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Enhanced Softwood Cellulose Accessibility by H_3PO_4 Pretreatment: High Sugar Yield without Compromising Lignin Integrity

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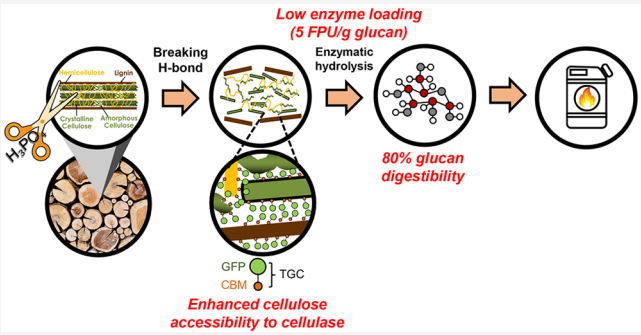
Supporting Information

ABSTRACT: Softwood lignocellulose is a potential feedstock for the production of biofuels and bioproducts. However, the highly cross-linked nature of softwood lignocellulose restricts enzyme access to its sugars. Thus, harsh pretreatment conditions (180−280 °C) and/or high enzyme loading are required to unlock sugars. These requirements negatively affect the economic viability of softwoods in biorefineries. Here we show that H_3PO_4 pretreatment of pine and Douglas fir under a mild reaction temperature (50 °C) and atmospheric pressure enabled a high (∼80%) glucan digestibility with low enzyme loading (5 filter paper units (FPU)/g glucan). The dissolution and regeneration of softwoods disrupted the hydrogen bonding between cellulose chains, thereby increasing the cellulose accessibility to cellulase (CAC) values by ∼38-fold (from ∼0.4 to 15 m^2/g biomass). Examination of H_3PO_4-pretreated softwoods by cross-polarization/magic angle spin (CP/MAS), ^{13}C- nuclear magnetic resonance (NMR), and Fourier-transform infrared spectroscopy (FTIR) revealed that breaking of the orderly hydrogen bonding of crystalline cellulose caused the increase in CAC (higher than 11 m^2/g biomass), which, in turn, was responsible for the high glucan digestibility of pretreated softwoods. The H_3PO_4 pretreatment process was feedstock independent. Lastly, 2D $^{13}C-^{1}H$ heteronuclear single quantum coherence (HSQC) NMR showed that the lignin was depolymerized but not condensed; thus, the lignin can be available for producing high-value products.



1. INTRODUCTION

Within 20 years, the number of automotive vehicles in use worldwide is projected to reach 2 billion[1] because of economic and population growth. Petroleum is the current feedstock for fuel production for these vehicles. Petroleum-derived fuels have two major drawbacks: (1) petroleum supply is limited, causing uncertainty in price and availability, and (2) processing petroleum generates carbon dioxide that contributes to global warming.[2,3] Clearly, an alternative fuel feedstock is urgently needed to supplement and/or replace petroleum.

Softwoods are woody biomass abundant in the northern hemisphere. Their high cellulose content, ∼40−48%,[4−6] makes them attractive renewable feedstocks for the production of biofuels and bioproducts.[7] The U.S. Department of Energy and the U.S. Department of Agriculture have estimated that ∼1.4 billion tons of woody biomass and agricultural wastes will be available by the mid-21st century, providing vast feedstocks for biorefineries.[8]

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The guaiacyl (G)-type lignin monomer is the main lignin monomer in softwoods.[9] The G-type lignin monomer has a C_4 atom available to form a bond with other G-type lignin monomer units, creating highly cross-linked lignin. The cross-linked lignin is a strong glue that seals the cellulose from enzymes and catalysts during upgrading.[4,10−15] Thus, typically, some types of pretreatment are required to provide cellulose accessibility to enzymes[16,17] to unlock sugars from lignocellulosic biomass.[18] For softwoods, severe pretreatment (e.g., 180−280 °C) conditions are needed to expose cellulose to enzymes. These conditions present three challenges: (1) sugar degradation, (2) a requirement for high enzyme loadings due to the generation of inhibitors (furfural, 5-hydroxymethylfurfural (HMF), acetic acid, levulinic acid, and formic acid),[12,27,16,17,19−23] and (3) difficulty in further lignin utilization due to lignin condensation.[24,25] Solving these challenges is important for the economics of softwood biorefineries.

Cellulose solvents, including H_3PO_4 and various types of ionic liquids, are effective at lignocellulose dissolution/pretreatment. The dissolution of lignocellulose unglues the lignocellulose components by disrupting the lignin-carbohydrate complex (LCC) linkages, and it decrystallizes the highly ordered cellulose structure by disrupting the intra/intermolecular hydrogen bonds between cellulose chains. Decrystallization of cellulose enhances its accessibility to acid/enzymes, enabling high glucan digestibility and fast hydrolysis rates with low enzyme loading.[26,27] In particular, pretreatment with the cellulose solvent H_3PO_4 (85%) breaks inter/intramolecular hydrogen bonds within lignocellulose and between cellulose chains under a mild condition (<60 °C and atmospheric pressure).[28,29] Thus, H_3PO_4 pretreatment has been explored recently for agricultural wastes, bioenergy crops, and hardwoods.[30−34] The pretreated lignocellulose samples had high glucan digestibilities of >73% with a low enzyme loading of 5 filter paper units (FPU) of enzyme/g glucan.[29,35,36] Various strategies have been developed to recycle these cellulose solvents.[37,38] Although H_3PO_4 pretreatment is effective at pretreating lignocellulose, its impact on softwoods and residual lignin after has not been assessed adequately.

In this contribution, we describe H_3PO_4 (85%) pretreatment at 50 °C and atmospheric pressure for <2 h on southern yellow pine and Douglas fir, followed by enzymatic hydrolysis. Pretreated pine and Douglas fir had high glucan digestibility of ∼80% with a low cellulase loading (5 FPUs/g glucan). We used a fusion protein to mimic and quantitate adsorption of cellulase enzymes onto the lignocellulose, cross-polarization/magic angle spin (CP/MAS) ^{13}C-nuclear magnetic resonance (NMR), and Fourier-transform infrared (FTIR) spectroscopy to characterize changes in the degree of crystallinity, and 2D ^{13}C−^{1}H heteronuclear single quantum coherence (HSQC) NMR spectroscopy to elucidate changes in chemical structure of lignin after H_3PO_4 pretreatment. H_3PO_4 pretreatment enhanced cellulose accessibility to cellulase. The process disrupted LCC linkages and the highly ordered hydrogen bonds of cellulose, thereby reducing the degree of crystallinity. The pretreated materials became more amorphous compared with the untreated biomass. Moreover, this process did not modify lignin, enabling its potential profitable use in biorefineries.

2. MATERIALS AND METHODS

2.1. Chemicals and Materials. All chemicals were reagent grade and purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.), unless otherwise noted. Phosphoric acid (85% w/w) and ethanol (95% v/v) were purchased from Fisher Scientific (Houston, TX, U.S.A.). Microcrystalline cellulose, Avicel PH105 (20 lm), was obtained from FMC Corp (Philadelphia, PA, U.S.A.). Regenerated amorphous cellulose (RAC) was prepared as described.[29,39] In short, (1) Avicel was mixed with water to make a slurry; (2) ice-cold 85% H_3PO_4 (∼4 °C) was added to the Avicel slurry and kept on ice for 1 h; and (3) deionized water was added to regenerate the RAC.[40] *Trichoderma reesei* cellulase (Novozyme 50013) and β-glucosidase (Novozyme 50010) were gifted by Novozymes North America (Franklinton, NC, USA). They had activities of 84 filter paper units (FPUs) of cellulase per mL and 270 units of β-glucosidase per mL. Southern yellow pine woodchips and Douglas fir were obtained from Brook Center at Virginia Tech (Blacksburg, VA, U.S.A.) and University of British Columbia (Vancouver, Canada), respectively. All naturally dried biomass samples were knife-milled by a Pallmann counter-rotating knife ring flaker (Clifton, NJ, U.S.A.). The resulting particulates with nominal sizes of 40−60 mesh (250−400 μm) were collected. They are referred to as untreated Douglas fir and pine and used as controls for all experiments.

