A coupled low temperature oxidative and ionic liquid pretreatment of lignocellulosic biomass

Samira Vasheghani Farahani^a, Yong Wah Kim^b, Constance A. Schall^{a*}

^a, University of Toledo, Department of Chemical & Environmental Engineering, Toledo, OH, USA

^b, University of Toledo, Department of Chemistry and Biochemistry, Toledo, OH, USA

samira.vasheghani@rockets.utoledo.edu

yong-wah.kim@utoledo.edu

*Corresponding Author, constance.schall@utoledo.edu

Abstract

An integrated pretreatment strategy consisting of a room temperature alkaline oxidation step coupled with ionic liquid (IL) incubation enables effective lignocellulosic biomass pretreatment at low temperatures (50°C). The IL, 1-ethyl-3-methyl-imidazolium acetate (EMIM-Ac), was used in pretreatment of a lignocellulosic hardwood feedstock, poplar. Glucose and xylose yields for 24 hour enzyme hydrolysis of pretreated poplar were measured to assess the efficacy of this pretreatment strategy. The proposed strategy resulted in high hydrolysis yields at a low enzyme loading of 9.5 filter paper units per gram of glucan. The low IL incubation temperatures were found to reduce undesired cellulose acetylation reactions.

Keywords

Ionic liquid, EMIM-Ac, Pretreatment, Acetylation, Cellulose, Lignin oxidation

Abbreviations

AHP: alkaline hydrogen peroxide

AMIM-Cl: 1-allyl-3-methyl imidazolium chloride

EMIM-Ac: 1-ethyl-3-methyl imidazolium acetate

IL: ionic liquid

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29 **1. Introduction**

30 Lignocellulosic biomass is a renewable source of carbon, consisting of three major components:
31 cellulose (30–45%), a highly crystalline polymer of the hexose sugar, glucose; hemicellulose (20–40%), a
32 complex amorphous polysaccharide comprised of pentose (often primarily xylose) and hexose sugars; and
33 lignin (5–25%), a polyphenyl-propanoid macromolecular assembly that is covalently cross-linked to
34 hemicellulose [1]. The complex and compact structure of lignocellulosic biomass renders it largely
35 impenetrable to water, catalysts, or enzymes used to hydrolyze its constituent polysaccharides to
36 monomeric sugars (saccharification). Because of this, pretreatment is necessary prior to saccharification
37 [2, 3].

38 Effective ionic liquid (IL) incubation of lignocellulosic biomass can generate an amorphous cellulosic
39 substrate enabling greater enzyme access for rapid hydrolysis of polysaccharides without the production
40 of fermentation inhibitors [4]. The crystalline structure of native cellulose, commonly called cellulose I
41 (referring to two naturally occurring allomorphs, I α and I β) [5, 6] is a major impediment to its hydrolysis
42 to monomeric sugars [7]. IL pretreatment typically involves a biomass incubation step followed by IL
43 displacement using an anti-solvent that precipitates the treated biomass. High IL incubation temperatures
44 (up to ~160°C) are often necessary for an effective biomass pretreatment that produces a readily
45 hydrolyzable substrate [8-10]. However, these high temperatures can result in thermal degradation of IL
46 and biomass and undesired cellulose derivatization reactions [11-15].

47 Imidazolium based ILs are effective solvents for dissolution of cellulose. Among 21 imidazolium-
48 based ILs screened, 1-ethyl-3-methyl imidazolium acetate (EMIM-Ac) and 1-allyl-3-methylimidazolium
49 chloride (AMIM-Cl) were found to be the most effective ILs in dissolution of cellulose and woodchips
50 [16]. In additional studies of 97 ionic liquids with a variety of cation moieties, 1-butyl-3-
51 methylimidazolium chloride (BMIM-Cl), AMIM-Cl and EMIM-Ac were found to be among the best
52 solvents for cellulose and lignocellulose [17]. However, halogenated ILs such as BMIM-Cl and AMIM-

