Effective Biomass Fractionation through Oxygen-Enhanced Alkaline-Oxidative Pretreatment

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Abstract

The high recalcitrance of plant cell walls is an obstacle for effective chemical or biological conversion into renewable chemicals and transportation fuels. Here, we investigated the utilization of both oxygen (O_2) and hydrogen peroxide (H_2O_2) as co-oxidants during alkaline-oxidative pretreatment to improve biomass fractionation and increase enzymatic digestibility.

The oxidative pretreatment of hybrid poplar was studied over a variety of conditions. Employing O_2 in addition to H_2O_2 as a co-oxidant during the two-stage alkaline pre-extraction/copper-catalyzed alkaline hydrogen peroxide (Cu-AHP) pretreatment process resulted in a substantial improvement in delignification relative to using H_2O_2 alone during the second-stage Cu-AHP pretreatment, leading to high overall sugar yields even at H_2O_2 loadings as low as 2% (w/w of original biomass). The presence of H_2O_2, however, was both critical and synergistic. Performing analogous reactions in the absence of H_2O_2 resulted in approximately 25% less delignification and a 30% decrease in sugar yields. The lignin isolated from this dual oxidant second stage had high aliphatic hydroxyl group content and reactivity to isocyanate, indicating that it is a promising substrate for the production of polyurethanes. To test the suitability of the isolated lignin as a source of aromatic monomers, the lignin was subjected to a sequential Bobbitt's salt oxidation followed by formic-acid catalyzed depolymerization process. Monomer yields of approximately 17% (w/w) were obtained, and the difference in yields was not significant between lignin isolated from our Cu-AHP process with and without O_2 as a co-oxidant. Thus, the addition of O_2 did not lead to significant lignin crosslinking, a result consistent with the two-dimensional heteronuclear single-quantum coherence (2D HSQC) NMR spectra of the isolated lignin.

Keywords: Alkaline hydrogen peroxide pretreatment; Aromatic monomers; Catalysis, Cellulosic biofuels, Copper; Lignin; Oxidative depolymerization; Polyurethanes

Introduction

Concerns over energy security and climate change have resulted in considerable effort focused on facilitating the transition of the fossil fuel-based economy to a renewable resource-based sustainable economy.[1-6] Among the renewable resources currently available, lignocellulosic biomass is an abundant, potentially carbon-neutral source and is considered to be a promising raw material for the production of biofuels and biomaterials at large scales.[4,5,7] The challenge of developing economic lignocellulosic biorefineries, however, is the high recalcitrance and complex structure of the plant cell walls which hinders both bioconversion and chemical conversion processes.[7] Thus, a pretreatment technology that can economically fractionate lignocellulosic biomass into renewable biopolymers is highly desirable. The fractionated polysaccharides can be converted into monomeric sugars and biofuels, while lignin can be used as the starting material for the production of various chemicals, materials, and fuels.[8-12] Thus, an effective pretreatment approach that simultaneously maximizes polysaccharide hydrolysis to fermentable sugars and extracts high-quality lignin for subsequent valorization would improve the economic viability of the lignocellulosic-to-biofuel industry.[13,14]

A sequential two-stage pretreatment process we developed previously consisting of an alkaline pre-extraction stage followed by a copper 2,2′-bipyridine-catalyzed alkaline hydrogen peroxide (Cu-AHP) pretreatment step can effectively fractionate lignin from the biomass as well as improve the enzymatic digestibility of the polysaccharides.[13,15,16] Our previous work demonstrated that following the two-stage alkaline pre-extraction/Cu-AHP pretreatment, woody biomass (including hybrid poplar, birch, and eucalyptus) was efficiently hydrolyzed by cellulolytic enzymes to recover more than 90% of the initial sugars.[13,17,18] Moreover, the two-stage alkaline pre-extraction/Cu-AHP pretreatment also generated a lignin stream with a low degree of condensation and minimal contaminating ash or polysaccharides that was suitable for the production of value-added products.[16,19-21] However, a challenge associated with this two-stage alkaline pre-extraction/Cu-AHP pretreatment process as well as many other oxidative pretreatment processes is the process economics, especially the cost of the H_2O_2.[13,22-24] To improve the economics of this oxidative lignocellulosic-to-biofuel technique, a reduction in the loading of expensive chemical inputs in conjunction with an increase in the recovery of biomass polymers is required.

One approach to improving the economics is to utilize an alternative oxidant that is inexpensive and can partially or completely substitute for the H_2O_2 while maintaining or increasing monomer sugar yields and lignin solubilization. Molecular oxygen is a highly appealing oxidant that is widely utilized in the pulp and paper industry as a delignification and bleaching agent.[25] During O_2 delignification of pulp, the free phenolic hydroxyl groups in lignin are ionized by the addition of alkali to generate phenolate ions, which are thought to initiate reactions with O_2 and result in the oxidation and solubilization of lignin into the liquid phase.[25-27] During this process, the addition of catalysts such as copper sulfate in the presence or absence of nitrogen-based ligands could further activate oxygen and promote the delignification performance.[28-32] Because H_2O_2 is generally more reactive than O_2 and can generate hydroperoxide anions that oxidize carbonyl groups and cleave lignin aromatic rings,[33,34] H_2O_2 has been utilized to improve the delignification performance of O_2 in pulp bleaching.[33,35]

Therefore, employing both H_2O_2 and O_2 as co-oxidants has the potential to improve delignification and the oxidation efficacy of the Cu-AHP pretreatment.

To date, limited work has been reported on utilizing the combination of O_2 and H_2O_2 as co-oxidants in the presence of copper catalysts during oxidative pretreatment to improve the delignification performance while providing highly digestible biomass.[32] In this work, we investigated using O_2 as a co-oxidant along with H_2O_2 during the two-stage alkaline pre-extraction/Cu-AHP pretreatment process to improve the recovery of biopolymers, including both polysaccharides and lignin. Our results indicate that the inclusion of O_2 as a co-oxidant led to enhanced lignin solubilization, a lower chemical input requirement, improved total sugar release by enzymatic hydrolysis, and reduced processing costs. Moreover, the properties of the lignin isolated from this process (high hydroxyl content, > 6.5 mmol/g) indicate that it could be an excellent renewable polyol in the formulation of lignin-based polyurethanes.[36] Furthermore, the susceptibility of the lignin towards oxidative depolymerization suggests that the use of O_2 as a co-oxidant did not result in the formation of additional lignin crosslinks,[37,38] and the lignin remains amenable to valorization via depolymerization into monomers. Together, these results highlight the utility of using O_2 as an inexpensive and environmentally friendly co-oxidant in conjunction with H_2O_2 during metal-catalyzed alkaline oxidative pretreatments.

