Scale-Up of a Two-Stage Cu-Catalyzed Alkaline-Oxidative Pretreatment of Hybrid Poplar

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ABSTRACT

A two-stage alkaline-oxidative pretreatment of hybrid poplar was investigated at scale (20 L reactor volume) with the goals of understanding how reaction conditions as well as interstage mechanical refining impact downstream process responses. The pretreatment comprises a first stage of alkaline delignification (alkaline pre-extraction) followed by a second delignification stage employing Cu-catalyzed alkaline hydrogen peroxide with supplemental O_2 (O_2-enhanced Cu-AHP). Increasing pre-extraction severity (*i.e.,* temperature and alkali loading) and pre-treatment oxidation (increasing H_2O_2 loading) were found to increase mass and lignin solubilization in each stage. Lignin recovered from the first stage was subjected to oxidative depolymerization and led to aromatic monomer yields as high as 23.0% by mass. Lignins recovered from the second stage Cu-AHP pretreatment liquors were shown to exhibit aliphatic hydroxyl contents more than six-fold higher than for a typical hardwood kraft lignin, indicating these lignins could serve as a biobased polyol for a range of polyurethane applications.

Keywords: Alkaline hydrogen peroxide (AHP) pretreatment, Copper, Hybrid Poplar, Oxidative delignification; Lignin; Biorefinery

1. INTRODUCTION

Woody biomass, including short rotation bioenergy crops such as hybrid poplar, represent a vast source of renewable carbohydrates and aromatics, and it is envisioned as a key feedstock for the sustainable production of biobased fuels and chemicals in the United States.[1] One approach to utilizing the carbon and energy contained in lignocellulosic biomass for fuels and chemicals includes chemical and biological deconstruction of the plant cell wall biopolymers to yield monosaccharides and modified lignins.[2] This deconstruction can involve a chemical pretreatment of the biomass to facilitate a subsequent enzymatic hydrolysis of the cell wall polysaccharides.[3] While technologies for cellulosic biofuels have seen efforts at commercialization over the last few years, there are still technological and economic challenges that limit widespread adoption of these biomass conversion approaches, particularly for woody biomass.[4]

Lignin represents one of the few renewable sources of aromatic chemicals.[5] Although well-documented lignin conversion challenges exist that derive from the low reactivity and high heterogeneity of lignin, this material represents an immense opportunity if process-derived lignin

streams can be generated that optimally couple to a lignin conversion approach.[6] Delignifying (including alkaline and alkaline-oxidative) pretreatments overcome cell wall recalcitrance by chemically modifying and/or depolymerizing lignin to increase its solubility in alkali. It is known that delignified biomass is highly susceptible to enzymatic hydrolysis[7, 8] and alkaline pretreatments can therefore be considered as biomass fractionation processes that have the capacity to provide a separate lignin stream that can be valorized to additional co-products such as aromatic monomers and polyurethanes.[9]

Oxidizing "post-treatments" coupled to an initial alkaline delignification are commonly employed in chemical bleaching or delignification in the pulp and paper industry, and they have been proposed as a component of two-stage pretreatments for cellulosic biofuels.[10] For example, alkaline pretreatment/pulping followed by oxygen delignification has been applied as a pretreatment for woody biomass.[11] Several catalytic oxidative treatments of biomass have recently been investigated that utilize features of successful biological approaches to plant cell wall deconstruction as technologies for pulp bleaching, delignification, or pretreatments for the production of biofuels.[12] As an example, an effective method for pretreatment has been developed using molecular oxygen treatment catalyzed by copper-diimine complexes.[13] Our recent work has focused on a two-stage pretreatment process that employs an initial alkali-only pretreatment followed by a second stage consisting of a metal-catalyzed alkaline-oxidative post-treatment.[14] This strategy is comparable to alkaline delignification coupled to an oxidative post-treatment used in the pulp and paper industry, and it offers the potential to increase or decrease the severity of either the pre-extraction or the post-treatment. In this approach, we identified that supplementation of H_2O_2 with O_2 during the catalytic alkaline-oxidative post-treatment stage resulted in a synergistic response that could not be achieved with either oxidant individually.[14] This approach is comparable to commercial peroxide-reinforced O_2 delignification stages with the significant difference that during pulp delignification, chelation stages are often applied to remove transition metals that will result in oxidative chain scission. For plant cell wall deconstruction, however, limited cellulose depolymerization is not problematic and, in fact, may be beneficial. Specifically, we demonstrated that the H_2O_2 loading could be significantly decreased from 80 mg H_2O_2/g biomass using only H_2O_2 as the oxidant to 20 mg H_2O_2/g biomass while still achieving 95% sugar monomer yields with the inclusion of 50 psig O_2 (3.45 bar).[15]

Our prior work demonstrated this two-stage alkaline-oxidative delignifying pretreatment is capable of yielding process lignins from hardwoods that are an ideal feedstock for co-products. Specifically, we've shown that these lignins can be coupled with an oxidative depolymerization to generate high yields of aromatic monomers or be used as a bio-based polyol replacement in the production of polyurethane foams and coatings.[16] Notably, the hybrid poplar lignins generated and recovered from Cu-catalyzed alkaline-oxidative pretreatment have been demonstrated to be superior to other sources of lignin (*e.g.*, kraft lignin and other biorefinery-derived lignins) with monomer yields greater than 20%[17] and aliphatic hydroxyl contents (*i.e.*, reactive sites for incorporation polyurethanes) more than 5-fold higher than those of kraft lignins.[14]

One important limitation of our prior work on this two-stage process is that the pretreatments were performed at the laboratory scale to facilitate screening of reaction conditions: ≤ 100 g for the alkaline pre-extraction stage and ≤ 5 g biomass for the Cu-catalyzed stage. An important implication of this small scale is that the reactions required finely knife-milled biomass (*i.e.*, ≤ 5 mm particle size) that would exhibit significantly different responses to pretreatment and enzymatic hydrolysis than in an industrial process.[18, 19] Notably, chemical pulping of woody biomass is performed commercially on wood chips and generally can include steps for chip "defiberization" by explosive depressurization at the termination of the cooking time[20] and/or coupled with mechanical refining that facilitates additional fibrillation. This fibrillation increases the surface area of fibers and for papermaking is important for fiber binding, but for cellulosic biofuels processes, this has been linked to increased cellulose accessibility for enzymatic hydrolysis.[21] Energy input for biomass size reduction is an important process parameter that can have a significant contribution to the operating costs of a biorefinery.[22] Importantly, because specific refining energy (*i.e.*, kWh/tonne) decreases with decreasing pulp yield (*i.e.*, increasing delignification),[23] particle size reduction and/or additional mechanical refining following the initial delignification stage (alkaline pre-extraction) would be the expected configuration for a scaled process.

In the present work, a series of studies were performed to investigate the performance of this two-stage alkaline-oxidative pretreatment process at scale using wood chips. Overall, the purpose of this work is to generate an improved understanding of process responses to changes in operating conditions and to improve the integration of the process components to yield a process that can achieve both high enzymatic hydrolysis yields and lignins with properties suitable for co-products

applications while simultaneously minimizing inputs to the process. Specific goals include identifying the relationships between pre-extraction conditions, interstage mechanical refining extent, second-stage Cu-AHP oxidant loading, and the subsequent process responses that include enzymatic hydrolysis yields, lignin solubilization, and lignin properties. First, we investigate the impact of alkaline pre-extraction conditions for alkaline delignification at the 20 L scale and subsequent degree of mechanical refining on several downstream process responses. This includes (1) performance of the second stage O_2-enhanced Cu-catalyzed alkaline hydrogen peroxide (Cu-AHP) pretreatment at the 100 mL scale, (2) enzymatic hydrolysis both before and after the Cu-AHP stage, and (3) properties of the lignin recovered from the alkaline pre-extraction. Next, using fixed conditions for alkaline pre-extraction and chip "disintegration" stages, we investigate the impact of H_2O_2 loading during O_2-enhanced Cu-AHP at the 20 L scale on solubilized and recovered lignin properties, fiber properties, and enzymatic hydrolysis yields.

2. MATERIALS AND METHODS

2.1 Biomass

Hybrid poplar (*Populus nigra* var. *charkoviensis*×*caudina* cv. NE-19) was collected from the University of Wisconsin Arlington Agricultural Research Station in 2012. The air-dried wood logs were chipped (Earthwise GS70016 Chipper Shredder) to yield approximately 0.5×2.0×2.5 cm wood chips. The chipped biomass was stored in airtight bags prior to use. Prior to composition analysis, biomass was knife-milled (Wiley Mini-Mill, Thomas Scientific, Swedesboro, NJ) to pass a 20-mesh screen (0.841 mm).

2.2 Alkaline Pre-Extraction and Lignin Recovery

An overall process flow diagram for the material flow for unit operations and materials used in this work is presented in **Figure 1**. Alkaline pre-extraction was performed using 1.8 kg (dry basis) wood chips (approximately 0.5×2.0×2.5 cm) in a 20 L, stainless-steel pulp digester (AU/E-20 with TP-2027 model lid, Regmed Indústria Técnica de Precisão Ltda., Osasco, São Paulo, Brazil) at a solids content of 15% (dry wt/liquid vol) corresponding to a liquor-to-wood ratio of 5.67. Reactor mixing was achieved by end-over-end tumbling of the vessel at a rate of 5.7 rpm. A range of reaction conditions were employed and include temperature over the range of 125 to 170

°C and alkali loadings of 100 to 150 mg NaOH/g biomass at a fixed reaction time of 60 min. During heat-up, reactors were heated at a rate of 3°C/min by an electrical resistance heater until the target temperature was reached. After the target time was reached, the heater was turned off and the reactor was allowed to cool overnight by heat loss to the ambient air. The temperature profile during cooling showed an exponential decrease approaching ambient temperature. At the completion of the reaction, the mass yield was calculated gravimetrically. For lignin recovery, the pre-extraction liquor was separated from treated wood chips by filtration and stored at 4 °C for 24 h. Pre-extracted wood chips were soaked in 12 L deionized water at room temperature for 24 h to recover liquor entrained in the biomass. Recovered liquor and the soaked wood chips liquor were combined and acidified to approximately pH 2 using sulfuric acid (96.4%, wt/wt) to precipitate the recoverable biopolymers. The acidified liquor was held at 4 °C for 24 h and then centrifuged at 8000 rpm (15,970 x g) for 10 minutes at 20 °C. The recovered biopolymers were washed with deionized water until the pH of the wash liquor was > 3.25. The recovered biopolymers were dried at room temperature for 48 h and then vacuum-oven dried at 50 °C and 0.68 bar pressure for 24 h prior to subsequent characterization.

