HIGH SOLIDS QUASI-SIMULTANEOUS ENZYMATIC SACCHARIFICATION AND FERMENTATION OF UN-DETOXIFIED WHOLE SLURRY OF **SPORL** PRETREATED DOUGLAS-FIR FOREST RESIDUE

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Forest residue is the most affordable feedstock for biofuel production as stated in a recent US National Research Council Report. Softwood forest residue represents a significant amount of woody biomass that can be sustainably used to produce biofuel. It also has very low contents of acetyl groups and 5-carbon polysaccharides, favorable for biofuel production through yeast fermentation. However, it is highly recalcitrant to enzymatic saccharification due to high bark and lignin content. Most existing pretreatment processes are unable to remove this recalcitrance. Sulfite pretreatment to overcome the recalcitrance of lignocelluloses (SPORL) has demonstrated unparalleled performance for bioconversion of softwoods. In this study, we evaluated SPORL process for bioconversion of un-detoxified Douglas-fir forest residue at a high solids loading to ethanol.

Keywords: forest residues, ethanol, pretreatment, high solids, enzymatic saccharification and fermentation

INTRODUCTION

Woody biomass offers significant advantages over herbaceous biomass and agriculture residue for bioconversion to biofuel and chemicals in terms of feedstock logistics for its high density and flexible harvesting.[1] This is especially true when using forest residue, a low cost forest biomass based on a recent US National Research Council Report by the National Academy of Sciences. Forest residue can be sustainably produced in North America and Nordic countries. To avoid the competition of feedstock with the traditional wood products and pulp and paper industries, using forest residue for biorefinery is an economical and logical choice. However, woody biomass is especially recalcitrant to microbial deconstruction of polysaccharides to sugars, one of the major pathways to converting lignocelluloses into biofuels and high value products. Few pretreatment processes can effectively remove the strong recalcitrance of woody biomass for efficient bioconversion to sugar and biofuels.[1] Although organosolv,[2-4] SO_2 steam explosion[5,6] pretreatments have been applied to woody biomass, limited success was achieved in terms of high titer and high yield biofuel production without detoxification. We have developed a Sulfite Pretreatment to Overcome the Recalcitrance of Lignocelluloses (SPORL) process that has demonstrated robust performance for converting woody biomass.[7-10]

Despite significant interest in co-product development through forest biorefinery,[11-13] the limited co-product market and significant amount of supply of lignin created a market mismatch. This suggests a large amount of forest biomass will be used for biofuel production to avoid the disruption of the co-product market. Therefore, this study applied SPORL to evaluate its performance for bioconversion of Douglas-fir forest residue to bio-ethanol. The study is to demonstrate the capability of SPORL for forest

biorefinery applications using low grade feedstock.

EXPERIMENTAL

The SPORL process

SPORL was developed based on sulfite pulping. It utilizes sulfite to achieve partial delignification in a range of approximately 10-50% under pH 1.5-5.0 through lignin sulfonation, as well as dissolution of 70-95% hemicelluloses. Lignin sulfonation is a key characteristic of the process. The dissolved lignin, i.e., lignosulfonate (LS), can be directly marketed as a co-product. Furthermore, LS has less affinity to cellulase and thereby results in negligible nonproductive binding to cellulase due to its strong hydrophilicity.[14] It was observed that enzymatic hydrolysis of the solid fraction can be enhanced when combined with pretreatment sulfite spent liquor (SSL) that contained LS.[15] This is because LS acts as a surfactant that can reduce nonproductive binding of cellulase to residual lignin on the solid substrate.[16-18] SPORL is conducted at a temperature higher than that used for sulfite pulping, but with a shorter time, because complete removal of lignin is not necessary and preservation of hemicelluloses is not favored for efficient enzymatic saccharification.[19] To illustrate the modification of sulfite pulping to develop the SPORL process, SPORL process conditions were compared with sulfite pulping in Figure 1.

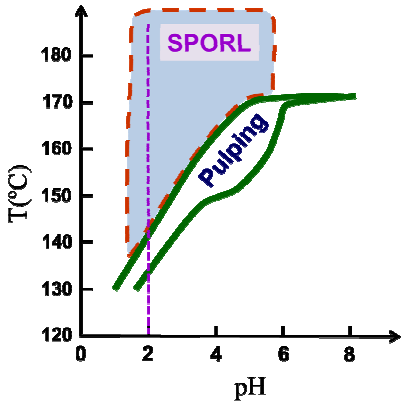

Figure 1: Comparisons of SPORL with sulfite pulping operating conditions

Materials

Forest residue and physical fractionation to reduce bark content

Forest residue of Douglas-fir was collected from roadside piles from a regeneration harvest using a Peterson Pacific 4710B horizontal grinder. The ground materials were screened to remove the fines smaller than 3.2 mm. The accepted Douglas-fir forest residue was labelled as FS-10. It was found that physical fractionation to reject the small size fractions was very effective to selectively remove bark and ash.[20] Approximately 30% of the bark and 40% of the ash were removed by rejecting particles less than 6.4 mm with the loss of biomass only about 10%.