Experimental

Materials

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 and hammer-milled (HammerHead, Muson Co., Inc. USA) to pass through a 5-mm screen. The milled biomass was stored in airtight bags prior to use. The chemical composition analysis[37] of the biomass was 45.5% glucan, 15.8% xylan (sum of xylan, galactan, and manan), 22.3% Klason lignin, 9.5% acid soluble lignin, 3.2% extractives, and 0.85% ash. The enzyme cocktails Cellic CTec3 (197.3 mg/g) and HTec3 (170.5 mg/g) were kindly provided by Novozymes A/S (Bagsværd, Denmark). Commercial Kraft hardwood lignin was provided by Suzano Papel e Celulose (São Paulo, Brazil). All other chemicals were reagent grade and purchased from Fisher Scientific (USA) unless otherwise noted.

Two-stage Cu-AHP pretreatment

Alkaline pre-extraction of biomass was performed at 10% NaOH loading on biomass (w/w based on the dry weight of the biomass) at 10% (w/v) solid consistency at either 90 °C or 120 °C for 1 h. For the alkaline pre-extraction at 90 °C, 100 g (dry basis) of hybrid poplar, 950 mL deionized water, and 50 mL 5 M NaOH were mixed and placed into a 2-L conical flask. Subsequently, the reaction vessel was immersed in a water bath preheated to the target temperature. The reaction mixture was subjected to intermittent shaking every 10 min for 30 seconds. The experiments for alkaline pre-extraction of poplar at 120 °C were performed using a 100-mL stainless steel Parr reactor (Parr Instruments Company, Moline, IL, USA). For each experiment, 5 g (dry basis) of hybrid poplar, 47.2 mL of deionized water, and 2.5 mL of 5 M NaOH were incubated at 200 rpm for 1 h. After alkaline pre-extraction, the remaining insoluble fraction was separated by filtration and thoroughly washed with deionized water (about 1 L). The washed biomass was stored at 4 °C for subsequent analysis and use. The chemical composition of the biomass both before and after pre-extraction under these two temperatures is summarized in Table 1.

Table 1. Chemical composition of poplar after alkaline pre-extraction

Substrate	Original	90 °C	120 °C
Solid remaining (%)[a]	N/A	87.1 ± 0.1	78.5 ± 0.1
Glucan (%)	45.5	50.1 (43.6)[b] ± 0.8	54.7 (42.9)[b] ± 1.2
Xylan (%)	15.8	16.4 (14.3)[b] ± 0.2	15.5 (12.2)[b] ± 0.2
Klason lignin (%)	22.3	23.3 (20.3)[b] ± 0.3	21.1 (16.6)[b] ± 0.4
Ash (%)	0.85	0.87 (0.76)[b] ± 0.1	0.86 (0.68)[b] ± 0.1

N/A: Not applicable. Values are expressed as an average ± standard deviation of triplicate experiments.

[a] Weight percentage (oven-dried weight) of the recovered biomass after alkaline pre-extraction relative to original biomass.

[b] Chemical composition of alkaline pre-extracted biomass relative to original biomass.

The second stage O_2-enhanced Cu-AHP pretreatment was conducted using the same Parr reactor described above (for the pre-extraction at 120 °C) at a biomass loading of 10% (w/v) (based on the weight of original biomass). On the basis of our earlier results,[22] the oxidative pretreatment was performed with several fixed conditions, including 10% NaOH (based on the weight of original biomass), 1 mM $CuSO_4$ (0.159% w/w, based on original biomass), and 1 mM 2,2'-bipyridine (bpy) (0.156% w/w, based on original biomass). The bpy ligand is important to improve the delignification performance of the oxidative pretreatment. The underlying mechanism is unknown, but we speculate that the aromatic N-donor ligand could improve the penetration of the copper ions into the cell wall to help localize the hydroxyl radicals formed from H_2O_2 and O_2.[16,21] The reactions were performed at 80 °C while mixing with a stirrer at 200 rpm for 12 h. The H_2O_2 loading was varied from 0 – 8% (w/w) (based on the dry weight of original biomass). When O_2 was used as a co-oxidant, the O_2 pressure was fixed at 50 psi (345 kPa). The O_2 charge was 45 kg per ton (oven-dried weight) original biomass with the reactor and experimental design utilized in this study. After the oxidative pretreatment stage, the reactor was cooled in an ice/water bath and depressurized at room temperature. The solid fraction was separated from the liquor via filtration, thoroughly washed with deionized water (approximately 1 L), and stored at 4 °C prior to compositional analysis and enzymatic hydrolysis. During the O_2-enhanced alkaline-oxidative pretreatment process, the O_2 consumption was ~20 kg per ton (oven-dried weight) of original biomass.

To recover the lignin solubilized during the alkaline-oxidative pretreatment process, the separated liquid phase was acidified to pH 2.0 with 72% (w/w) H_2SO_4. The precipitate was recovered by centrifugation (10 min at 11269 × *g*), washed 2 times with aqueous H_2SO_4 (pH 2.0), and finally washed one time with cold deionized water. After centrifugation, the precipitated (lignin) was frozen at -80 ℃, lyophilized, and stored in the dark at 4 °C (see Table S1 for lignin yields).

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). Reactions were performed at 5% (w/v) solid loading (based on the weight of original, untreated biomass) and 50 ℃ for 72 h in 50 mM sodium citrate buffer (pH 5) 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 released into solution was measured using a high-performance liquid chromatography (HPLC) system (Agilent 1260 Series) following a National Renewable Energy Laboratory (NREL) protocol.[39]

Chemical composition analysis

The moisture content of the biomass was determined by drying at 105 ± 2 °C to a constant weight. Before and after the pretreatment, the chemical composition of biomass was measured following an NREL two-stage hydrolysis protocol.[39] In brief, the air-dried biomass was ground with a Wiley Mill to pass through 20 mesh screen. A sample (0.1 g) of the ground material was digested by the two-step H_2SO_4 hydrolysis protocol.[39] After hydrolysis, the acid-insoluble lignin (Klason lignin) was separated by filtration, dried at 105 ± 2 °C, and weighed. The content of carbohydrates was quantified by an HPLC system (Agilent 1260 Series equipped with an infinity refractive index detector) fitted with a Bio-Rad Aminex HPX-87H column (Bio-Rad Laboratories, USA) using 5.0 mM sulfuric acid as the mobile phase with a flow rate of 0.6 mL min^{-1} and an operation temperature of 65 °C. The xylose content reported is actually the combination of xylose, mannose, and galactose because the HPX-87H column cannot separate these three sugars. Sugar quantification was accomplished by comparing the peak area to a standard curve prepared using pure glucose and xylose. The ash content of solid samples was determined by combustion of approximately 0.5 g of 20-mesh milled dry biomass at 550 °C for 12 h.