2.2. H_3PO_4 Pretreatment of Softwoods. Pine and Douglas fir were pretreated with 85% H_3PO_4 as described.[40,41] In short, approximately 1.05 g of natural-dried biomass (∼5% moisture) was mixed with 8 mL of 85% (w/w) H_3PO_4 in a 50 mL plastic centrifuge tube and heated at 50 °C at atmospheric pressure for 105 min, unless otherwise noted. The pretreatment reaction was stopped and regenerated by adding 20 mL of 95% (v/v) ethanol. Pretreated solid was separated by a swing bucket centrifuge at 4500 rpm for 10 min. The supernatant was removed. The pretreated solid was washed and centrifuged in a sequence of 40 mL of 95% (v/v) ethanol and 40 mL of deionized water two times, respectively. The resulting solid was neutralized with a 2 M sodium carbonate solution and stored in the presence of 0.1% (w/v) NaN_3 at 4 °C to prevent microbial growth prior to enzymatic hydrolysis. These resulting pretreated solids after H_3PO_4 pretreatment, regeneration, and solvent wash are referred to as pretreated pine and Douglas fir throughout this study. The severity of the H_3PO_4 pretreatment of softwoods was expressed as the severity factor (R_o).[42] The R_o combined reaction temperature and time and in the following calculation:

$$\log R_o = \left[t \cdot \exp\left(\frac{T - 100}{14.75}\right)\right]$$

where t and T are pretreatment time (min) and temperature (°C), respectively. This calculation did not account for an effect of pH because pH information was lacking during pretreatment processes. The R_o values of other pretreatment processes were also calculated for comparison.

2.3. Enzymatic Hydrolysis. The pretreated softwood was suspended to obtain 10 g of glucan per liter in a 50 mM sodium citrate buffer (pH ∼4.8) supplemented with 0.1% (w/v) NaN_3 (to prevent microbial growth) in a 50 mL centrifuge tube. Enzyme loadings were the following: (1) 5 FPUs of cellulase and 10 units of β-glucosidase per gram of glucan or (2) 15 FPUs of cellulase and 30 units of β-glucosidase per gram of glucan, unless otherwise noted. The cocktail

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(pretreated samples + buffer solution + enzyme) was suspended in an incubator shaker at 50 °C and 600 rpm; samples were collected for 72 h. The cocktail samples were centrifuged at 13 000 rpm for 5 min to separate clear solution from the suspended solids. Exactly 500 μL of the supernatant was transferred to a 2 mL microcentrifuge tube maintained at room temperature for 30 min to allow cellobiose to be continuously converted to glucose by the residual enzyme. This step was important to ensure accurate enzymatic hydrolysis results. The supernatant was then acidified by adding 30 μL of 10% (w/w) sulfuric acid, followed by freezing overnight. The frozen sample was thawed, mixed well, and centrifuged at 13 000 rpm for 5 min to remove any precipitated solids. The supernatant, containing soluble sugars after enzymatic hydrolysis, was processed with an 1110 Agilent high-pressure liquid chromatography system (HPLC, Agilent Technologies, Santa Clara, CA, U.S.A.). Enzymatic glucan digestibility was calculated as follows:

$$\text{glucan digestibility}(\%) = \frac{\text{glucan}_{\text{supernatant}}}{\text{glucan}_{\text{pretreated biomass}}} \times 100$$

After 72 h enzymatic hydrolysis, the residual solid was washed with 20 mL of deionized water to remove soluble sugars and enzyme prior to freeze-drying overnight. All experiments were conducted in triplicate, and the standard deviations were calculated. The composition of the residual solid was then analyzed to construct the mass balance.

Environmental factor (E-factor)[43] was calculated to describe the sustainability aspect of the pretreatment, followed by enzymatic hydrolysis of softwoods. The inputs and outputs from the mass balance were used in the E-factor calculation. The E-factor is the ratio between the amount of waste produced (residual biomass, chemicals, and solvents/water) and the amount of desired products (glucose or glucose equivalent, in this case) produced:

$$\text{E-factor} = \frac{\text{amount of waste}}{\text{amount of desired product}}$$

The E-factors of the pretreatment alone and pretreatment + enzymatic hydrolysis of the selected processes were calculated for comparison (see the Supporting Information).

2.4. Compositional Analysis of Softwoods and Glucose Determination. Compositional analysis of softwoods and residual enzymatic hydrolysis solids was performed by the NREL LAP procedure.[44,45] Briefly, 0.2 g of the sample was dissolved in 2 mL of 72% H_2SO_4 at 30 °C for 1 h in an incubator shaker prior to dilution of the H_2SO_4 concentration to 4% with deionized water. The sample was autoclaved at 121 °C, 15 psi for 1 h and then filtered by a ceramic filter crucible. The filtrate was analyzed by HPLC for soluble sugars. Residual solids were dried at 105 °C overnight. The weight of the dried solids corresponded to the amount of lignin and ash in the sample. The weight loss of the dried sample and residual weight after calcination at 575 °C for 8 h corresponded to the amount of lignin and ash, respectively, in the sample.

Soluble sugars were measured by HPLC equipped with a refractive index detector (RID) and diode array detector (DAD). A Bio-Rad Aminex HPX-87P column (Richmond, CA, U.S.A.) was used to analyze soluble sugars with a rate of 0.6 mL/min of 4 mM H_2SO_4 as a mobile phase at 60 °C. All sugars were calibrated against certified standards (Absolute Standards Inc., Hamden, CT, U.S.A.).

2.5. Characterization of Softwoods before and after H_3PO_4 Pretreatment. The total substrate accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and non-cellulose accessibility to cellulase (NCAC) were determined based on the maximum adsorption of the TGC fusion protein. This fusion protein contained thioredoxin (T), green fluorescent protein (GFP), and three cellulose-binding modules (C). The fusion protein was used in the presence and absence of bovine serum albumin (BSA).[40,46] TSAC was calculated by the maximum adsorption of TGC in the absence of BSA, whereas CAC was calculated by the maximum adsorption of TGC in the presence of BSA. NCAC was calculated by the difference between TSAC and CAC. TGC fusion protein was produced in *Escherichia coli* BL21 (pNT02), purified by adsorption onto regenerated amorphous cellulose (RAC), and desorbed with ethylene glycol (EG).[47] EG was removed by dialysis in a 50 mM sodium citrate buffer (pH 6.0) and the TGC solution was concentrated using Millipore 10 kDa molecular weight cutoff centrifugal ultrafilter columns (Spectrum Lab, Billerica, MA, USA). Confocal microscopy was performed to confirm the presence of TGC on biomass. The confocal images were recorded on the Zeiss Axio Observer Z1 inverted microscope for fluorescence or brightfield microscopy (Carl Zeiss Microscopy, LLC, Thornwood, NY, U.S.A.).

2.5.1. Scanning Electron Microscopy (SEM). SEM images of the biomass materials were taken with a Zeiss-DSM940 (Carl Zeiss, Okerkochen, Germany), as described.[36] All samples were sputter-coated with gold prior to SEM imaging.

2.5.2. Attenuated Total Reflection (ATR) Fourier-Transform Infrared (FTIR) Spectroscopy. ATR-FTIR was conducted using a Thermo Nicolet 6700 ATR/FT-IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.). A total of 256 scans at a resolution of 6 cm^{-1} were averaged for each sample. The samples were scanned in the spectral range between 400 and 4000 cm^{-1}. The absorbances of the bands were resolved using Voigt distribution function by PeakFit1 4.12 software (Systat *Software* Inc., Chicago, IL, U.S.A.).

2.5.3. Cross-Polarization/Magic Angle Spin (CP/MAS) ^{13}C-Nuclear Magnetic Resonance (NMR). The CP/MAS ^{13}C NMR spectra of all samples were acquired with a Bruker Avance I 500-MHz NMR spectrometer operating at the resonance frequencies of 500.23 MHz for ^{1}H and 125.80 MHz for ^{13}C, using a double-resonance Bruker 4.0 mm broad-band CP-MAS probe spinning at 13−14 kHz. Cross-polarization for 2 ms contact time was achieved using a ^{1}H 90° pulse width of 4.2 μs at 60-kHz two-pulse phase-modulated proton decoupling field and 2-s recycle delay. Total accumulation time was between 1000 and 3000 transients. All spectra were collected at room temperature and referenced against the chemical shifts of adamantane at 38.48 and 29.45 ppm. According to the C_4 peak-deconvolution method, the degree of crystallinity was determined and expressed as the crystallinity index (CrI) (see Supporting Information for a detailed calculation of CrI).[48]

2.5.4. 2D ^{13}C-^{1}H heteronuclear single quantum coherence (HSQC) NMR spectroscopy. Softwood samples were extracted to remove extractives and ball-milled as described.[49,50] The ball-milled softwood sample (∼20−30 mg) was mixed with DMSO-d_6 (600 uL) in a microcentrifuge tube. A minute amount of 1-ethyl-3-methylimidazolium acetate (∼10 uL) was added as a cosolvent to help dissolve softwood samples.[51,52] This solution was sonicated in a Branson 2510 table-top cleaner (Branson Ultrasonic Corporation, Danbury, CT, USA)

