R., Overview of biomass pretreatment for cellulosic ethanol production. *International journal of agricultural and biological engineering* **2009,** *2* (3), 51-68. Doi.org/10.3965/j.issn.1934-6344.2009.03.051-068

5. Zhu, J.; Pan, X., Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. *Bioresource technology* **2010,** *101* (13), 4992-5002. doi.org/10.1016/j.biortech.2009.11.007

6. Blanch, H. W.; Simmons, B. A.; Klein-Marcuschamer, D., Biomass deconstruction to sugars. *Biotechnology Journal* **2011,** *6* (9), 1086-1102. doi.org/10.1002/biot.201000180

7. Baral, N. R.; Sundstrom, E. R.; Das, L.; Gladden, J.; Eudes, A.; Mortimer, J. C.; Singer, S. W.; Mukhopadhyay, A.; Scown, C. D., Approaches for more efficient biological conversion of lignocellulosic feedstocks to biofuels and bioproducts. *ACS Sustainable Chemistry & Engineering* **2019,** *7* (10), 9062-9079. doi.org/10.1021/acssuschemeng.9b01229

8. Das, L.; Li, W.; Dodge, L. A.; Stevens, J. C.; Williams, D. W.; Hu, H.; Li, C.; Ray, A. E.; Shi, J., Comparative Evaluation of Industrial Hemp Cultivars: Agronomical Practices, Feedstock Characterization, and Potential for Biofuels and Bioproducts. *ACS Sustainable Chemistry & Engineering* **2020,** *8* (16), 6200-6210. doi.org/10.1021/acssuschemeng.9b06145

9. Rogers, R. D.; Seddon, K. R., Ionic liquids--solvents of the future? *Science* **2003,** *302* (5646), 792-793. DOI: 10.1126/science.1090313

10. Brandt, A.; Gräsvik, J.; Hallett, J. P.; Welton, T., Deconstruction of lignocellulosic biomass with ionic liquids. *Green chemistry* **2013,** *15* (3), 550-583. DOI: 10.1039/C2GC36364J

11. Tu, W.-C.; Hallett, J. P., Recent advances in the pretreatment of lignocellulosic biomass. *Current Opinion in Green and Sustainable Chemistry* **2019,** *20*, 11-17. doi.org/10.1016/j.cogsc.2019.07.004

12. Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D., Dissolution of cellose with ionic liquids. *Journal of the American chemical society* **2002,** *124* (18), 4974-4975. doi.org/10.1021/ja025790m

13. Sun, N.; Rahman, M.; Qin, Y.; Maxim, M. L.; Rodríguez, H.; Rogers, R. D., Complete dissolution and partial delignification of wood in the ionic liquid 1-ethyl-3-methylimidazolium acetate. *Green Chemistry* **2009,** *11* (5), 646-655. DOI: 10.1039/B822702K

14. Halder, P.; Kundu, S.; Patel, S.; Setiawan, A.; Atkin, R.; Parthasarthy, R.; Paz-Ferreiro, J.; Surapaneni, A.; Shah, K., Progress on the pre-treatment of lignocellulosic biomass employing ionic liquids. *Renewable and Sustainable Energy Reviews* **2019,** *105*, 268-292. doi.org/10.1016/j.rser.2019.01.052

15. Socha, A. M.; Parthasarathi, R.; Shi, J.; Pattathil, S.; Whyte, D.; Bergeron, M.; George, A.; Tran, K.; Stavila, V.; Venkatachalam, S., Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. *Proceedings of the national academy of sciences* **2014,** *111* (35), E3587-E3595. doi.org/10.1073/pnas.1405685111

16. Wang, H.; Gurau, G.; Rogers, R. D., Ionic liquid processing of cellulose. *Chemical Society Reviews* **2012,** *41* (4), 1519-1537. DOI: 10.1039/C2CS15311D