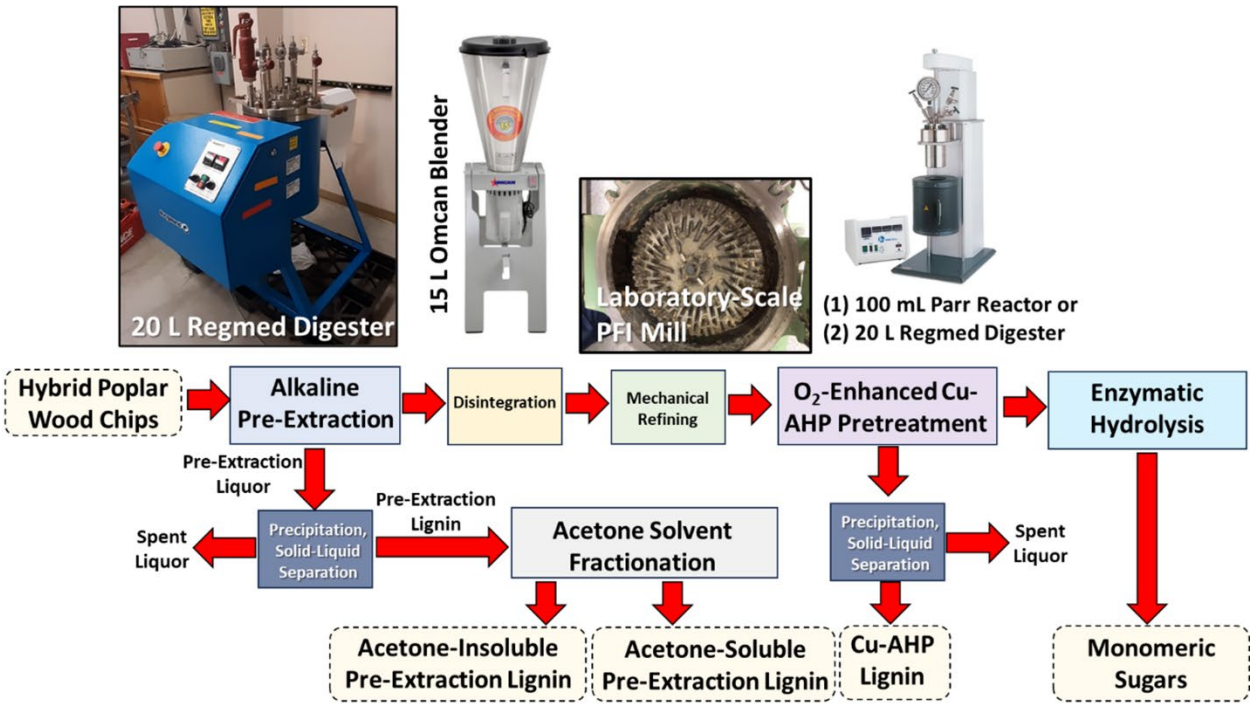

Fig. 1 Process flow diagram illustrating material flows and unit operations utilized in this study.

2.3 Pulp Disintegration and PFI Milling

After the recovery of liquor entrained in the biomass by soaking the alkaline pre-extracted biomass in 12 L deionized water, biomass was further washed with approximately 100 L deionized water until it reached a neutral pH and then subjected to disintegration prior to mechanical refining and/or O_2-enhanced Cu-AHP pretreatment. Disintegration was performed using a 15 L stainless-steel cup tilting blender (BL-BR-0015, Omcan Inc., Mississauga, Ontario, Canada) for 5 minutes at a consistency of 15% (dry wt/liquid volume) at 3500 rpm. Washed and disintegrated biomass was filtered using a 45 x 60 cm, 120 µm nylon mesh home brewing mesh filter bag (Xiamenshi Shanghaocha Trading Company, Ltd., Xiamen, China) and stored in zip-lock bag at 4 °C. Moisture content and pre-extracted solids remaining were determined after this stage as a preparation for mechanical refining or the secondary oxidative stage. The mechanical treatment of the disintegrated biomass was performed using a laboratory-scale PFI mill in accordance with TAPPI standard T 248. Approximately 30 g of the disintegrated biomass were evenly positioned along the inner wall of the bedplate within the PFI mill. Following that, the disintegrated biomass was subjected to mechanical impacts via the beater roll at different revolution counts (3000, 6000, and 10000).

2.4 O_2-Enhanced Cu-AHP Pretreatment

The O_2-enhanced Cu-AHP pretreatment was conducted at two different scales: (1) a 100 mL stainless-steel Parr reactor (Parr Instruments Company, Moline, IL, USA) using disintegrated and mechanically refined biomass at a biomass loading of 5% (wt/vol) based on the weight of original biomass and (2) in the 20 L Regmed AU/E-20 laboratory digester used for alkaline pre-extraction with disintegrated biomass at a biomass loading of 10% (wt/vol) based on the dry weight of original biomass. The O_2-enhanced Cu-AHP pretreatment was performed under the optimized conditions based on our prior work,[14] including 10% NaOH (wt/wt original biomass), 1.0 mM $CuSO_4$ (0.159% wt/wt original biomass), 1.0 mM 2,2′-bipyridine (bpy) (0.156% wt/wt original biomass), and an O_2 pressure of 50 psig (3.45 bar). The H_2O_2 loadings varied from 6.0 to 40 mg H_2O_2/g original biomass. The reactions were performed at 80 °C for 12 h with mixing at 200 rpm for the 100 mL scale and end-over-end tumbling at a rate of 5.7 rpm for the 20 L scale. For the 100 mL scale reaction, the reactor was cooled to room temperature in a water bath (100 mL scale) or in ambient air (20 L scale) and depressurized. Temperature and pressure profiles for 20 L batches are presented in **Figure S1, Supporting Information**. For the 100 mL scale, the solid fraction was separated from the liquor via filtration, thoroughly washed with approximately 1 L

deionized water and stored in a sealed bag at 4 °C. For the 20 L scale, the solid fraction was separated from the liquor using the same 120 µm nylon mesh home brewing mesh filter bags from the pre-extraction and pressed from the biomass using a 19 L stainless-steel fruit press (MacIntosh, Pleasant Hill Grain, Hampton, Nebraska, USA). Pre-extracted wood chips were soaked in 12 L deionized water at room temperature for 24 h to recover additional liquor entrained in the biomass. The pretreated biomass was thoroughly washed with approximately 100 L deionized water and stored in a sealed zip-lock bag at 4 °C prior to composition analysis and enzymatic hydrolysis. Lignins were recovered from the alkaline liquor using the same approach as the pre-extraction liquors.

2.5 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in 15 mL pre-autoclaved (121 °C for 15 min) Falcon tubes using an enzyme cocktail consisting of CTec3 and HTec3 at a protein ratio of 1:1 and a total enzyme loading of 15 mg protein/g glucan (based on initial glucan content). The enzyme cocktails Cellic CTec3 (protein content of 197.3 mg/g) and HTec3 (protein content of 170.5 mg/g) were kindly provided by Novozymes A/S (Bagsværd, Denmark). Bovine serum albumin (BSA; Sigma-Aldrich Corp., St. Louis, MO) was added to select enzymatic hydrolysis flasks to make up the reduced protein content of cellulases and hemicellulases before the addition of the hydrolytic enzymes with total protein loadings (CTec3 + HTec3 + BSA) summing to 15 mg protein/g glucan. Reactions were performed at 5% (wt/vol) solid loading (based on the weight of original, untreated biomass) and 50 °C for 72 h in 50 mM sodium citrate buffer (pH 5.0) with orbital shaking at 80 rpm (C24KC Incubator Shaker, New Brunswick Scientific, NJ, USA). Following enzymatic hydrolysis, the liquid phase was separated from the reaction mixture through centrifugation (10 min at 3075 × g). The concentration of glucose and xylose generated by hydrolysis was measured by an HPLC (Agilent 1260 Series) equipped with an Aminex HPX-87H (Bio-Rad Laboratories, Hercules, CA) using aqueous 0.05 M H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min and detection by refractive index with the "xylose" measurement comprising the sum of xylose, mannose, and galactose, which co-elute on this column.

2.6 Acetone Fractionation of Recovered Biopolymers

Solubility in acetone and solvent fractionation of biopolymers recovered from alkaline pre-extraction liquors was performed by first suspending 12.0 g (dry basis) of the recovered biopolymers in 120 mL of acetone. This suspension was incubated at 50 °C for 30 min in a water

bath and then centrifuged at 4000 rpm for 10 min to separate soluble and insoluble fractions. The insoluble components were dried at room temperature for 24 h and subsequently dried in a vacuum oven at 50 ℃ at 0.68 bar pressure for 24 h. The fraction of solubilized lignin was determined gravimetrically.

2.7 Cell Wall Composition Analysis

Compositional analysis was performed on the raw biomass, pre-extracted and pretreated biomass, as well as recovered lignins using approximately 100 mg (dry basis) of material. Polysaccharide and Klason lignin contents were determined according to NREL LAP TP-510-42618[24] with minor modifications as outlined in our prior work.[25] Moisture content was determined using a moisture analyzer (MB90, Ohaus Corporation, USA), while ash content was determined following NREL/TP-510-42622.[26]

2.8 Lignin Characterization

The number and weight average molar masses of unfractionated (recoverable biopolymers), acetone-soluble, and acetone-insoluble fractions were determined by gel permeation chromatography (GPC) using acetylated samples as described in our prior work[25] using an Agilent HPLC 1260 series equipped with a Waters Styragel HR 4 (7.8 × 300 mm, Milford, MA, USA) column. HPLC grade THF was used as a mobile phase with a 0.5 mL/min flow rate with UV detection at 280 nm and a column temperature of 35 ℃. Monodisperse polystyrene standards in the range of 484 to 65,000 Da (Readycal, Fluka Analytical) were used as a reference. The molar masses were calculated in Excel using a "direct standard calibration" method reported elsewhere.[27] A Bruker 600 MHz Avance III NMR spectrometer was used to collect ^{13}C and ^{1}H–^{13}C correlation 2-D heteronuclear single-quantum coherence (HSQC) NMR spectra of unfractionated recovered biopolymers as well as acetone-soluble and acetone-insoluble fractions. The sample preparation and analysis were performed as in our previous work.[23] ^{31}P NMR was performed on select samples using sample functionalization and analysis as reported in our prior work.[14] Lignins obtained from the various fractions of the pretreatment liquors were subjected to Cu-catalyzed oxidative alkaline depolymerization using the optimized condition as described in the **Supporting Information**.

2.9 Fiber Analysis

Prior to fiber analysis, approximately 15 g of disintegrated pulps were suspended in 1.0 L of water (1.5% consistency) and passed through a 10 mesh (2.0 mm) screen with an additional 20 L of water to remove large particle "rejects". The screened biomass was filtered (Whatman 40) and the recovered fibers were subjected to image analysis using an FS5 Fiber Image Analyzer (Valmet Oyj, Espoo, Finland). For this, approximately 50 mg (dry basis) of screened biomass was suspended in 500 mL of water. Image acquisition was conducted at a pump flow rate of 20 mL/s. Total number of particles per sample characterized ranged from approximately 500,000 to $2.75x10^6$ from which particle features (length, width, and percent fines) were determined.

3. RESULTS AND DISCUSSION

This work explores the scale up of a two-stage alkaline-oxidative pretreatment process with interstage mechanical refining to yield cellulosic sugars and lignin streams amenable to upgrading in co-products applications. The purpose of this work is two-fold. Primarily, this work investigates the impact of changing process variables during biomass fractionation on processing outcomes (mass, lignin, and hydrolysis yields; lignin properties; fiber properties). Secondarily, the series of biomass pretreatment runs generated kilogram quantities of lignins that were the subject of separate studies on using these lignins as feedstocks for materials applications (polyurethane coatings and foams) and as chemicals (lignin depolymerization) and motivates the lignin fractionation and characterization work in the current study.