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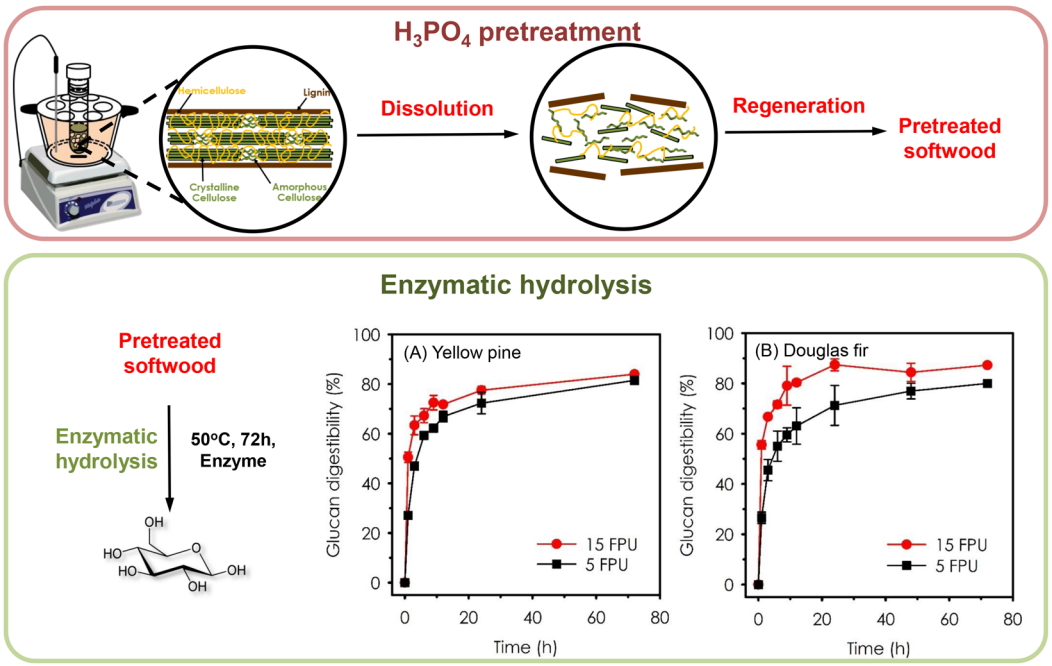

Figure 1. Hydrolysis profiles of H_3PO_4-pretreated yellow pine samples (A) and Douglas fir (B) at 5 and 15 FPU/g glucan.

until it became a homogeneous gel. The temperature of the sonication bath was maintained below 55 °C. The homogeneous gel was transferred to the NMR tube. HSQC spectra were acquired at 25 °C using a Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient $^1H/^{13}C$ cryoprobe and the q_hsqcetgp pulse program (ns = 200, ds = 16, number of increments = 256, d_1 = 1.0 s).[53] Chemical shifts were referenced to the central DMSO peak (δ_C/δ_H 39.5/2.5 ppm). Assignments of the HSQC spectra were described elsewhere.[49] A semiquantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin 3.1 (Windows) processing software. A Gaussian apodization in F_2 (LB = −0.50, GB = 0.001) and squared cosine-bell in F_1 (LB = −0.10, GB = 0.001) were applied prior to 2D Fourier Transformation. The semiquantitative evaluation of the interunit linkages of lignin was expressed as (1) the relative abundance of the interunit linkages of lignin and (2) the amount of interunit linkages per 100 aromatic (Ar) units (C_9 units) in lignin. The calculations were performed as follows:

$$\text{the relative abundance}(\%) = \frac{\text{volume Integral of interunit linkage}}{\text{total volume integral of all interunit linkage}} \times 100$$

$$\text{amount of interunit linkages}(\text{per 100 Ar units}) = \frac{\text{volume Integral of interunit linkage}}{\text{volume Integral of correlation peak of } G_2} \times 100$$

3. RESULTS AND DISCUSSION

The pine and Douglas fir samples consisted of ∼35−39 wt % glucan, 9−12 wt % mannan, 3−6 wt % xylan, and 33−34 wt % lignin (Table S1). The high mannan content was a signature of softwoods. Our softwood compositions agreed with previously reported values.[20,54] High lignin content in softwoods and its highly cross-linked G-type lignin structure are barriers for enzymes and/or catalysts to access cellulose. Our strategy to circumvent this barrier was to employ H_3PO_4 pretreatment to disrupt the lignin-carbohydrate complex (LCC) linkages and intra/intermolecular hydrogen bonds between crystalline cellulose chains of softwoods.

3.1. Effect of H_3PO_4 Pretreatment of Softwood on Enzymatic Hydrolysis. Untreated pine and fir showed a low glucan digestibility of <7% after 72 h with 15 FPUs/g glucan of enzyme loading. These results illustrated the high recalcitrance of softwoods. Conversely, pretreated pine and Douglas fir had a fast hydrolysis rate and their glucan digestibilities were >72% after 12 h, reaching a maximum of 87% after 72 h for Douglas fir at 15 FPU/g glucan enzyme loading. A 3-fold reduction in enzyme loading (5 FPU/g glucan) still yielded a fast hydrolysis rate and high glucan digestibilities of >63% after 12 h and >80% after 72 h for both softwoods (Figure 1). These results confirmed that H_3PO_4 pretreatment was efficient for softwoods; thus, we obtained a high glucan digestibility (>63%) after 12 h at a low cellulase loading.

Pretreatment cost represents about 20% of the total cost of biomass conversion processes.[18] The harsh reaction conditions (high temperature: 170−200 °C and 6.5−7.2 bar) and/or high enzyme loading add significantly to capital expenditure (CAPEX) and operating expenditure (OPEX) of the overall process. Moreover, a harsh reaction condition (high temperature >120 °C) possibly cleaves β-O-4 lignin linkage and forms undesired condensed C−C linkages,[55] limiting lignin's potential upgrading to coproducts.

To obtain a high glucan digestibility, typically, severe pretreatment conditions (high log R_0) are required to disrupt the highly ordered crystalline cellulose of softwoods (Table 1). Previous high log R_0 (3.54−5.00) studies with organosolv, sulfite, and physicochemical pretreatments did demonstrate high sugar yields.[19,56−58] The high log R_0 suggested that the pretreatments were energy-intensive, contributing to the high CAPEX and OPEX of the overall processes. For example,

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Table 1. Selected Pretreatment Technologies for Softwoods

	pretreatment	feedstock	pretreatment condition	enzyme loading (FPU/g glucan)[a]	protein conc. (mg protein/g glucan)[a]	glucan digestibility	E-factor[b]	severity (log R_o)	ref
cellulose solvent	H_3PO_4 pretreatment	pine	50 °C, 1.45−2 h	5	10	78% @48 h	168.9	0.91	this study
		Douglas fir		15	30	80% @48 h			
	cholinium lysinate ([Ch][Lys])	pine	140 °C, 1 h	14.8	20	23% @72 h	171.4	2.96	68
	1-butyl-3-methylimidazolium chloride ([C_4C_1 im]Cl)	pine	130 °C, 18 h	17.5	37	97% @96 h	133.1	3.92	69
	N,N-dimethylbutylammonium hydrogen sulfate [DMBA][HSO_4])	pine	170 °C, 30 min	16.4	20	75% @168 h	111.6	3.54	6
physical	ball-milling	Douglas fir	10 min milling/10-min pausing, 240 min	114	173	~ 63% @72 h	12.3	0.17	56
chemical	dilute acid	spruce	180 °C, 30 min	15	60	59.5% @48 h	105.2	3.83	19
	ethanol organosolv	Douglas fir	188 °C, 15 min	14	35	>90% @24 h	140.3	3.77	70
	Ethanol organosolv	pine	170 °C, 1h	8	32	61% @80 h	100.0	3.84	57
	Wet explosion	Pine	170 °C, 7.2 bar O_2, 22 min	44	66	96% @72 h	10.7	3.54	58
Physico-chemical	sulfite pretreatment	spruce	180 °C, 30 min	15	59	91% @ 24 h	100.3	3.83	19
				5	20	70% @48 h			
	steam pretreatment	Douglas fir	160 °C, 10 min	n.a.[d]	n.a.[d]	~ 80% @72 h	56.9	2.77	71
	SO_2-steam explosion	pine	215 °C, 5 min	20	114	94% @48 h	94.4	4.08	72
	SO_2-steam explosion	Douglas fir	210 °C, 5 min	60	218	>95% @72 h	48.2	3.94	73
	SO_2-steam explosion + alkaline peroxide treatment	Douglas fir	195 °C, 4.5 min +80 °C, 45 min	10	37	90% @48 h	174.0	4.49[c]	74
	SO_2-steam explosion + alkaline peroxide treatment	Douglas fir	195 °C, 4.5 min +80 °C, 45 min	20	50	>95% @48 h	141.3	4.49[c]	70
	SO_2-steam explosion	Douglas fir	195 °C, 4.5 min	20	79	81% @48 h	107.7	5.06[c]	75
	+ alkali-oxygen treatment		+ 110 °C, 3h	40	158	90% @48 h	96.8		

[a]Enzyme loading and protein concentration (see Table S2 and the Supporting Information for detailed calculations). [b]E-factor (E-factor$_{pretreatment+hydrolysis}$, see Table S3 and the Supporting Information for detailed calculations). [c]Severity factor (see Table S3 and the Supporting Information). [d]n.a. = not available.