Lignin characterization

The molecular weight distributions of lignin were analyzed using an Agilent 1260 Infinity HPLC equipped with a G1315D 1260 diode array detector VL, monitoring at 280 nm and recording 190-400 nm, a G1362A refractive index detector, and a Waters Ultrahydrogel 250 7.8×300 mm gel permeation chromatography (GPC) column attached to a Waters Ultrahydrogel 6×40 mm guard column. The temperature was held constant at 40 °C with 0.7 mL/min isocratic flow of 5 mM NaOH in 80/20 0.1 M aqueous sodium nitrate/acetonitrile as the mobile phase. Extracted lignin (20 mg dry weight) was dissolved in 1 mL eluent, and 25 µL of the sample was injected onto the HPLC. The retention times were compared to a sodium polystyrene sulfonate kit from Scientific Polymer Products (Mn 1,440-85,600 g/mol). The number average molecular weight (Mn), weight average molecular weight (Mw), and polydispersity (PDI) were calculated according to the method described by Malawar and Senak.[40]

The hydroxyl content of the lignin samples was measured using phosphorous-31 nuclear magnetic resonance (^{31}P NMR) spectroscopy.[26] Approximately 40 mg of the dry lignin was dissolved in 325 µL of a mixture of anhydrous pyridine and $CDCl_3$ (volume ratio of 1.6:1) and 300 µL anhydrous dimethylformamide (DMF). After completely dissolving the lignin, 100 µL cyclohexanol (stock concentration of 22 mg/mL) in anhydrous pyridine and $CDCl_3$ (volume ratio of 1.6:1) was added into the solution as an internal standard. Chromium (III) acetylacetonate (50 µL of a 5.6 mg/mL stock solution) in anhydrous pyridine and $CDCl_3$ (volume ratio of 1.6:1) was added into the mixture as a relaxation reagent. Phosphorylation of the lignin hydroxyl groups was initiated by the addition of 100 µL of the phosphitylation reagent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane. Analysis of the solution was performed using an Agilent DDR2 500 MHz NMR spectrometer (relaxation delay of 5 s and 128 scans) equipped with a 7600AS autosampler and VnmrJ 3.2A software. After obtaining the ^{31}P NMR spectra, the hydroxyl content of the lignin sample was calculated based on the ratio of the cyclohexanol internal standard (145.3.1-144.9 ppm) peak areas to the sample peak areas as described by Brandt et al.[41]

To evaluate the appropriateness of using the lignin in polyurethane formulations, we tested the reactivity of the lignin towards isocyanate (a key reagent that reacts with polyols in the manufacture of polyurethane).[42] The reactivity of the lignin was determined using a titration method following a modified version of the American Society for Testing and Materials standard ASTM-D5155-14.[43] Briefly, 1.0 g of methylene diphenyl diisocyanate (MDI) and 1.0 g of oven-dried lignin were dissolved in 6.0 g of DMF (dried with 4A molecular sieves, Fisher Scientific) and heated at 50 °C for 60 min. Then, 0.5 g of the solution was added to 25 mL of a dibutylamine solution (2 M) in toluene and mixed at 23 °C for 15 min at 150 rpm, followed by the addition of 110 mL of 2-propanol. After mixing, the solution was titrated with 1 M HCl to pH 4.2. A separate experiment was performed without the addition of the lignin-isocyanate sample as the control. Polyethylene glycol 400 (PEG 400) was used as a reference petroleum-based polyol. The amount of free isocyanate (unreacted isocyanate) was calculated according to equation (1):

$$\%NCO = \frac{4.202\,(V_1 - V_2) \times M}{m} \tag{1}$$

where $\%NCO$ is the fraction of free (unreacted) isocyanate, V_1 is the volume of HCl required to reach pH 4.2 for the control sample (mL), V_2 is the volume of HCl required to reach pH 4.2 for lignin-isocyanate sample (mL), *M* is the molarity of HCl, and *m* is the weight (g) of the lignin/isocyanate sample added to the analysis mixture.

^{1}H-^{13}C-gradient heteronuclear single quantum coherence (HSQC) spectra were recorded on a 500 MHz Bruker NMR spectrometer equipped with a 5 mm iProbe (BBO probe) at room temperature using pulse sequence "hsqcedetgpsisp2.3". Spectra were recorded with spectral widths of 8013 Hz (^{1}H) and 20 kHz (^{13}C) with an acquisition time of 63.9 ms (F2, 512 complex points for ^{1}H) and 63.9 ms (F1, 1024 increments for the ^{13}C dimension) and 48 scans were taken per increment using a delay of 1.5 s.

Depolymerization of lignin

Lignin oxidation was conducted according to modified literature procedures.[19,37,44] Briefly, 35 mg of lignin, 80 mg of Bobbitt's salt (4-acetamido-2,2,6,6-tetramethyl-1-oxopiperidinium tetrafluoroborate), 5 mL of 80:20 (v/v) acetonitrile:water, and 0.5 mL of 0.5 M HCl were placed into a 20-mL pressure tube. The reaction tube was sealed with a Teflon-lined cap and placed into a heating block at 80 °C for 2 h with magnetic stirring at 500 rpm. After cooling to room temperature, the solution was transferred into a 6-dram vial and concentrated under high vacuum (0.06 MPa) at 40 °C in a rotational vacuum concentrator (Thermo Scientific, SpeedVac Concentrator) to collect the oxidized lignin. The oxidized lignin was then subjected to formic acid-mediated lignin depolymerization to generate aromatic monomers as previously described.[19] Specifically, the dried lignin residue was dissolved in 5 mL of formic acid with sonication, and the solution was transferred into a 20 mL pressure tube. Sodium formate (40 mg) was added to the solution, and the tube was sealed and heated at 110 °C with magnetic stirring for 24 h. The pressure tube was then cooled to room temperature, the solution transferred to a round-bottom flask, and the solvent removed using a rotary evaporator (Buchi Rotavapor). The lignin residue was suspended in water and the monomers were extracted 3 times with ethyl acetate. The organic fractions were combined and dried via rotary evaporation. The residue was dissolved in methanol and analyzed using a Prominence HPLC system (Shimadzu Scientific Instruments, USA) equipped with a RestekUltra C18 column (150×4.6 mm ID-3 µm particle size) and an SPD-M20A diode array detector. The system was maintained at 35 °C and the mobile phase was an $CH_3CN:H_2O$ mixture containing 0.1% (v/v) formic acid. Monomers were detected and quantified by measuring the optical absorbance at 280 nm and 310 nm using 1,4-dimethoxybenzene (10 mM) as the internal standard loading control.