A summary of the reaction conditions used in this series of scale-up studies is provided in **Table 1** and can be summarized as comprising three integrated studies. In the first study (conditions 1 through 9 in **Table 1**), we investigated the impact of the first stage pre-extraction conditions in the 20 L digester, specifically temperature and alkali loading, on mass and lignin solubilization as well as impacts on performance of downstream unit operations. These downstream impacts include (1) response to varying levels of mechanical refining using a laboratory PFI mill, (2) response to enzymatic hydrolysis after only pre-extraction and refining, and (3) subsequent response to O_2-enhanced Cu-AHP pretreatment (performed at the 100 mL scale) and enzymatic hydrolysis. The second study (conditions 10 through 12 in **Table 1**) investigated the impacts of operating conditions, specifically H_2O_2 loading during the scaled up second stage O_2-enhanced Cu-AHP pretreatment, on downstream process responses. These impacts include subsequent mass and lignin extraction and recovery yields, enzymatic hydrolysis

yields, and fiber properties. The final series of studies involved characterization of lignins recovered by acidification from the liquors of both the first stage alkaline pre-extraction and the second stage O_2-enhanced Cu-AHP pretreatment. Specifically, the lignins from the first stage alkaline pre-extraction were subjected to solvent fractionation in acetone since solvent fractionation can enrich select functionalities or properties (*e.g.*, molar mass) to allow for optimal utilization in target applications such as polyurethane coatings and phenolic adhesives[28] or potentially generate fractions that are optimal for depolymerization to aromatic monomers. Finally, the lignins recovered from the second stage O_2-enhanced Cu-AHP pretreatment were subjected to characterization for properties advantageous for their use as a biobased polyol in polyurethane resins.

Reaction Condition	First Stage: Alkaline Pre-Extraction (all at 20 L Scale)		Interstage Refining		Second Stage: O_2-Enhanced Cu-AHP Pretreatment		Enzymatic Hydrolysis	
	NaOH Loading (mg/g biomass)	T (°C)	Chip Disinte-gration	PFI Refining (rev)	Reactor Scale	H_2O_2 Loading (mg/g biomass)	After Refining	After Cu-AHP
1	150 mg/g	170 °C	+	3000	100 mL	20 mg/g	+	+
2			+	6000	Not performed		+	–
3			+	10000			+	–
4	150 mg/g	145 °C	+	3000			+	–
5			+	6000	100 mL	20 mg/g	+	+
6			+	10000	Not performed		+	–
7	100 mg/g	125 °C	+	3000			+	–
8			+	6000			+	–
9			+	10000	100 mL	20 mg/g	+	+
10	100 mg/g	120 °C	+	None	20 L	6.0 mg/g	–	+
11			+	None	20 L	12 mg/g	–	+
12			+	None	20 L	40 mg/g	–	+

Table 1 Summary of reaction conditions and scales used in this study. Operating conditions that were unvaried are provided in the **Materials and Methods**.

3.1 Alkaline Pre-Extraction and Mechanical Refining Impact on Downstream Process Performance

Our prior work screened a range of reaction conditions for the alkaline pre-extraction stage, including temperature, time, NaOH loading, solid-to-liquid ratio, and poplar genotype at the small scale (0.5 to 5.0 g) using finely knife-milled (≤0.841 mm particle size)[25] or hammer-milled (≤5.0 mm particle size)[14] biomass. Chemical pulping of woody biomass is nearly universally

performed on wood chips in batch or continuous reactors, with chip size and shape optimized for penetration of pulping chemicals and circulation of liquor between chips.[29] Specifically, the chip is longest in the axial dimension (*i.e.*, parallel to the fibers in the fluid transport direction of the lumen) up to approximately 30 mm and must be thin (minimum of approximately 2 mm to prevent excess generation of fines) in one of the other two dimensions to facilitate optimal diffusion of alkali into the chip.[30] Chemical pulping of sawdust is performed commercially but can have challenges with respect to uniform impregnation of pulping chemicals, flowability, and plugging of screens or process lines,[31] necessitating the use of different digester systems.[32] In the present study, pre-extraction temperature and alkali loading were screened on hybrid poplar wood chips in a 20 L digester with the goal of generating a "high", "medium", and "low" severity for the pre-extraction stage (**Table 1**). The measured mass extracted (**Fig. 2A**) and the calculated solubilized lignin (**Fig. 2B**) for the three different reaction conditions show the general trends of increasing solubilization with increasing pre-extraction severity. Complete composition and mass yields for all conditions are available in **Supporting Information Table S1**. This trend of increasing mass and lignin solubilization with increasing pre-extraction severity is not surprising, and mass solubilizations (15.2% to 35.0%) are in the range of pulp yields utilized for semichemical pulping[23] and could be considered to reduce the specific mechanical refining energy required for defiberization and/or additional refining.[33]

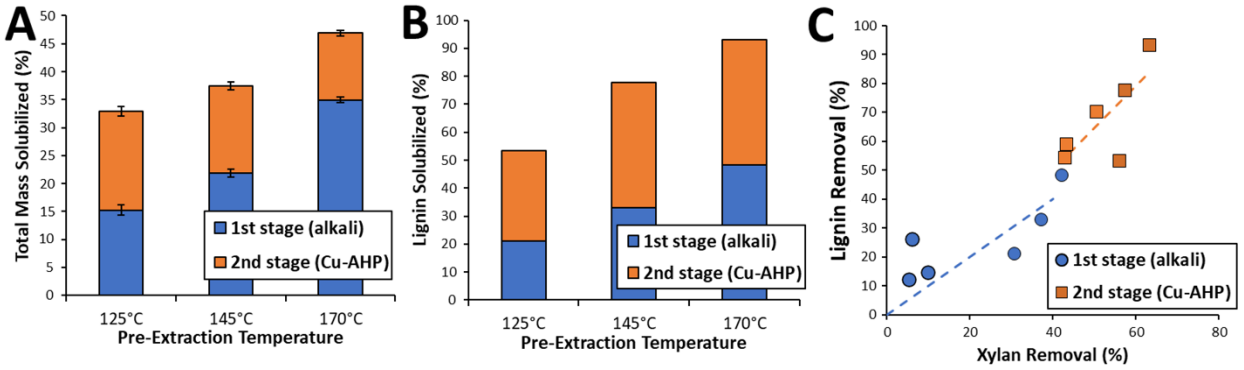

Fig. 2 Impact of first stage pre-extraction temperature and subsequent refining and O_2-enhanced Cu-AHP pretreatment under "standard" conditions on (**A**) total mass and (**B**) lignin solubilization corresponding to conditions 1, 5, and 9 in **Table 1**. Additional data on (**C**) lignin versus xylan solubilization during both stages of delignification for all twelve reaction conditions from **Table 1**.

After subjecting alkali pre-extracted poplar chips to disintegration and mechanical refining in a PFI mill (corresponding to conditions 1, 5, and 9 in **Table 1**), the second stage of delignification

was performed. Our earlier work has demonstrated that increasing the reaction temperature and using both O_2 and H_2O_2 as co-oxidants allowed us to significantly reduce the loading of H_2O_2 and bipyridine while still maintaining high sugar yields (≥ 90%).[14-15] This second stage Cu-AHP pretreatment can be considered to operate as a delignification stage comparable to an extended oxygen delignification in pulping and shows that a significant fraction of the remaining mass (**Fig. 2A**) and lignin (**Fig. 2B**) can be removed during this stage (*i.e.*, 32.1% to 44.8% more lignin). Results for xylan removal versus lignin removal for all conditions screened show equal lignin removal and xylan removal (slope of trendline = 1.00 %/%) for the first stage pre-extraction (up to 42% xylan removal, **Fig. 2C**). In the second stage of the pretreatment, the selectivity for lignin removal increases (**Fig. 2C**) with a slope of 1.42 lignin-to-xylan removal (%/%). The reason for this is likely two-fold. First, the highly extractable xylan has already been removed. Second, the alkaline-oxidative stage is more suited for targeted delignification (*i.e.*, high lignin/xylan selectivity) through lignin oxidation and solubilization.[34]

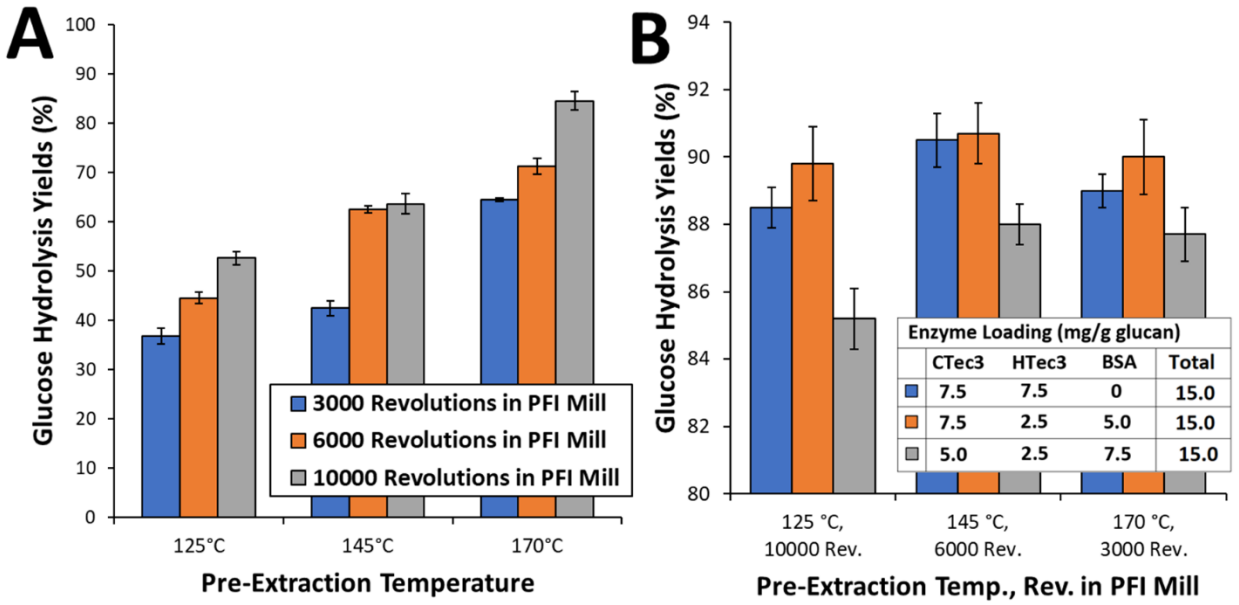

Fig. 3. Impact of pre-extraction temperature and mechanical refining on (**A**) glucose enzymatic hydrolysis yields (no second stage Cu-AHP was performed) based on conditions 1 through 9 in **Table 1**. Glucose yields following (**B**) second stage Cu-AHP and enzymatic hydrolysis at an enzyme loading of 15 mg protein per g glucan (based on initial composition) corresponding to conditions 1, 5, and 9 in **Table 1**.