Shuai et al. studied sulfite pretreatment to overcome recalcitrance of ligno-cellulose (SPORL) and by dilute acid (DA) pretreatment of spruce at 180 °C for 30 min (log R_0 of 3.83) and obtained 91% and 55% glucan digestibility, respectively, with 15 FPUs/g glucan enzyme loading.[19] A decrease in enzyme loading from 10 to 5 FPUs/g glucan dropped the glucan digestibility of SPORL-pretreated spruce from 91% to 70%.[19] Nonetheless, this and similar approaches do not mitigate the high economic cost of conversion.

The green chemistry environmental factor (E-factor) is another important indicator of the sustainability of a process.[59,60] A high E-factor indicates that a process produces more waste with a negative environmental impact.[61] The ideal E-factor is zero, but zero is not usually attainable because most chemical processes generate some waste. For softwood pretreatment processes, E-factors have ranged from 10.7 to 174.0 (Table 1; see also the Supporting Information). Ball milling and wet explosion pretreatments have low E-factors (10.7−12.3) because they use low amounts of chemicals. Although the ball-milling samples gave a low E-factor of 12.3, they achieve only a moderate glucan digestibility of 63% after 72 h with a high enzyme loading of 114 FPU/g glucan (173 mg protein/g glucan).

The foregoing examples emphasize that a balance must be struck between minimizing sugar degradation and/or inhibitory compound formation (low log R_0) and minimizing waste (low E-factor). However, low R_0 and low E-factor do not necessarily go hand-in-hand. From a cost and sustainability standpoint, a mild and environmentally friendly pretreatment process would revolutionize the future biorefinery.

Our H_3PO_4 pretreatment process had an E-factor of 168.9. However, several advantages of our approach offset this high E-factor. First, we achieved a high glucan digestibility of ∼78−80% with a low enzyme loading (5 FPUs/g glucan) and a high reaction rate. The enzyme cost in biofuels production is ∼$0.68/gal (based on theoretical sugar yields from lignocellulosic biomass) or $1.47/gal (based on saccharification and fermentation yields) of bioethanol.[62,63] Hence, the reduction of enzyme loading while maximizing sugar yield is an important economic feature of our process. Second, the H_3PO_4 pretreatment was conducted at a mild reaction condition of 50 °C and atmospheric pressure, with an

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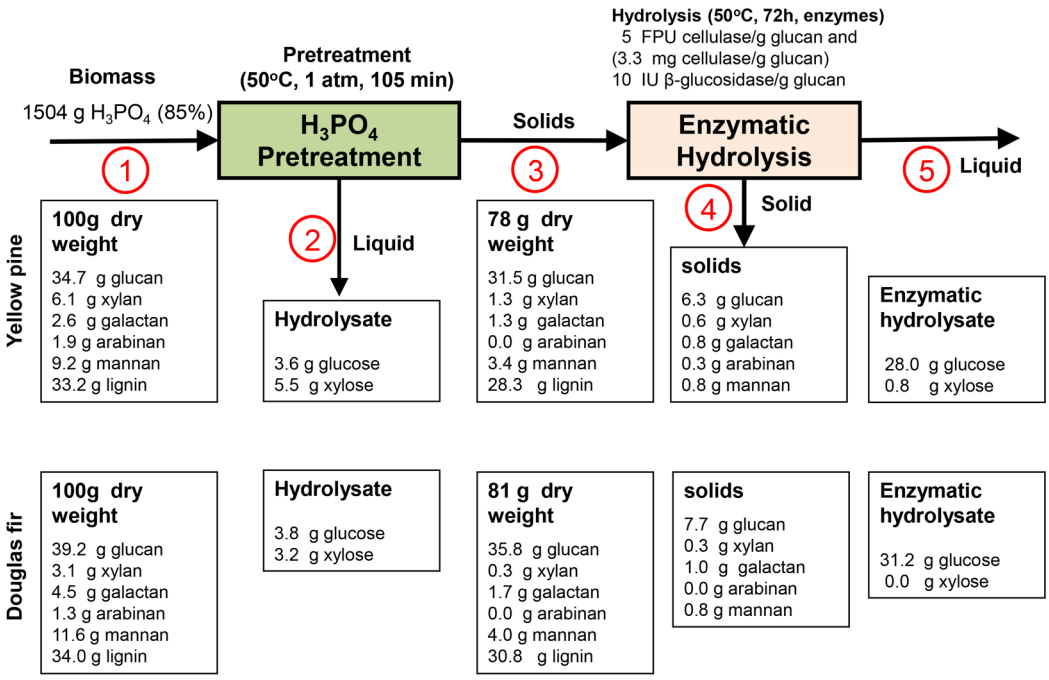

Figure 2. Mass balance of H_3PO_4-pretreated yellow pine (A) and H_3PO_4-pretreated Douglas fir (B) at 5 FPU/g glucan.

associated low severity factor of 0.91. More commonly, high severity pretreatments are used to achieve high glucan digestibility of softwoods (Table S3 and Figure S2), but high severity pretreatments cause sugar degradation and formation of inhibitors (furfural, 5-hydroxymethylfurfural (HMF), acetic acid, levulinic acid, and formic acid) to downstream fermentation step.[12,27,16,17,19−23] Third, H_3PO_4 recycling can be considered as a means to further enhance the economic benefit of our process. Liquid extraction[64,65] and diffusion dialysis[66] show potential in recycling H_3PO_4 with high purity (80−90% acid recovery). Moreover, spent H_3PO_4 can be used to produce fertilizer.[67] In sum, the H_3PO_4 pretreatment process reduces energy consumption, reduces enzyme loading, and likely will reduce the operating cost for biorefineries.

3.2. Mass Balance of the Pretreatment and Enzymatic Hydrolysis of Pine and Douglas Fir. To understand the economics of the process, we constructed the mass balance on the basis of 100 g of softwood (Figure 2). The solid recovery was 78% and 81% for pretreated pine and Douglas fir. We obtained a high glucan recovery, ∼90%, from pretreated pine and Douglas fir, whereas xylan recovery was low (∼10%; stream 3). These results suggested that ethanol could efficiently recover most dissolved glucan and simultaneously fractionate xylan in the liquid stream. We observed a slight decrease (∼15%) in lignin content after H_3PO_4 pretreatment (stream 3), suggesting that most lignin remained in the solid. After enzymatic hydrolysis, we achieved 80% glucan digestibility for pretreated pine (28.0 g of glucose/100 g dry weight) and 79% (31.2 g of glucose/100 g dry weight) for pretreated Douglas fir (stream 5). The overall glucose yields were 82% for pine and 80% for Douglas fir (streams 2 + 5). Moreover, the overall xylose yield was ∼90% for both pretreated softwoods.

The main barriers to softwood conversion are (1) inter/intramolecular hydrogen bonding networks within cellulose chains that form highly ordered crystalline cellulose and (2) lignin-carbohydrate complex (LCC) linkages.[40,76] To design and optimize the process for other types of softwood lignocellulose, we need to more completely understand the factors that contribute to high glucan digestibility. Accordingly, as described next, we characterized the morphology and accessibility of cellulose, the cellulose surface functionalities, changes in cellulose crystallinity, and changes in the chemical structure of lignin of pretreated softwoods.

