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1 # Comparisons of high titer ethanol production and lignosulfonate properties
2 by SPORL pretreatment of lodgepole pine at two temperatures

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12 ## Abstract

13 Mountain pine beetle killed lodgepole pine wood chips were pretreated by SPORL (Sulfite
14 Pretreatment to Overcome the Recalcitrance of Lignocelluloses) at 180°C for 25 min and
15 165°C for 75 min using the same chemical loadings, which represent the same pretreatment
16 severity. The pretreated whole slurries were used to produce lignosulfonate and ethanol
17 through simultaneous enzymatic saccharification and combined fermentation (SSCombF) up
18 to solids loading of 18% *without detoxification*. Low temperature pretreatments reduced
19 furan formation, which facilitated ethanol production as measured by ethanol productivity and
20 sugar consumption. The improved carbohydrate yields at 165°C also produced high ethanol
21 yields (liter per tonne wood) at all SSCombF solids loadings. An ethanol yield and titer of 306
22 L tonne^{-1} wood, or approximately 72% theoretical, and 47.1 g L^{-1}, respectively, were achieved
23 without detoxification at 165°C. Lignosulfonates (LS) produced from the two SPORL runs
24 are highly sulfonated but have lower molecular weight than a commercial high purity softwood
25 LS. Both infrared and NMR spectra of LS from SPORL treated wood chips were compared
26 with those of the commercial LS. The LSs from SPORL treated wood chips were found to
27 have better dispersion property than the commercial LS.

28 **Keywords:** High solids processing and fermentation, Enzymatic saccharification/hydrolysis,
29 Woody biomass, Lignosulfonate, Inhibitor formation
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32 This work was conducted while Haifeng Zhou was visiting students and Shao-Yuan Leu was a
33 postdoctoral fellow at the USDA Forest Products Laboratory and on official government time
34 of Zhu, Gleisner.

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36 ## 1. Introduction

37 High titer biofuel production from lignocelluloses is critically important to process energy
38 efficiency and economics [1, 2]. While microbial fermentation is a proven route for biofuel
39 production from lignocelluloses using the sugar platform, microbial inhibition arising from
40 degradation products produced during the lignocellulose pretreatment step limits the final
41 product titer [3, 4]. Studies reporting high titer ethanol production are limited to either low yields
42 because mild pretreatment conditions were used to reduce inhibitor formation[5], or using a
43 feedstock with very low recalcitrance such as corncob [6], or just using the washed solids without
44 including inhibitor containing pretreatment spent liquor [7]. Washing is not practical because of
45 water consumption is an environmental concern. Without washing the solids, a detoxification
46 step is often necessary for fermentation [4, 8]. The concept of low temperature pretreatment was
47 developed to avoid detoxification for high solids fermentation; however pretreatment duration
48 was arbitrary determined and severity was reduced. As a result, sugar yield was maintained at
49 the expense of increased energy input through additional processing steps such as alkali
50 extraction and mechanical refining, and xylanase supplementation [9].

51 We proposed the concept of applying a lower pretreatment temperature for longer times than
52 is typical so as to maintain pretreatment severity and therefore the resultant substrate enzymatic
53 digestibility in a previous study [10]. This is based on the fact that the activation energy of sugar
54 degradation to microbial inhibitors is greater than that for hemicellulose dissolution; therefore
55 hemicellulose dissolution can be favored over sugar degradation by designing a low
56 temperature pretreatment with longer reaction time. Furan formation was found significantly

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57 reduced at a low temperature compared with a high temperature pretreatment at the same
58 severity [10]. Furthermore, by maintaining the same pretreatment severity, the substrate
59 enzymatic saccharification efficiency was not negatively affected [10].
60 The objective of this study is to demonstrate the advantage of this low temperature
61 pretreatment with longer reaction time for high titer ethanol production through fermentation
62 using a *Saccharomyces cerevisiae.* Two pretreatments with the same severities but at different
63 temperatures were compared in terms of ethanol productivity, yield, and the quality of the
64 lignin co-product. Lodgepole pine wood chips were pretreated by SPORL (Sulfite
65 Pretreatment to Overcome the Recalcitrance of Lignocelluloses) [11] at 180°C for 25 min and
66 165°C for 75 min using identical liquid to wood ratio and chemical loadings. SPORL was
67 chosen because of its robust performance in removing the strong recalcitrance of softwoods
68 and softwood forest residues [10, 12, 13]. The pretreated whole slurry were first liquefied and then
69 fermented *without detoxification* at three unwashed solids loadings of 12, 15, and 18%.
70 Results from this study expand upon a prior study and confirmed that lodgepole chips
71 pretreated at the lower temperature (165°C, 75 min) could be converted to ethanol without
72 conditioning the whole slurry beforehand [14]. An additional benefit of the SPORL process is
73 the co-production of lignosulfonate (LS), which has an existing commercial market.
74 Therefore, the properties of lignosulfonate (LS) dissolved in the SPORL pretreatment spent
75 liquor were also compared.
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78 ## 2. Materials and Methods

79 ### 2.1 Materials

80 A mountain pine beetle-killed tree (labeled as BD4) was harvested from the Canyon Lakes
81 Ranger District of the Arapaho-Roosevelt National Forest, Colorado, USA, and debarked on
82 site. Logs were wrapped in plastic bags before shipping to the USDA Forest Products
83 Laboratory (FPL), Madison, WI. Detailed information about the tree, harvesting, and
84 transportation have been described previously [15, 16]. Wood chips were produced at FPL and
85 the chips were screened to retain chips between 6 and 38 mm. The screen chips were frozen at
86 approximately -16°C until use.