Enzymatic hydrolysis was next performed on the pre-extracted wood chips (*i.e.*, first stage only) subjected to disintegration and differing extents of mechanical refining in a PFI mill to assess the impact on sugar yields (**Fig. 3A**). Several key findings can be highlighted from these data.

First, as expected, with the increase in both the pre-extraction severity and refining level, the hydrolysis yields of glucose and xylose increased. For the pre-extraction impact, this increase is likely due to the increased accessibility of carbohydrates to enzymes by removing more lignin when increasing the severity of alkaline pre-extraction (**Fig. 2B**) and is a well-known correlation.[14] The shearing and compression forces exerted by the beater roll give rise to multiple effects on the disintegrated biomass, encompassing intra-fiber bond breakage, fibrillation, and fiber cutting.[35, 36] Increasing the extent of refining (number of revolutions) facilitates increased fibrillation, which increases both the surface area of biomass and, consequently, the accessibility of the glucan and xylan.[21] A final observation is that relatively high hydrolysis yields with higher extents of refining can be achieved at the modest levels of delignification achieved in the first stage (*i.e.*, 21% to 48%, **Fig. 2B**). This is in agreement with our prior work on alkaline pretreatment that demonstrated for a variety of hardwoods, including hybrid poplar, that extents of delignification ranging from 50% to 80% could result in glucose hydrolysis yields of 80% to 90% at modest enzyme loadings (*i.e.*, 10 mg/g glucan).[37] While beyond the scope of this work, the sugar streams are expected to be highly fermentable in a subsequent biological conversion step to products such as ethanol since the majority of the inhibitors (*i.e.*, inorganics, soluble aromatics/lignin, acetate, Cu catalyst) are removed in the solid-liquid separation prior to enzymatic hydrolysis.

Following alkaline pre-extraction, disintegration, and refining at one select condition (conditions 1, 5, and 9 in **Table 1**), the biomass was subjected to O_2-enhanced Cu-AHP pretreatment at the 100 mL scale under identical conditions (20 mg H_2O_2/g biomass, 3.45 bar O_2, 100 mg NaOH/g biomass, 1.0 mM Cu(bpy), 80 °C, 12 h). Liquors were recovered and the pretreated biomass was subjected to enzymatic hydrolysis using a range of enzyme combinations and cellulase loadings (**Fig. 3B**). Specifically, the impact on hydrolysis yields was assessed as a function of enzyme loading and supplementation with bovine serum albumin (BSA), which has been demonstrated to improve hydrolysis yields at low enzyme loadings, presumably by binding to lignin and preventing non-productive absorption of cellulases due to hydrophobic interactions.[36, 38] The results showed that near-theoretical glucose hydrolysis yields (>88%) can be achieved for all conditions using the highest enzyme loading of 15 mg/g glucan. Furthermore, the high sugar yields, especially the glucose yields, could be preserved even by reducing the hemicellulase loading from 7.5 to 2.5 mg protein per g glucan by adding 5 mg BSA instead. However, further reducing cellulase loading from 7.5 to 5 mg protein per g glucan slightly reduced the sugar yields.

These results suggested that as an inexpensive protein, BSA could be used to replace or supplement the enzyme protein for enzymatic hydrolysis.

3.2 Impact of H_2O_2 Loading on Second Stage Cu-AHP Performance at Scale

Next, H_2O_2 loading was varied during O_2-enhanced Cu-AHP pretreatment at the 20 L scale and its impact mass yields, lignin solubilized, and hydrolysis yields (**Fig. 4**) was determined. These results demonstrated that both the total mass (**Fig. 4A**) and lignin (**Fig. 4B**) solubilized increase with increasing H_2O_2 loading with a strong selectivity for lignin removal over xylan removal (**Fig. 2C**). Next, glucose hydrolysis yields were incrementally increased with increasing H_2O_2 loading (**Fig. 3C**). As H_2O_2 loading on biomass is one of the key contributors to process operating costs,[15] it could be considered that minimizing the H_2O_2 input into the process would be the preferred process configuration if sugar hydrolysis yields and lignin yields and co-product utility can be maintained. Finally, it can be observed from all the hydrolysis results that xylose enzymatic hydrolysis yields following the O_2-enhanced Cu-AHP step were generally in the range of 40% to 60% (**Fig. S2, Supporting Information**). Considering that sugar yields are one of the key drivers of process economics, these yields seemingly need to be maximized. It must be remembered, however, that these xylose yields represent the xylose released *per original xylan*. Because 40% to 60% of the xylan is solubilized during the first and second stage of delignification (**Fig. 2C**) and can theoretically be recovered during the lignin precipitation stage, the total xylose yields could be nearly quantitative.

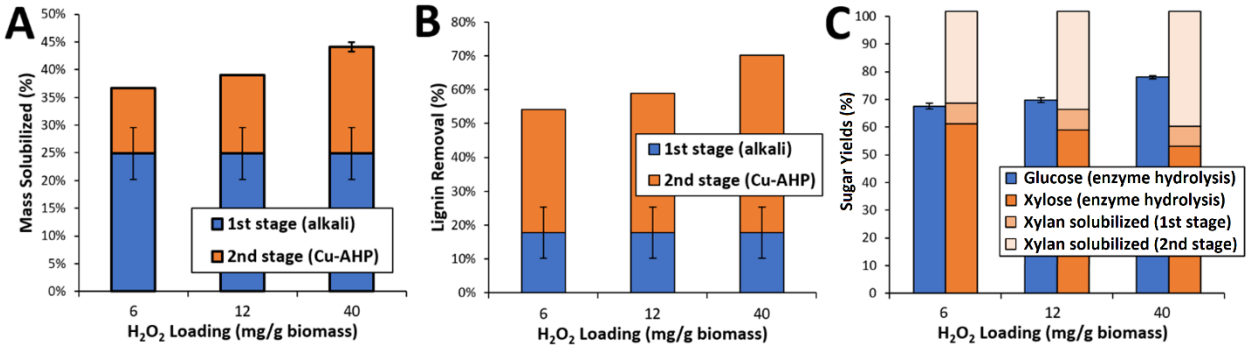

Fig. 4 Impact of H_2O_2 loading during O_2-enhanced Cu-AHP pretreatment at the 20 L scale on (**A**) total mass solubilization, (**B**) lignin solubilization, and (**C**) enzymatic hydrolysis yields corresponding to conditions 10, 11, and 12 in **Table 1**. Percents represent the amount released relative to original biomass. Pre-extraction data represent averages of four replicates, while the 40 mg/g H_2O_2 loading represents the averages of duplicate pretreatments.

A series of characterizations of disintegrated, delignified poplar were next performed to assess how pretreatments impacted particle morphology, response to defiberization, and, consequently, how these differences may impact the subsequent processing response (*i.e.*, delignification during the second stage and enzymatic hydrolysis). Macroscopic differences in alkaline pre-extracted poplar before and after disintegration can be observed **(Fig. S3, Supporting Information**) that result from liberation of individual "fibers" (*i.e.*, individual cells) from the wood chips. Removal of lignin, particularly from the lignin-rich middle lamella, is well-understood to facilitate fiber liberation with mechanical disruption. Fiber liberation has been shown to start at ~30% delignification and is complete by ~80% delignification for alkaline-pulped Douglas-fir (*Pseudotsuga menzeisii*).[40] However, there are import differences in the objective of difiberization/refining for a papermaking process versus a cellulosic biofuels process. Namely, a key goal for a papermaking process is to increase surface area and fibrillation to facilitate fiber-fiber bonding to yield improvements in mechanical strength properties for the resulting paper.[41] For cellulosic biofuels processes, the goal is to facilitate accessibility of cell wall polysaccharides to cellulolytic enzymes.

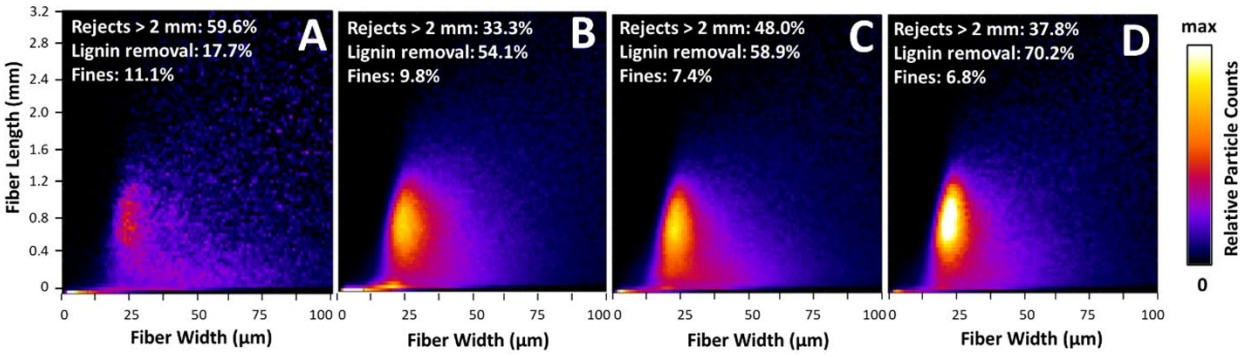

Fig. 5 Number average fiber particle size distributions for disintegrated particles following (**A**) alkali pre-extraction only or Cu-AHP pretreatment at peroxide loadings of (**B**) 6 mg H_2O_2/g Cu-AHP pretreated biomass, **(C)** 12 mg H_2O_2/g Cu-AHP pretreated biomass, and **(D)** 40 mg H_2O_2/g Cu-AHP pretreated biomass.

To better understand how increasing delignification impacts free fiber morphological properties following (a) alkaline pre-extraction, (b) defiberization, and (c) Cu-AHP pretreatment, property distributions of individual particles following disintegration were characterized using automated image analysis of fiber suspensions with number distributions of fiber length versus width plotted in **Figure 5** along with selected key metrics. Several key trends can be observed from this data. First, as described in the **Materials and Methods**, the disintegrated biomass was

screened in the wet state to pass a 10 mesh (2.0 mm) screen to remove "rejects" and prevent plugging of the lines of the Valmet Fiber Image Analyzer during image analysis. The fraction of rejected material on a mass basis ranged from 33.3% to 59.6% (mass basis) and was not a clear function of treatment conditions. One discernable trend is that with increasing delignification (*i.e.*, **Fig. 5A** < **Fig. 5B** < **Fig. 5C** < **Fig. 5D**) the relative abundance of visually distinct "fibers" with 0.2 mm < length < 1.5 mm and 12.5 µm < width < 37.5 µm increases substantially. The expected value for length-weighted fiber length for unbeaten hardwood kraft fibers has been measured as 885 µm.[42] The correlation between increasing abundance of fibers within the typical range of hardwood kraft pulps with increasing delignification can be interpreted as increasing delignification promoting fiber liberation to yield a tighter distribution of free fibers, whereas the higher lignin particles were more polydisperse and contained more multi-fiber particles. Finally, "dust-like fines" (*i.e.*, length < 0.2 mm, width < 0.2 mm) can be observed in the bottom left side of each figure, while other "fines" with length > 0.2 mm are also present with the total fines content presented in the figure. The trend of decreasing fines content with increasing delignification may be a consequence of solid-liquid separations following the second stage Cu-AHP pretreatment.