Statistical analysis

Data was analyzed by a one-way analysis of variance with Unscrambler v10.5 software (CAMO software, Japan) followed by Tukey's post-hoc test.[45] Significant differences between treatment conditions were evaluated at $p \leq 0.05$.

Results

Effect of alkaline-oxidative pretreatment conditions on lignin and xylan solubilization

During the two-stage alkaline pre-extraction/alkaline-oxidative pretreatment process, the objective of the first stage (alkaline pre-extraction) is to solubilize some of the lignin and hemicellulose and remove extractives, thereby increasing biomass porosity and removing some of the compounds that inhibit the subsequent enzymatic hydrolysis.[16] Indeed, 25.7% and 9.0% of the lignin and 23.0% and 9.6% of the xylan was solubilized and removed during alkaline pre-extraction at 120 and 90 °C, respectively. Concomitantly, 5.6% and 4.1% of the glucan was also removed (Table S2).

To understand the effect of O_2 as a co-oxidant on the biomass fractionation performance during the second stage of the pretreatment process, *i.e.*, during the alkaline-oxidative pretreatment stage, a series of oxidative pretreatments with alkaline pre-extracted poplar was performed. After oxidative pretreatment, the chemical composition of the remaining solid substrates was determined (Table 2). A substantial amount of lignin and xylan were solubilized during the oxygen-enhanced Cu-AHP process, resulting in an increased glucan percentage in the remaining solid (Table 2). Figure 1 shows the solubilized lignin and xylan obtained following the two-stage alkaline pre-extraction/alkaline-oxidative pretreatment process. In the first set of experiments, shown on the far left of Figure 1, we performed three control oxidative pretreatments, including Cu-AHP with H_2O_2 alone, copper 2,2'-bipyridine [Cu(bpy)]-catalyzed pretreatment with O_2 alone, and O_2-enhanced AHP [*i.e.*, no Cu(bpy)]. Compared to the catalyzed pretreatment with H_2O_2 or O_2 alone, the utilization of both O_2 and H_2O_2 as co-oxidants removed more lignin and xylan even without the presence of Cu(bpy) as a catalyst. While the specific mechanism that led to this improvement is unknown, it has been hypothesized that H_2O_2 in the absence of Cu(bpy) may improve O_2 delignification in the pulp and paper industry by increasing the formation hydroxyl radicals.[26,33]

Table 2. Chemical composition of poplar following the two-stage alkaline-oxidative pretreatment process performed under various conditions.

Experiment[a]	Solid Remaining (%)[b]	Glucan (%)[c]	Xylan (%)[c]	Acid-Insoluble Lignin (%)[c]
Untreated	N/A	45.5	15.8	22.3
120 °C – Cu-AHP 8% H_2O_2	68.6 ± 0.7	58.1 ± 0.6	14.2 ± 0.5	16.1 ± 0.5
120 °C – Cu(bpy) + O_2	69.6 ± 0.7	56.8 ± 0.8	13.6 ± 0.3	16.3 ± 0.3
120 °C – AHP 8% H_2O_2 + O_2	65.8 ± 1.0	61.0 ± 0.8	14.1 ± 0.4	11.8 ± 0.2
120 °C – Cu-AHP 8% H_2O_2 + O_2	52.2 ± 0.6	74.7 ± 1.1	12.9 ± 0.6	8.2 ± 0.5
120 °C – Cu-AHP 6% H_2O_2 + O_2	52.6 ± 0.6	74.4 ± 0.9	13.1 ± 0.5	8.9 ± 0.4
120 °C – Cu-AHP 4% H_2O_2 +O_2	53.2 ± 0.4	73.7 ± 1.2	13.0 ± 0.6	10.1 ± 0.5
120 °C – Cu-AHP 2% H_2O_2 + O_2	54.1 ± 0.8	73.3 ± 1.3	12.9 ± 0.5	10.8 ± 0.4
90 °C – Cu-AHP 8% H_2O_2	76.3 ± 0.6	54.6 ± 0.9	15.1 ± 0.6	20.3 ± 0.6
90 °C – Cu-AHP 4% H_2O_2 + O_2	71.4 ± 0.8	57.9 ± 1.1	13.1 ± 0.3	16.7 ± 0.8

N/A: Not applicable.

[a] 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment. Values are expressed as average ± standard deviation of triplicate experiments.

[b] Weight percentage (oven-dried weight) of the recovered biomass after two-stage Cu-AHP pretreatment relative to original biomass.

[c] Weight percentage based on oven-dried weight of two-stage pretreated biomass.

In the second series of experiments, shown in the middle of Figure 1, we performed O_2-enhanced Cu-AHP pretreatment with various H_2O_2 loadings [2, 4, 6, and 8% (w/w)]. The solubilized lignin and xylan during O_2-enhanced Cu-AHP pretreatment was significantly higher than those from the control reactions ($p \leq 0.05$). As the H_2O_2 loading decreased from 8% (w/w) to 2% (w/w), the removal of lignin and xylan dropped slightly from approximately 55% and 33% to 48% and 32%, respectively, providing a reduction in the chemical inputs without sacrificing performance. In fact, the removed lignin and xylan during O_2-enhanced Cu-AHP pretreatment using only 2% (w/w) H_2O_2 loading was still much higher than the oxidative pretreatment with either 8% H_2O_2 only or 50 psi O_2 only. These results indicate that O_2 and H_2O_2 exhibit a synergistic effect in improving delignification and pretreatment efficacy. Although the specific mechanism for this synergy is unknown, we speculate that the addition of O_2 could enhance the formation of reactive species, including free hydroxyl (OH·) radicals, superoxide hydroxyl ($O_2^{-}\cdot$) radicals, hydroperoxide anions (HOO^{-}), which attack the phenolic hydroxyl groups of the main lignin molecular chains and break up the polymeric structure of lignin, thereby facilitating the degradation and solubilization of lignin.[46]