Cl pose potential corrosion issues in processing equipment. Moreover, the melting points of BMIM-Cl and AMIM-Cl are reported to be $\sim 70^{\circ}\text{C}$ [18, 19] and $\sim 52^{\circ}\text{C}$ [20, 21], significantly higher than EMIM-Ac (a liquid below room temperature). Some cholinium-carboxylate ionic liquids such as choline acetate and choline propionate are reported to have qualities similar to EMIM-Ac for pretreatment of lignocellulosic materials [22]. However, the high melting point of choline acetate (between $51\text{--}72^{\circ}\text{C}$) and high viscosity of choline-based ionic liquids, ranging between 630-8500 centipoise, render these ILs less satisfactory than EMIM-Ac (160cp) [23-25]. EMIM-Ac exhibits a high capacity for cellulose dissolution [26]. In addition to its low melting point ($<-20^{\circ}\text{C}$) and relatively low viscosity [27], the acetate anion is less corrosive than comparable halides [28]. These characteristics make EMIM-Ac one of the most effective solvents for IL pretreatment. However, acetylation of cellulose has been reported in EMIM-Ac at temperatures in the range used for pretreatment of biomass [12]. Acetylation of cellulose can impede enzyme hydrolysis and produce undesired acetylated glucose [29, 30]. Furthermore, it can result in loss of the acetate group of EMIM-Ac and reduce the recyclability and compatibility of this ionic liquid within industrial applications. In studies presented herein, acetylation of Avicel, a crystalline cellulose model compound, was monitored for samples incubated in EMIM-Ac at high and low temperatures. Reduction in this undesired derivatization reaction was observed at decreased IL incubation temperatures.

Prior work indicated that cellulose I, found in lignin free substrates was transformed into more easily hydrolyzed cellulose II or amorphous cellulose with incubation in IL at 50°C . In contrast, higher temperatures of $\sim 120^{\circ}\text{C}$ were required to affect similar transitions in a lignocellulosic biomass [31]. This suggested that removal or partial decomposition of lignin from biomass can result in effective IL pretreatment at lower temperatures. Therefore, we employed a '*coupled method*' consisting of a room temperature alkaline hydrogen peroxide (AHP) oxidation step for partial removal of lignin, followed by IL incubation in EMIM-Ac at 50°C . The glucose and xylose yields for 24 hour enzyme hydrolysis of a pretreated lignocellulosic hardwood substrate, poplar, were measured to assess the efficacy of this pretreatment strategy using a low enzyme loading of ~ 9.5 filter paper units (FPU) per gram of glucan.

2. Materials and Methods

2.1. Materials

Poplar was provided by the National Renewable Energy Laboratory (NREL). D-xylose (99%), dimethyl sulfoxide-d₆ (99.9% D), 1-ethyl-3-methyl imidazolium acetate (>97%), 1-allyl-3-methyl imidazolium chloride (>97%), 1-methylimidazole (>97%), Avicel and birchwood xylan were purchased from Sigma-Aldrich. D-glucose (99+%), and hydrogen peroxide (35 wt%) were purchased from Acros Organics. Sodium hydroxide, sodium citrate dihydrate and citric acid monohydrate were purchased from Fisher Scientific. The commercial enzyme mixture, Cellic Ctec2, was provided by Novozymes.

2.2. Biomass compositional analysis and quantitation of monomeric sugars

The compositional analysis of the poplar biomass samples was carried out in triplicate using NREL standard LAP 002 protocols using a concentrated and dilute acid digestion [32]. Sugar concentrations of the digests were determined via high performance liquid chromatography (HPLC), with refractive index detection using a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis ion-exchange column. The HPLC was operated at 80°C in an isocratic mode with a mobile phase of deionized water at 0.6mL/min. Mixed sugar standards of known concentrations were used to generate standard curves to calculate the concentration of released sugars. Glucose and xylose released from glucan and xylan, respectively, in enzyme hydrolysis experiments were reported as a percentage of theoretical yield of monomeric sugars based on glucan and xylan analysis of untreated substrates.