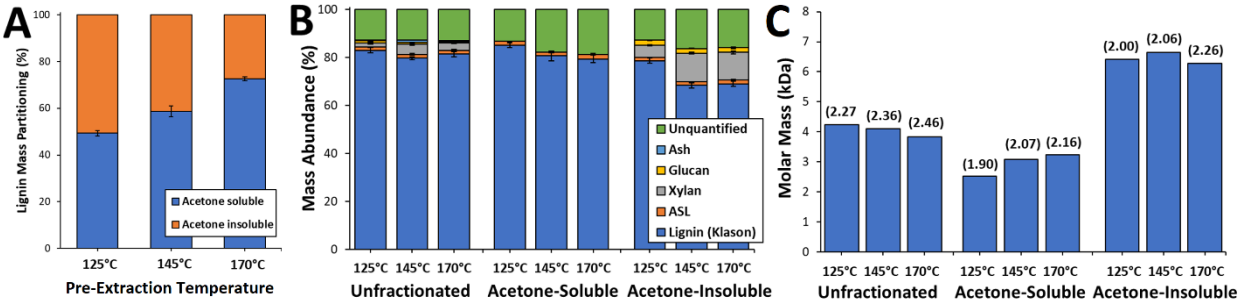

Fig. 6 Characterization of acetone fractionation of lignin-rich biopolymers recovered from differing alkaline pre-extraction conditions: (**A**) fractionation yields/solubility, (**B**) composition of unfractionated and acetone-fractionated lignins, and (**C**) number average molar mass of unfractionated and acetone-fractionated lignins. Number in parentheses indicate polydispersity indices. ASL: Acid soluble lignin.

3.3 Properties of Lignins Recovered from Alkaline Pre-Extraction Stage

In the next part of this work, the lignin-rich biopolymers recovered from the alkaline pre-extraction stage for varying conditions (*i.e.*, pre-extraction only conditions 1 through 9) were subjected to solvent fractionation, and the recovered biopolymers were characterized for composition, molar mass by GPC, β-O-4 content by ^{13}C NMR, phenolic hydroxyl content by ^{31}P NMR, and response to Cu-catalyzed depolymerization. Solubility in an organic solvent is a

necessary prerequisite for many applications such as the use of lignin as a polyol for incorporation into polyurethanes.[28] Additionally, this solubilization can act as a fractionation step and has the potential to remove impurities in the lignins such as ash and polysaccharides. The results show substantial differences in the solubility of the three lignins recovered from different pre-extraction conditions (**Fig. 6A**). Specifically, increasing pre-extraction severity increases the fraction of soluble lignin. This outcome is likely a consequence of chemical modifications of the lignins during pre-extraction, and it is well-established that structural differences in lignin polymers impact its solubility in a particular solvent.[43, 44] This result was further explored through a series of characterization methods used to analyze the unfractionated and acetone-fractionated lignins.

The composition results for the unfractionated versus the acetone-fractionated lignins showed that the unfractionated lignins exhibited a carbohydrate content of less than 5% and that these carbohydrates were selectively enriched in the acetone-insoluble phase (**Fig. 6B**). This finding of poor polysaccharide solubility in acetone has been reported in prior work.[45, 46] Another key finding is that the ash content of the unfractionated lignins was all less than 1.0% (**Fig. 6B**) and that this ash did not partition into the acetone-soluble fraction, such that acetone fractionation can act as a de-ashing step for applications that may have poor tolerance for inorganic impurities (*e.g.*, carbon fibers). Molar mass distribution of the raw and acetone-fractionated lignins showed that the acetone preferentially solubilizes the low molar mass lignins (**Fig. 6C**), leaving the high molar mass lignins in the acetone-insoluble residue, which agrees with prior literature.[47, 48]

The impact of pre-extraction conditions and acetone fractionation on lignin functional groups and linkages were assessed using quantitative ^{13}C NMR, ^{31}P NMR, 2D HSQC NMR, and antioxidant activity index (**Fig. S4, Supporting Information**) and correlations within the data sets were observed (**Fig. S5, Supporting Information**). Specifically, trends include clear decreases in β-O-4 linkage content (**Fig. S4A, Supporting Information**) and β-O-4 relative abundance (**Fig. S4C, Supporting Information**) with increasing pre-extraction severity (*i.e.*, increasing temperature and alkali loading) as cleavage of ether linkages is a key outcome of alkaline delignification processes.[49] It can also be observed that acetone fractionation resulted in lignins with lower β-O-4 content partitioning into the acetone-soluble fraction and the higher β-O-4 content lignins partitioning into the acetone-insoluble phase (**Fig. S4A, Supporting Information**). This result agrees with the molar mass results (**Fig. 6C**) where acetone solubilization enriches

lower molar mass lignins because β-O-4 linkages are one of the primary linkages in hardwoods such as hybrid poplar and are targeted during alkaline delignification processes. Finally, analysis of β-O-4 linkage abundance and ^{31}P NMR-determined functional groups within the lignin also show clear correlations (**Fig. S5, Supporting Information**). Notably, a linear correlation between β-O-4 content and phenolic hydroxyl content is reasonable since cleavage of a β-O-4 bond generates a phenolic hydroxyl group. The phenolic hydroxyl content is also shown to be inversely correlated with the aliphatic hydroxyl content (**Fig. S5, Supporting Information**). This can be understood as decreasing the relative abundance of aliphatic hydroxyl functional groups with increasing depolymerization through cleavage of β-O-4 linkages (*i.e.*, increasing phenolic hydroxyl content).

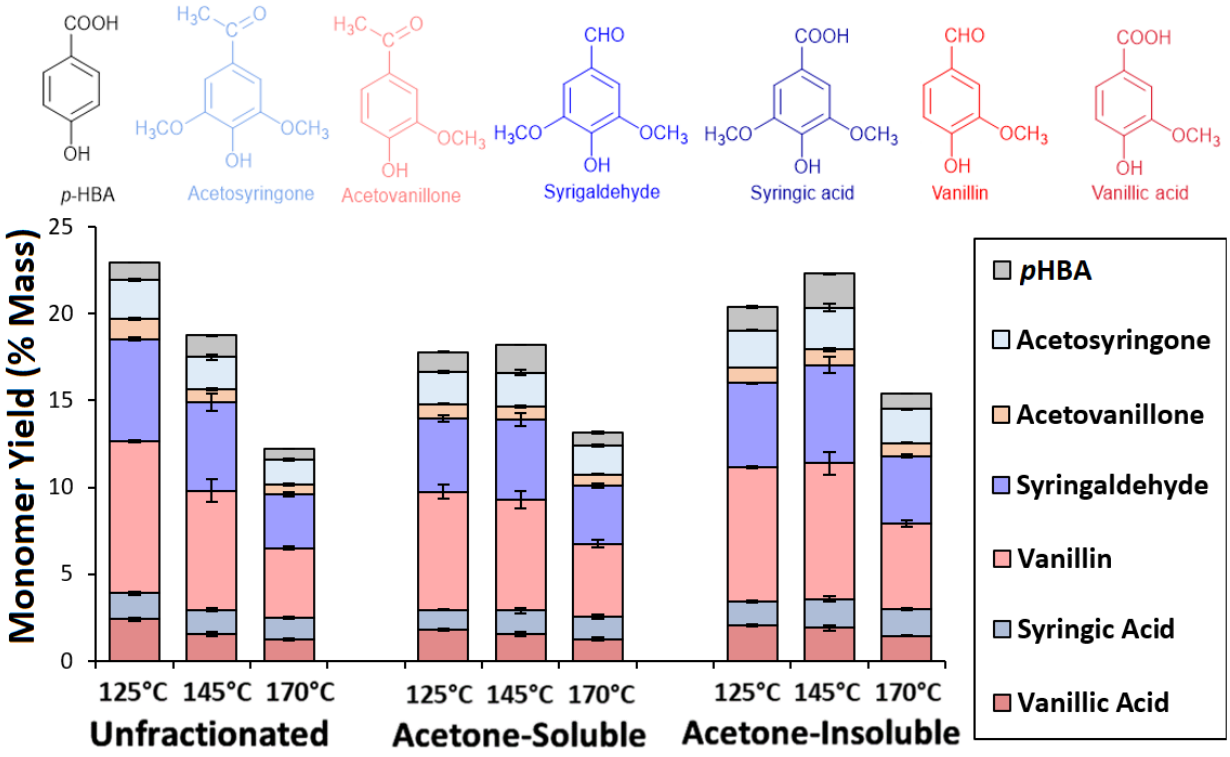

Fig. 7 Monomer yields from Cu-catalyzed alkaline oxidative depolymerization of unfractionated and acetone-fractionated lignins recovered from pre-extraction at differing severities. *p*HBA: *p*-hydroxybenzoate.

As the final component of the characterization of the lignins recovered from the alkaline pre-extraction liquors, the lignins were subjected to Cu-catalyzed depolymerization (**Fig. 7**). These results showed that increasing pre-extraction temperatures decreased the monomer yield obtainable and that the acetone-soluble fraction generally showed lower yields than the acetone-

insoluble fraction. Because ethers such as β-O-4 linkages are privileged targets of this depolymerization, these results are logical as lignin characterization results show that increasing pre-extraction temperature decreases molar mass (**Fig. 6C**) and β-O-4 content (**Supporting Information Fig. S4A**).

3.4 Properties of Lignins Recovered from Cu-AHP Stage

In the final part of this work, lignins derived from the second stage Cu-AHP delignification (corresponding to conditions 10, 11, and 12 in **Table 1**) were characterized for purity, molar mass distribution, and functional groups (**Fig. 8**). These results show several notable trends. First, the composition results demonstrate that with increasing H_2O_2 loading during the second stage Cu-AHP pretreatment, the content of polysaccharides (predominantly xylan) and acid soluble lignin increases (**Fig. 8A**). A potential explanation for the acid soluble lignin increase is that with increasing oxidation, the amount of partially oxidized, water-soluble lignins is increased. For the polysaccharide trend, one possible explanation for the increased xylan abundance is that while xylan solubilization increases with increasing lignin removal (**Fig. 2C**), the mild conditions for the Cu-AHP pretreatment preserve more of the soluble xylan relative to the harsher alkaline pre-extraction conditions with the result that more xylan ends up in the precipitates. The results for molar mass show that with increasing H_2O_2 loading, the recovered lignins decrease in number average molar mass and significantly increase in polydispersity (**Fig. 8B**). This same trend was observed for the lignins from the pre-extraction stage for increasing pre-extraction severity (**Fig. 6C**). This outcome can be explained as increasing lignin solubilization results in increased depolymerization. A final observation from the Cu-AHP lignin characterization results is that the lignins recovered from this stage exhibit the highest aliphatic hydroxyl content (**Fig. 6C**) of any of the lignins characterized in this study (5.7 to 6.5 mmol/g). These aliphatic hydroxyl groups are the target site for reaction with an isocyanate group in lignin-based polyurethane applications,[50] and these results suggest that the high aliphatic hydroxyl content as well as the light color of the lignins (**Fig. S6, Supporting Information**) should render these lignins as an ideal polyol for incorporation into biobased polyurethanes resins.