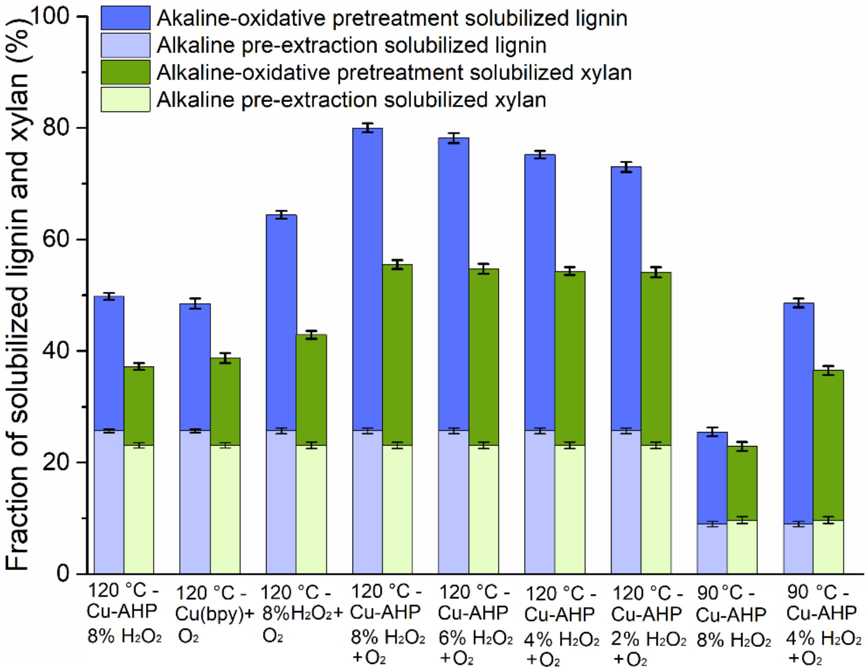

Figure 1. Solubilized lignin and xylan from poplar following alkaline pre-extraction and the second-stage alkaline-oxidative pretreatment. All reactions were performed for 13 h at 10% (w/v) solids. The data are the average of triplicate experiments, and error bars represent ± standard deviation of the means. 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment.

In the third set of experiments, we examined the impact of alkaline pre-extraction temperature on delignification performance of the oxidative pretreatment, shown on the far right of Figure 1. The reduction of alkaline pre-extraction temperature reduced the solubilization of lignin and xylan during the Cu-AHP alkaline-oxidative process. This is not surprising that pre-extraction at higher temperature removed more lignin and xylan from biomass, resulting in higher accessibility of residual biomass components to the chemicals utilized during the oxidative pretreatment stage. Moreover, the results obtained from biomass pre-extracted at 90 °C confirm the synergistic effect of using both O_2 and H_2O_2 as co-oxidants during alkaline oxidative processes, even at low H_2O_2 loadings. Following the two-stage alkaline pre-extraction/O_2-enhanced Cu-AHP pretreatment, up to 75% of lignin and 55% of xylan were removed, which can be expected to significantly increase the enzymatic digestibility of the pretreated biomass.

Overall sugar release following enzymatic hydrolysis

Enzymatic hydrolysis was performed on the pretreated biomass to determine the extent of recovered sugars following delignification. Figure 2 shows the combined of glucose and xylose released during each stage of the process and the subsequent enzymatic hydrolysis. Sugar release was calculated based on the initial glucan and xylan content. The carbohydrates solubilized during the alkaline pre-extraction stage (glucan = 5.6% at 120 °C and 4.1% at 90 °C; xylan = 23.0% at 120 °C and 9.6% at 90 °C) can theoretically be recovered. Furthermore, we have demonstrated that the carbohydrates solubilized during the second-stage alkaline oxidative pretreatment (glucan = 4-9% and xylan = 14-34%) can be directly recovered by adding the pretreatment liquor into the enzymatic hydrolysis step. It is notable that the combination of O_2 and H_2O_2 as co-oxidants achieved much higher overall glucose release compared to using H_2O_2 or O_2 individually (Figure 2a). When conducting O_2-enhanced Cu-AHP pretreatment on the 120 °C alkaline pre-extracted poplar, the reduction of the H_2O_2 loading from 8% to 2% (w/w) only slightly decreased the overall glucose release (from ~97% to ~93%). In contrast, when conducting oxidative pretreatments of the same 120 °C alkaline pre-extracted poplar using either 50 psi O_2 or 8% (w/w) H_2O_2 independently as oxidants, the overall glucose release was in the range of 79-82% (based on initial glucan content). This not only indicates the efficacy of using both O_2 and H_2O_2 as co-oxidants in alkaline oxidative pretreatments, it also provides an opportunity to improve the processing economics by reducing the chemical (H_2O_2) inputs while simultaneously increasing sugar release.

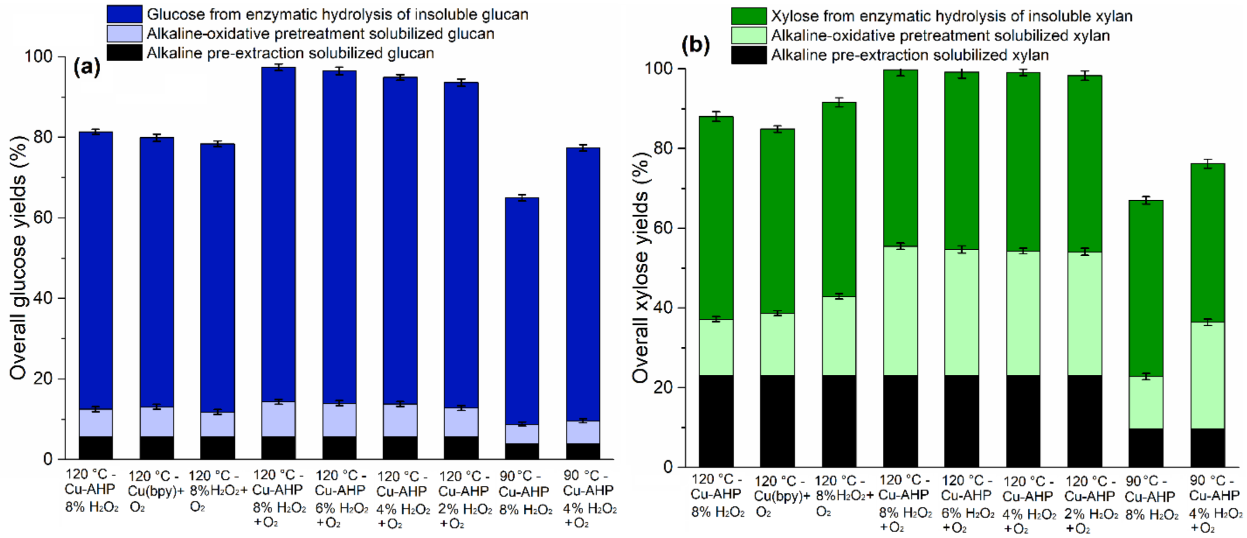

Figure 2. Impact of alkaline-oxidative pretreatment conditions on overall sugar release of (a) glucose and (b) xylose following enzymatic hydrolysis of hybrid poplar. Enzymatic hydrolysis was performed at 50 °C and pH 5 using 15 mg of total protein per g of glucan (based on initial glucan content) for 72 h. The data are an average of triplicate experiments, and error bars represent ± standard deviation of the means. The pre-extracted glucan (5.6% for 120 °C pre-extraction and 4.1% for 90 °C pre-extraction) and xylan (23.0% for 120 °C pre-extraction and 9.6% for 90 °C pre-extraction) during alkaline pre-extraction stage are indicated in black. 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment.