87 Commercial cellulase enzymes Cellic® CTec2 (abbreviated CTec2) were complimentary
88 provided by Novozymes North America (Franklinton, North Carolina, USA). The cellulase
89 activity of 147 FPU mL^{-1} was calibrated using a literature method [17]. All chemicals including
90 sodium acetate buffer, sulfuric acid, and sodium bisulfite were ACS reagent grade and
91 purchased from Sigma-Aldrich (St. Louis, MO). High purity sodium LS (D748) from sulfite
92 pulping of softwood was donated by LignoTech USA (Rothschild, WI).

93 Titanium dioxide (TiO_2) powder was purchased from Kermel Chemistry Co. Ltd.
94 (Tianjin, China) with purity of 99%. The manufacture specified average particle diameter of
95 TiO_2 was 5.43 μm. An EyeTech Laser particle size analyzer (Ankersmid Co. Ltd., Holland)
96 was used to analysis TiO_2 particle size in aqueous suspensions.

97 *Saccharomyces cerevisiae* YRH400 was engineered from a fungal strain for xylose
98 fermentation [18]. YPD agar plates containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1}

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99 glucose, and 20 g L^{-1} agar, was used to grow the strain at 30°C. A liquid YPD medium was
100 used to culture the strain in a flask on a shaking bed at 90 rpm (Thermo Fisher Scientific,
101 Model 4450, Waltham, MA) overnight at 30°C. The cultured medium was monitored using
102 optical density at 600 nm (OD_{600}) by a UV-Vis spectrometer (Model 8453, UV-visible
103 spectroscopy system, Agilent Technologies, Palo Alto, CA) and used to inoculate the
104 fermentation culture.

105 **2.2 SPORL pretreatment**

106 SPORL pretreatments of lodgepole pine BD4 wood chips were conducted in a 23 L
107 laboratory wood pulping digester using dilute sodium bisulfite solution of pH approximately
108 2.0 at two temperatures of 180ºC and 165ºC for 25 min and 75 min, respectively. The required
109 reaction time of 75 min at the lower pretreatment temperature of 165ºC was determined using
110 equation (1) when the same chemical loadings of C_A and C_B were maintained based on constant
111 pretreatment severity measured by the combined hydrolysis factor (CHF; Eq. (2)) [10].

112 $$t^{T165} = \exp\left[-\frac{E}{R}\left(\frac{1}{T^{165}} - \frac{1}{T^{180}}\right)\right] \cdot t^{T180} \quad (1)$$

113 $$CHF = e^{\left(\alpha - \frac{E}{RT} + \beta\, C_A + \gamma\, C_B\right)}(C_A + C_B)t \quad (2)$$

114 Where E = 100,000 J/mole is apparent activation energy, R=8.314 is universal gas constant, and t^{T180} =
115 25 min. The digester was heated by a steam jacket and rotated at 2 rpm for mixing. Both
116 pretreatments used 2000 g wood chips in oven dry (od) weight with fixed liquor to wood ratio
117 (L/W) of 3:1. The sulfuric acid and sodium bisulfite charge on wood as mass fraction was 2.2%
118 and 8.0%, respectively. These pretreatment conditions resulted in the same pretreatment
119 severity of CHF = 22.5 to produce the same level of hemicellulose dissolution [10, 19]. Therefore,

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120 comparisons can be made between these two pretreatments for ethanol production. At the end
121 of each pretreatment, the digester was cooled down by flushing the heating jacket with tap
122 water to terminate the reaction. The pretreated solids and spent liquor were directly
123 transferred to a disk mill for size reduction without adding water. The resultant whole slurry
124 was pressed in a screen box to separate solids from liquor for component mass balance
125 determination. The yield of solids (unwashed) was determined from the oven dry weight of
126 the material. Both the volume and weight of the collected pretreatment spent liquor were
127 recorded. Duplicate pretreatments were conducted at each pretreatment temperature and the
128 solid and liquor samples from replicate runs were respectively combined for downstream
129 saccharification and fermentation evaluation.