3.3. Fusion Protein Assessment of Morphology and Cellulose Accessibility of Pretreated Softwoods. The pretreatment of softwoods disrupted their fibrous structure. (Figure 3). SEM Figure 3A,B shows the clear fibrous structures of untreated pine and Douglas fir, and Figure 3C,D shows disrupted structures after H_3PO_4 pretreatment. Disruption of

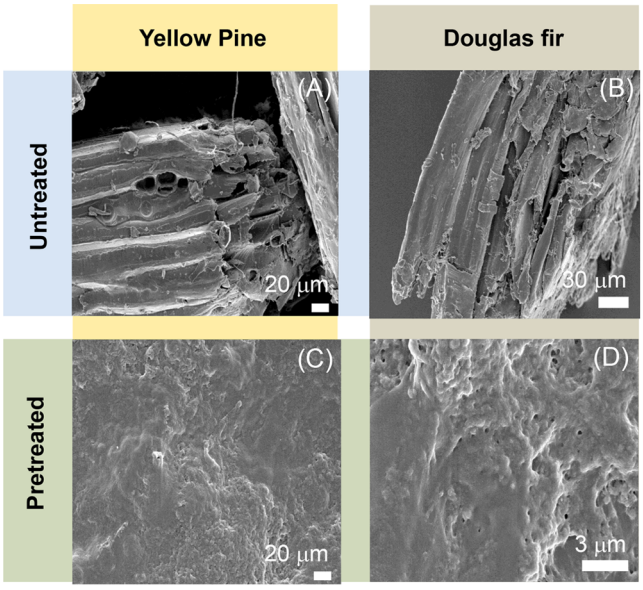

Figure 3. SEM micrographs of untreated and pretreated yellow pine (A and C) and untreated and pretreated Douglas fir (B and D).

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the fibrous structure may have been due to swelling of the cellulose induced by phosphate ions adding to hydroxyl groups. Phosphate addition would form cellulose phosphate bridges (cellulose-O-PO_3H_2).[77−79] These bridges stretch cellulose chains away from each other in the crystalline cellulose, resulting in swelling and disruption of the fibrous structure. The addition of antisolvent (ethanol or water) converted cellulose phosphate to free phosphate and amorphous cellulose,[78,79] which was more susceptible to enzymatic hydrolysis.

The formation of amorphous cellulose also suggested enhanced surface accessibility. A common method to determine the surface accessibility of materials is the N_2 adsorption−desorption technique.[80,81] For our purpose, the drawback of this technique is that the N_2 molecule is small compared with the cellulase enzyme. Hence, N_2 adsorption/desorption may overestimate the surface accessibility to enzymes. A more accurate quantitative assay to determine cellulose accessibility to enzymes is based on adsorption of a nonhydrolytic fusion protein (TGC) to the cellulose surface.[82,83] The TGC protein is 62 kDa, similar in size to *Trichoderma reesei endo*-glucanase I.[83,84] Moreover, TGC had the cellulose-binding module (CBM) to mimic cellulase adsorption onto cellulose. The green fluorescent protein (GFP) moiety in TGC reports the adsorbed area under fluorescence microscopy (Figure S1A). TGC nonspecifically adsorbs onto lignocellulosic biomass (cellulose, hemicellulose, and lignin) (Figure S1B). Thus, we first blocked nonspecific TGC adsorption by incubating untreated and pretreated softwoods with bovine serum albumin (BSA), which was allowed to bind onto the noncellulosic portions of the softwoods. Then TGC could bind to the cellulosic portion (Figure S1C). TGC adsorption followed a Langmuir adsorption isotherm and enabled the calculation of cellulose accessibility to cellulase (CAC).[83] TGC adsorption onto the untreated and pretreated softwoods without BSA blocking enabled calculation of the total substrate accessibility to cellulase (TSAC). Hence, the noncellulose accessibility to cellulase (NCAC) is calculated from the difference between TSAC and CAC.

Untreated Douglas fir had a low TSAC value of ∼0.58 m^2/g of biomass and untreated pine had a similarly low value of 0.75 m^2/g of biomass (Figure 4). The CAC value was 0.40 m^2/g of biomass for Douglas fir and 0.43 m^2/g of biomass for pine. Because TSAC measurement accounted for the noncellulose accessibility to cellulase (NCAC), the TSAC value of untreated and pretreated softwoods was higher than CAC. After pretreatment, TSAC values increased 27-fold (from 0.6 to 16 m^2/g of biomass) for Douglas fir and 20-fold (0.8 m^2/g of biomass to ∼16 m^2/g of biomass) for pine (Figure 4). Similarly, CAC values increased after pretreatment, ∼38-fold (from ∼0.4 to 15 m^2/g of biomass) for both Douglas fir and pine. This 38-fold increase in CAC value explains the high susceptibility of pretreated pine and Douglas fir (>80% glucan digestibility) to enzymatic hydrolysis at a low digestibility under 15 FPUs cellulase/g of glucan). These increases in TSAC and CAC also explained the disappearance of the fibrous structure observed in the SEM micrographs after pretreatment.

A high CAC value is important to achieve high glucan digestibility. Typically, untreated biomass has a low CAC value due to the crystalline nature of cellulose, hemicellulose, and lignin.[29] After pretreatment, the CAC value increases because

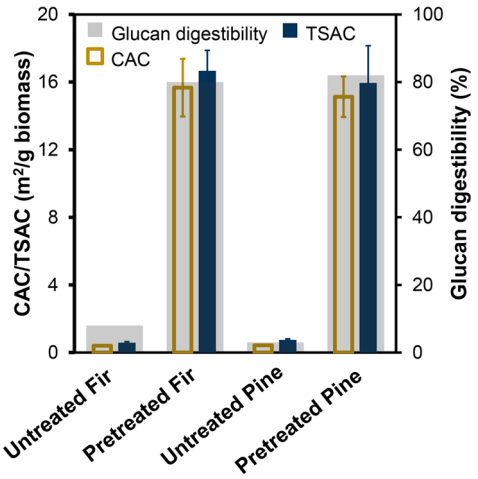

Figure 4. Total surface accessibility to cellulase (TSAC), cellulose accessibility to cellulase (CAC), and glucan digestibility after 72 h with 5 FPU of cellulase and 10 units of β-glucosidase per gram of glucan.

of improvement in the accessible surface area from breaking the highly ordered hydrogen bonding of crystalline celluloses and/or removal of hemicellulose/lignin. Thus, the mode of pretreatment is important. Lignin glues plant components together. It was commonly believed that lignin removal would increase the surface accessibility of enzymes to cellulose substrate. Rollin et al. compared two pretreatment techniques that either targeted lignin removal or increased cellulose accessibility.[40] They found that increasing cellulose accessibility was more important than lignin removal to achieve a high glucan digestibility. We also found a correlation between CAC and glucan digestibility (Figure S3). An increase in CAC >11 m^2/g biomass from H_3PO_4 pretreatment improved glucan digestibility to >80%.

3.4. Changes in Surface Functionalities of Softwoods after H_3PO_4 Pretreatment. Pretreatment with H_3PO_4 improved cellulose accessibility by converting crystalline cellulose to amorphous cellulose and unwrapping hemicellulose and lignin from the cellulose. This finding was established by FTIR investigation of changes in surface chemical functionality. Normalized FTIR spectra of pine and Douglas fir showed similar bands at 808 (in-phase ring glucomannan), 895 (anomeric vibration at β-glycosidic linkage), 1263 (C−O stretching in guaiacyl ring), 1430 (CH_2 bending vibration from cellulose and lignin), 1451 (C−H deformations of lignin), 1507 (aromatic ring stretch in lignin), 1595 (aromatic skeletal vibrations and C = O stretch), and 1730 (ketone/aldehyde C═O stretching; Figure 5 and Table S4).[85−88] Galactoglucomannan is the principal hemicellulose in softwoods.[89] The hydroxyl groups at the C_2- and C_3-positions in the backbone units are partly substituted by *O*-acetyl groups, giving rise to the acetyl group band at 1730 cm^{-1}. The vibration mode related to the acetyl groups at 1730 cm^{-1}[85] disappeared after pretreatment, suggesting deacetylation. The disappearance of the acetyl groups corroborated the 70% hemicellulose removal in the mass balance during pretreatment (Figure 2). The intensity of the in-phase ring glucomannan peak at 808 cm^{-1} was reduced after pretreatment, confirming the removal of hemicelluloses.[90] Other absorbance regions related to lignin, including[57] in-plane C−H

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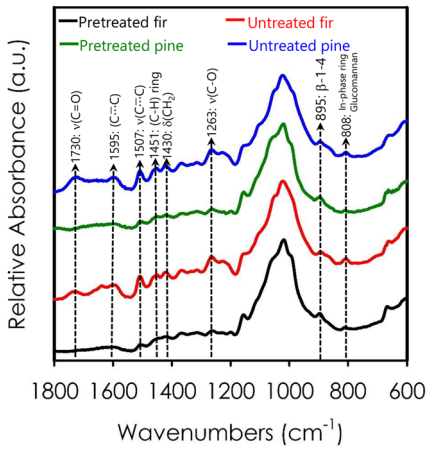

Figure 5. FTIR spectra of yellow pine and Douglas fir before and after H_3PO_4 pretreatment.