Decreasing the alkaline pre-extraction temperature from 120 to 90 °C led to a predictable reduction in overall sugar release (Figure 2). A plot of glucose release versus extent of lignin removal revealed a strong positive correlation (Figure S1), further supporting that lignin removal during delignifying pretreatments can be a good indicator of glucose release. Xylose release displayed a similar trend, revealing a decrease in yield using the lower pre-extraction temperature as well as much higher release for the O_2-enhanced Cu-AHP pretreatment relative to Cu-AHP with H_2O_2 only (Figure 2b). Taken together, these data highlight that O_2 is a promising co-oxidant in combination with H_2O_2 to enhance alkaline-oxidative pretreatment efficacy.

Impact of pretreatment on recovered lignin properties

Valorization of the isolated lignin is critical to improve the process economics of an integrated biorefinery. One of the proposed value-added applications of lignin streams is in the formulation of polyurethanes because the hydroxyl groups in lignin can react with isocyanate to form urethane linkages. The use of lignin can reduce or even eliminate the need for petroleum-based polyols during the production of polyurethanes. For example, lignin was recently used to replace 100% of the phenol during the production of phenol-formaldehyde resin.[47] Critical polyol parameters that impact polyurethane properties are the number, types (*e.g.*, aliphatic versus phenolic), and accessibility of hydroxyl groups, the number of carboxylates, the solubility of the polyol, and its molecular weight distribution.[48-50] In the case of lignin, these properties are a complex function of the type of biomass, the isolation method, and the conditions (pH and temperature) used to extract lignin.[51]

Table 3. Molecular weight distribution of the lignin samples from different alkaline-oxidative pretreatment conditions.

Pretreatment conditions[a]	Mn (g/mol)	Mw (g/mol)	PDI
120 °C – Cu-AHP 8% H_2O_2	16180	86870	5.4
120 °C – Cu(bpy) + O_2	4950	50560	10.2
120 °C – Cu-AHP 8% H_2O_2 + O_2	8280	47330	5.7
120 °C – Cu-AHP 4% H_2O_2 +O_2	6940	42050	6.1
90 °C – Cu-AHP 4% H_2O_2 + O_2	6690	57150	8.5

[a] 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment.

Lignin isolated from both the Cu-AHP and O_2-enhanced Cu-AHP process was characterized in terms of molecular weight, hydroxyl group content, and reactivity to isocyanate. Cu-AHP pretreatment employing H_2O_2 as the sole oxidant results in higher average molecular weight (M_n) lignin than that of the pretreatments with O_2 alone or the combination of O_2 and H_2O_2 (Table 3). The combination of O_2 and H_2O_2 as co-oxidants only slightly altered the Mn compared to pretreatment with O_2 only. This suggests that O_2 plays a more important role than H_2O_2 in cleaving lignin chains into small pieces and/or reducing lignin condensation/crosslinking during the reaction. This conclusion is supported by the Mw values, which account for the fraction of large molecular weight chains in the polymer. One potential explanation is that the oxidation of lignin hydroxyl groups by O_2 occurred on the main molecular chain of lignin, while the oxidation of chromophores by H_2O_2 occurred through the cleavage of side chains.[34,35,46,52] Analysis of the lignin precipitated following O_2-enhanced Cu-AHP by HSQC NMR indicated that this lignin is essentially identical to the previously characterized lignin obtained using the original two-stage Cu-AHP strategy (*i.e.*, no O_2),[21] although the β-O-4 content might be slightly reduced in lignin precipitated following O_2-enhanced Cu-AHP pretreatment (Figure S2).

To assess the suitability of the lignin as a polyol replacement for the production of polyurethane, the hydroxyl content, the type of hydroxyl group, and the reactivity of the lignin towards isocyanate were determined. As shown in Figure 3a, compared to a commercial Kraft hardwood lignin which has aliphatic and phenolic hydroxyl groups of 1.04 and 4.45 mmol/g, respectively, lignin isolated from the Cu-AHP pretreatment process had significantly ($p \leq 0.05$) higher aliphatic hydroxyl content (3.62-5.26 mmol/g) and lower phenolic hydroxyl content (0.55-0.79 mmol/g). This is probably due to the significant depolymerization of Kraft hardwood lignin during the pulping process that yields a high number of phenolic hydroxyl content,[53,54] while the metal-catalyzed alkaline oxidative pretreatment only results in minimal lignin condensation or depolymerization. The utilization of both O_2 and H_2O_2 as co-oxidants resulted in a slight reduction in the total hydroxyl content (Figure 3a).

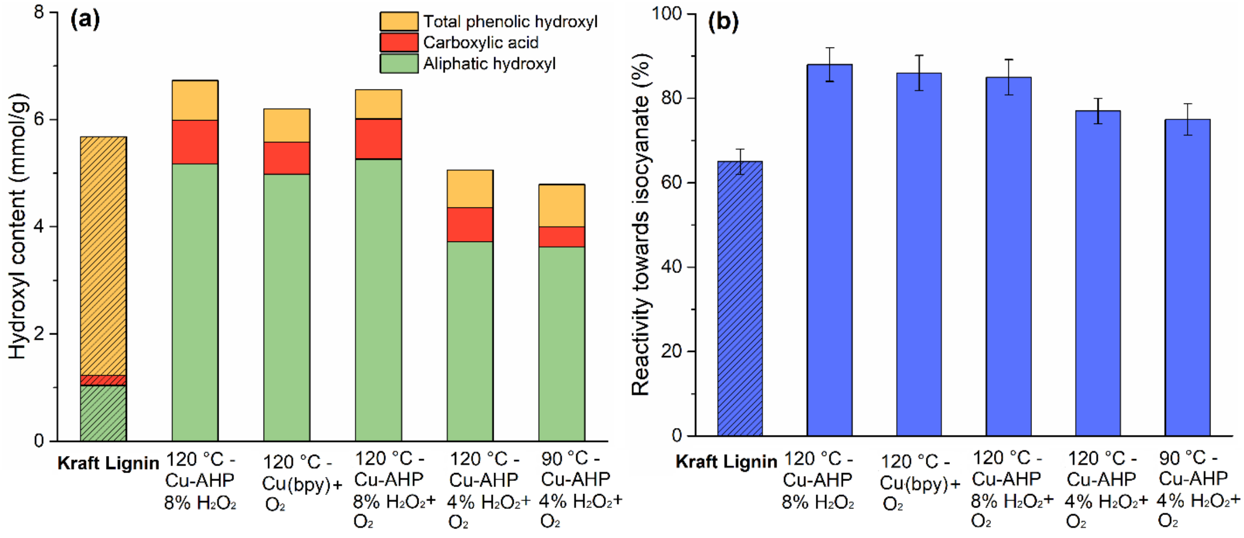

Figure 3. Impact of Cu-AHP pretreatment conditions on (a) the hydroxyl group content and (b) the reactivity towards isocyanate (MDI) of the lignin samples. The data shown in Figure 3b are the average of triplicate measurements of the same batch lignin, and error bars represent ± standard deviation of the means. 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment.

