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Bioconversion of Beetle-Killed Lodgepole Pine Using SPORL: Process Scale-up Design, Lignin Coproduct, and High Solids Fermentation without Detoxification

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Supporting Information

ABSTRACT: Mountain pine beetle killed Lodgepole pine (*Pinus contorta* Douglas ex Loudon) wood chips were pretreated using an acidic sulfite solution of approximately pH = 2.0 at a liquor to wood ratio of 3 and sodium bisulfite loading of 8 wt % on wood. The combined hydrolysis factor (CHF), formulated from reaction kinetics, was used to design a scale-up pretreatment on 2000 g wood chips at a relatively low temperature of 165 °C that reduced furan formation and facilitated high solids saccharification and fermentation. The pretreated solids and liquor were disk milled together to result in a biomass whole slurry of 25% total solids. The whole biomass slurry was directly used to conduct simultaneous enzymatic saccharification and combined fermentation (SSCombF) using a commercial cellulase and *Saccharomyces cerevisiae* YRH400 without detoxification. A terminal ethanol titer of 47.1 g L^{-1} with a yield of 306 L (tonne wood)$^{-1}$, or 72.0% theoretical, was achieved when SSCombF was conducted at an unwashed solids loading of 18%. The lignosulfonate (LS) from SPORL was highly sulfonated and showed better dispersibility than a high purity commercial softwood LS, and therefore has potential as a directly marketable coproduct.

INTRODUCTION

Forest biomass can be sustainably produced in large quantities globally as a viable feedstock for producing biofuel and bioproducts through the biorefinery concept.[1,2] The successful development of a forest biorefinery industry can reduce our reliance on fossil fuel, mitigate climate change, and help sustainable rural economic development in many countries. The sugar platform pathway that uses enzymes to saccharify wood structural carbohydrates has many advantages; for example, it can leverage the technological advances, infrastructure, and human capital from the mature pulp and paper industry. Furthermore, sugar as a key building block molecule has flexibility for different conversion options in producing fuel and chemicals.[3] Developing effective forest biomass pretreatment technology is critical because sugar production is the most expensive step and dictates not only downstream processing but also lignin coproduct potentials.[1,4] Several pretreatment processes demonstrated potential for forest biomass conversion after decades of research and development;[5−8] however, high solids processing for high-titer biofuel production, to reduce separation cost and improve energy efficiency, remains a challenge.[9,10] The concentrations of both the soluble lignin and sugar degradation products produced during pretreatment are significantly increased in high solids processing, which reduces cellulase activity due to increased nonproductive binding of cellulase to lignin as well as strong inhibition by the sugar degradation products to enzymatic saccharification and fermentation or catalytic conversion.[11−14] Few studies have reported on high-titer biofuel production with high yield using moderate cellulase loadings because of these difficulties. High-titer biofuel productions were often conducted using only the washed pretreated solids and without including the pretreatment spent liquor (the hemicellulosic sugar stream) that contains lignin and sugar degradation products.[15,16] Furthermore, value-added lignin utilization is another challenge in developing a forest biorefinery. Lignin condensation significantly limits the lignin coproduct potential when using SO_2-catalyzed steam pretreatment.[6]

In this study, we present the potential of the sulfite pretreatment to overcome recalcitrance of lignocelluloses (SPORL)[7] as a viable technology for forest biorefinery as schematically shown in Figure 1. SPORL was developed based on sulfite pulping, and offers a significant advantage over competing technologies for process scale-up. Lignin sulfonation using SPORL produced lignosulfonate (LS) that has very low

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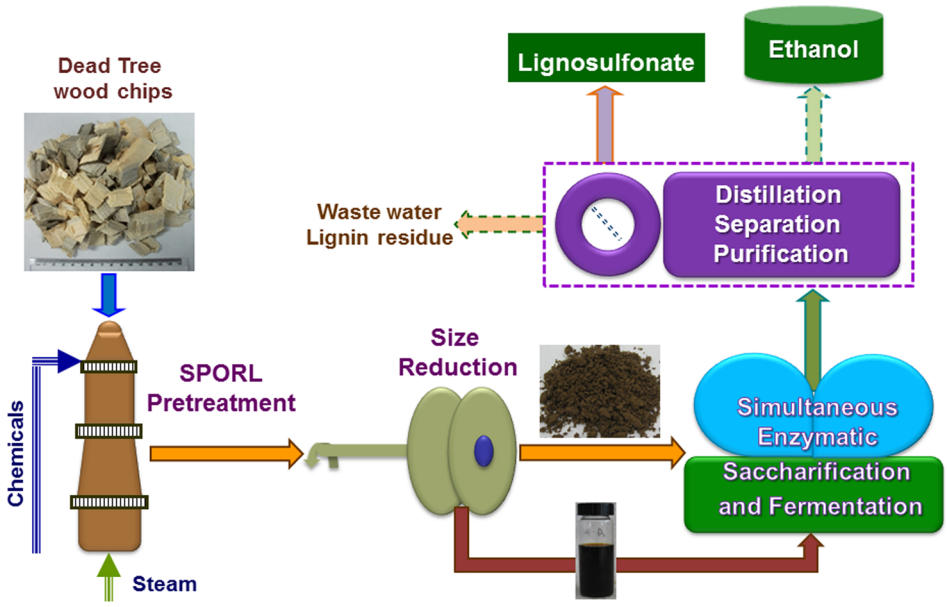

Figure 1. A schematic diagram shows the process flow of scale-up SPORL pretreatment and downstream saccharification and fermentation. The process boxed by dashed lines was partially conducted.

affinity to cellulase and can act as a surfactant to enhance enzymatic saccharification,[17] which makes SPORL uniquely suitable for bioconversion of forest biomass with high lignin content. It also significantly facilitated simultaneous enzymatic saccharification of pretreated solids and combined fermentation (SSCombF) with inclusion of the hemicellulosic sugar stream, thereby avoiding solids washing and separation of solids and liquid. Although robust performance of SPORL for bioconversion of both hardwoods and softwoods has been demonstrated,[15,18] high-titer ethanol production using SPORL pretreated whole biomass slurry remained difficult due to accumulated inhibitor levels at high-solids fermentation, and was achieved only with fed-batch operation and some level of detoxification as reported previously.[10] LS from SPORL pretreatment was never characterized, and its properties and utility are not known for commercial applications. Furthermore, SPORL process scale-up has not been explored.