130 **2.3 Enzymatic hydrolysis**

131 Washed pretreated solid substrate at 20 g L^{-1} loading was used in enzymatic hydrolysis in a
132 50 mL of 50 mM acetate buffer setting on a shaking incubator (Thermo Fisher Scientific,
133 Model 4450, Waltham, MA) at 50°C and 200 rpm. Elevated pH 5.5, higher than commonly
134 used pH 4.8-5.0, is to significantly reduce nonproductive cellulase binding to lignin to enhance
135 lignocellulose saccharification [20-22]. NaOH of 5 wt% or acetic acid was used to adjust the pH
136 of the substrate suspension to pH 5.5. The CTec2 loading was 10 FPU or 0.067 mL g^{-1} glucan
137 that is lower than most studies using softwoods. Aliquots of 1 mL enzymatic hydorlysate
138 were taken periodically (3, 6, 9, 24, 48, and 72 h) for glucose analysis after centrifuging at
139 13000 g for 5 min. Each data point is the average of two analyses. The data from replicate runs
140 were used to calculate the mean value and standard deviation used as error bars in plots.

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141 **2.4 Quasi-simultaneous enzymatic saccharification and combined fermentation**

142 **(SSCombF)**

143 Quasi-simultaneous enzymatic saccharification and combined fermentation (SSCombF)

144 of the enzymatic hydrolysate of the pretreated lodgepole pine solids and spent liquor were

145 carried out in 250 mL Erlenmeyer flasks using a shaking incubator (Thermo Fisher Scientific,

146 Model 4450, Waltham, MA) at three levels of unwashed solids mass fractions, 12, 15, and 18%.

147 The matched amount of pretreatment spent liquor determined based on the collected amounts

148 of unwashed solids and liquor based on their respective yields from pretreatment was added to

149 the flasks. The mixture (or the pretreated lodgepole pine whole slurry) was adjusted to pH 6.2

150 with solid calcium hydroxide. Acetic acid/sodium acetate buffer (0.05 mole L^{-1}) of pH 5.5 was

151 added into the pH adjusted mixture to liquefy the water insoluble solids using CTec2 at 20 FPU

152 g^{-1} glucan (doubled the amount used in saccharification study discussed above). Elevated pH

153 5.5 is again used to reduce nonproductive cellulase binding to lignin and enhance

154 saccharification [20-22]. Liquefaction of solid substrate was observed in about 18-22 h at 50°C

155 and 200 rpm. The mixture was then cooled down to 35°C and the shaker speed was reduced to

156 90 rpm and inoculated with 2 mL of yeast seed. The initial optical density of the yeast for all

157 fermentation experiments was controlled at OD_{600} = 5. No nutrients were applied during

158 fermentation. Aliquot samples of fermentation broth were taken periodically for

159 monosaccharides, inhibitor, and ethanol analyses. Reported results are the average of

160 duplicate analyses. Replicate fermentation runs were conducted to ensure experimental

161 repeatability. The standard deviations were used as error bars in plotting.

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162 **2.5 Analytical methods**

163 The chemical compositions of the untreated and pretreated lignocelluloses were analyzed
164 as described previously [15]. All lignocellulosic samples were Wiley milled (Model No. 2,
165 Arthur Thomas Co, Philadelphia, PA, USA) to 20 mesh (~1 mm) and hydrolyzed in two stages
166 using sulfuric acid volumetric concentration of 720 mL L^{-1} at 30 °C for 1 h and 36 mL L^{-1} at
167 120 °C for 1 h. Carbohydrates of the hydrolysates were analyzed by high performance anion
168 exchange chromatography with pulsed amperometric detection (ICS-5000, Dionex). Klason
169 lignin (acid insoluble) was quantified gravimetrically [23]. For fast analysis, glucose in the
170 enzymatic hydrolysates were measured using a commercial glucose analyzer (YSI 2700S, YSI
171 Inc., Yellow Springs, OH, USA).

172 The pretreatment spent liquor and fermentation broth samples were analyzed using a
173 Dionex HPLC system (Ultimate 3000) for glucose, mannose, xylose, arabinose, and galactose
174 using an RI (RI-101) detector and BioRad Aminex HPX-87P column (300×7.8 mm) operated
175 at 80°C. Double distilled water (d.d.w.) was used as eluent at a flow of 0.6 mL min^{-1}. The
176 same HPLC was used to analyze fermentation inhibitors (acetic acid, furfural and
177 5-Hydroxymethylfurfural (HMF)) and ethanol using a UV detector (VWD-3400RS) and a
178 BioRad Aminex HPX-87H column (300×7.8 mm) operated at 60°C. Diluted sulfuric acid
179 solution of 0.005 mole L^{-1} was used as eluent at a flow rate of 0.6 mL min^{-1}. Samples were
180 centrifuged at 13000 g for 5 min. The supernatant was diluted by deionized water, and then
181 filtrated by a 0.22 μm filter prior to injection to the column. All sample injection volume was
182 20 μL.