The content and type of hydroxyl group is known to be strongly correlated with the reactivity of the lignin,[36,55] and it was therefore not unexpected that Cu-AHP lignin generally demonstrated a much higher reactivity than Kraft hardwood lignin (Figure 3b). This is presumably because Cu-AHP lignin has a significantly higher aliphatic hydroxyl content (Figure 3a), and aliphatic hydroxyl groups are more reactive than aromatic hydroxyl groups towards isocyanate.[55,56] In fact, a plot of aliphatic hydroxyl group content (Figure 3a) versus reactivity (Figure 3b) indicates a correlation with an R^2 of 0.94. Importantly, the reactivity of all the Cu-AHP lignins tested using a variety of oxidation conditions ranged from 75-85%, which are significantly ($p \leq 0.05$) higher than the reactivity of commercial Kraft hardwood lignin (65%), and closer to the reactivity of petroleum-based polyol (PEG 400) that we measured using similar method (90.5%). This indicates the lignin isolated through the Cu-AHP process is a promising candidate to entirely replace petroleum-based polyol in the production of rigid polyurethane foams and coatings.

Depolymerization of recovered lignin

To further examine the impact of oxidative pretreatment on the lignin properties, the monomer yields of the lignin were determined using a sequential Bobbitt's salt-based oxidation and formic acid-mediated depolymerization process (Figure 4).[37,44] The concentration of obtained aromatic monomers was also determined (Table S4). Following sequential oxidation and depolymerization, the S/G ratio of the depolymerized monomers from different Cu-AHP lignin samples was consistently in the range of 1.23-1.32, which is in a good agreement with previous results reported on poplar lignin.[57] Moreover, our results indicate that Cu-AHP pretreatment employing O_2 and H_2O_2 as the oxidant(s) either individually or in combination does not selectively cleave or crosslink S or G units of the lignin, which is favorable for further lignin depolymerization into aromatic monomers. The dominant monomers generated through this process are diketones, attributed to the cleavage of the β-O-4 bond through an aryl vinyl ether intermediate that is formed in the formic acid catalyzed depolymerization step.[19] Importantly, following the sequential oxidation and depolymerization process, the monomer yields of the Cu-AHP lignin samples were not appreciably different from one another ($p > 0.05$), further highlighting that using both O_2 and H_2O_2 as co-oxidants does not negatively impact lignin quality or the overall depolymerization efficiency. Lignin samples prepared using different pre-extraction temperature (120 °C versus 90 °C) also gave similar monomer yields, except that the lower pre-extraction temperature led to slightly higher yields of *p*-hydroxybenzoic acid, consistent with increased retention of the corresponding ester linkage at the lower pretreatment temperature. Thus, the higher alkaline pre-extraction temperature that resulted in substantially increased sugar yield did not simultaneously lead to a decrease in the quality of the residual lignin. Taken together, these results demonstrate that the use of O_2 as a co-oxidant in the Cu-AHP pretreatment process maintains the high quality of the lignin for efficient depolymerization and further valorization.

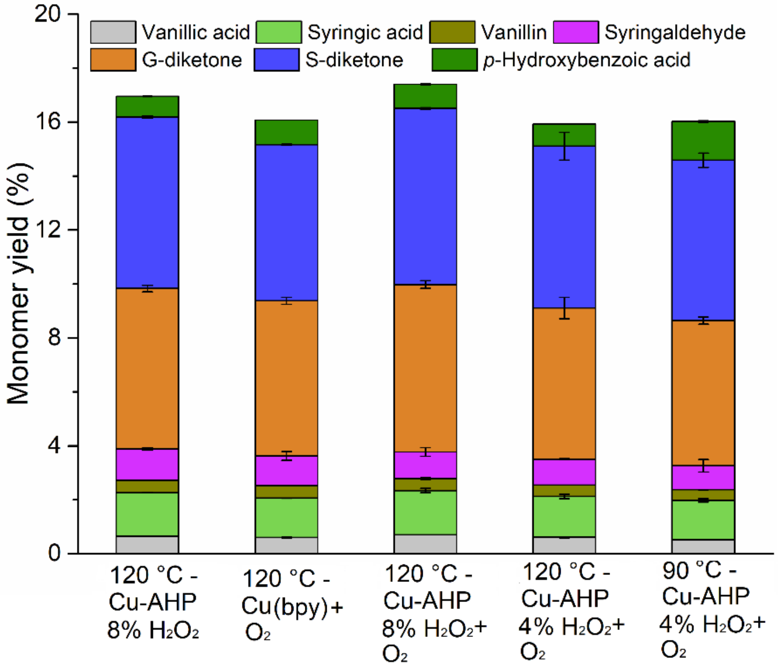

Figure 4. Monomer yields (on a lignin basis) from a sequential oxidation and depolymerization of lignin isolated using different alkaline-oxidative pretreatment conditions. 120 °C and 90 °C: alkaline pre-extraction step conducted at 120 °C and 90 °C, respectively. Cu-AHP H_2O_2: Cu-

AHP pretreatment; Cu(bpy) + O_2: Cu(bpy)-catalyzed alkaline-oxidative pretreatment with O_2 as the only oxidant; Cu-AHP H_2O_2 + O_2: O_2-enhanced Cu-AHP pretreatment.

Discussion

In this study, we have identified an improved alkaline oxidative pretreatment using both O_2 and H_2O_2 as co-oxidants that efficiently delignifies biomass for sugar production while also providing a lignin stream amenable to subsequent valorization. Following enzymatic hydrolysis of the O_2-enhanced Cu-AHP pretreated biomass, total net sugar yields of ~93% for glucose and ~100% for xylose (based on the initial composition) can be achieved using a relatively low H_2O_2 loading (2% w/w). Compared to our earlier studies of the two-stage Cu-AHP pretreatment using H_2O_2 as the only oxidant,[13,16,18,22] using a combination of O_2 and H_2O_2 as co-oxidants substantially improved pretreatment efficiency and enabled reduced chemical inputs. Moreover, integrating the published data on lignin removal and monomeric sugar yields during Cu-AHP pretreatment employing different H_2O_2 loadings [0, 2, 4, 6% (w/w)][22] with the results obtained in this study (Figs. 1 and 2) indicates a synergistic effect of using both O_2 and H_2O_2 as co-oxidants during the Cu-catalyzed alkaline-oxidative pretreatment of lignocellulosic biomass. Although there is literature precedent for enhancing O_2-dependent delignification of pulp by the addition of H_2O_2 or $CuSO_4$,[30,32] to our knowledge there are no published reports describing the simultaneous use of O_2 and H_2O_2 as co-oxidants during the oxidative pretreatment of lignocellulosic biomass to recover both lignin and monomeric C-5 and C-6 sugars.