We hypothesize that sugar degradation reactions have a stronger dependence on temperature than hemicellulose dissolution reactions. Therefore, reducing temperature and increasing duration of SPORL pretreatment can reduce the formation of sugar degradation products such as furans without changing the pretreatment severity and, therefore, cellulose enzymatic saccharification.[19] Because low temperature pretreatment is also advantageous in terms of improving process energy efficiency and reducing capital cost, we will use a relatively low temperature for SPORL pretreatment of 165 °C to produce substrates from lodgepole pine (*Pinus contorta* Douglas ex Loudon). The substrates will be used in high solids processing to achieve one-step high-titer ethanol production with high yield without detoxification. We will demonstrate how to use a combined hydrolysis factor (CHF) as a pretreatment severity measure[12] to develop low-temperature SPORL pretreatment. We will also characterize LS molecular weight distribution, degree of sulfonation, as well as dispersion properties in comparison with a commercial high purity LS. Therefore this work represents a significant contribution to the development of viable forest biorefinery technologies.

MATERIALS AND METHODS

Materials. Lodgepole pine wood chips were produced at the Forest Products Laboratory, Madison, Wisconsin, from a mountain pine beetle-killed tree (abbreviated BD4) harvested from the Canyon Lakes Ranger District of the Arapaho-Roosevelt National Forest, Colorado, USA. Detailed information about the tree, harvesting, and transportation have been described previously.[20,21] The wood chips were screened to retain chips between 6 and 38 mm. The screened chips were stored frozen at approximately −16 °C until use.

Commercial cellulase enzymes Cellic CTec2 (abbreviated CTec2) was generously provided by Novozymes North America (Franklinton, North Carolina, USA). The cellulase activity is 147 FPU/mL as calibrated by a literature method.[22] Sodium acetate, acetic acid, sulfuric acid, and sodium bisulfite were used as received from Sigma-Aldrich (St. Louis, MO). All chemicals were ACS reagent grade.

Titanium dioxide (TiO_2) was a commercial product in powder form from Kermel Chemistry Co. Ltd. (Tianjin, China) with a purity of 99% and was used as received. The size analysis of TiO_2 was carried out using an EyeTech Laser particle size analyzer (Ankersmid Co. Ltd., Holland). The manufacture-specified average particle diameter of TiO_2 was 5.43 μm.

High purity sodium LS (D-748) from acid sulfite pulping of softwood was donated by LignoTech USA (Rothschild, WI).

Saccharomyces cerevisiae YRH400 is an engineered fungal strain for xylose fermentation.[23] The strain was grown at 30 °C for 2 days on YPD agar plates containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose, and 20 g L^{-1} agar. A colony from the plate was transferred by loop to liquid YPD medium in a flask and cultured overnight at 30 °C with agitation at 90 rpm on a shaking bed incubator (Thermo Fisher Scientific, model 4450, Waltham, MA). The biomass concentration was monitored using optical density at 600 nm (OD_{600}) by a UV−vis spectrometer (model 8453, UV−visible spectroscopy system, Agilent Technologies, Palo Alto, CA). The cultured medium was used to inoculate the fermentation medium.

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SPORL Pretreatment. Pretreatments were conducted at two scales using lodgepole pine BD4 wood chips: 150 and 2000 g oven dry weight, using dilute solutions of sulfuric acid and sodium bisulfite. The liquid to wood ratio (L/W) was fixed at 3 (v/w) for all pretreatments. Fifty nine (59) small scale (150 g) pretreatments were conducted in 1 L bomb-type reactors with a wide range of sulfuric acid and sodium bisulfite loading, temperature, and duration (Supporting Information, Table S1). Three bomb reactors were placed in a 23 L laboratory wood pulping digester in an autoclave configuration as described previously.[20,24] The reactor bombs were directly heated by steam. Scale-up pretreatment (2000 g) was only conducted at 165 °C for 75 min using the same 23 L rotating wood pulping digester and heated by a steam jacket.[25] The sulfuric acid and sodium bisulfite charge on wood was 2.2% (w/w) and 8.0% (w/w), respectively. The methodology for selecting these conditions will be discussed in the next section.

At the end of each pretreatment, the digester was cooled down by flushing the heating jacket with tap water to terminate the reaction. The pretreated wood chips and pretreatment spent liquor were separated using a screen box. The solid contents of the pretreated wood chips were approximately 30%. For all small scale pretreatments, the solids were milled with the application of water, acting as a washing step, to reach a discharge solids consistency of approximately 10%. For the scale-up (2000 g) pretreatment, however, the pretreated solids and spent liquor were directly transferred to a disk mill for size reduction without adding water (Figure 1). This is to facilitate high solids fermentation for high-titer ethanol production without diluting the milled whole biomass slurry. The resultant whole slurry was pressed in a screen box to separate solids from liquor for component mass balance determination. The yield of the solids (unwashed) was determined from the oven dry weight of the material. Both the volume and weight of the collected pretreatment spent liquor were recorded. A previous study indicated that the disk milling of SPORL pretreated lodgepople pine significantly reduced energy consumption for size reduction,[26] which is important to improve the energy efficiency of a forest biorefinery.