Chemicals and cellulase

Commercial cellulase Cellic® CTec3 (abbreviated CTec3) was generously provided by Novozymes North America (Franklinton, NC, USA). The cellulase activity was 217 FPU/mL as calibrated by a literature method.[21] 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.

Pretreatment

A previous study using lodgepole pine indicated that a pretreatment at 180 ºC for 30 min could produce optimal sugar yield.[8] Furthermore, we were able to balance fermentation inhibitor formation and enzymatic digestibility by using a low temperature pretreatment because the activation energy for sugar degradation is higher than hemicelluloses dissolution.[22] This is critical to facilitate high solids fermentation without detoxification. In designing the low temperature pretreatment at 165 ºC, the pretreatment severity measured by the combined hydrolysis factor, CHF (Eq. (1), was maintained at approximately 20 based on the optimal condition of T = 180 ºC for t^T = 30 min by using a longer pretreatment time to ensure similar hemicellulose removal and substrate enzymatic digestibility.[8,22] The required pretreatment time at *Tup* = 165 ºC was determined to be approximately t^{Tup} = 75 min using eq. (2) when the same chemical loadings were maintained.

$$CHF = e^{\left(\alpha - \frac{\beta}{RT} + \beta C_A + \gamma C_B\right)}(C_A + C_B)t \quad (1)$$

$$t^{T}Tup = \exp[E/R(1/Tup - 1/T)]\ (t^{\dagger}T \quad (2)$$

where C_A and C_B are the concentrations of chemical A (acid) and chemical B (bisulfite) used in pretreatment,

respectively; α = 28.5, β = 17, γ = -10 are adjustable parameters, E = 100,000 J/mole is the apparent activation energy, R is the universal gas constant of 8.314 J/mole/K, and T is absolute temperature (K).

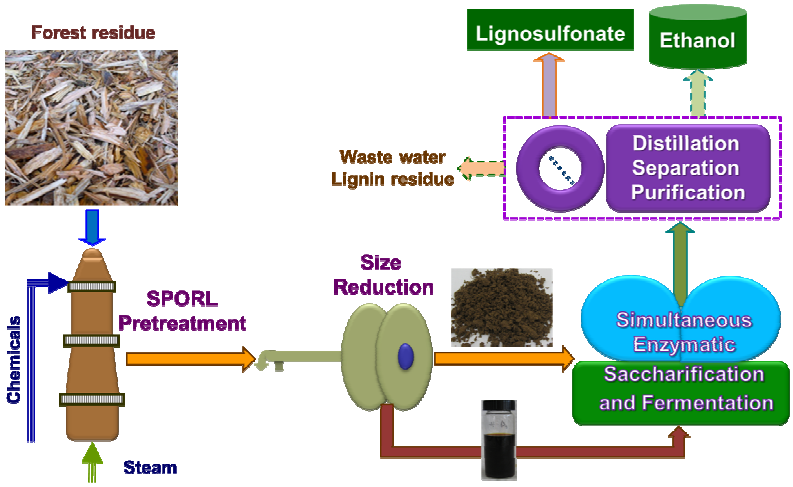

Figure 2: Schematic flow diagram of the experiments

The pretreatment, enzymatic saccharification and fermentation for ethanol production were carried out according to the schematic flow diagram shown in Figure 2. The screen accepted forest residue FS-10 was directly pretreated using a dilute sulfite solution. The dilute sulfite solution is often prepared by bubbling sulfur dioxide (SO_2) into a hydroxide solution in commercial pulp mills. The pH of the dilute sulfite solution is adjusted by varying the amount of excess or free SO_2. However, for easy preparation of sulfite solution in laboratory practice, we used sodium bisulfite ($NaHSO_3$) from a commercial source to prepare aqueous sulfite solution with the application of sulfuric acid (H_2SO_4) to adjust the pH of the solution to the desired level around 1.9. It should be pointed out that this laboratory practice requires a higher sulfite charge on wood to achieve equivalent effectiveness because H_2SO_4 behaves differently from H_2O-SO_2. The sodium bisulfite and sulfuric acid charges on wood were 12% and 2.2%, respectively, in making the pretreatment sulfite solution. FS-10 of 2 kg in oven dry weight (OD) was pretreated at 165 ºC for 75 min in a laboratory wood pulping digester of 23 L heated by a steam jacket and rotated at 2 rpm. The solid to sulfite solution ratio was 1:3 (w/v). At the end of the pretreatment, the digester was cooled down using tap water through the jacket. The pretreated solids together with spent liquor were then fed to a pressurized disk refiner (Andritz Sprout–Bauer Refiner, Springfield, Ohio) for size reduction as described previously.[23,24] The refined whole slurry was neutralized by lime ($Ca(OH)_2$) and directly used for subsequent enzymatic saccharification and fermentation without detoxification.