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183 **2.6 LS purification and separation and LS dispersion properties**

184 Lignin purification and separation was achieved through dialysis using Spectra/Pro®
185 Biotech Cellulose Ester Dialysis Membrane bag (MWCO: 100-500 Da, Spectrum Laboratories,
186 Inc., Rancho Dominguez, CA). The dispersion property of different LS was evaluated by
187 examining the stability of TiO_2 particle in a dilute aqueous solution using a Turbiscan Lab
188 Expert (Formulaction, France). Detailed procedure for LS separation and dispersion
189 measurements can be found in our previous study [14].

190 **2.7 Gel permeation chromatography (GPC)**

191 Aqueous GPC was conducted using columns Ultrahydrage™ 120 and Ultrahydrage™ 250
192 and a UV detector 2487 at 280 nm (Waters Corp., Milford, MA). Polystyrene sulfonate with
193 molecular weight ranged from 500 to 10,000 g $mole^{-1}$ was used for calibration. A 0.10 mole
194 L^{-1} $NaNO_3$ solution was used as eluent at 0.50 mL min^{-1}. All LS samples after dialysis
195 (M_w<500), filtrated by a 0.22 μm filter, were used for GPC analysis.

196 **2.8 IR and ^{1}H-NMR analyses**

197 Infrared (IR) spectra of LS were recorded between 4000 and 400 cm-1 with 32 scans on a
198 Nexus spectrometer (Thermo Nicolet, USA). Disks were prepared by mixing 2 mg of dried LS
199 (after dialysis) with 200 mg KBr (for spectroscopy) in an agate mortar.

200 The ^{1}H-NMR spectra of LS were recorded with 30 mg LS (after dialysis) dissolved in 0.5
201 mL of dimethylsulfoxide (DMSO-d6) using a Bruker DRX-400 (maker and manufacture)
202 spectrometer at 400 MHz.

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204 # 3. Results and Discussion

205 ## 3.1 Comparison of wood component recovery

206 Wood chips were pretreated at 165°C for 75 minutes or 180°C for 25 minutes with the
207 same chemical loadings; these two reaction conditions were chosen because they sum to the
208 same combined severity factor. The pretreated wood chips were pressed and recovered as
209 unwashed solids and spent liquor. Total solids recovery as initial untreated wood chip mass
210 fraction was 96.6 and 85.7% for pretreatment conducted at 165°C and 180°C, respectively.
211 Most of the total solids were retained with the unwashed wet solids because it contains all
212 water insoluble components and approximately two thirds (67%) of the pretreatment spent
213 liquor. High pretreatment temperature reduced the recovery of water insoluble solids, due to
214 increased dissolution, and slightly increased recovery of dissolved solids including
215 lignosulfonate (Table 1). Glucan recovery as wood glucan from the unwashed solids were 97
216 and 83% for pretreatment conducted at 165 and 180°C, respectively. The total recoveries of
217 individual saccharides following pretreatment at 165°C were approximately 10% more than
218 the respective value from pretreatment conducted at 180°C.

219 The HMF and furfural production due to carbohydrate degradation as mass fractions of
220 wood xylan and mannan were 5.2 and 7.6% at 165°C, respectively, which is significantly
221 lower than 9.6 and 11.1% at 180°C. The ratio of the measured furan (HMF + furfural)
222 concentration between 180°C and 165°C is 1.64, close to the predicted value of 1.75 as
223 calculated using the kinetic model developed previously [10].

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225 **3.2 Comparison of enzymatic saccharification of washed solids**

226 Substrate enzymatic digestibility (SED) for each pretreated sample was measured under

227 standard conditions using washed solids at 20 g L^{-1} solids loading, and defined as the

228 percentage of substrate glucan enzymatically saccharified to glucose. While these conditions

229 deviate from process conditions, they allow for comparison with literature values. SED at

230 72 h reduced from 87% to 75% when pretreatment temperature was reduced from 180°C to

231 165°C (Fig. 1). This suggests slightly reduced delignification and dissolution of

232 hemicelluloses at 165°C negatively impacted *SED* even though these two pretreatment were

233 conducted at the same severities of *CHF* = 22.5.

234 **3.3 Comparison of fermentation performance**

235 The whole slurries produced from the wood chips were converted to ethanol using

236 enzymatic liquefaction followed by simultaneous saccharification and combined fermentation

237 (SSCombF). At 12% solids, pretreatment at the higher temperature (180°C) facilitated

238 greater cellulose digestion during the liquefaction step than 165ºC. The initial glucose

239 concentrations following liquefaction were 52 g L^{-1} and 44 g L^{-1} for the higher and lower

240 temperatures, respectively (Fig. 2a, yeast added at t = 0). Within the first 18 h of fermentation,

241 the initial glucose had been consumed and glucose concentration was constant for both runs;

242 indicating that the rate of glucose release and consumption were equal thereafter. The

243 higher initial glucose concentration resulted in improved ethanol production throughout the

244 SSCombF. A final ethanol concentration of 32 g L^{-1} was obtained from the slurry pretreated

245 at 180°C compared with 29 g L^{-1} for 165°C. The average glucose consumption and ethanol