Reaction Kinetics for Pretreatment Scale-up. To balance sugar yield against degradation to inhibitory compounds for achieving maximal biofuel productivity through high solids processing, we relied on data from small scale pretreatments to design the scaled-up pretreatment using the previously developed combined hydrolysis factor (CHF), expressed by eq 1.[12] This can lessen the numerous and tedious pretreatment trials at large scales, especially at the commercial scale, needed to reduce cost and improve efficiency without arbitrarily selecting pretreatment conditions for reducing sugar degradation.[27]

$$\mathrm{CHF} = \exp(\alpha - E/RT + \beta C_A + \gamma C_B) \times (C_A + C_B)t \tag{1}$$

Where C_A and C_B are the concentrations of chemical A (sulfuric acid) and chemical B (sodium bisulfite) used in pretreatment, respectively; α, β, and γ are adjustable parameters, E is the apparent activation energy, R is universal gas content of 8.314 J/mol/K, t is in min, and T is absolute temperature (K). Hemicellulose dissolution by pretreatment can be predicted from first order kinetics with the consideration of slow and fast hemicelluloses[12] as follows,

$$X_R = (1 - \theta)e^{-\mathrm{CHF}} + \theta e^{-f\mathrm{CHF}} \tag{2}$$

where X_R is fraction of hemicellulose remaining on pretreated solids, f is the ratio of the hydrolysis reaction rate constants between the slow and fast hemicelluloses, and θ is the initial fraction of slow reacting hemicelluloses.

Equation 2 suggests that hemicellulose removal is only dependent on CHF. Furthermore, CHF also correlates well to enzymatic saccharification based on our previous study using aspen;[12] therefore CHF is a true severity measure. When the same chemical loadings, C_A and C_B, from a small scale experiment are used in a scale-up pretreatment with the same severity CHF, the pretreatment time, t^{Tup}, or pretreatment temperature Tup, for scale-up pretreatment can be determined using eq 1

$$t^{\mathrm{Tup}} = \exp\left[-\frac{E}{R}\left(\frac{1}{\mathrm{Tup}} - \frac{1}{T}\right)\right]\cdot t^T \tag{3}$$

We can use first order reaction kinetics to model the amount of sugar degradation as demonstrated previously.[19] All degradations were lumped into one pool. Hydroxymethylfurfural (HMF) and furfural were used as indicator species neglecting further degradation of furan to acids. Then the rate of furan formation D is expressed as

$$\frac{dD}{dt} = k_d(1 - X_R) \tag{4}$$

The degradation reaction rate constant can be expressed by Arrhenius kinetics,

$$k_d = e^{(\alpha_d - E_d/(RT))} \tag{5}$$

where α_d is an adjustable parameter and E_d is the apparent activation energy. By substituting eq 2 into eq 4, the amount of furan formation can be obtained by

$$D = k_d t\left[1 - \frac{1-\theta}{\mathrm{CHF}}(1 - e^{-\mathrm{CHF}}) - \frac{\theta}{f\cdot\mathrm{CHF}}(1 - e^{-f\mathrm{CHF}})\right] \tag{6}$$

The ratio of furan formation between two pretreatments with identical severity CHF and chemical loadings but at different temperatures, $T1 > T2$, can be determined from eq 6 as follows,[19]

$$\frac{D_{T1}}{D_{T2}} = \frac{k_d^{T1}}{k_d^{T2}}\cdot\frac{t^{T1}}{t^{T2}} = \exp\left[\frac{E - E_d}{R}\left(\frac{1}{T1} - \frac{1}{T2}\right)\right] \tag{7}$$

Enzymatic Hydrolysis. Enzymatic hydrolysis of the lignocellulosic substrate was conducted at 2% (w/v) in 50 mL of 50 mM acetate buffer (pH 5.5) on a shake/incubator (Thermo Fisher Scientific, model 4450, Waltham, MA) at 50 °C and 200 rpm. An elevated pH of 5.5, higher than commonly used pH 4.8−5.0, can significantly reduce nonproductive cellulase binding to lignin, enhancing lignocellulose saccharification as demonstrated in our recent studies.[17,28,29] NaOH of 5% (wt) or acetic acid was used to adjust the pH of the substrate suspension to pH 5.5. The CTec2 loading was varied. Aliquots of 1 mL enzymatic hydolysate were taken periodically (3, 6, 9, 24, 48, and 72 h) and centrifuged at 13000 g for 5 min for glucose analysis. Each data point is the average of two analyses. The data from replicate runs were used to calculate

the mean value and standard deviation that is used as error bars in plots.

Quasi-simultaneous Enzymatic Saccharification and Combined Fermentation (SSCombF). SSCombF of the enzymatic hydrolysate of the pretreated lodgepole pine solids and spent liquor were carried out in 250 mL Erlenmeyer flasks using a shaker/incubator (Thermo Fisher Scientific, model 4450, Waltham, MA). The matched amount of pretreatment spent liquor, based on the ratio of collected unwashed solids and liquor, was added to the flasks. The mixture (or the pretreated lodgepole pine whole slurry) was adjusted to pH 6.2 with solid calcium hydroxide. Acetic acid/sodium acetate buffer (50 mM) of pH 5.5 was added into the pH adjusted mixture to conduct enzymatic hydrolysis using CTec2 at 20 FPU/g glucan. An elevated pH 5.5, higher than the commonly used pH 4.8−5.0, and the lignosulfonate in SPORL pretreatment spent liquor can significantly reduce nonproductive cellulase binding to lignin and enhance lignocellulose saccharification.[17,28,29] Liquefaction of solid substrates was observed in about 18−22 h at 50 °C and 200 rpm. The mixture was then cooled down to 35 °C, and inoculated while the shaker speed was reduced to 90 rpm. The initial optical density of the yeast was controlled at OD_{600} = 5. No additional nutrients were applied during fermentation. Each flask was covered by aluminum foil to maintain fermentation under anaerobic conditions. Samples of the fermentation broth were taken periodically for analysis of monosaccharides, inhibitors, and ethanol. Reported results are the average of duplicate analyses. Replicate fermentation runs were conducted to ensure experimental repeatability. The standard deviations were used as error bars in plotting.