In the proposed two-stage pretreatment process, following solubilization of ~25% of the original lignin in an alkaline pre-extraction stage (conducted at 120 °C), the Cu-catalyzed alkaline-oxidative pretreatment using the combination of O_2 and H_2O_2 as co-oxidants removes another ~50% of the lignin. The latter process stage remains effective even at a H_2O_2 loading as low as 2% (w/w based on initial biomass). The removal of such high amount of lignin (~75% of the initial lignin) results in the significant increase of the enzymatic digestibility of the biomass;[58] this is part of the reason for high sugar yields following enzymatic hydrolysis. Moreover, the dissolved lignin in the liquid phase can theoretically be recovered at high purity and employed in a variety of lignin valorization strategies, including the production of polyurethanes or depolymerization into aromatic monomers.

The lignin isolated from Cu-AHP pretreatment with or without the addition of O_2 was essentially identical. While the 2D HSQC NMR spectra of the lignin precipitated from the O_2-enhanced Cu-AHP process indicated the presence of a small fraction of oxidized benzylic OH in the β-O-4-units, the extent of this lignin oxidation was very similar to lignin without using O_2 as a co-oxidant (Figure S2). In addition, the intensities of other interunit linkages (β-β and β-5) remained essentially identical, further indicating that the O_2-enhanced Cu-AHP process yielded lignin that was very similar to that obtained from the standard Cu-AHP process. Importantly, all Cu-AHP lignin samples had higher hydroxyl group content (especially aliphatic hydroxyl group) and reactivity with isocyanate (MDI) relative to commercial Kraft hardwood lignin (Figure 3), similar to lignin obtained from other oxidative pretreatment processes, such as magnesium sulfate-catalyzed alkaline oxygen pretreatment of *Eucalyptus*.[33] In addition, compared to lignin isolated from alkaline oxygen or alkaline hydrogen peroxide pretreatment of rice straw or wheat straw,[59,60] lignin isolated during the O_2-enhanced Cu-AHP pretreatment of poplar has much higher aliphatic hydroxyl content. These results suggest that O_2-enhanced Cu-catalyzed alkaline oxidative pretreatment can provide a lignin stream that may be very amenable to the formulation of polyurethanes.

We also evaluated the impact of delignification conditions on lignin properties through a sequential oxidation and formic acid depolymerization method. Oxidation of C_α-OH to C_α=O through Bobbitt's salt oxidation is an efficient approach to decrease β-O-4 ether bond dissociation energy, making it susceptible to subsequent formic acid-catalyzed depolymerization.[19] The monomer yield variation of different Cu-AHP lignin samples was very minor, suggesting that the addition of O_2 as a co-oxidant did not have a significant impact on the lignin properties. Thus, the combination of O_2 and H_2O_2 as co-oxidants during alkaline oxidative pretreatment consistently produced high-quality lignin samples that can be effectively depolymerized for further valorization.

Conclusion

In this study, we demonstrated that using both O_2 and H_2O_2 as co-oxidants under alkaline conditions is an effective biomass fractionation strategy which can simultaneously generate both highly digestible biomass for sugar production and a high-quality lignin stream amenable to subsequent valorization. In addition, employing O_2 as a co-oxidant enables a substantially reduced H_2O_2 loading to be used while still achieving high yields of monomeric sugar following enzymatic hydrolysis. The lignin isolated from the O_2-enhanced Cu-AHP pretreatment process exhibits a high content of hydroxyl groups (especially aliphatic hydroxyls) and substantial reactivity to isocyanate, suggesting that the lignin might be very useful for the production of polyurethanes. In addition, the lignin was also susceptible to sequential oxidation and depolymerization, indicating that the lignin might also be useful for the production of aromatic monomers. These data demonstrate the potential of employing both O_2 and H_2O_2 as co-oxidants during alkaline-oxidative processes, and a detailed technoeconomic analysis (TEA) of this promising new pretreatment process is in progress.

Supporting Information

The following information is available free of charge at http://pub.acs.org:

Experimental results, (Table S1) Lignin precipitation yield from different alkaline-oxidative pretreatment liquors; (Table S2) solubilized poplar components during alkaline pre-extraction; (Table S3) monomer concentration from lignin depolymerization; (Figure S1) correlation between lignin removal and enzymatic hydrolysis glucose yields; (Figure S2) HSQC NMR spectra of lignin samples.

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recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

Notes

The authors declare the following competing financial interest(s): E.L.H., Z.Y., D.B.H., S.S.S., and B.D.B. are listed as inventors on a related pending patent application (Economical Methods for Performing Oxidative Catalytic Pretreatment of Plant Biomass Using a Homogeneous Catalyst System); US Publication No.: US 2020/0332376 A1. As holders of this patent, we may benefit financially from advances in the technology discussed in this article.

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Abbreviations

AHP: Alkaline hydrogen peroxide
bpy: 2,2'-bipyridine
Cu-AHP: copper 2,2'-bipyridine-catalyzed alkaline hydrogen peroxide
DMF: dimethylformamide
GPC: gel permeation chromatography
HPLC: high-performance liquid chromatography
MFSP: minimum fuel selling price
MDI: methylene diphenyl diisocyanate
NREL: National Renewable Energy Laboratory
PDI: polydispersity
^{31}P NMR: Phosphorous-31 nuclear magnetic resonance
TEA: technoeconomic analysis

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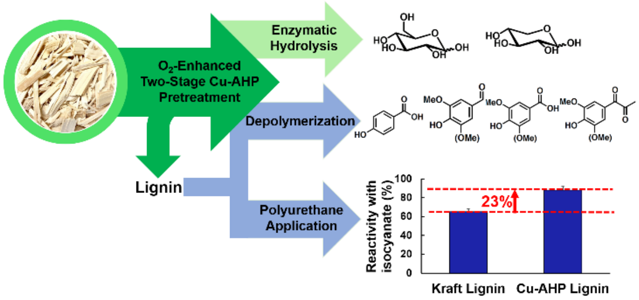
TOC Graphic:



Using O_2 as a synergistic co-oxidant in combination with H_2O_2 during metal-catalyzed alkaline oxidative pretreatment provides a green and cost-effective method to fractionate the biopolymers of lignocellulosic biomass.