17. Rigual, V.; Ovejero-Pérez, A.; Rivas, S.; Domínguez, J. C.; Alonso, M. V.; Oliet, M.; Rodriguez, F., Protic, Aprotic, and Choline-Derived Ionic Liquids: Toward Enhancing the Accessibility of Hardwood and Softwood. *ACS Sustainable Chemistry & Engineering* **2019,** *8* (3), 1362-1370. doi.org/10.1021/acssuschemeng.9b04443

18. Bhalla, A.; Cai, C. M.; Xu, F.; Singh, S. K.; Bansal, N.; Phongpreecha, T.; Dutta, T.; Foster, C. E.; Kumar, R.; Simmons, B. A., Performance of three delignifying pretreatments on hardwoods: hydrolysis yields, comprehensive mass balances, and lignin properties. *Biotechnology for biofuels* **2019,** *12* (1), 213. doi.org/10.1186/s13068-019-1546-0

19. Sun, N.; Parthasarathi, R.; Socha, A. M.; Shi, J.; Zhang, S.; Stavila, V.; Sale, K. L.; Simmons, B. A.; Singh, S., Understanding pretreatment efficacy of four cholinium and imidazolium ionic liquids by chemistry and computation. *Green Chemistry* **2014,** *16* (5), 2546-2557. DOI: 10.1039/C3GC42401D

20. Xu, F.; Sun, J.; Konda, N. M.; Shi, J.; Dutta, T.; Scown, C. D.; Simmons, B. A.; Singh, S., Transforming biomass conversion with ionic liquids: process intensification and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. *Energy & Environmental Science* **2016,** *9* (3), 1042-1049. DOI: 10.1039/C5EE02940F

21. Hossain, M. M.; Rawal, A.; Aldous, L., Aprotic vs protic ionic liquids for lignocellulosic biomass pretreatment: anion effects, enzymatic hydrolysis, solid-state NMR, distillation, and recycle. *ACS Sustainable Chemistry & Engineering* **2019,** *7* (14), 11928-11936. doi.org/10.1021/acssuschemeng.8b05987

22. Sun, J.; Konda, N. M.; Parthasarathi, R.; Dutta, T.; Valiev, M.; Xu, F.; Simmons, B. A.; Singh, S., One-pot integrated biofuel production using low-cost biocompatible protic ionic liquids. *Green Chemistry* **2017,** *19* (13), 3152-3163. DOI: 10.1039/C7GC01179B

23. Socha, A. M.; Plummer, S. P.; Stavila, V.; Simmons, B. A.; Singh, S., Comparison of sugar content for ionic liquid pretreated Douglas-fir woodchips and forestry residues. *Biotechnology for biofuels* **2013,** *6* (1), 61. doi.org/10.1186/1754-6834-6-61

24. White, E. M., *Woody biomass for bioenergy and biofuels in the United States: A briefing paper*. DIANE Publishing: 2010.

25. Lauri, P.; Havlík, P.; Kindermann, G.; Forsell, N.; Böttcher, H.; Obersteiner, M., Woody biomass energy potential in 2050. *Energy Policy* **2014,** *66*, 19-31. doi.org/10.1016/j.enpol.2013.11.033

26. CEC, https://ww2.energy.ca.gov/2019publications/CEC-500-2019-033/CEC-500-2019-033.pdf. Last accessed 01/10/2021

27. Yoo, C. G.; Pu, Y.; Ragauskas, A. J., Ionic liquids: Promising green solvents for lignocellulosic biomass utilization. *Current Opinion in Green and Sustainable Chemistry* **2017,** *5*, 5-11. doi.org/10.1016/j.cogsc.2017.03.003

28. Zhang, Q.; Hu, J.; Lee, D.-J., Pretreatment of biomass using ionic liquids: research updates. *Renewable Energy* **2017,** *111*, 77-84. doi.org/10.1016/j.renene.2017.03.093

29. Dutta, T.; Papa, G.; Wang, E.; Sun, J.; Isern, N. G.; Cort, J. R.; Simmons, B. A.; Singh, S., Characterization of lignin streams during bionic liquid-based pretreatment from grass, hardwood, and softwood. *ACS sustainable chemistry & engineering* **2018,** *6* (3), 3079-3090. doi.org/10.1021/acssuschemeng.7b02991