2.3. Enzyme hydrolysis

Enzymatic hydrolysis of pretreated and native substrates followed NREL standard LAP 009 protocol [33] with enzyme loadings of 9.5 FPU per gram of glucan (4.5 mg protein / g glucan) using a commercial cellulase enzyme mixture, Cellic Ctec2 (Novozymes). The hydrolysis was run at 1% (w/v) solid loadings at 50°C in 50mM sodium citrate buffer, pH 4.8 for 24 hours with mixing in a rotary shaker water bath. The protein concentration of Cellic Ctec2 was determined using the

colorimetric Bradford protein assay with bovine serum albumin standards [34]. Activity of the cellulase was measured and reported in filter paper units [35].

2.4. Biomass pretreatment

A ‘coupled method’ of pretreatment consisted of a room temperature AHP oxidation step followed by IL incubation at 50°C. Selected samples were pretreated via AHP oxidation or IL incubation alone.

2.4.1. Room temperature AHP oxidation step

For the oxidation step, native poplar was mixed with alkaline hydrogen peroxide (AHP) at 10% (w/v) biomass solid loading. Samples were incubated for 1 to 24 hours at room temperature. The AHP solution consisted of a 25 mM NaOH solution with hydrogen peroxide added at a ratio of 12.5 % (w/w) H₂O₂ with respect to native poplar. Oxidation reactions were stopped by addition of deionized water, followed by centrifugation and removal of filtrate. Samples were dried to a constant weight at 40°C, unless otherwise noted.

2.4.2. IL incubation

For the IL incubation step, samples were mixed with 1-ethyl-3-methyl imidazolium acetate (EMIM-Ac) at a 5 to 20% (w/w) loading of biomass to IL. Samples were incubated between 0.5 to 4 hours at 50°C (unless otherwise noted). After IL incubation, deionized water was added to the IL incubation containers to precipitate polysaccharides and displace the IL from the biomass. Samples were centrifuged, and the supernatant was removed. Precipitated biomass was repeatedly washed and centrifuged until a clear supernatant was observed. The precipitated solids (regenerated biomass) were then filtered and hydrolyzed.

2.5. ¹³C NMR sample preparation and data acquisition conditions

Samples of Avicel, a microcrystalline cellulose model compound, were incubated in EMIM-Ac at 50 and 140°C for 4 hours with a biomass solid loading of 5% (w/w). Deionized water was added to the IL incubation containers to precipitate the Avicel and displace IL as previously described for biomass samples. Avicel samples were then dried to a constant weight under vacuum at 40°C. The EMIM-Ac

incubated Avicel samples and a control Avicel sample (not exposed to EMIM-Ac) were each separately combined with 1-allyl-3-methyl imidazolium chloride (AMIM-Cl) at a biomass solid loading of 5% (w/w) and stirred at 30°C until complete dissolution was visually observed. DMSO (~20% v/v) was mixed with samples in order to reduce the sample viscosity.

Liquid state ^{13}C NMR was used to detect evidence of acetylation of cellulose by EMIM-Ac: ^{13}C NMR (DMSO- d_6) for acetylated cellulose (structure shown in Figure 1) exhibited chemical shifts in ppm at: $\delta=170.6$ (acetate CO or C-7), 102.4 (C-1), 79.5–72.6 (C2-5), 60.2 (C-6, not substituted), 21.1 (C-8), AMIM-Cl (structure shown in Figure 1b) at: $\delta=137.6$ (C-1'), 132.4. These assignments are in agreement with those previously reported [12, 36].

NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer. It has a dual channel 5mm DCH CryoProbe that was optimized for the ^{13}C sensitivity at 150.92 MHz. The data were recorded at 65°C with 1024 transients. A delay time of 2.0s and a 30° flip angle of 3.2 μs was used in the ^{13}C pulse sequence.

2.6. X-ray Powder Diffraction (XRD)

Pretreated samples were dried at 40°C overnight then ground into a fine powder using a mortar and pestle. XRD data were collected at room temperature with a X'PERT PRO powder diffractometer PAN188 analytical equipped with an X'celerator detector using nickel-filtered Cu-K α radiation. Samples were scanned from 5 to 35° (2 θ) with a step size of 0.05 and a step time of 10s. The XRD data were interpreted based on XRD studies on cellulose I and cellulose II allomorphs reported by Isogai *et al.* [37]. The crystallinity index, CrI, was calculated based on the method described by Segal *et al.* [38].