Analytical Methods. The chemical compositions of the untreated and pretreated lignocelluloses were analyzed as described previously.[20] All solid lignocellulosic samples were Wiley milled (model No. 2, Arthur Thomas Co, Philadelphia, PA, USA) to 20 mesh (~1 mm) and hydrolyzed in two stages using sulfuric acid of 72% (v/v) at 30 °C for 1 h and 3.6% (v/v) at 120 °C for 1 h. Carbohydrates of the hydrolysates were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (ICS-5000, Dionex). Klason lignin (acid insoluble) was quantified gravimetrically.[30] For fast analysis, glucose in the enzymatic hydrolysates was measured using a commercial glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, OH, USA).

Monosaccharides (glucose, mannose, xylose, arabinose, and galactose) in liquid samples were determined with a Dionex HPLC system (Ultimate 3000) equipped with an RI (RI-101) and UV (VWD-3400RS) detector and using a BioRad Aminex HPX-87P column (300 mm × 7.8 mm) operated at 80 °C. Double distilled water (d.d.w.) was used as eluent at a flow of 0.6 mL/min. Inhibitors (acetic acid, furfural, and HMF) and ethanol were measured by the same HPLC system equipped with a BioRad Aminex HPX-87H column (300 mm × 7.8 mm) operated at 60 °C. A 5 mM sulfuric acid solution was used as eluent at a flow rate of 0.6 mL/min. All sample injection volumes were 20 μL. Samples were diluted in deionized water and filtered by a 0.22 μm syringe filter prior to injection.

Lignosulfonte (LS) Purification and Separation. LS in the pretreatment spent liquor was separated and purified by dialysis. A Spectra/Pro Biotech Cellulose Ester Dialysis Membrane bag (MWCO: 100−500 Da, Spectrum Laboratories, Inc., Rancho Dominguez, CA) was use to dialyze 10 mL of spent liquor in a DI water bath. The DI water was replaced every 12 h for the first 2 days and every 24 h thereafter. The conductivity of the water was monitored using a YSI Conductance Meter (model 35, YSI Inc., Yellow Springs, OH, USA). The dialysis was terminated after approximately 7 days when the conductance of the DI-water reached a constant 2.3 μohm for 24 h, the same as that of the fresh DI water. The dialysis membrane had a cutoff molecular weight of 500 Da, allowing the removal of impurities, such as furfural, HMF, and acetic acid.

Gel Permeation Chromatography (GPC). Aqueous GPC was conducted using Ultrahydrogel 120 and Ultrahydrogel 250 columns and measured with a Waters UV detector 2487 at 280 nm (Waters Co., USA). Polystyrene sulfonates with molecular weights ranging from 500 to 10000 g/mol were used for calibration. A 0.10 mol/L $NaNO_3$ solution was used as eluent at 0.50 mL/min. All LS samples used for GPC analysis were obtained from dialysis (M_w < 500) and syringe filtered (0.22 μm).

Elemental Analysis. The LSs were freeze-dried after dialysis (M_w < 500) to remove salt and other impurities before elemental analysis was conducted using a vario Element Analyzer (EL III, Elementar, Germany). The measurement was carried out in triplicate. The average results and the standard deviation are reported.

Dispersion Properties. The dispersion property of different LSs was evaluated by examining the stability of TiO_2 particles in a dilute aqueous solution using a Turbiscan Lab Expert instrument (Formulaction, France). The TiO_2 powder (3 wt %) was slowly mixed with 0.5 wt % LS in double DI water and stirred at 1000 rpm for 5 min before measurements. The Turbiscan measures backscattering and transmission intensities of a laser light to detect particle size change.[31]

RESULTS AND DISCUSSION

Combined Hydrolysis Factor (CHF) for Designing Scale-up SPORL Pretreatment. Using CHF produced good predictions of the dissolution of hemicelluloses (sum of mannan and xylan) of lodgepole pine based on 59 small scale SPORL pretreatments (Supporting Information, Table S1) (Figure 2a). The apparent activation energy E = 100 000 and adjustable parameters α = 28.5, β = 17.0, and γ = −9.8 were obtained on the basis of fitting experimental data (Figure 2a). Furthermore, CHF was also found to predict enzymatic hydrolysis glucose yield (EHGY) well as shown in Figure 2b, even though delignification becomes a more important factor than removal of hemicellulose to the saccharification of lodgepole pine with a higher lignin content as compared to a hardwood such as aspen.[12] Here EHGY is defined as the glucose yield from enzymatic hydrolysis as a percentage of the theoretical glucose in untreated wood. Moreover, EHGY reached a theoretical asymptotic value of approximately 70% at CHF ≈ 20 (Figure 2b). This is also confirmed by previous studies that produced good ethanol yields from the same lodgepole pine (BD4) wood chips using a SPORL pretreatment with a severity CHF = 18.9 at 180 °C for 20 min and sulfuric acid and sodium bisulfite charge of 2.2 and 8.0%, respectively, on oven dry (od) wood.[10,24] A slightly higher CHF = 22.5 (>18.9) was used to design the scaled-up SPORL pretreatment of 2000 g. This CHF (= 22.5) corresponds to a slightly longer pretreatment time of 25 min at 180 °C than that used in the two previous studies.