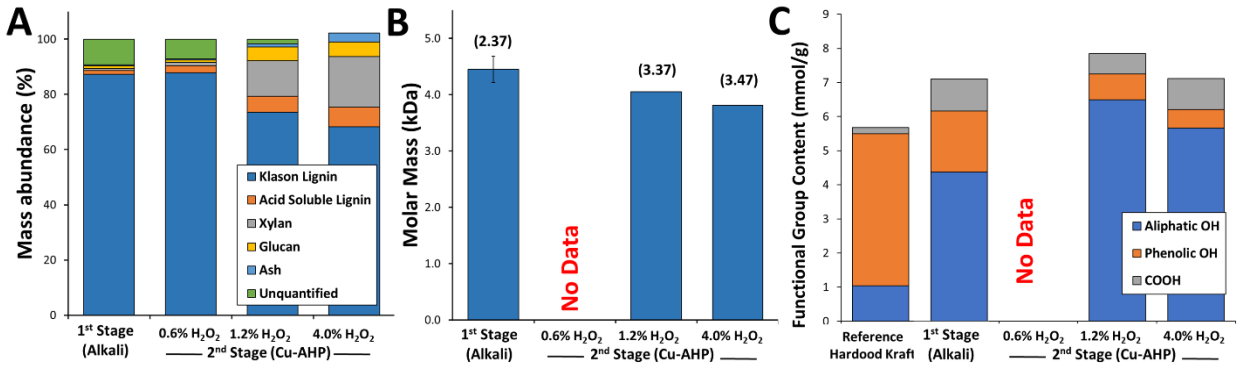

Fig. 8 Properties of lignins from second stage O_2-enhanced Cu-AHP including: **(A)** composition, **(B)** number average molar mass, and (**C**) phenolic and aliphatic hydroxyl content from ^{31}P NMR. Number in parentheses indicate polydispersity indices.

4. CONCLUSIONS

Overall, this work demonstrated that the two-stage alkaline-oxidative pretreatment can be scaled to the 20 L reactor scale while maintaining key process performance metrics that include high sugar hydrolysis yields at low enzyme loadings and lignins suitable for co-products applications. Not surprisingly, increasing severity in each stage of the pretreatment, defined by increasing temperature and alkali loading first stage pre-extraction and increasing H_2O_2 loading during the second stage Cu-AHP pretreatment, was demonstrated to increase the lignin and xylan solubilization and increase the enzymatic hydrolysis yields. Importantly, while a complete techno-economic analysis would be necessary to identify the economically optimal process configuration, conditions with high levels of delignification (*i.e.*, high lignin co-product yield), high enzymatic hydrolysis yields at low enzyme loadings, and low oxidant loadings should be the more economically promising of the conditions screened. For example, condition 1 in **Table 1** results in >90% lignin solubilization, approximately 90% glucose hydrolysis yields, and 100% solubilization of xylan by pretreatment or enzymatic hydrolysis.

A key difference from prior work was performing the alkaline pre-extraction with large particle wood chips rather than finely milled biomass, which necessitated a particle size reduction prior to the second stage Cu-AHP delignification. The impact of this interstage "defiberization" coupled with mechanical refining was shown to have a number of quantifiable impacts as well as additional hypothesized benefits. First, enzymatic hydrolysis yields after only a single stage of pretreatment (alkaline pre-extraction) were shown to improve with increasing refining and it is

hypothesized that delignification impacts cellulose accessibility at the nanoscale by allowing enzyme penetration within cell walls and that this delignification further impacts particle size reduction or fiber liberation during disintegration and refining, resulting in increased surface area and cellulose accessibility. Finally, delignification impacts fibrillation during mechanical refining resulting in increased surface area and cellulose accessibility. Using automated image analysis, it was shown that increasing levels of delignification resulted in more monodisperse distributions of particle sizes with respect to both length and width with sizes approaching expected dimensions of kraft hardwood fibers.

Lignin utility in co-products is a critical component of the economic feasibility of cellulosic biorefining technologies. The final component of the work characterized lignin properties from both the alkaline pre-extraction stage and the Cu-AHP pretreatment. The alkaline pre-extraction lignins were subjected to fractionation in acetone, and it was shown that the ash and polysaccharide impurities were concentrated in the acetone-insoluble fraction. In addition, these data indicate that increasing pre-extraction severity resulted in lower molar masses, higher phenolic hydroxyl contents, lower β-O-4 contents, and lower yields of aromatic monomers when subjected to depolymerization. Notably, the aromatic monomer yields as high as 23.0% by mass could be achieved from the lignin recovered from the mildest pre-extraction conditions. The lignins recovered from the second stage Cu-AHP pretreatment liquors were shown to exhibit decreasing molar mass and increasing polysaccharide content with increasing H_2O_2 loading during pretreatment. Also notably, the aliphatic hydroxyl content, which is critical for incorporation into polyurethane resins is more than six-fold higher than for a typical hardwood kraft lignin, indicating these lignins could serve as a biobased polyol for a range of polyurethane applications.

SUPPORTING INFORMATION

Additional information is included in the **Supporting Information** about the methods for lignin depolymerization, lignin fluorescence imaging, lignin lightness measurements, and measurement of lignin antioxidant capacity. Additional data is presented that shows temperature and pressure profiles during the second stage Cu-AHP pretreatment (**Fig. S1**), impact of reaction conditions and PFI milling on xylose hydrolysis yields (**Fig. S2**), macroscopic morphological differences in biomass after treatments (**Fig. S3**), characterization results for acetone-fractionated pre-

598 extraction lignins (**Fig. S4**), correlation between lignin properties in from acetone-fractionated
599 lignins (**Fig. S5**), color and fluorescence of lignins recovered from the second stage Cu-AHP
600 pretreatment (**Fig. S6**), and summary of mass yields and compositions for all conditions (**Table**
601 **S1**).

602

603 **ACKNOWLEDGEMENTS**

604 This work was funded by the U.S. Department of Energy (DOE) EERE under Contract No. DE-
605 EE0008148. Mingfei Li was funded by the China Scholarship Council from the Chinese Ministry
606 of Education.

607

608 **COMPETING INTERESTS**

609 The authors declare the following competing financial interest(s): E.L.H. and D.B.H. are listed as
610 inventors on a related patent (US Patent 11,530,458: Methods of using multi-ligand metal
611 complexes to perform oxidative catalytic pretreatment of lignocellulosic biomass) and E.LH.
612 D.B.H., Z.Y., and S.S.S are listed as inventors on a related patent application (US16/903,598:
613 Economical methods for performing oxidative catalytic pretreatment of plant biomass using a
614 homogeneous catalyst system). As holders of this patent and patent application, we may benefit
615 financially from advances in the technology discussed in this article.

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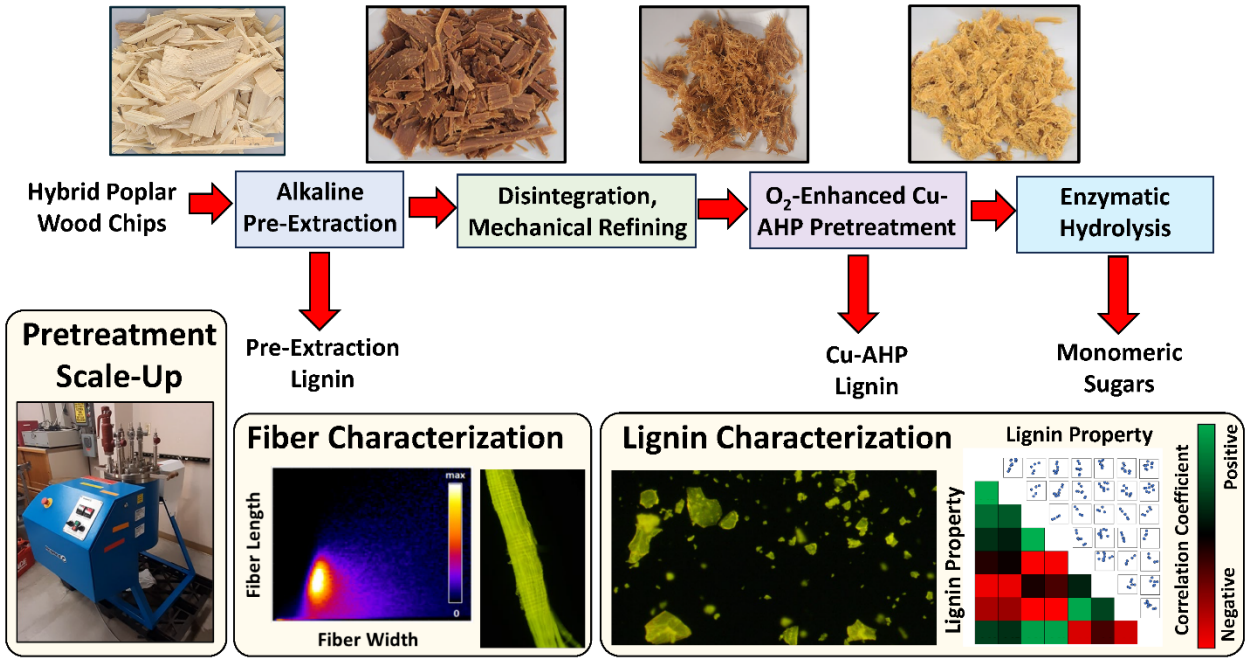

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750 **Table of Contents Graphic**

SUPPORTING INFORMATION

Scale-Up of a Two-Stage Cu-Catalyzed Alkaline-Oxidative Pretreatment of Hybrid Poplar

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1. SUPPLEMENTARY MATERIALS AND METHODS

1.1 Lignin Depolymerization

Lignins obtained from the various fractions of the pretreatment liquors were subjected to Cu-catalyzed oxidative alkaline depolymerization using the optimized condition as described in prior work.[1] Briefly, approximately 50 mg of the solid lignin was transferred into a PTFE reaction vessel

equipped with a stir bar containing 5.0 mg of $CuSO_4 \cdot 5H_2O$ in 10 mL of 2.0 M NaOH. The mixture was stirred briefly at room temperature until the lignin dissolved. All the vessels were then transferred to a 1 L stainless steel Parr reactor, after which the reactor was pressurized with 25 bar of air and heated to a temperature of 160 °C over 45 min. The reaction was then quenched by placing the reactor in a bucket of ice and allowed to cool to room temperature. After de-pressurizing the reactor, the product mixture was acidified to *p*H 2 using concentrated HCl followed by extraction using ethyl acetate. The ethyl acetate solvent was removed using a rotary evaporator after which the crude product was diluted with methanol, transferred to a 10 mL volumetric flask, and brought to volume for analysis by a UPLC system (Waters Acquity class H equipped with a BEH C18 1.7 µm, 2.1 x 50 mm column, with PDA detector) equipped with Empower software. The UPLC was maintained at 40 °C and the mobile phase was an acetonitrile/water mixture containing 0.1% formic acid. All the monomers were quantified by measuring the absorbance of the product mixture at 280 nm using a 1,4-dimethoxybenzene internal standard and the appropriate calibration curves.