3. Results and Discussion

3.1. Acetylation of cellulose in EMIM-Ac

Cellulose can undergo undesired acetylation at high IL incubation temperatures [12], impeding enzyme hydrolysis of glucose [29, 30]. Hence, acetylation of Avicel, a crystalline model compound, was monitored for a high (140°C) and low (50°C) IL temperature incubation. The cellulose samples were incubated in neat EMIM-Ac for four hours, then washed with water to displace IL. Dried samples were

dissolved in neat AMIM-Cl (an IL lacking an acetate group) and ^{13}C NMR spectra were collected. A spectrum of an Avicel control unexposed to EMIM-Ac and dissolved in neat AMIM-Cl was also collected (Figure 1).

The spectrum for cellulose incubated in EMIM-Ac at 140°C (Figure 1 a) exhibited a chemical shift at ~ 170.6 ppm, corresponding to the C-7 atom of the carbonyl group of acetylated cellulose [12, 36]. This chemical shift was absent for Avicel incubated in EMIM-Ac at 50°C and the Avicel control dissolved in AMIM-Cl alone (Figure 1 b & c). These results suggest that acetylation of cellulose by EMIM-Ac was reduced with incubation at 50°C in comparison to incubation at 140°C . The lack of observable cellulose acetylation via NMR at the reduced IL incubation temperature of 50°C indicates merit in the development of a low temperature ionic liquid processing scheme.

The ratio of integrated signals for the acetate carbon signal (C-7) and the anomeric sugar carbon (C-1) provides an estimate of the degree of acetylation although this measure is not expected to be quantitative. For a 20 minute incubation of cellulose in EMIM-Ac at 150°C this ratio was previously reported to be 0.015 [36]. A similar integration was performed in our studies for the 4-hour incubation of cellulose in EMIM-Ac at 140°C and resulted a ratio of 0.15. The higher ratio may indicate that extended IL incubation times at high temperature can lead to significant increases in cellulose acetylation.

To assess the effect of acetylation on saccharification of cellulose, Avicel samples were incubated in EMIM-Ac at 50 and 140°C for four hours followed by enzyme hydrolysis of the regenerated cellulose. Glucose yields were approximately 100 and 71%, for samples incubated in IL at 50 and 140°C respectively. The reduction in glucose yield at 140°C suggests that acetylation of cellulose can impede efficient enzymatic hydrolysis.

3.2. Coupled method - AHP and IL pretreatment

The coupled method consisting of a room temperature alkaline hydrogen peroxide (AHP) oxidation step for partial removal of lignin, followed by IL incubation in EMIM-Ac at 50°C resulted in higher 24 hour glucose yields as compared to IL incubation alone (Figure 2). Sugar yields were reported as a

percentage of the theoretical yield of monomeric sugars based on the initial mass and composition of native poplar (Table 1). All hydrolysis experiments were run at a low enzyme loading of 9.5 FPU/g glucan. For all combinations of oxidation time and IL incubation time and solid loading shown in Figure 2, glucose yields for the coupled method are 49 to 87% higher than selected controls of AHP oxidation or IL incubation alone. As seen in Figure 2 and Table 1, standard deviations for samples run in triplicate were small. Glucose yields for an IL incubation control were 18% (for a 50°C, 4 hour incubation with 5% solids). AHP controls had 12 and 13% glucose yields for 6 hour and 24 hour AHP oxidation, respectively. The glucose yield of 83% for the coupled method (6 hour AHP, followed by 4 hour IL incubation at 5% solids) was greater than the sum of yields for AHP and IL incubation alone (~30%), indicating a synergistic effect of combining the pretreatments.

In order to achieve glucose yields approaching that of the coupled method, for IL incubation alone, the IL incubation temperature must be raised substantially above 50°C. For example, glucose yields for 4 hour IL incubation of poplar (5% solid loading) at 75 and 120°C were 41 and 69%, respectively. Preceding IL incubation with AHP oxidation improves effectiveness of IL pretreatment at reduced temperatures.