The first order kinetic model (eq 6) provided a good prediction of furan formation as shown in Figure 2c. The apparent activation energy for sugar degradation E_d = 160930

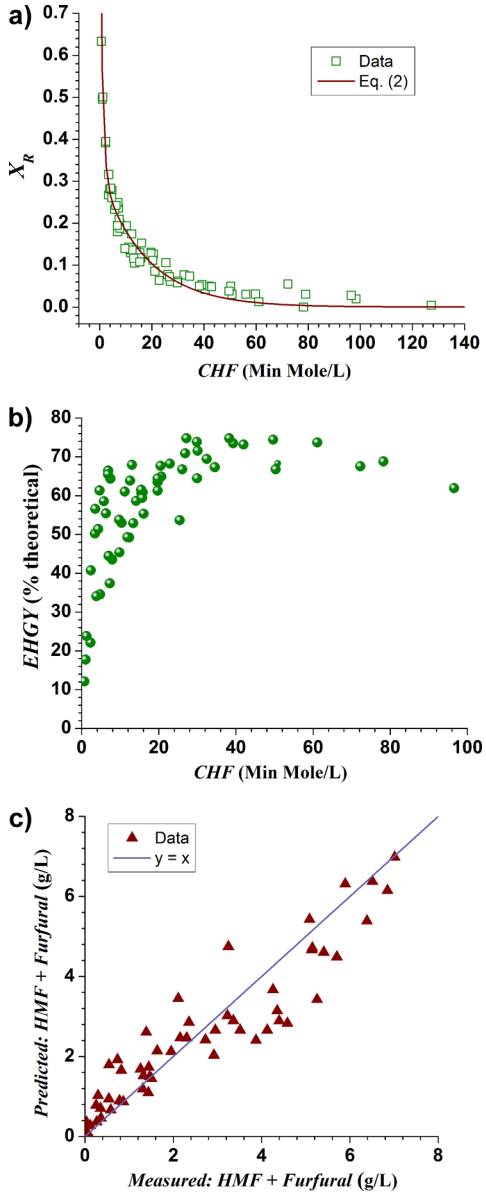

Figure 2. Results from 59 small scale SPORL pretreatments each using 150 g of BD4 wood chips. (a) Predicted fraction by the combined hydrolysis factor (CHF) of hemicelluloses (mannan + xylan) remaining on solids; (b) correlation between enzymatic hydrolysis glucose yield (EHGY) and CHF; (c) comparison between measured and predicted (eq 6) furan (HMF + furfural) formation.

and adjustable parameter α_d = 41 were used from our previous study.[19] Because the activation energy for sugar degradation, E_d, is higher than that for hemicellulose dissolution, E, furan formation will be lower when pretreatment is conducted at a lower temperature and constant severity CHF, as clearly indicated by eq 7. Specifically, furan formation can be reduced by 43% when pretreatment temperature is reduced from 180 to 165 °C on the basis of eq 7 predictions. Equation 7 was experimentally validated using paired pretreatments with the same CHF but at different temperatures.[19] Therefore, scale-up SPORL pretreatment was conducted at 165 °C to facilitate high solids enzymatic saccharification and fermentation. On the basis of eq 3, an extended pretreatment duration of 75 min is required to achieve CHF = 22.5 at 165 °C. The chemical loadings of 2.2 and 8% for acid and bisulfite, respectively, were the same as used previously[10,24] to achieve a similar amount of delignification as sulfite and pH are the dominant factors in controlling delignification. This is because cellulose saccharification is also dependent on delignification for softwoods as indicated by the data scattering at the same hemicellulose dissolution or CHF (Figure 2b) where there are variations in delignification.

Wood Component Recovery in a Scaled-up Pretreatment. The moisture content and the chemical composition of the untreated lodgepole pine along with initial wood wet and dry mass are listed in Table 1. As shown in Figure 1, after disk milling and pressing, each component derived from the SPORL pretreatment of 2000g BD4 lodgepole pinewood chips was recovered in two streams: (1) the unwashed wet solids that consist of all water-insoluble materials along with pretreatment spent liquor retained after pressing, and (2) remaining spent liquor collected from pressing. Total solids recovery was 96.6%. Lignin was only measured from the unwashed solids as Klason lignin. The lignin in the pretreatment liquor, in the form of LS, was not analyzed, but rather calculated from the balance of wood Klason lignin. The majority of each component was recovered from the unwashed wet solids because it contains all water-insolubles and approximately two-thirds (67%) of the pretreatment spent liquor. Glucan recovery as saccharides was 98.4%, indicating minimal loss. Recoveries of hemicelluloses as saccharides, excepting arabinan, are approximately 60−65%. The productions of HMF and furfural, expressed as the amounts of mannan and xylan reacted, were 5.2 and 7.6%, respectively.

Enzymatic Saccharification of Washed Solids from Scale-up Pretreatment. Enzymatic hydrolysis of the washed pretreated solids from the scale-up pretreatment at 165 °C were carried out at several CTec2 loadings between 5 and 20 FPU/g cellulose to evaluate the effectiveness of SPORL pretreatment in removing lodgepole pine recalcitrance. The results indicate that 80% cellulose saccharification, represented by the substrate enzymatic digestibility (SED) defined as the percentage of substrate glucan enzymatically saccharified to glucose, can be achieved at a CTec2 loading of 15 FPU/g cellulose as shown in Figure 3. The SED of xylan and mannan reached 70 and 60%, respectively, after 72 h. These results suggest that the pretreatment is effective for efficient saccharification of the lodgepole pine wood chips.

Sugar Consumption, Ethanol Production, and Furan Metabolization during SSCombF. SSCombF of unwashed solids at 18% loading with additional pretreatment spent liquor showed robust production of ethanol using *S. cerevisiae* YRH400 as shown in Figure 4a. The unwashed solids were liquefied prior to yeast inoculation, which is the source of the initial glucose. Ethanol concentration was linearly proportional to fermentation time in the first 24 h (Figure 4a is in log scale to show initial fermentation). Similar linear relations were also observed in the first 24 h for glucose, mannose, and xylose consumptions as well as HMF and furfural metabolization (Figure 4b). Linear regressions resulted in an average ethanol productivity of 1.03 g L^{-1} h^{-1} in the first 24 h (Table 2).