Enzymatic saccharification and fermentation

Separate enzymatic hydrolysis experiments of washed solid substrate were conducted at 50 ºC at CTec3 loading of 15 FPU/g glucan to evaluate the effectiveness of pretreatment for saccharification.

Quasi-simultaneous enzymatic saccharification and combined fermentation (SSCombF) of the pretreated whole slurry was carried out at total solids of 21% in 100 mL Erlenmeyer flasks on a shaker/incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA). The whole slurry was adjusted to pH 6.2 with solid calcium hydroxide. Acetic acid/sodium acetate buffer (50 mM) of pH 6.0 was used for enzymatic hydrolysis using CTec3 at 24 FPU/g glucan. Using an elevated pH of approximately 5.5 (which is higher than the commonly used pH 4.8-5.0) and lignosulfonate in the SPORL pretreatment liquor could significantly reduce nonproductive cellulase binding to lignin to enhance lignocellulose saccharification.[14,16,25] Liquefaction of solid substrate was conducted at 50 ºC and 200 rpm. The mixture was then cooled down to 35 °C and the shaker speed was reduced to 90 rpm and inoculated with 1 mL of yeast seed, i.e. *Saccharomyces cerevisiae* YRH400. The YRH400 was grown at 30 °C for 2 days on YPD agar plates containing 10 g/L yeast

extract, 20 g/L peptone, 20 g/L glucose, and 20 g/L 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 cultured yeast seed was used to inoculate the fermentation medium.

The optical density of the yeast seed was OD_{600} = 30 measured by a UV-Vis spectrophotometer (Model 8453, Agilent Technologies, Palo Alto, CA). No additional nutrients were applied during fermentation. Samples of the fermentation broth were taken periodically for analysis of monosaccharides, inhibitors, and ethanol. Replicate fermentation runs were conducted to ensure experimental repeatability. The standard deviations were used as error bars in plotting.

RESULTS AND DISCUSSION

Cell wall modification by SPORL pretreatment

SPORL pretreatment significantly enriched the glucan content in the pretreated substrate by removing 40% of the total solids. Approximately 40% lignin and 80% of hemicelluloses were dissolved (Table 1), which significantly improved the substrate enzymatic digestibility (SED), defined as the percentage of substrate glucan enzymatically saccharified into glucose. The SED of the washed SPORL pretreated FS10 reached 90% in 24 h (Figure 3).

Ethanol production through fermentation

Both glucose consumption and ethanol production were linearly proportional to fermentation time in the early stage of fermentation (Figure 4). The overall fermentation performance data were calculated (Table 2). Terminal ethanol concentration reached approximately 40 g/L. The recalcitrant nature of forest residue required severe pretreatment that increased furan production. As a result, the final ethanol production was lower than that obtained in our previous study using lodgepole pine under the same pretreatment conditions.[8]

Table 1
Cell wall chemical composition before and after SPORL pretreatment

Sample label	K Lignin (%)	Arabinan (%)	Galactan (%)	Glucan (%)	Xylan (%)	Mannan (%)	Water insoluble solid yield (%)
Untreated residue	29.30	1.04	2.00	40.97	5.70	9.67	100.0
Treated washed residue	27.80	ND	0.23	63.39	2.23	2.58	61.3

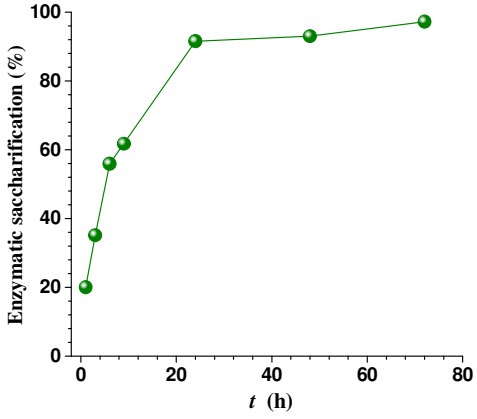

Figure 3: Substrate enzymatic digestibility

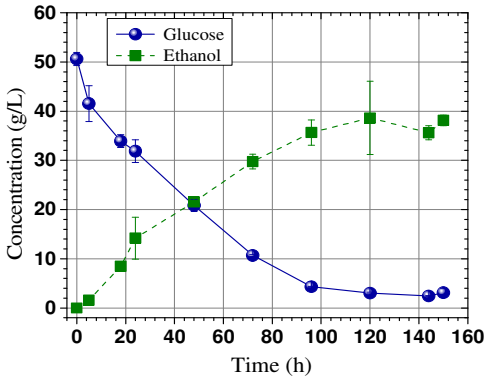

Figure 4: Glucose consumption and ethanol production in quasi-simultaneous enzymatic saccharification and fermentation