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246 production at 24 h were -2.48 and 1.01 g L^{-1} h^{-1}, respectively, for the slurry produced at
247 180°C compared with -2.27 and 0.89 g L^{-1} h^{-1} for the slurry produced at 165°C (Table 2).
248 However, the average mannose consumption in the 24 h for the slurry produced at 180°C was
249 -0.45 g L^{-1} h^{-1}, only slightly lower -0.54 g L^{-1} h^{-1} for the slurry produced at 165°C (Table 2) as
250 can be clearly seen from Fig. 3a. The HMF and furfural concentrations in the slurry from
251 180°C were consistently higher than those in the slurry from 165°C until all the furans were
252 metabolized by YRH400 (Fig. 4a is shown in logarithmic scale for clarity).
253 At 15% solids loading, initial glucose production in SSCombF slurries from the 180°C and
254 165°C pretreatments were similar (Fig. 2b). Initial glucose concentrations following
255 liquefaction were approximately 60 g L^{-1} for both slurries. Glucose consumption and
256 ethanol production for fermenting the slurry pretreated at 165°C were -2.61 g L^{-1} h^{-1} and 1.06
257 g L^{-1} h^{-1}, respectively, slightly higher than the corresponding values for the slurry from 180°C,
258 -2.27 g L^{-1} h^{-1} and 0.87 g L^{-1} h^{-1} (Table 2). This reversed the trend observed from the
259 fermentation runs at 12% solids loadings, suggesting the increased furan concentrations may
260 have slowed ethanol production for the slurry from 180ºC pretreatment. Furthermore, both
261 glucose consumption and ethanol production for the slurry pretreated at 165°C in the first 24
262 h were higher than the corresponding value for fermentation of the slurry from 180°C at 15%
263 (Table 2). No obvious differences in mannose consumption between the two fermentations
264 were observed (Fig. 3b). The same final ethanol concentration of approximately 38 g L^{-1}
265 was produced from both slurries after 96 h fermentation.
266 When the solids loading was increased to 18% in SSCombF, the advantage of pretreating

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267 at 165°C over 180°C became readily apparent. Both glucose consumption and ethanol
268 production rates for the slurry produced at165°C are significantly higher than the
269 corresponding value for the slurry produced at 180°C (Fig. 2c). For the slurry produced at
270 180°C, the average glucose consumption within the first 24 h was reduced from -2.27 to
271 -0.76 g L^{-1} h^{-1} or by almost 70%. The average ethanol production within the first 24 h was
272 reduced from 0.87 to 0.40 g L^{-1} h^{-1} or more than 50%. However, for the slurry produced at
273 165°C, both glucose consumption and ethanol productivity were essentially unchanged when
274 fermentation solids loading was increased for 15 to 18%. Mannose consumption was also
275 significantly reduced when fermenting the slurry pretreated at 180°C versus 165°C (Fig. 3c);
276 the average mannose consumption rates within the first 24 h were -0.14 and -0.54 g L^{-1} h^{-1} for
277 the slurries from 180ºC and 165ºC, respectively (Table 2). Both fermentation runs have a
278 lag phase in mannose consumption of 8 and 6 h for slurry from 180°C and 165°C
279 pretreatment, respectively. The same terminal ethanol concentration of approximately 47 g
280 L^{-1}, however, was produced from both slurries.

281 No significant difference in xylose consumption was observed between fermenting the
282 slurry from 180 and 165°C at all solids levels as shown in Figs. 3a-3c, perhaps due to the low
283 xylose concentration of below 7 g L^{-1}. *Saccharomyces* yeasts do not express xylose-specific
284 transporters and uptake of xylose into the cell is inefficient, especially at low xylose
285 concentrations. Additionally, xylose fermentation is severely inhibited by acetate used in
286 the buffer solution. It does not take much acetate to cause problems (<2 g L^{-1} h^{-1}). The
287 average rates of furan metabolization were approximately the same for all fermentation runs

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288 (Table 2 and Figs 4a-4c).

289 **3.4 Comparison of ethanol yield**

290 Ethanol yield in terms of g per g sugar in the pretreated whole slurry as well as liter per
291 tonne wood base were calculated as shown in Table 2. The results indicate that improved
292 substrate digestibility by using a higher pretreatment temperature of 180°C increased the
293 ethanol yield (g per g sugar) at a low SSCombF solids loading of 12%, i.e., 0.43 compared
294 with 0.39 for the slurry pretreated at 165°C. This advantage disappeared at the higher solids
295 loading of 18% most likely due to the elevated level of fermentation inhibitors. Higher
296 temperature pretreatment at 180°C also reduced carbohydrate yield (Table 1) compared with
297 that at 165°C, which negatively impacted ethanol yield in terms of liter per tonne wood.
298 The ethanol yield in L $tonne^{-1}$ wood from the 180°C pretreated slurry is consistently lower
299 than that from the 165°C at all three solids fermentation loadings. A maximum ethanol
300 yield of 306 L $tonne^{-1}$ wood and final titer of 47.1 g L^{-1} was achieved when SSCombF was
301 conducted at 18% unwashed solids. Examining the time-dependent furan concentration
302 profile, a further increase in solids loading for SSCombF is probably feasible as both HMF
303 and furfural were completely metabolized within 48h. Using the furan level for the slurry
304 from 180°C pretreatment as the limit for effective fermentation without detoxification, we
305 can estimate the maximum limit for SSCombF solids loading of the slurry produced at165°C
306 to be approximately 30%. A potential ethanol titer of 75 g L^{-1} can be achieved *without*
307 *detoxification* assuming a similar fermentation efficiency.