Ethanol concentration reached 41 g L^{-1} within 48 h of inoculation. Glucose concentration remained low throughout most of the fermentation, which indicates that YRH400 fermented the glucose as readily as it was released by the cellulases. Average glucose and mannose consumption were −2.12 and −0.50 g L^{-1} h^{-1}, respectively. Clearly, mannose consumption is significantly slower than glucose consumption.

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Table 1. Chemical Composition of the Untreated Lodgepole Pine BD4 Wood Chips and Component Recovery from Scale-up SPORL Pretreatment at 165°C for 75 min with Sulfuric Acid and Sodium Bisulfite Loading on Wood 2.2 and 8%, Respectively

	untreated wood	unwashed solids[a]	collected spent liquor[a]	total recovery
wet weight (kg)	2.281	6.227	1.726	
solids content (%)	87.7	31.86	8.37	
solids (kg)[b]	2.0	1.984; 90.0%	0.144; 6.6%	96.6%
Klason lignin (%)	28.6	28.31; 99.0%	0.29; 1.0%	100.0%
arabinan (%)	1.7	0.58; 34.2%	0.06; 3.3%	37.6%
galactan (%)	2.9	1.43; 49.3%	0.40; 13.9%	63.1%
glucan (%)	41.9	40.64; 97.0%	0.60; 1.4%	98.4%
mannan (%)	11.7	5.98; 51.1%	1.70; 14.5%	65.6%
xylan (%)	5.5	2.86; 52.0%	0.53; 9.7%	61.6%
HMF (%)[c]		0.43; 3.7%	0.18 (1.7); 1.5%	5.2%
furfural (%)[c]		0.31; 5.7%	0.13 (1.1); 2.3%	8.0%
acetic acid (%)		1.87	0.76	

[a]The numbers after after the semicolon represent the theoretical (based on untreated wood) carbohydrate content in wt %. [b]In oven dry (od) weight. [c]Reported as mannan and xylan for HMF and furfural, respectively, representing percent of mannan and xylan degraded to HMF and furfural, respectively. The numbers in the parentheses are concentration measured in the collected spent liquor in g L^{-1}

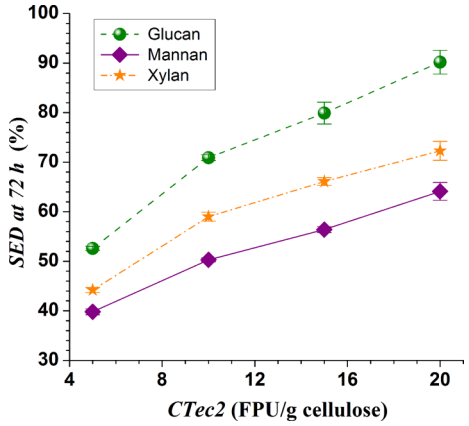

Figure 3. Effect of cellulase CTec2 loading on washed solid substrate enzymatic digestibility (SED) at 72 h.

Xylose consumption by YRH400 is very limited only at 0.07 g L^{-1} h^{-1}, an order of magnitude slower than mannose consumption. This is partially due to the low initial xylose concentration of only approximately 7 g L^{-1} (Figure 4a). *S. cerevisiae* has not evolved xylose-specific transporters and instead relies on hexose transporters. The highest affinity transporter measured for xylose only has a Km of approximately 190 mm,[32] which means xylose is imported into the cell slowly and requires a higher concentration than for sugars that are natively fermented. Consumptions of arabinose and galactose are negligible; strain YRH400 is unable to consume arabinose. The maximal ethanol titer was 47.1 g L^{-1} achieved after 120 h fermentation (Table 2). The calculated ethanol yield based on this titer was 0.396 g/g sugar or 306 L/tonne wood, using the final measured volume of the fermentation broth of 46.5 mL. The initial HMF and furfural concentrations in the pretreatment spent liquor were low with values of approximately 1.7 and 1.1 g L^{-1} (Table 1), respectively. This is significantly lower than the 2.7 and 2.2 g L^{-1} produced at 180C° for 20 min with a pretreatment severity *CHF* = 18.9.[24] The low concentrations of HMF and furfural facilitated YRH400 metabolism of these two inhibitors. The average rate of consumption of HMF and furfural were −0.05 and −0.14 g L^{-1} h^{-1}, respectively (Table 2). This observation is in agreement with an earlier study using the parental strain of

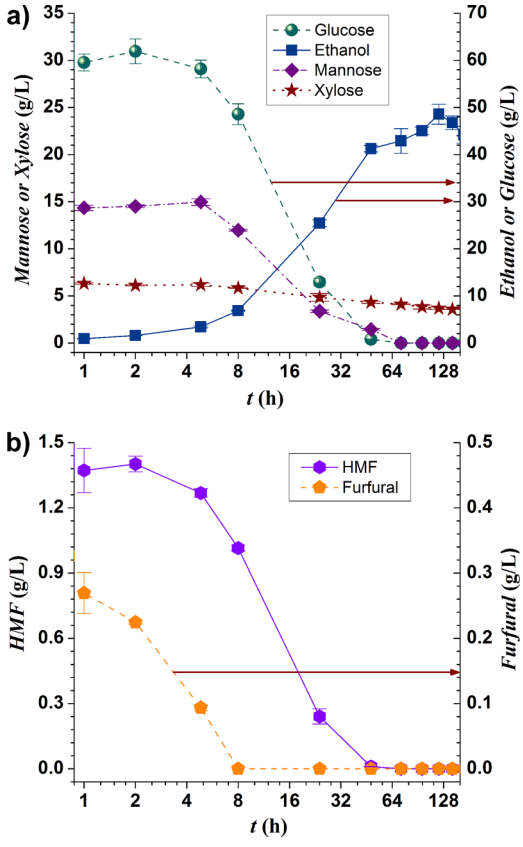

Figure 4. Time-dependent sugar, ethanol, and furan concentration profiles in the fermentation broth using the whole biomass slurry from the scale-up SPORL pretreatment at 18% unwashed solids loading: (a) sugar and ethanol; (b) furans.