1.2 Lignin Fluorescence Imaging

Fluorescence imaging of lignin samples was carried out using an OMAX 40X-2500X 18MP USB 3.0 Digital EPI-Fluorescence Trinocular Compound Biological Lab Microscope (Model M837ZFLR-C180U3) which was equipped with an OMAX A35180U3 18-megapixel digital camera, featuring the resolution of 4912 x 3684 pixels. The fluorescence imaging process incorporated a blue excitation filter within the 410 to 490 nm range, combined with a barrier (emission) filter set at 515 nm. Image acquisition was facilitated through ToupLite software. To quantify fluorescence intensities in ImageJ, regions of interest (ROIs) were selected using the rectangular selection tool. Before proceeding with intensity measurements, the background correction step was performed to account for any potential influence from non-specific signals that might influence the results. Subsequently, gray values are calculated using the "measure" function in ImageJ to obtain fluorescence intensity values for the selected ROIs. Within the context of ImageJ, the term "gray value" refers to the numerical value associated with the pixel intensity in grayscale images with range from 0 (black) to 255 (white) in an 8-bit grayscale image.

1.3 Lignin Lightness

Lignin samples were subjected to color measurements using an FRU WR18 handheld colorimeter, with the measurements conducted in triplicate. Prior to the measurements, the colorimeter underwent calibration using the manufacturer's calibration standards, including a blackboard and a whiteboard. To measure the lignin samples, the colorimeter's measurement window was placed in close contact with each sample to ensure accurate readings. Thorough cleaning of the colorimeter was carried out between measurements. The color data, represented by the L, a, and b values, were recorded within the CIELAB color space, where L represents the lightness, ranging from 0 to 100. These measurements utilized a 4.0 mm caliber measuring window, a standard D65 light source using a 10° CIE standard observer, and an 8/d illumination system with a photodiode array sensor.

1.4 Lignin Antioxidant Capacity

Antioxidant capacity analysis of lignins were performed as reported elsewhere.[2] For this, 0.1 mL of a known quantity of lignin sample in 90% dioxane by volume in water solution was reacted with 3.9 mL ethanol solution containing 25 mg/L of 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a

free radical source in darkness for 30 min. The drop of the absorbance of the reaction system was determined using a UV spectrometer at 517 nm. The radical scavenging capacity of the sample was calculated as: antioxidant activity index (%) = $(A_0 - A_1)/A_0 \times 100\%$, where A_0 is the absorbance of the control containing no lignin, A_1 is that from lignin sample.

2. SUPPLEMENTARY FIGURES AND TABLES

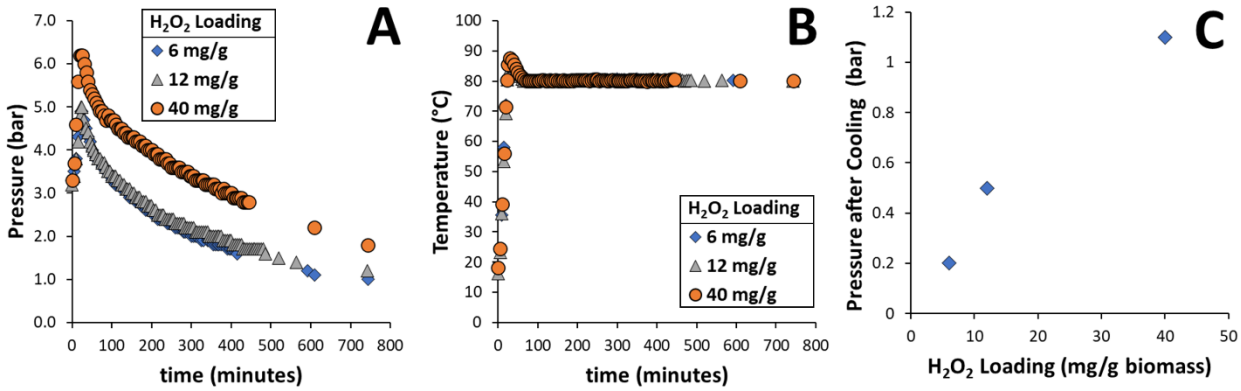

Figure S1. (**A**) Pressure and (**B**) temperature profiles during the second stage Cu-AHP pretreatment at the 20 L scale, and (**C**) final pressure after cooling to room temperature.

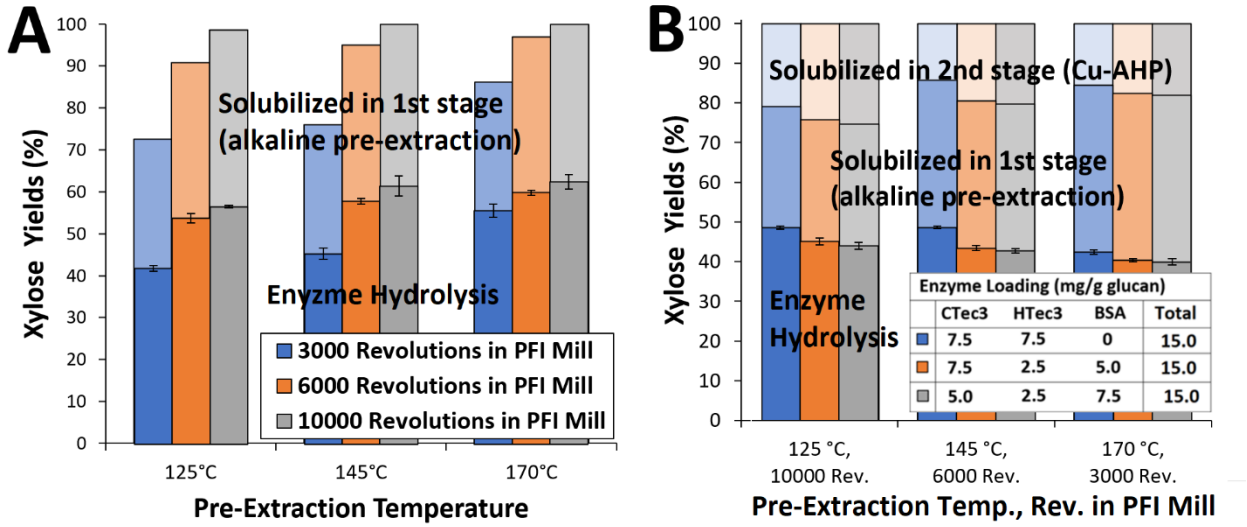

Figure S2. Impact of pre-extraction temperature (first stage only) and mechanical refining on (**A**) xylose hydrolysis yields based on conditions 1 through 9 in **Table 1**. Xylose yields following (**B**) second stage Cu-AHP and enzymatic hydrolysis at an enzyme loading of 15 mg protein per g glucan (based on initial composition) corresponding to conditions 1, 5, and 9 in **Table 1**.

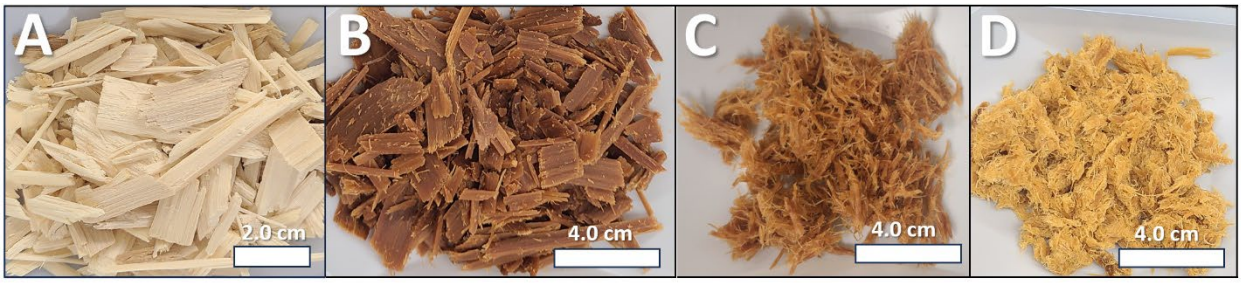

Figure S3. Macroscopic morphological differences in alkali pre-extracted hybrid poplar (**A**) before pretreatment, (**B**) before disintegration, (**C**) after disintegration (conditions 10 to 12 from **Table 1**), and (**D**) after Cu-AHP pretreatment (condition 12 from **Table 1**).

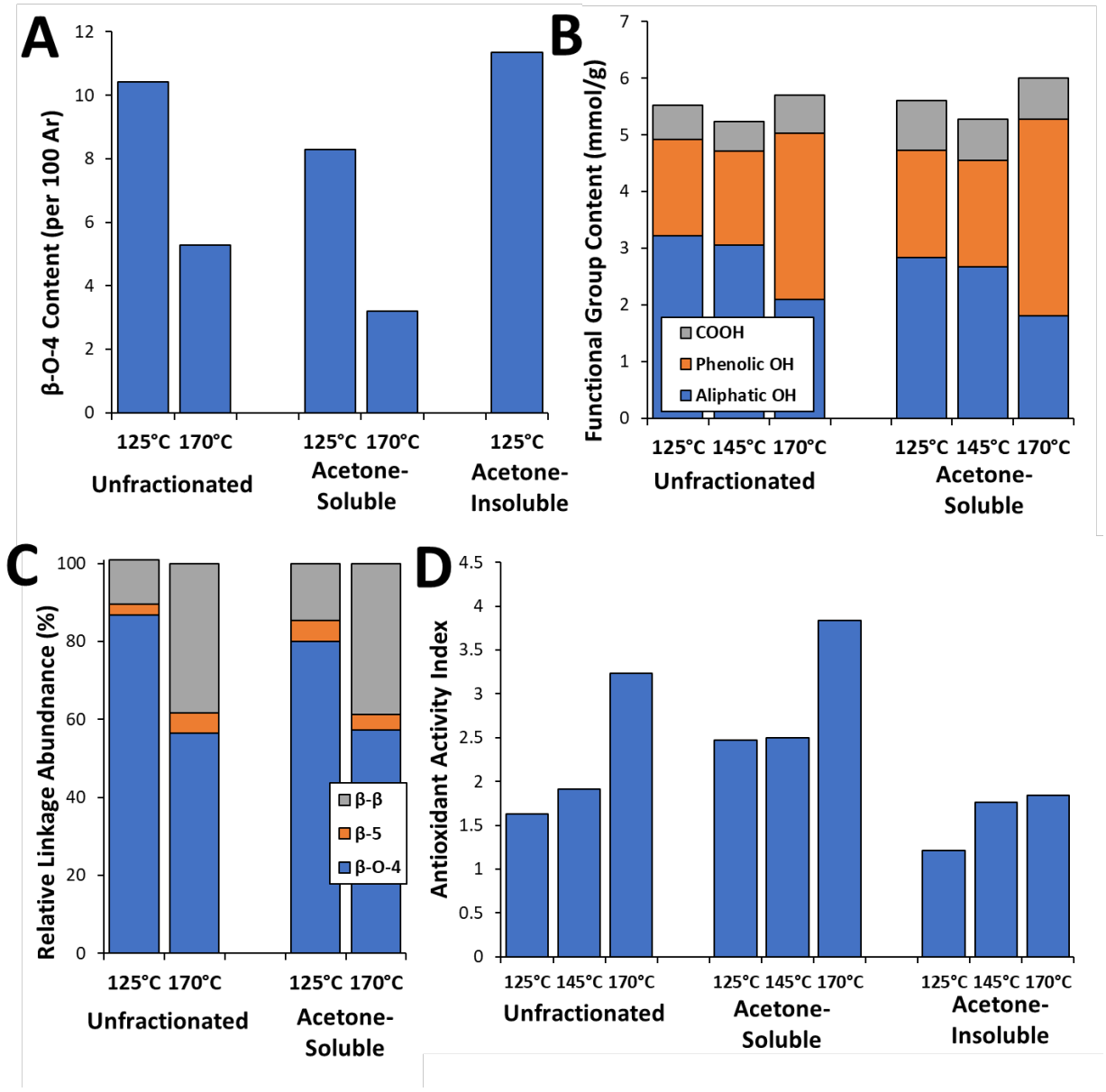

Figure S4. Characterization of acetone fractionation of lignin-rich biopolymers recovered from differing alkaline pre-extraction conditions: (**A**) β-O-4 content determined by ^{13}C NMR, (**B**) ^{31}P NMR-determined functional groups, (**C**) relative abundance of major quantified linkages by 2-D HSQC NMR, and (**D**) antioxidant activity index.