30. Mansfield, S. D.; Kim, H.; Lu, F.; Ralph, J., Whole plant cell wall characterization using solution-state 2D NMR. *Nature protocols* **2012,** *7* (9), 1579-1589. doi.org/10.1038/nprot.2012.064

31. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. *Laboratory analytical procedure (LAP): NREL*; TP-510-42618. Golden, Colorado: National Renewable Energy Laboratory: 2008.

32. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. *Determination of extractives in biomass: NREL*; TP-510–42619. National Renewable Energy Laboratory (NREL), Golden, Colorado: 2005.

33. Eudes, A.; Juminaga, D.; Baidoo, E. E.; Collins, F. W.; Keasling, J. D.; Loqué, D., Production of hydroxycinnamoyl anthranilates from glucose in Escherichia coli. *Microbial cell factories* **2013,** *12* (1), 62. doi.org/10.1186/1475-2859-12-62

34. Eudes, A.; Juminaga, D.; Baidoo, E. E.; Collins, F. W.; Keasling, J. D.; Loqué, D., Erratum to: Production of hydroxycinnamoyl anthranilates from glucose in Escherichia coli. *Microbial cell factories* **2014,** *13* (1), 1-1. doi.org/10.1186/1475-2859-13-8

35. Bertaud, F.; Crampon, C.; Badens, E., Volatile terpene extraction of spruce, fir and maritime pine wood: supercritical CO2 extraction compared to classical solvent extractions and steam distillation. *Holzforschung* **2017,** *71* (7-8), 667-673. doi.org/10.1515/hf-2016-0197

36. Fernández-Agulló, A.; Freire, M. S.; Ramírez-López, C.; Fernández-Moya, J.; González-Álvarez, J., Valorization of residual walnut biomass from forest management and wood processing for the production of bioactive compounds. *Biomass Conversion and Biorefinery* **2020**, 1-10. doi.org/10.1007/s13399-019-00598-9

37. Tyśkiewicz, K.; Konkol, M.; Kowalski, R.; Rój, E.; Warmiński, K.; Krzyżaniak, M.; Gil, Ł.; Stolarski, M. J., Characterization of bioactive compounds in the biomass of black locust, poplar and willow. *Trees* **2019**, 1-29. doi.org/10.1007/s00468-019-01837-2

38. Yoo, C. G.; Meng, X.; Pu, Y.; Ragauskas, A. J., The critical role of lignin in lignocellulosic biomass conversion and recent pretreatment strategies: A comprehensive review. *Bioresource Technology* **2020,** *301*, 122784. doi.org/10.1016/j.biortech.2020.122784

39. Liao, J. J.; Abd Latif, N. H.; Trache, D.; Brosse, N.; Hussin, M. H., Current advancement on the isolation, characterization and application of lignin. *International Journal of Biological Macromolecules* **2020,** 162, 985-1024. doi.org/10.1016/j.ijbiomac.2020.06.168

40. Glasser, W. G., About making lignin great again-some lessons from the past. *Frontiers in chemistry* **2019,** *7*, 565. doi.org/10.3389/fchem.2019.00565

41. Stewart, D., Lignin as a base material for materials applications: Chemistry, application and economics. *Industrial crops and products* **2008,** *27* (2), 202-207. doi.org/10.1016/j.indcrop.2007.07.008

42. Liu, E.; Li, M.; Das, L.; Pu, Y.; Frazier, T.; Zhao, B.; Crocker, M.; Ragauskas, A. J.; Shi, J., Understanding lignin fractionation and characterization from engineered switchgrass treated by an aqueous ionic liquid. *ACS Sustainable Chemistry & Engineering* **2018,** *6* (5), 6612-6623. doi.org/10.1021/acssuschemeng.8b00384