stretch at 1451 cm^{-1}[86] and C−O ring stretches at 1263 cm^{-1}, were greatly reduced after H_3PO_4 pretreatment.[86,91]

The band at 1430 cm^{-1} showed the dominant cellulose I region. Cellulose I is the native form of cellulose in plants.[92,93] The band at 895 cm^{-1} showed dominant cellulose II and amorphous cellulose regions. After H_3PO_4 pretreatment, the pretreated softwoods showed an intensity reduction of the 1430 cm^{-1} peak and an increase in the intensity of the 895 cm^{-1} peak, indicating that H_3PO_4 had disrupted the highly ordered hydrogen bonding in cellulose and converted cellulose I to amorphous cellulose and/or cellulose II. Amorphous cellulose and cellulose II accelerated the overall rate of enzymatic hydrolysis because they are more accessible to enzymes compared with cellulose I.[93]

These findings coincided with the disappearance of the fibrous structure shown by SEM and an increase in the CAC values after pretreatment. Moreover, hemicellulose and lignin, glued around the cellulose structure, were unwrapped after H_3PO_4 pretreatment, corresponding with the reduction of spectral intensities in acetal groups and lignin-carbohydrate linkages. In sum, H_3PO_4 pretreatment converted crystalline cellulose to amorphous cellulose by removing hemicellulose and lignin, and improving cellulose accessibility.

3.5. Changes in the Degree of Crystallinity of Softwoods after H_3PO_4 Pretreatment. Intra/intermolecular hydrogen bonding within cellulose chains affects its chemical structure and the strength to hold the structure together. Cellulose crystallinity has an important effect on enzymatic hydrolysis. High crystallinity equals low cellulose accessibility by cellulase. Thus, we used CP/MAS ^{13}C NMR to determine the crystallinity index (CrI) of untreated and pretreated softwoods. Typically, changes in the C_4 region of cellulose informed us of the CrI of cellulose (Figure S4, blue region, adapted from ref 94). Changes in the C_6 region suggested the breaking of hydrogen bonding between cellulose chains (Figure S4, orange region).[48,95] To establish the baseline, we first used Avicel (crystalline cellulose) and RAC (amorphous cellulose) as controls. The Avicel spectrum had doublets at C_4 and C_6 regions. At the C_4 region of Avicel, the peak ∼88.90 ppm indicated crystalline cellulose, whereas the peak at ∼84.20 ppm indicated amorphous cellulose (Figure S5) The C_6 region peak at 65.35 ppm indicated strong hydrogen bonding between cellulose chains. We also observed a small shoulder peak ∼62.80 ppm, indicating disordered hydrogen bonding between cellulose chains.[48] The CrIs of Avicel, RAC, untreated, and H_3PO_4-treated softwoods are shown in Figure 6. Avicel had 51% CrI, suggesting that Avicel

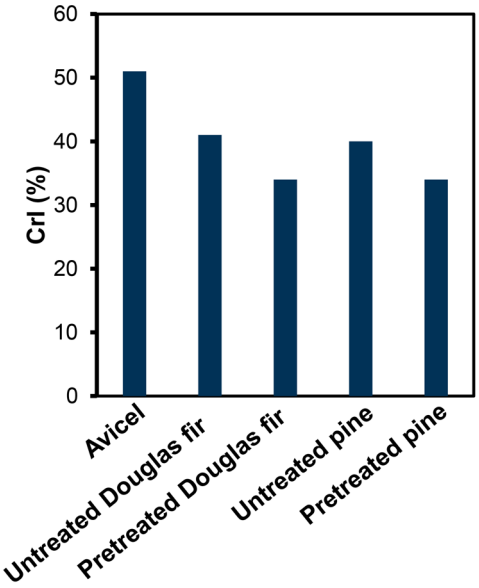

Figure 6. Crystallinity index of Avicel, untreated softwoods, and pretreated softwoods.

contained both crystalline and amorphous cellulose. The RAC spectrum showed a broad peak at the C_4 region, suggesting disruption of hydrogen bonding and an amorphous state (compared with Avicel; Figure S5). The C_6 region of RAC showed a disappearance of the peak at 65.35 ppm, causing a peak at 62.80 ppm to become more pronounced. The disappearance of the peak at 65.35 ppm suggested the breaking of hydrogen bonding between cellulose chains and RAC becoming more amorphous. However, we could not determine the CrI of RAC because we did not observe a crystalline cellulose peak after H_3PO_4 pretreatment.[29,96] Comparing Avicel with RAC, we found the difference in the C_4 region agreed with the difference in the C_6 region, suggesting that dissolving Avicel in 85% H_3PO_4 disrupted inter/intramolecular hydrogen bonding within the cellulose structure.

Untreated pine and fir showed doublets at C_4 and C_6 regions (Figure 7), suggesting that their cellulosic portion contained

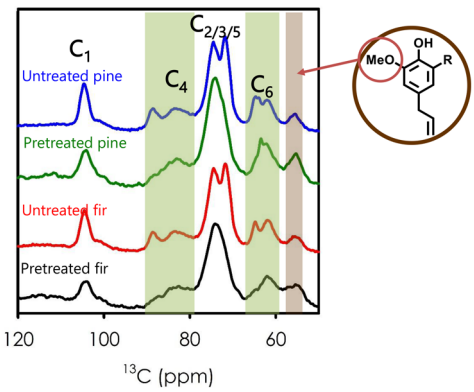

Figure 7. CP/MAS ^{13}C NMR spectra of yellow pine and Douglas fir samples before and after H_3PO_4 pretreatment

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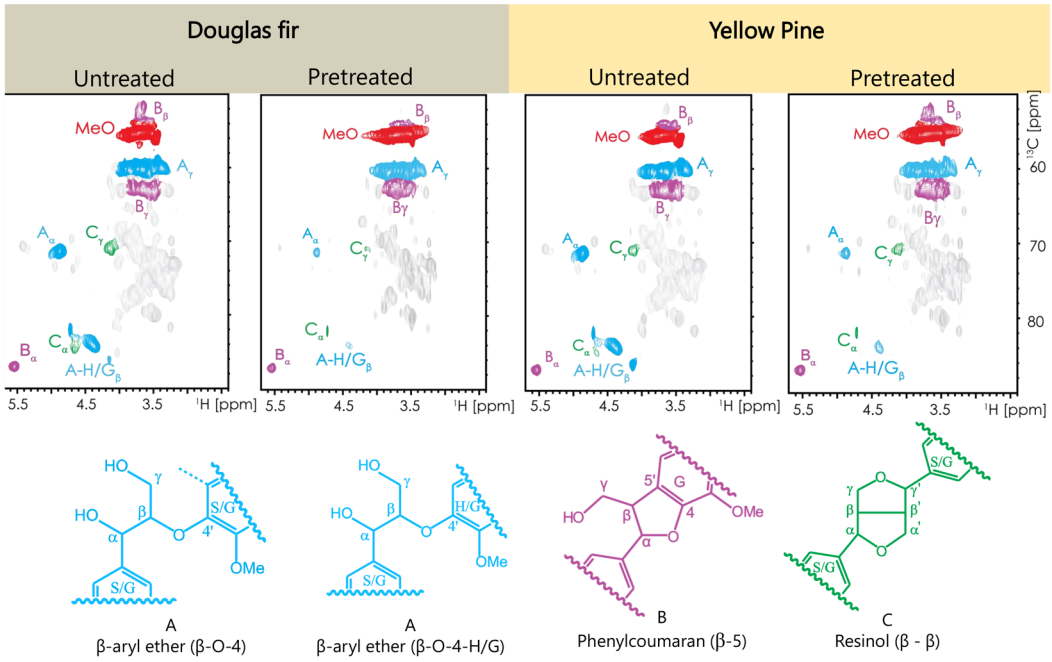

Figure 8. 2D ^{13}C-^{1}H HSQC NMR spectra in aliphatic region of Douglas fir and yellow pine before and after H_3PO_4 pretreatment.