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309 **3.5 Comparison of LS molecular weight distribution**

310 The dissolved lignin in spent liquor is in the form of LS, a directly marketable co-product.
311 The sulfonic acid group content and molecular weight (*MW*) are two properties that can affect
312 the performance of LS for a given application. The LS from SPORL-pretreated lodgepole
313 pine at 165ºC, LS-SP165, and at 180ºC, LS-SP180, both have very high sulfonic acid group
314 contents as measured by sulfur content (Table 3). The sulfur content of LS-SP180 is much
315 higher than that of the high purity commercial softwood LS D-748. However, both LSs from
316 SPORL pretreatment have much smaller molecular weights than the commercial product (Fig.
317 5). Pretreatment at 165ºC for 75 min produced a LS with a higher MW than that produced at
318 180ºC for 25 min. The peak MW of LS-SP165 is approximately 10% of that of D-748.

319 **3.6 Spectral characteristics LS from SPORL**

320 Infrared (IR) spectroscopy is a useful technique for studying lignin structure. The IR
321 spectra of the LSs from SPORL spent liquor along with high purity commercial LS D-748 are
322 shown in Fig. 6a. Based on literature assignments [24-26], the broad band at 3440 cm^{-1} is
323 attributed to the O-H stretching in phenolic and aliphatic structures. The bands at 2937 and
324 1465 cm^{-1} are C-H stretching in methyl and methylene of side chains. Weak shoulder peak at
325 1714 cm^{-1} is associated with the unconjugated carboxyl stretching; peak at 1642 cm^{-1} originates
326 from conjugated carboxyl stretching. The peak at 1515 and 1420 cm^{-1} are attributed to
327 aromatic skeletal vibrations. However, there is no obvious vibrations characterization for
328 guaiacyl (G) and syringyl (S) ring, such as the G ring and C=O stretch around 1270 cm^{-1}, the S
329 ring around 1328 cm-1. A shoulder of the G ring and C=O stretch around 1270 cm^{-1} can be

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330 seen form the D-748 spectrum. The peaks at 1140 and 1113 cm^{-1} arise from the C-H in-plate
331 deformation in G ring and S ring, respectively. Moreover, the carbohydrate arising vibrations
332 are associated with other vibrations in the region of 1300-500 cm-1. The peak at 1042 cm^{-1} is
333 attributed to the S=O stretching, C-H deformation and C-OH bending. The peak at 621 cm^{-1} is
334 due to C-S stretching. All peak intensities for LS-SP165 from 165°C SPORL pretreatment are
335 lower than those for LS-SP180 from 180°C pretreatment. The commercial LS D-748 that has
336 much sharper peaks at 1515 and 1420 cm^{-1} from aromatic skeletal vibrations, as well as at 1642
337 cm^{-1} from conjugated carboxyl stretching than LSs from SPORL. The peaks at 1140 and 1113
338 cm^{-1} of D-748 spectrum from the C-H in-plate deformation in G ring and S ring were shifted to
339 high wavenumbers.

340 The ^{1}H-NMR spectra were obtained from the same amount of samples (30 mg LS in 0.5
341 mL DMSO-d_6). The signal strength in Table 4 are normalized by the signal of DMSO-d_6
342 (2.56~2.44 ppm). As shown in Fig. 5 and Table 4, the resonance between 7.06 and 6.78 ppm
343 and between 6.78 and 6.41 ppm is attributed to aromatic protons in guaiacyl (G) and syringyl
344 (S) units, respectively. The signal strength represents the presence of relative contents of S
345 and G units [27]. The low resonance of methoxyl protons between 3.58-3.16 ppm for the D-748
346 spectrum suggests the difference in demethylation reactions of aromatic methoxyl groups
347 between sulfite pulping and SPORL pretreatment. The signal at 5.00-4.35 ppm, assigned to
348 the H_α and H_β in β-O-4 units [28], is decreased as pretreatment temperature increases, suggesting
349 increased cleavage of β-O-4 linkages at high temperatures. Although the cleavage of α-aryl
350 ether bonds is mainly responsible for fragmentation of lignin, acid-induced hydrolysis of