YRH400 (D5A) to ferment dilute acid pretreated switchgrass.[33] *Saccharomyces* yeast does not fully metabolize furans, but instead the aldehydes are converted to their less toxic alcohol forms by the action of aldehyde dehydrogenase.[34] Cultures with an inhibitory furan concentration are marked by a prolonged phase which lasts until the furan concentration has been sufficiently lowered to allow for growth and fermentation.[33]

Molecular Weight Distribution and Sulfur Content of SPORL Produced LS. LS is produced commercially through

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Table 2. Fermentation of Lodgepole Pine Whole Slurry from Scale-up SPORL Pretreatment at 165 °C for 75 min: Average Ethanol Productivity, Rates of Sugar Consumption and Furan Metabolization in the first 24 h, along with Maximal Ethanol Production at 120 h

Average fermentation performance measure in the first 24 h (g L^{-1} h^{-1})	
ethanol productivity	1.03
glucose consumption	−2.10
mannose consumption	−0.54
xylose consumption	−0.02
HMF metabolization	−0.05 (18 h)
furfural metabolization	−0.06 (5h)
Maximal ethanol production at 120 h	
ethanol concentration (g L^{-1})	47.1 ± 2.1
ethanol yield (g g $sugar^{-1}$)[a]	0.396 ± 0.018
ethanol yield (L tonne $wood^{-1}$)	306 ± 14
ethanol yield (% theoretical)[b]	71.8 ± 3.5

[a]Based on the total of glucan, mannan, xylan in the pretreated-solids and glucose, mannose, and xylose in the pretreatment spent liquor. [b]Theoretical yield (426 L tonne $wood^{-1}$) based on total glucan, mannan, xylan in the untreated wood.

sulfite pulping and has a variety of applications.[35] The effectiveness of LS is strongly influenced by the sulfonic acid group content, which is often dependent in turn on its molecular weight (MW) when it is produced through low temperature sulfite pulping. SPORL was conducted at 165 °C in this study, a higher temperature than used for sulfite pulping, but also for a much shorter duration of only 75 min. The LS from SPORL-pretreated lodgepole pine, LS-SP165, has a much smaller molecular weight compared with a high purity commercial softwood LS D-748 as listed in Table 3. The

Table 3. Comparisons of Molecular Weights and Sulfur Content between the LS from SPORL Pretreated Lodgepole Pine at 165°C, LS-SP165, and a High Purity Commercial LS

LS	M_n (Da)	M_w (Da)	M_w/M_n	sulfur (wt %)
LS-SP165	810	1440	1.77	6.04 ± 0.33
D-748	4800	14000	2.92	6.01 ± 0.39

maximum molecular weight of LS-SP165 is 10 times lower than the D-748. On the other hand, LS-SP165 has a much narrower molecular weight distribution as the ratio between the number averaged (M_n) and weight averaged (M_w) molecular weight was only 1.8 compared with 2.9 for D-748.

Most sulfur in LS is associated with the sulfonic acid group that contribute to surface properties, and therefore sulfur content can be a direct measure of LS dispersing properties. LSs with higher sulfur content were found to be better than those of low sulfur content as a dispersant for coal−water slurry and cement.[36,37] A similar finding was obtained in applying LS as a dispersant to gypsum paste.[38] The sulfur content of LS-SP165 was approximately equal to that of D-748.

Dispersibility of LS from SPORL. TiO_2 is one of the most widely used mineral oxides with a variety of industrial applications in paints, plastics, papermaking, ceramics, fibers, and inks.[39,40] The dispersion stability of TiO_2 is very important in these applications. Because of their large surface area and strong surface properties, TiO_2 particles often tend to form agglomerates which negatively impact its end-use performance. Using commercial LS to improve TiO_2 dispersion is a common industry practice. We can evaluate the effectiveness of LS as dispersant by examining the colloidal stability of TiO_2 particle suspensions. A linear increase in mean particle size with time from 17 μm at time zero to 25 μm in 60 min, or an increase of approximately 50%, was observed for the control as shown in Figure 5, suggesting continued particle agglomeration. The

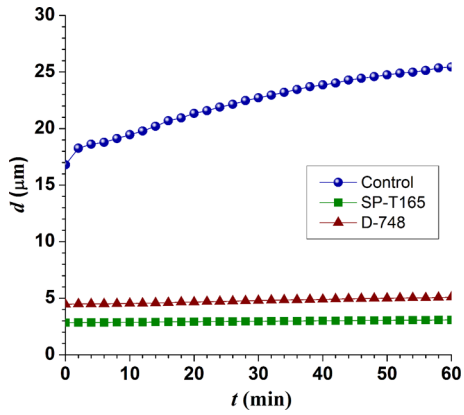

Figure 5. Comparison of time-dependent TiO_2 particle size with and without application of LS.

mean particle size was increased only by 14% and 8% with the application of D-748 and LS-SP165, respectively, in the same 60 min period (Figure 5). Furthermore, the initial particle size was 4.5 and 3.0 μm with the application of D-748 and LS-SP165, respectively, and were significantly lower than the 17 μm for the control, suggesting significant reduction in TiO_2 particle agglomeration during sample preparation by LS. LS-SP-165 produced by the present SPORL pretreatment showed better performance in dispersing TiO_2 particles and stabilizing its suspension than a high purity commercial LS D-748 despite its much smaller molecular weight.