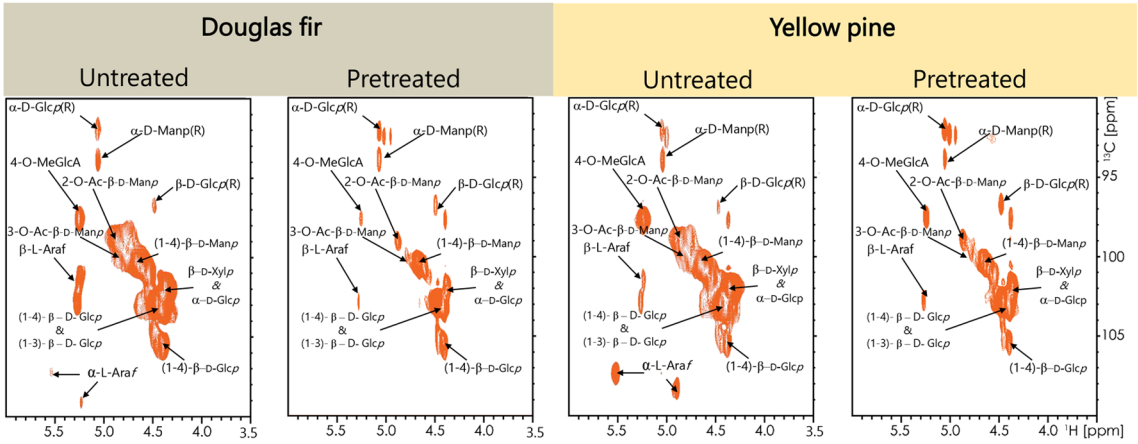

Figure 9. 2D ^{13}C-^{1}H HSQC NMR spectra in anomeric region of Douglas fir and yellow pine before and after H_3PO_4 pretreatment.

both crystalline and amorphous fractions. After pretreatment, the crystalline cellulose peaks at 88.90 and 65.35 ppm of both pine and Douglas fir disappeared, indicating that crystalline cellulose became amorphous. We observed ∼18% and 16% decrease in CrI of pine and Douglas fir after H_3PO_4 pretreatment. The decrease in CrI of pretreated pine and Douglas fir coincided with FTIR results that crystalline cellulose became more amorphous after H_3PO_4 pretreatment. Moreover, untreated pine and fir showed a peak near ∼56 ppm, assigned to methoxyl groups in lignin,[57] and we observed this peak after pretreatment. These results suggested that H_3PO_4 pretreatment did not remove much of the lignin, in agreement with the mass balance calculation that indicated 9–15% lignin removal.

3.6. 2D ^{13}C-^{1}H HSQC NMR Spectra of Softwood Samples before and after Pretreatment. To understand changes in the chemical structure of softwood lignins, we used 2D ^{13}C-^{1}H HSQC NMR to characterize their aliphatic (lignin side-chain units, Figure 8), anomeric (Figure 9), and aromatic regions pre- and post-treatment (Figure 10). The peak assignments of aliphatic, anomeric, and aromatic regions are summarized in Table S5.[49]

3.6.1. Anomeric Region. The HSQC spectra in the anomeric region of pine and Douglas fir provided information about the configuration and glycosidic linkages between sugar monomeric units.[50] Signals in these spectra are associated with oligosaccharides from cellulose and hemicelluloses. We assigned the cellulose peaks based on previously reported values.[97,98] The internal cellulose unit, (1→4)-β-D-Glc$p$, (δ_C/δ_H = 102.5/4.5 ppm) is the most important cellulosic component of the plant cell wall. The α- and β-anomeric reducing-end correlations of cellulose were well separated from the internal cellulose unit. For example, the α-D-Glc$p$(R) was at δ_C/δ_H 92.7/5.2 ppm and β-D-Glc$p$(R) was at δ_C/δ_H 97.1/4.44 ppm. In general, we observed reducing end unit cross-peaks in the anomeric region after pretreatment, but the internal cellulose peak became weaker, suggesting cleavage of glycosidic linkages by pretreatment.

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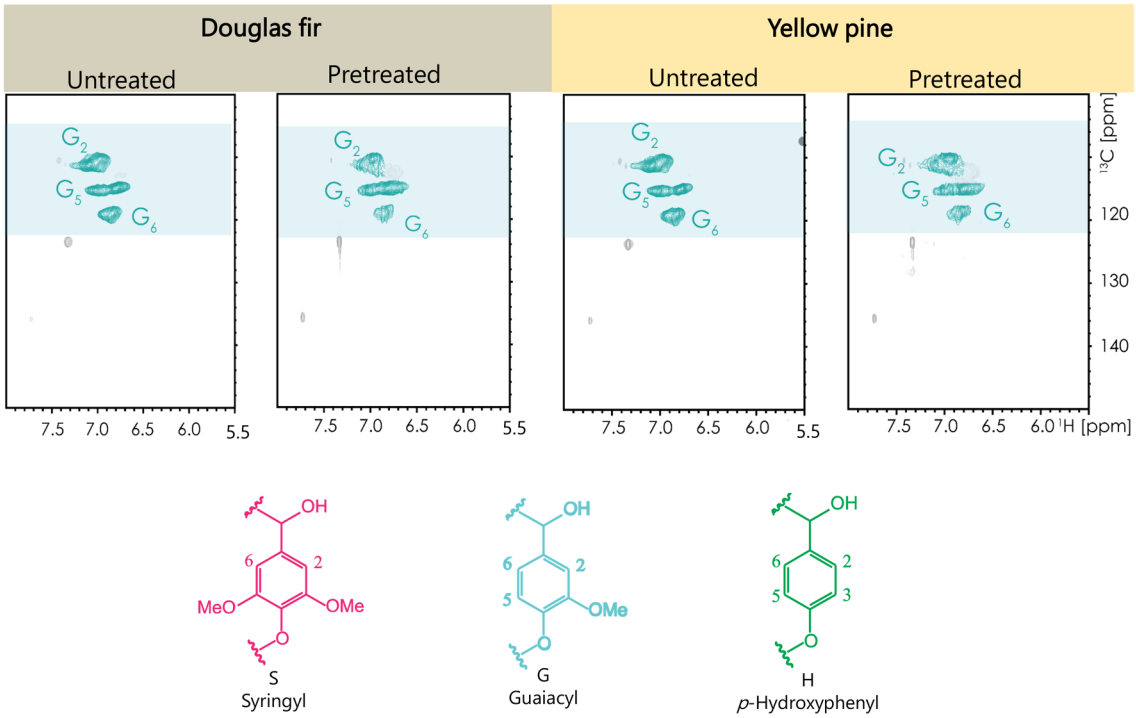

Figure 10. 2D ^{13}C-^{1}H HSQC NMR spectra in aromatic region of Douglas fir and yellow pine before and after H_3PO_4 pretreatment.

In general, the higher frequency of reducing end correlations in the anomeric region suggests depolymerization of carbohydrates. Hence, we could estimate the cellulose degree of polymerization (DP) by integrating the cross-peaks of reducing end units and internal anomeric cellulose.[98,99] Our calculated cellulose DP was ∼9 for untreated pine and ∼5 for untreated Douglas fir. These low DP values might have been due to the overestimation of reducing end units, and the actual cellulose DP could have been higher.[98] For this study, we sought to investigate the relative change of the cellulose DP. After pretreatment, the cellulose DP decreased by 62% for pine and by 36% for Douglas fir. These decreases in cellulose DP suggested cleavage of hydrogen bonding. These results also coincided with the pretreatment changes in C_4 and C_6 regions observed by CP/MAS ^{13}C NMR (Figure 7), suggesting that inter/intramolecular hydrogen bonding between cellulose chains was disrupted. The disruption of hydrogen bonding resulted in an increase in cellulase accessibility to cellulose (Figure 4).

Hemicellulose is another component that we observed in the anomeric region. Softwood hemicellulose is present as galactoglucomannans.[100] These galactoglucomannans contain acetyl groups on the (1→4)-*β*-mannosyl units at C_2 (2-*O*-Ac-*β*-D-Man*p*) and C_3 (3-*O*-Ac-*β*-D-Man*p*) positions.[49,101] Our untreated pine and Douglas fir samples showed the cross-peak of 2-*O*-Ac-Man*p* (M_2), but we did not observe 3-*O*-Ac-Man*p*(M_3)peak. The disappearance of the M_3 peak might have occurred because it overlapped with the *β*-aryl ether (A_α). The M_2 cross-peak became weaker after pretreatment, suggesting that hemicelluloses were removed. These results corroborated our mass balance (Figure 2), which showed that we removed ∼79% of the xylan from pine and 90% from Douglas fir. We also removed ∼63% mannan from pine and 66% from Douglas fir.

3.6.2. Aliphatic Region. Lignin linkages of untreated softwoods consisted mainly of *β*-aryl ether (*β*-O-4 **A**) with resinol (*β*–*β* **B**) and a trace amount of phenylcoumaran (*β*-5 **C**). Pretreatment weakened the *β*-O-4 cross-peak, suggesting that the ether bonds were cleaved and lignin fragments were released. Using semiquantitative analysis, we integrated these cross-peaks to determine the relative abundance of these lignin linkages (per 100 aromatic units) (Table 2). These calculated values of softwood lignin interunit linkages agreed with previously reported values for softwoods.[68,102−104] Our calculated values of lignin's interunit linkages revealed that *β*-O-4 was the most abundant, followed by *β*-5 and *β*–*β*. The pretreated softwoods showed ∼45% reduction in *β*-O-4 **A** and a slight increase in *β*-5 **B**, whereas *β*–*β* **C** remained unchanged.