Table 2
Fermentation performance of SPORL pretreated forest residue of Douglas-fir (FS10)

Average fermentation performance (g L^{-1} h^{-1})	
Glucose consumption (48 h)	-0.59 ± 0.08
Ethanol productivity (48 h)	0.53 ± 0.03
Glucose consumption (24 h)	-0.85 ± 0.10
HMF metabolization (24 h)	-0.05 ± 0.00
Maximal ethanol production	
Ethanol concentration (g L^{-1})	38.6 ± 7.5
Ethanol yield (g g sugar $^{-1}$) [a]	0.39 ± 0.08
Ethanol yield (L tonne wood $^{-1}$)	215 ± 42

[a] based on the total of glucan, mannan, xylan in the pretreated-solids and glucose, mannose, and xylose in the pretreatment spent liquor

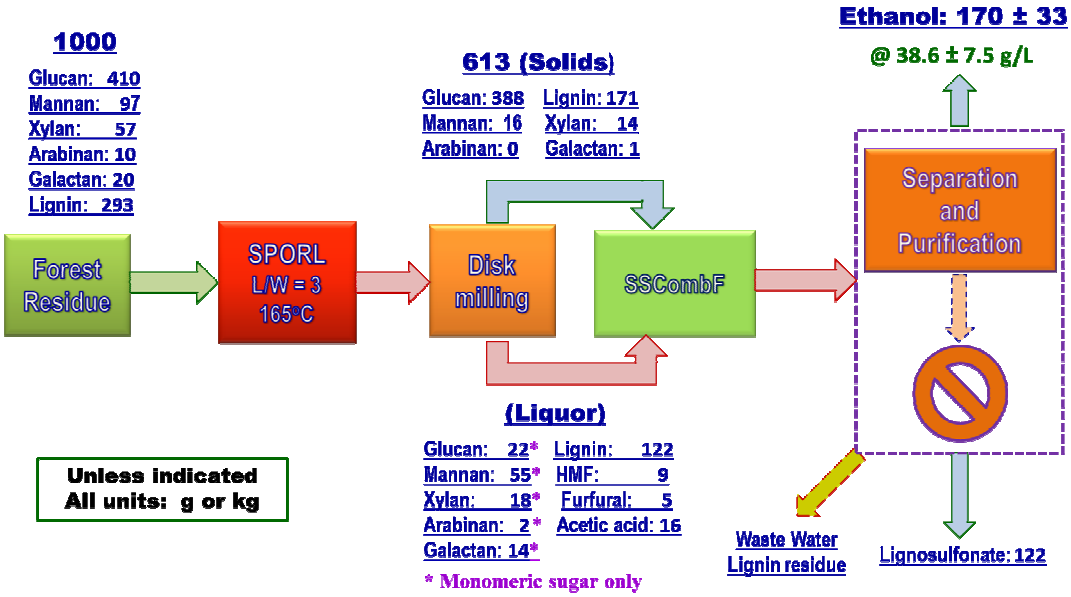

Figure 5: Overall mass balance of the present study

The overall mass balance was conducted based upon the collected process data (Figure 5). Total ethanol production was 214 ± 42L (or 170 ± 33 kg) per metric tonne FS10 based on the average of two fermentation runs conducted two weeks apart with a relatively large standard deviation. This yield is equivalent to 53 ± 10% theoretical based on glucan, mannan, and xylan content of FS10. This yield can be improved by modifying pretreatment conditions to reduce furan formation, as will be reported in a future study. The LS yield was 122 kg/tonne FS10 based on the balance of the amount of Klason lignin removed from the pretreatment. Our previous study demonstrated that LS from SPORL pretreated lodgepole pine has better dispersion properties than commercial softwood LS.

CONCLUSION

We have demonstrated that the sulfite based pretreatment – SPORL – is an effective process to convert softwood Douglas-fir forest residue to ethanol at high titer through enzymatic saccharification and yeast fermentation with good yield without detoxification. The combined hydrolysis factor (CHF) was successfully used to scale pretreatment at relatively low temperature of 165 °C to reduce sugar degradation into inhibitors

to facilitate fermentation at high solids 21% of the whole biomass slurry without detoxification. The lignosulfonate (LS) produced by SPORL, as a co-product, is a potential revenue stream. The SPORL process fits well with the pulp and paper industry with existing sulfite mills around the world. We conclude that SPORL is a viable technology for forest biorefinery. Co-products of the residual lignin from enzymatic hydrolysis, such as activated carbon, need to be developed in the future.

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