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351 arylglycerol-β-arylether substructures also exists during sulfite pulping [29, 30]. This can be
352 clearly seen from the very low signals between 5.00-4.35 for D-748 from sulfite pulping. The
353 signal between 5.75-5.45 ppm and 3.20-2.90 ppm is attributed to the H_α and H_β in β-5 and H_β in
354 β-β structure, respectively. Lignin condensation reactions can result in the formation of
355 diphenylmethane substructures involving C-1 or C-6 and C-5 of aromatic nuclei, while some
356 substructure in lignin can also undergo internal condensation to form a cyclohexene ring, such
357 as β-O-4 and β-β units [31]. Apparently, lignin condensation occurred in SPORL pretreatment
358 but pretty much absent in sulfite pulping due to low temperature based on the signal strength
359 between 5.75-5.45 ppm and 3.20-2.90 ppm (Table 4). It is also possible that the large MW of
360 D748 may prevent differentiation of lignin structure using ^{1}H-NMR.

361 **3.7 Dispersibility of LS from SPORL**

362 LS is used as a dispersant in many commercial applications. We compared the
363 dispersion properties of the two LSs from SPORL pretreatment with that of commercial LS
364 D-748 by evaluating the mean particle size of TiO_2 in an aqueous solution. Without the
365 application of LS (control), TiO_2 mean particle size increased linearly with time due to
366 agglomeration as shown in Fig. 7. The increase was approximately 50%, from 17 to 25 μm
367 in 60 min. The mean particle size was increased only by 5.5% and 8.6 % with the
368 application of LS-SP180 and LS-SP165, respectively, in the same 60 min period. These
369 increments are lower than the 14.1% achieved applying a high purity commercial LS D-748.
370 More importantly, the initial particle size were 2.6 and 2.8 μm with the application of
371 LS-SP180 and LS-SP165, respectively, smaller than the 4.5 μm when applying D-748.

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372 These results indicate that LSs from the present SPORL pretreatments have better dispersion
373 properties than high purity commercial softwood LS D-748 despite their smaller molecular
374 weight.
375

376 ## 4. Conclusions

377 At similar pretreatment severities, as determined by the combined hydrolysis factor (CHF)
378 for hemicellulose dissolution, a lower temperature pretreatment at 165°C with a longer
379 duration of 75 min produced less sugar degradation products such as furan compared with
380 pretreating at 180°C for 25 min. The lower temperature SPORL pretreatment at 165°C
381 produced a solid substrate with slightly less enzymatic digestibility as measured by enzymatic
382 cellulose saccharification using 2% washed solids compared with the substrate produced at
383 180°C. As a result, glucose consumption and ethanol production was reduced when
384 simultaneous enzymatic saccharification and combined fermentation (SSCombF) of the
385 pretreated whole slurry was conducted at low unwashed solids loadings of 12%. However, the
386 reduced amount of furan formation associated with low pretreatment temperature using
387 SPORL facilitated SSCombF at high solids loadings of 18%. SPORL pretreatment at 165°C
388 also produced a higher carbohydrate yield than that at 180°C and an overall higher ethanol
389 yield per tonne wood at all solids loadings. SSCombF at 18% solids loading produced an
390 ethanol yield of 306 L/tonne wood at titer of 47.1 g/L without detoxification using a lodgepole
391 pine tree killed by mountain pine beetles. Lignosulfonates (LSs) produced by SPORL at 180
392 and 165°C are highly sulfonated though they have a lower molecular weight than a commercial

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393 high purity softwood LS. LSs from SPORL treated wood chips had better dispersity than the
394 commercial LS from softwood.
395

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Fig. 2 Time-dependent glucose consumption and ethanol production during simultaneous enzymatic saccharification and combined fermentation (SSCombF) of SPORL-pretreated lodgepole pine whole slurry at three solids loadings. Comparison between two substrates produced at 180ºC and 165ºC. Unwashed solids loadings: (a) 12%; (b) 15%; (c) 18%

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Table 1. Chemical composition of the untreated lodgepole pine BD4 wood chips and wood component recoveries from SPORL pretreatments at two temperatures. Pretreatments were conducted at sulfuric acid and sodium bisulfite loading on wood are 2.2 and 8%, respectively, for 25 and 75 min at 180°C and 165°C, respectively.