Table 2. Changes in Lignin's Interunit Linkages of Lignin before and after H_3PO_4-Pretreatment of Pine and Douglas Fir[a]

	relative abundant (%)		
sample\lignin interunit linkages	*β*-O-4 A	*β*-5 B	*β*–*β* C
untreated pine	74 (48)	19 (12)	6 (4)
pretreated pine	61 (26)	33 (14)	6 (3)
untreated Douglas fir	73 (47)	18 (12)	9 (6)
pretreated Douglas fir	56 (26)	35 (16)	9 (4)

[a]Values in parentheses indicate the relative amount of interunit linkages per 100 aromatic units.

The bond dissociation enthalpy (BDE) of these lignin interunit linkages are in the following order: $BDE_{\beta\text{-O-4}}$(65 kcal/mol) < $BDE_{\beta-\beta}$(117 kcal/mol) < $BDE_{\beta\text{-5}}$ (125 kcal/mol).[105] These BDE values agree with our results, showing that the *β*-O-4 was easier to be cleaved compared with *β*-5and *β*–*β*. After pretreatment, a significant decrease in relative abundance of *β*-O-4 and maintenance of the *β*-5 and *β*–*β* similar to those of

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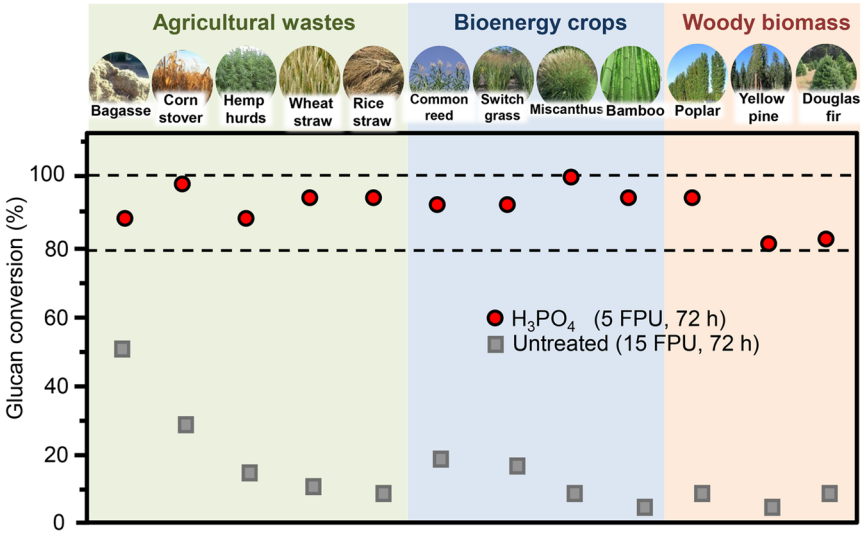

Figure 11. H_3PO_4 pretreatment accommodates many types of feedstocks: agricultural wastes, bioenergy crops, and woody biomass.

untreated softwoods suggested that lignin depolymerization was the dominant reaction as evidenced by a great decrease in the β-O-4 **A** with minimal carbon−carbon linkage degradation reaction (breaking β−β **C**) and condensation reaction (formation of β-5 **B**).[106] The residual lignin from this pretreatment was depolymerized with minimal formation of C−C bonds, an outcome that provides the opportunity to upgrade the resulting lignin into high-value products (aromatic chemicals, battery components, and carbon fibers)[107,108] and to make this process economically viable.[109]

3.6.3. Aromatic Region. Lignin has three aromatic units: syringyl (S) unit, guaiacyl (G) unit, and *p*-hydroxyphenyl (H) unit. The aromatic region of untreated pine and Douglas fir showed that both contained only guaiacyl (G) units, which was a signature of softwoods.[19] Examination of the aromatic region (Figure 10) demonstrated that pretreated softwoods did not show any change in the signals of major aromatic units compared to that of untreated softwoods. After ionic liquid pretreatment of pine, the condensed lignin structure has shown C−H correlations between the 2- and 6-positions (G_2-condensed structure at δ_C/δ_H 112.5/6.65 ppm and G_6-condensed structure at δ_C/δ_H 120.5/6.55 ppm) of the guaiacyl units and the 5-position of other lignin side chains.[110,111] These G_2- and G_6-condensed structures were absent from the aromatic region of our pretreated softwoods, which was consistent with the maintenance of the β-5 and β−β linkages in the aliphatic region after pretreatment. These results suggested that, although H_3PO_4 pretreatment could hydrolyze lignin's β-aryl ether linkages into small lignin fragments, the remaining lignin in pretreated samples was not modified and/or condensed. Note that, by the 2D HSQC NMR technique, we could not observe C_5-substituted condensed phenolic compounds (5−5′ linkage)[112] because C−H bonds did not exist in the 5−5′ linkages. We are in the process of using ^{31}P NMR spectroscopy to analyze the C_5-substituted condensed phenolic linkages.

We have applied our H_3PO_4 pretreatment process to a variety of lignocellulosic feedstocks at a mild reaction temperature (50 °C) and atmospheric pressure, including agricultural waste, bioenergy crops, and woody biomass (hardwoods and softwoods). H_3PO_4 pretreatment improved cellulose accessibility to cellulase, resulting in a high glucan digestibility of ∼80−100% with a low enzyme loading of 5 FPUs/g glucan (Figure 11, adapted from ref 113). This process was biomass species-independent. Its mild reaction condition, high sugar yield at a low enzyme loading, and preservation of lignin will have a great impact on the economic viability of biorefineries.

4. CONCLUSION

We examined the H_3PO_4 pretreatment of pine and Douglas fir at low temperature (50 °C) and atmospheric pressure. This process showed a low severity factor (0.91) and E-factor (168.9) compared with other pretreatment processes. After 48 h, we achieved a high glucan digestibility of 78% for pine and 80% for Douglas fir, even at a low enzyme loading of 5 FPUs/g glucan. H_3PO_4 pretreatment produced cellulose accessibility to cellulase (CAC) values higher than the 11 m^2/g threshold, yielding >78% glucan digestibility at a low enzyme loading (5 FPU/g glucan). Examination of pretreated softwoods by CP/MAS, ^{13}C NMR, and FTIR revealed breaking of the orderly hydrogen bonding of crystalline cellulose, which was responsible for an enhanced CAC value. The process was feedstock-independent. NMR revealed that lignin was depolymerized without being condensed, providing the opportunity to upgrade the pretreated lignin to value-added chemicals. In summary, the mild reaction condition, high sugar yield at a low enzyme loading, and preservation of lignin of our H_3PO_4 pretreatment process will have a great impact on the economics of the biorefinery.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.iecr.9b05873.

Composition of Douglas fir and yellow pine, determination of enzyme activity and protein concentration, determination of the severity factor and the environmental factor (E-factor), characteristic FTIR peaks of softwood, ^{13}C-^{1}H correlation peaks in aliphatic, anomeric, and aromatic regions of softwoods, illustration of the adsorption behavior of the TGC fusion protein and BSA on pretreated softwoods, correlation of severity

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index and cellulose accessibility to cellulase (CAC) on glucan digestibility, calculation of crystallinity index (CrI), crystalline and amorphous regions in CP/MAS ^{13}C NMR spectra, CP/MAS ^{13}C NMR spectra of Avicel and regenerated amorphous cellulose (RAC) (PDF)

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The manuscript was written with contributions by all authors. All authors have approved the final version of the manuscript.

Notes

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■ ABBREVIATIONS

CAC, cellulose accessibility to cellulase; CP/MAS, cross-polarization/magic angle spin; NMR, nuclear magnetic resonance; FTIR, Fourier-transform infrared spectroscopy; HSQC, heteronuclear single quantum coherence, CAPEX, capital expenditure; OPEX, operating expenditure; LCC, lignin-carbohydrate complex; HMF, 5-hydroxymethylfurfural; FPU, filter paper units; HPLC, high-pressure liquid chromatography; E-factor, environmental factor; RID, refractive index detector; DAD, diode array detector; TSAC, total substrate accessibility to cellulase; NCAC, non-cellulose accessibility to cellulase; BSA, bovine serum albumin; GFP, green fluorescent protein; EG, ethylene glycol; CrI, crystallinity index; DP, degree of polymerization; RAC, regenerated amorphous cellulose; S, syringyl; G, guaiacyl; H, hydroyphenyl

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