Xylose yields for the coupled method varied from 34 to 42% for pretreatment conditions shown in Figure 2. These yields are significantly greater than that of IL incubation alone (9% for 50°C and 4 hour incubation) and comparable to AHP alone (45% for 6 hour and 24% for 24 hour AHP oxidation) for all combinations of oxidation/IL incubation times and solid loadings shown (Figure 2).

3.2.1. IL incubation time and solid loading

Increasing the biomass solid loading in IL from 5 to 15% (w/w) for 6 hour AHP followed by 4 hour IL incubation resulted in minor changes in glucose and xylose yields (Figure 2). Similarly small differences were found in glucose and xylose yields for biomass solid loading of 5 and 20% for 24 hour AHP followed by 4 hour IL incubation. In this case, glucose yields were 84 and 87% and xylose yields were 35 and 42% for biomass loadings of 5 and 20%, respectively. Additionally, no drop in yield was

observed at increased IL solid loading for 24 hour AHP, followed by 2.5-hour IL incubation. These results indicate that increasing the solid loading from 5 to ~20% (w/w) for the IL incubation step is feasible.

Glucose yields for 6 hour AHP oxidation, followed by 4, 2, 1 and 0.5 hour IL incubations with 5% (w/w) biomass loading were 83, 79, 66, and 49%, respectively (Figure 2). These results suggest that a minimum two-hour incubation time is necessary to obtain high glucose yields. The decrease in glucose yields may be the result of limited mass transfer between the IL and biomass associated with short incubation time. Respective xylose yields were relatively constant with decreasing IL incubation times.

3.2.3. AHP Oxidation time

As AHP oxidation time increased from 6 to 24 hours, both glucan and xylan losses increased (Table 1). However, lignin removal from the native poplar did not significantly increase with the extended oxidation time. Since a goal of the AHP step in the coupled method is lignin removal with minimal losses of polysaccharides (to maximize sugar yields), the AHP oxidation time was systematically varied at constant IL incubation conditions to determine if the AHP step could be further shortened.

For the coupled method, AHP oxidation time was varied from 1 to 24 hours (Figure 3). The treated substrate was then directly incubated in IL at constant conditions (4 hours at 50°C with solid loading of 5%). Glucose yields displayed little variation for oxidation times between 6 and 24 hours (86 to 88%). These results indicate that oxidation times greater than 6 hours do not improve glucose yields in a coupled pretreatment. As noted previously, glucose yields in the coupled method for AHP times between 6 and 24 hours were substantially higher than that of IL incubation alone at 50°C (18%) or 120°C (69%). At shorter oxidation times of 4, 2 and 1 hours followed by IL incubation at 50°C, glucose yields decreased to 71, 67 and 60%, respectively.

Xylose yields increased monotonically from 34 to 65% with decreased AHP oxidation times (Figure 3). These results suggest reduced degradation and loss of hemicellulose at shorter oxidation times. These increased xylose yields at reduced AHP oxidation times may reflect decreased losses of xylan during the

pretreatment steps. Gravimetric and compositional analysis of poplar oxidized in AHP alone for 6 and 24 hours indicated increased xylan losses (~9 to 13%) at the extended oxidation time (Table 1). Partial loss of xylan due to alkaline oxidation has been reported in prior studies [39, 40]. However, a more substantial xylan loss of ~42% occurred with the coupled method of six-hour AHP oxidation followed by IL treatment (Table 1). No measurable losses of xylan and other biomass components were detectable with poplar pretreated via IL incubation at 50°C alone. AHP oxidation can cleave ester linkages between lignin and hemicellulose and fragment these components [41, 42]. These changes can render hemicellulose more soluble in the IL/anti-solvent stream as reflected in increased xylan losses after coupled pretreatment compared to IL treatment alone.