Mass Balance for the Scale-up Pretreatment. The overall mass balance of the scale-up pretreatment was conducted on the basis of the collected process data. As shown in Figure 6, total ethanol production was 241 ± 11 kg/tonne wood or 306 ± 14 L/tonne wood at 47.1 ± 2.1 g L^{-1} based on the average of two fermentation runs conducted two weeks apart. This yield is equivalent to 72% theoretical yield based on glucan, mannan, and xylan content of the lodgepole pine. The LS yield was low at 20 kg/tone wood for the pretreatment conditions used; however, the yield can be improved by slightly increasing sodium bisulfite loading to facilitate delignification as demonstrated previously.[19]

CONCLUSIONS

This study demonstrated that SPORL is an effective pretreatment to remove the strong recalcitrance of softwood lodgepole pine for efficient enzymatic saccharification. The combined hydrolysis factor (CHF) was successfully used to design scale-up pretreatment at a relatively low temperature of 165 °C to reduce sugar degradation into fermentation inhibitors. This facilitated high solids simultaneous enzymatic saccharification and combined fermentation (SSCombF) of the enzymatic and pretreatment hydrolysates (spent liquor). A final ethanol yield of 306 L/tonne wood, at a titer of 47.1 g L^{-1}, was achieved using *S. cerevisiae* when SSCombF was conducted at 18% solids loading without washing of the pretreated solids or detoxification of the pretreatment hydrolysate. The lignosulfo-

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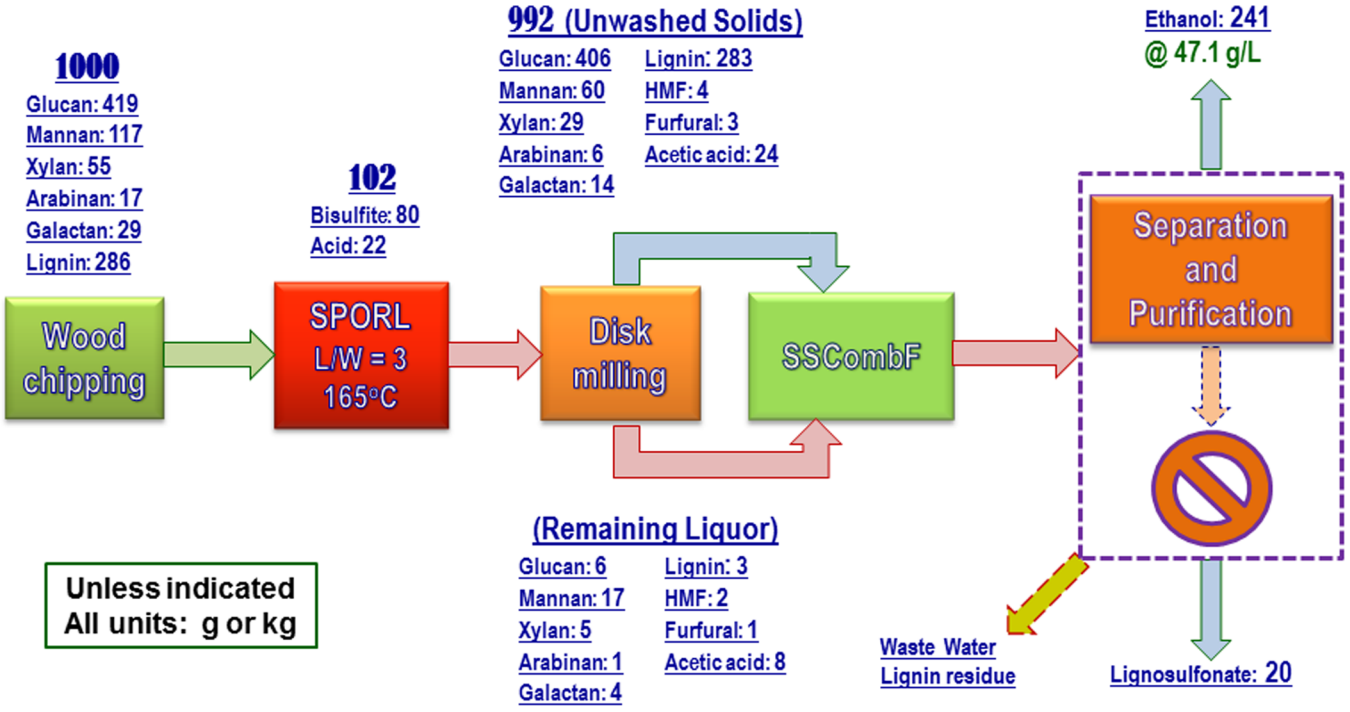

Figure 6. A block diagram shows process mass balance for the scale-up pretreatment at 165 °C for 75 min with subsequent saccharification and fermentation. The process boxed by dashed lines was partially conducted.

nate (LS) produced by SPORL has a low molecular weight distribution. However, it is highly sulfonated and demonstrated better performance as a dispersant than a commercial high purity LS. Therefore, the SPORL process has been shown to facilitate conversion of woody biomass to ethanol at a high yield and high final titer and production of a directly marketable lignin coproduct. Given that SPORL has a lower level of technical risk than other competing technologies because its unit operations are based upon classical sulfite pulping, we conclude that SPORL is a viable technology for forest biorefinery. Utilization of the enzymatic hydrolysis residue lignin such as activated carbon needs to be developed in future studies.

■ ASSOCIATED CONTENT

Supporting Information

Table S1: List of SPORL pretreatments at 150 g scale using mountain pine beetle killed lodgepole pine BD4 wood chips along with key chemical components of the pretreated solids. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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