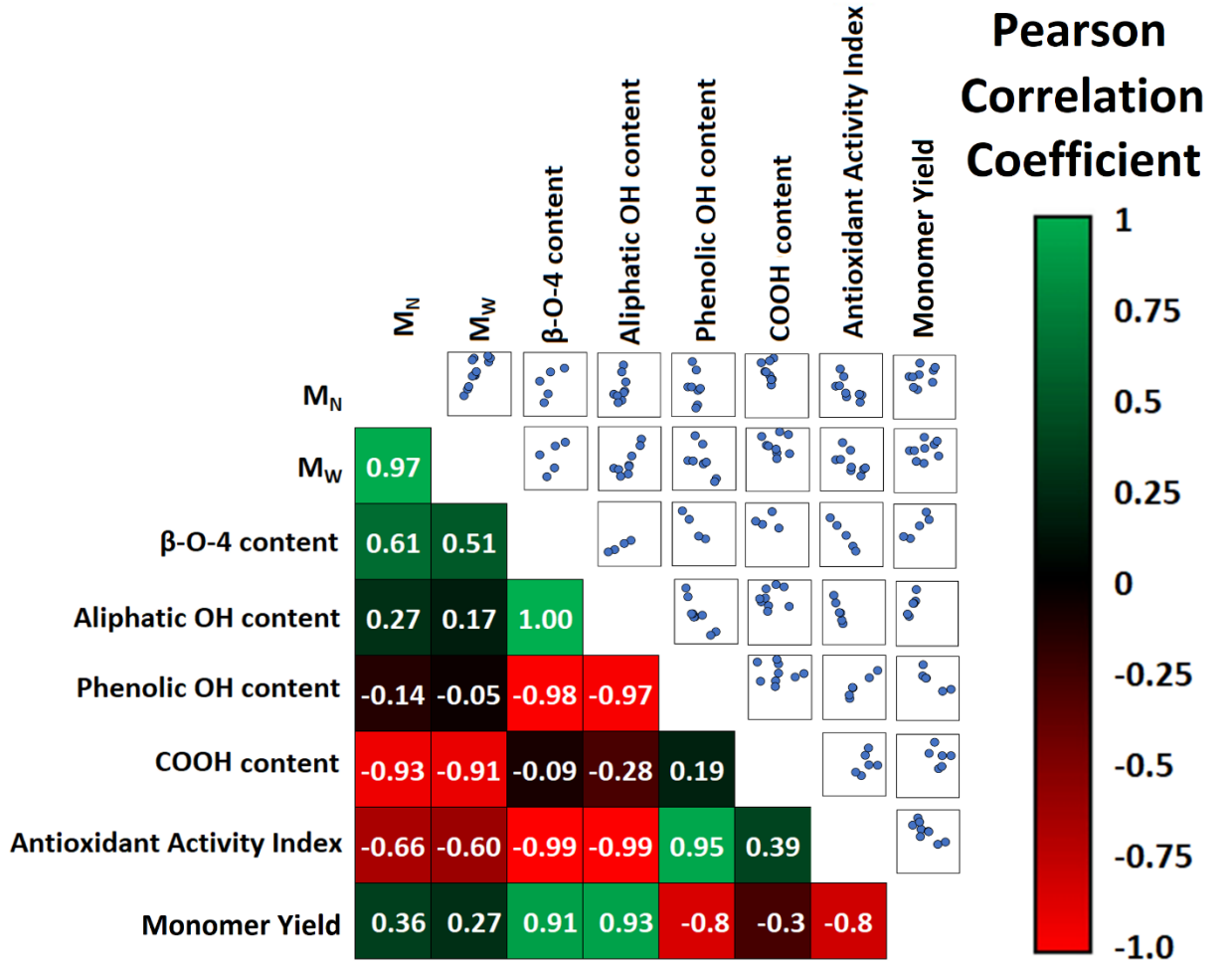

Figure S5: Pearson correlation coefficients between characterized lignin properties from acetone-fractionated lignins from conditions 1 through 9 from **Table 1**.

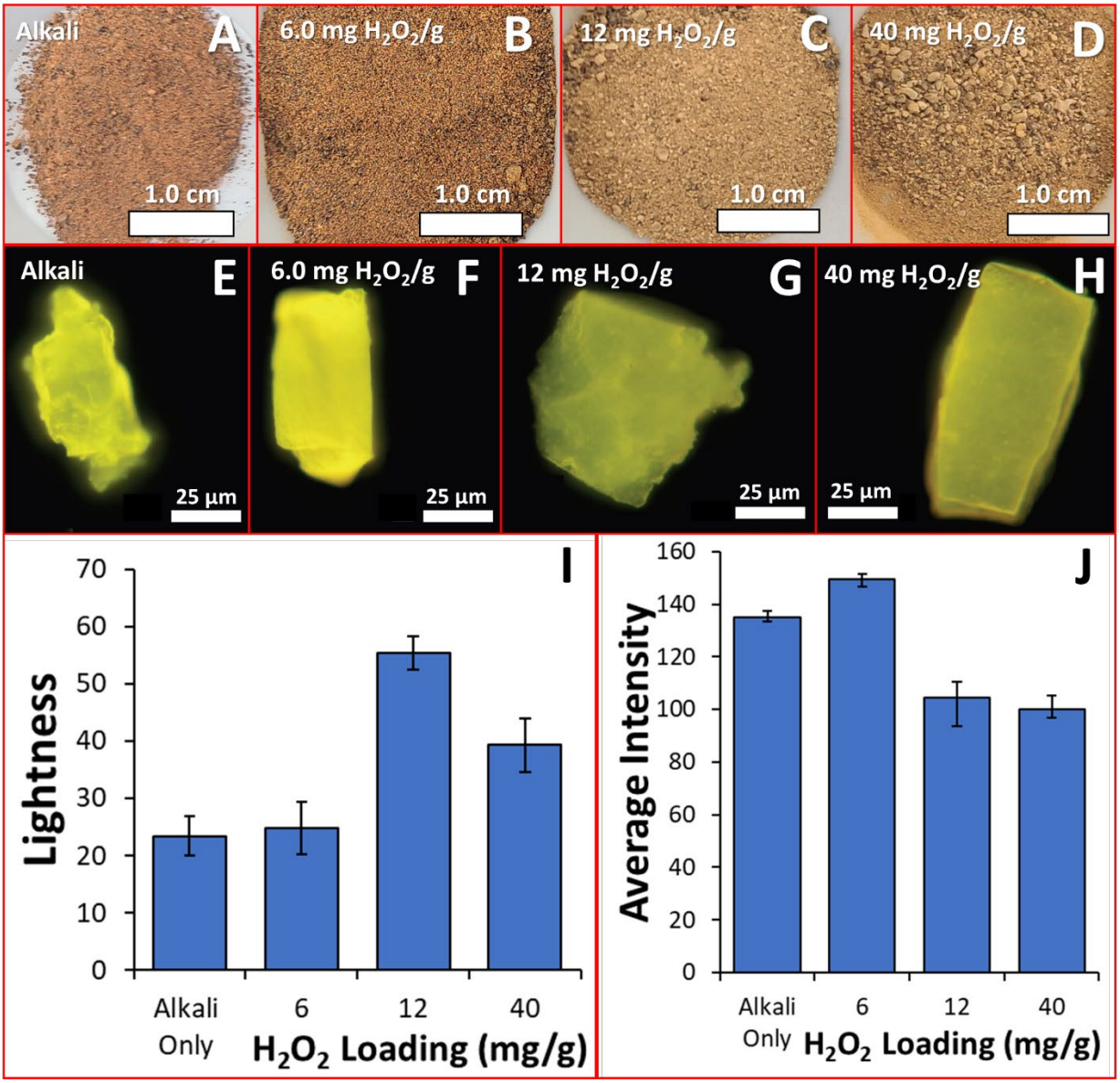

Figure S6: Lignins recovered from the first stage alkaline pre-extraction **(A, E)** and second stage Cu-AHP pretreatment **(B, C, D, F, G, H)** visualized at the macroscopic scale **(A, B, C, D)** and at the microscopic scale **(E, F, G, H)** demonstrating autofluorescence. Lignin lightness **(I)** and fluorescence intensity **(J)**.

Treatment Condition		Yield (%)	Glucan (%)	Xylan (%)	Klason (%)	ASL (%)	Ash (%)	Unquantified (%)
Untreated		-	43.3	16.6	23.0	1.3	1.0	14.7
1	1st Stage	65.0	60.8	14.8	18.3	1.6	1.7	2.8
	2nd Stage	53.1	73.8	11.5	3.0	ND	ND	11.7
5	1st Stage	78.1	51.4	13.4	19.8	1.4	1.4	12.6
	2nd Stage	62.6	66.0	11.3	8.2	ND	ND	14.5
9	1st Stage	84.8	50.1	13.6	21.4	1.4	1.6	11.9
	2nd Stage	67.1	61.8	10.9	16.0	ND	ND	11.3
10	1st Stage	72.9	49.4	21.4	23.2	ND	ND	3.0
	2nd Stage	63.3	55.3	15.0	16.6	ND	ND	13.0
11	1st Stage	80.9	49.7	19.5	25.0	ND	ND	5.9
	2nd Stage	61.0	59.7	15.4	15.5	ND	ND	9.4
12	1st Stage	76.6	49.9	19.6	25.6	ND	ND	5.0
	2nd Stage	55.9	66.7	14.7	12.3	ND	ND	6.3

Table S1: Summary of mass yields (based on initial dry mass) and composition (based on actual dry mass) for all experimental conditions. ND: Not determined. ASL: Acid soluble lignin.

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