3.2. Role of lignin in IL treatment

Effective IL pretreatment transforms the native cellulose allomorph, cellulose I, to the more digestible crystal allomorph, cellulose II (mercerization) or amorphous cellulose [31, 43]. In lignocellulosic substrates, this transition is dependent upon IL incubation temperature and typically requires temperatures up to 160°C [8-10]. In contrast, transitions to more digestible cellulose allomorphs have been observed at temperatures of 50°C or below for lignin free substrates such as ramie cotton or Avicel, a commercially available micro-crystalline cellulosic material [31]. Lignin is known to impede mercerization of cellulose [44]. These observations suggest that full or partial removal of lignin from biomass can decrease the temperature required for effective IL pretreatment.

In order to assess linkages between lignin content and efficacy of IL pretreatment at low incubation temperatures (50°C), substrates with varied lignin content were pretreated via 4 hour IL incubation. The substrates included: (a) native poplar; (b) Avicel (lignin free); (c) a poplar-mimic (lignin free); and (d) oxidized poplar (substrate with partial removal of lignin). The lignin-free poplar-mimic was prepared by combining Avicel, and birchwood xylan to match the glucan to xylan ratio of native poplar (Table 1). Oxidized poplar was prepared by 6 hour oxidation of native poplar using AHP. Efficacy of IL treatment

was assessed through measurements of twenty-four hour hydrolysis yields at enzyme loadings of 9.5 FPU per gram of glucan (Figure 4).

Glucose yield was 18% for native poplar pretreated with IL at 50°C. This is in contrast to IL treated poplar mimic and oxidized poplar with glucose yields of 93 and 82%, respectively (Figure 4). These substrates contain xylan (a hemicellulose component) and either no or reduced lignin. Avicel, a substrate with no lignin and negligible xylan, exhibited the highest glucose yield of ~100%. These results indicate that reduction or removal of lignin improves efficacy of IL pretreatment at 50°C.

The glucose yields of the substrates which contained xylan, poplar mimic and oxidized poplar, were less than the essentially xylan free Avicel. The lower glucose yields can be attributed, in part, to the xylan content of these substrates, which can hinder glucan hydrolysis [42, 45]

Xylose yields were 9, 52, and 45% for native poplar, poplar mimic, and oxidized poplar, respectively, indicating greater yields for lignin-free and reduced-lignin substrates. AHP oxidation can cleave ester linkages between lignin and xylan, rendering it more digestible [41, 42].

In order to monitor the structural transitions of cellulose and changes in crystallinities of these substrates, X-ray powder diffraction (XRD) data were collected prior to, and after IL incubation at 50°C. The crystallinity index (CrI) was estimated from the XRD data (Figure 5). Crystallinity of Avicel decreases with low temperature IL treatment as does AHP oxidized poplar incubated in IL (the coupled method). This is consistent with effective IL treatment and the increased digestibility seen in Figure 4. However, CrI increases for native poplar after IL pretreatment. Residual presence of cellulose I may promote nucleation and transformation of amorphous regions of native cellulose to cellulose I. Crystallinity of AHP oxidized poplar (without IL treatment) also increases. This may be due in part to removal of the more amorphous xylan and lignin components (Table 1).

Cellulose I exhibits a singlet dominant reflection at $2\theta \approx 22.5^\circ$ and a doublet signal centered at $2\theta \approx 15.5^\circ$. The XRD pattern for cellulose II, exhibits a broad singlet at $2\theta \approx 12^\circ$ and a doublet centered at $2\theta \approx 20.5^\circ$. XRD patterns for Avicel, native poplar, and oxidized poplar prior to IL incubation exhibit

reflections characteristic of cellulose I. Poplar incubated in IL at low temperature also exhibits reflections characteristic of cellulose I. The diffraction pattern for IL-treated Avicel exhibits a pattern corresponding to cellulose II. Poplar treated via the coupled method of AHP oxidation followed by IL incubation more closely resembles amorphous cellulose with a shift in diffraction peaks towards the cellulose II allomorph. Conversion of cellulose I to cellulose II or amorphous cellulose is an essential characteristic of effective IL treatment and is an attribute that increases biomass digestibility [31]. This conversion and increased digestibility is observed in substrates with disrupted lignin (oxidized poplar) or no lignin (Avicel) (Figures 4 and 5).