43. Shi, J.; Balamurugan, K.; Parthasarathi, R.; Sathitsuksanoh, N.; Zhang, S.; Stavila, V.; Subramanian, V.; Simmons, B. A.; Singh, S., Understanding the role of water during ionic liquid pretreatment of lignocellulose: co-solvent or anti-solvent? *Green Chemistry* **2014,** *16* (8), 3830-3840. DOI: 10.1039/C4GC00373J

44. Hu, J.; Mok, Y. K.; Saddler, J. N., Can we reduce the cellulase enzyme loading required to achieve efficient lignocellulose deconstruction by only using the initially absorbed enzymes? *ACS Sustainable Chemistry & Engineering* **2018,** *6* (5), 6233-6239. doi.org/10.1021/acssuschemeng.8b00004

45. Pallapolu, V. R.; Lee, Y.; Garlock, R. J.; Balan, V.; Dale, B. E.; Kim, Y.; Mosier, N. S.; Ladisch, M. R.; Falls, M.; Holtzapple, M. T., Effects of enzyme loading and β-glucosidase supplementation on enzymatic hydrolysis of switchgrass processed by leading pretreatment technologies. *Bioresource technology* **2011,** *102* (24), 11115-11120. doi.org/10.1016/j.biortech.2011.03.085

46. Zhang, H.; Li, J.; Huang, G.; Yang, Z.; Han, L., Understanding the synergistic effect and the main factors influencing the enzymatic hydrolyzability of corn stover at low enzyme loading by hydrothermal and/or ultrafine grinding pretreatment. *Bioresource technology* **2018,** *264*, 327-334. doi.org/10.1016/j.biortech.2018.05.090

47. Liu, Z.-H.; Qin, L.; Pang, F.; Jin, M.-J.; Li, B.-Z.; Kang, Y.; Dale, B. E.; Yuan, Y.-J., Effects of biomass particle size on steam explosion pretreatment performance for improving the enzyme digestibility of corn stover. *Industrial Crops and Products* **2013,** *44*, 176-184. doi.org/10.1016/j.indcrop.2012.11.009

48. Yang, Y.; Sun, M.; Deines, T.; Zhang, M.; Li, J.; Wang, D. Effects of Particle Size on Biomass Pretreatment for Biofuel Production, International Manufacturing Science and Engineering Conference, American Society of Mechanical Engineers: 2019; p V002T03A011. doi.org/10.1115/MSEC2019-2916

49. Shi, J.; Thompson, V. S.; Yancey, N. A.; Stavila, V.; Simmons, B. A.; Singh, S., Impact of mixed feedstocks and feedstock densification on ionic liquid pretreatment efficiency. *Biofuels* **2013,** *4* (1), 63-72. doi.org/10.4155/bfs.12.82

50. Nguyen, Q. A.; Yang, J.; Bae, H.-J., Bioethanol production from individual and mixed agricultural biomass residues. *Industrial crops and products* **2017,** *95*, 718-725. doi.org/10.1016/j.indcrop.2016.11.040

51. Yu, Z.; Gwak, K. S.; Treasure, T.; Jameel, H.; Chang, H. m.; Park, S., Effect of lignin chemistry on the enzymatic hydrolysis of woody biomass. *ChemSusChem* **2014,** *7* (7), 1942-1950. doi.org/10.1002/cssc.201400042

52. Bay, M. S.; Karimi, K.; Esfahany, M. N.; Kumar, R., Structural modification of pine and poplar wood by alkali pretreatment to improve ethanol production. *Industrial Crops and Products* **2020,** *152*, 112506. doi.org/10.1016/j.indcrop.2020.112506

53. Li, C.; Tanjore, D.; He, W.; Wong, J.; Gardner, J. L.; Sale, K. L.; Simmons, B. A.; Singh, S., Scale-up and evaluation of high solid ionic liquid pretreatment and enzymatic hydrolysis of switchgrass. *Biotechnology for biofuels* **2013,** *6* (1), 154. doi.org/10.1186/1754-6834-6-154