4. Conclusions

An effective low temperature pretreatment strategy was developed to avoid high temperature IL incubation commonly required for biomass pretreatment. This pretreatment strategy is a coupled method consisting of a room temperature oxidation step using alkaline hydrogen peroxide followed by IL incubation at 50°C. Application of this low temperature pretreatment scheme resulted in high hydrolysis yields of lignocellulosic polysaccharides at a low enzyme loading of ~9.5 FPU/g glucan. One of the advantages of low temperature IL incubation is reduction of cellulose acetylation in EMIM-Ac. Low temperature IL treatment also reduces degradation of both the IL and feedstock and facilitates ionic liquid recycle.

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Figure 1. ¹³C NMR spectra for: (a) Avicel incubated in EMIM-Ac at 140°C; (b) Avicel incubated in EMIM-Ac at 50°C; (c) Avicel without EMIM-Ac incubation. All samples were dissolved in AMIM-Cl.

Figure 2. Twenty-four hour enzyme hydrolysis yields of glucose and xylose for poplar pretreated with a coupled method of AHP oxidation followed by 50°C IL incubation. Conditions are defined in the legend as: ih (iih - iii %w/w) (i) Oxidation time, (ii) IL incubation time and (iii) IL incubation solid loading. The first set of histograms represents a control of IL incubation alone. Controls of AHP oxidation alone are indicated with a 0h IL incubation time. Error bars (one standard deviation) are shown for samples run in replicates of three or more. Other reported yields are either for single experiments (for two data sets) or the average of duplicate experiments.

325 **Table 1.** Native (untreated) and pretreated poplar biomass compositions.

Pretreatment	<i>Composition</i>			<i>Losses</i>		
	Glucan (%)	Xylan (%)	Lignin (%)	Glucan (%)	Xylan (%)	Lignin (%)
Native poplar	43±0.4	13±0.7	26±0.6	-	-	-
<i>Coupled method</i>						
6h AHP + IL incubation	48±2.3	9±0.6	24.8±2	6.9±3.9	42.2±1	21.8±3.4
<i>Controls</i>						
IL incubation	43±0.1	13±0.2	25.3±0.8	0±0.1	0±0.3	2.7±1
6h AHP	47.6±0.3	13±0.2	23.7±1	0±0.9	9.6±0.6	19.2±2.8
24h AHP	47±0.6	13±0.2	24.2±0	5.3±0.7	13.4±0.2	20.9±1.1

326 Loss of components after pretreatment are tabulated for samples run in triplicate (average ± one standard
327 deviation). Losses reflect mass loss of samples after IL treatment. Lignin composition is a total of acid
328 soluble and insoluble lignin. For samples incubated in IL, incubation time and temperature were 4 hours
329 and 50°C.

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Figure 3. Twenty-four hour enzyme hydrolysis yields of glucose and xylose for poplar pretreated with a coupled AHP oxidation and IL incubation for varying oxidation times from one to 24 hours. IL incubation time was kept constant at 4 hours with a constant IL loading of 5% (w/w). Error bars (one standard deviation) are shown for samples run in replicates of three or more.

Figure 4. Twenty-four hour enzyme hydrolysis yields for substrates of varied lignin content: native poplar; poplar mimic (lignin free); oxidized poplar (substrate with partial removal of lignin), and Avicel (lignin free). All substrates were incubated in EMIM-Ac at 50°C for 4 hours with 5% (w/w) solid loading. Enzyme loading was 9.5 FPU/g glucan. Error bars (one standard deviation) are shown for samples run in replicates of three or more.

Figure 5. XRD data with crystallinity index, CrI, for: (a) Avicel, (b) IL treated Avicel, (c) native poplar, (d) IL treated poplar, (e) AHP oxidized poplar and (f) poplar pretreated by the coupled method (AHP followed by IL incubation). All substrates with IL treatment were incubated for four hours at 50°C. Untreated Avicel (a) has an XRD pattern characteristic of cellulose I. IL-treated Avicel (b) has a pattern characteristic of cellulose II.

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Graphical Abstract