54. Modenbach, A. A.; Nokes, S. E., The use of high-solids loadings in biomass pretreatment—a review. *Biotechnology and Bioengineering* **2012,** *109* (6), 1430-1442. doi.org/10.1002/bit.24464

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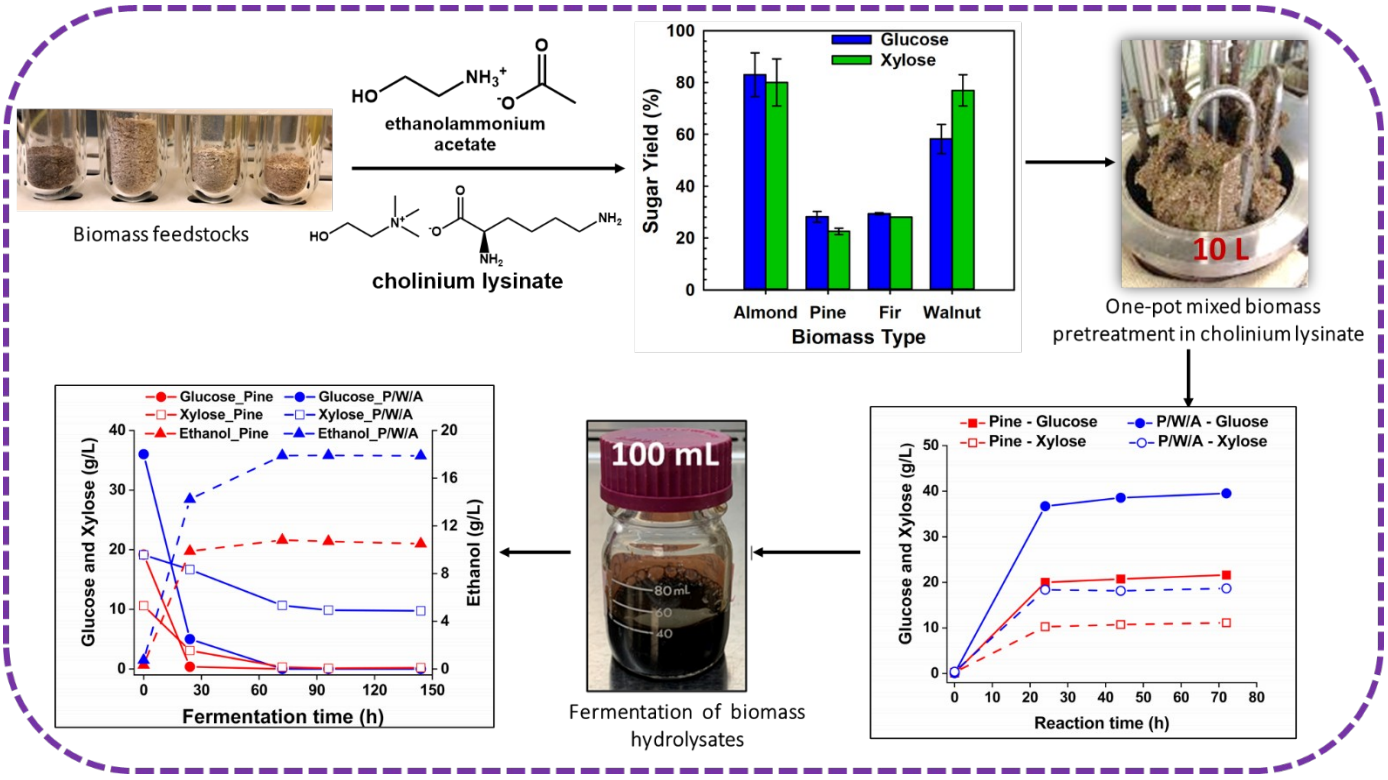


**Synopsis:** Deconstruction of woody biomass via ionic liquids and conversion of hydrolysates to ethanol using a C5-utilizing strain of *Saccharomyces cerevisiae.*