	Untreated wood[d]	**Unwashed solids** [a]		**Collected Spent Liquor** [a]		**Total recovery (%)**	
Temperature (°C)		**165**[d]	**180**	**165**[d]	**180**	**165**[d]	**180**
Wet weight (kg)	2.281	6.227	5.620	1.726	2.250		
Solids content (%)	87.7	31.86	29.98	8.37	9.09		
Solids (kg) [b]	2.0	1.984; 90.0%	1.685; 76.4%	0.144; 6.6%	0.205; 9.3%	96.6%	85.7%
Klason lignin (%)	28.6	28.31; 99.0%	24.45; 85.5%	0.29; 1.0%	4.15; 14.5%	100.0%	100.0%
Arabinan (%)	1.7	0.58; 34.2%	0.39; 23.0%	0.06; 3.3%	0.07; 3.9%	37.6%	26.9%
Galactan (%)	2.9	1.43; 49.3%	1.04; 35.9%	0.40; 13.9%	0.55; 18.9%	63.1%	54.8%
Glucan (%)	41.9	40.64; 97.0%	34.68; 82.8%	0.60; 1.4%	1.03; 2.5%	98.4%	85.2%
Mannan (%)	11.7	5.98; 51.1%	4.21; 36.0%	1.70; 14.5%	2.39; 20.4%	65.6%	56.4%
Xylan (%)	5.5	2.86; 52.0%	1.87; 33.9%	0.53; 9.7%	0.70; 12.7%	61.6%	46.6%
HMF (%) [c]		0.43; 3.7%	0.72; 6.1%	0.18 (1.7); 1.5%	0.41 (3.0); 3.5%	5.2%	9.6%
Furfural (%) [c]		0.31; 5.7%	0.40; 7.3%	0.13 (1.1); 2.3%	0.23 (1.6); 4.2%	8.0%	11.5%
Acetic acid (%)		1.87	2.05	0.76	1.17		

[a] The numbers after ";" is wt% of theoretical based on untreated wood carbohydrate content
[b] in oven dry (od) weight
[c] reported as of xylan and mannan for furfural and HMF, represent percent of xylan and mannan converted to furfural and HMF, respectively. The numbers in the parenthesis are concentration measured in the collected spent liquor in g/L
[d] compositional data for wood that was untreated and SPORL pretreated at 165°C are reported previously [14]

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Table 2. Comparisons of the performance of SSCombF at three solids loadings between two lodgepole pine BD4 whole slurry samples pretreated at 180 and 165°C.

SSCombF solids (%)	12%		15%		18%	
Pretreatment T (°C)	165	180	165	180	165 [c]	180
Average fermentation performance measure in the first 24 h unless indicated (g/L/h)						
Ethanol Productivity	0.89	1.01	1.06	0.87	1.03	0.40
Glucose consumption	-1.94	-2.48	-2.61	-2.27	-2.10	-0.76
Mannose consumption	-0.54	-0.45	-0.41	-0.40	-0.54	-0.14
HMF metabolization	-0.056 (18h)	-0.063	-0.039	-0.061	-0.051	-0.046
Furfural metabolization	-0.059 (2h)	-0.088 (5h)	-0.059 (5h)	-0.070 (8h)	-0.058 (5h)	-0.060 (8h)
Terminal maximal ethanol production						
Ethanol concentration (g/L)	29.1 ± 0.4	32.4 ± 1.2	36.0 ± 1.7	38.0 ± 0.3	47.1 ± 2.1[c]	47.2 ± 1.8
Ethanol yield (g/g sugar) [a]	0.391 ± 0.005	0.431 ± 0.017	0.375 ± 0.045	0.393 ± 0.003	0.396 ± 0.018	0.394 ± 0.015
Ethanol yield (L/tonne wood)	302 ± 4	285 ± 11	290 ± 13	260 ± 2	306 ± 14	260 ± 10
Ethanol yield (% theoretical) [b]	70.8 ± 0.9	67.0 ± 2.6	68.0 ± 3.1	60.9 ± 0.5	71.8 ± 3.3	61.1 ± 2.3

[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) based on total glucan, mannan, xylan in the untreated wood
[c] data appearing in this subcolumn was earlier published previously as part of a different study [14].

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Table 3. Comparisons of elemental contents between a high purity commercial softwood LS and LSs from SPORL-pretreated lodgepole at 180 and 165ºC.

LS	N (%)	C (%)	H (%)	Sulfur (wt%)
LS-SP180	< 0.14	24.99 ± 0.050	3.44 ± 0.003	11.33 ± 0.32
LS-SP165	0.51 ± 0.121	41.93 ± 0.392	5.15 ± 0.090	6.04 ± 0.33
D-748	0.12 ± 0.006	44.07 ± 0.032	5.45 ± 0.051	6.01 ± 0.39

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Table 4. Signal assignment of ^{1}H-NMR spectra of LSs

Signal (ppm)	Assignment	LS-SP180	LS-SP165	D-748
7.06~6.78	H_2, H_5, H_6 in guaiacyl (G) units	0.11	0.71	0.43
6.78~6.41	H_2, H_6 in syringyl (S) units	0.47	1.12	0.84
5.75~5.45	H_α, H_β in β-5' substructures	0.07	0.61	-
5.00~4.75	H_α in β-O-4' structures	1.95	2.65	0.13
4.75~4.35	H_β in β-O-4' structures	1.2	1.8	0.25
3.58~3.16	H in methoxyls	8.01	8.43	6.7
3.20~2.90	H_β in β-β' structures	1.85	1.36	-
2.56~2.44	DMSO	1	1	1
2.15~2.00	H in aromatic acetates	0.47	0.33	0.32
1.97~1.71	H in aliphatic acetates	0.82	0.51	0.49
1.62~0.75	Aliphatic H	1.53	1.34	1.2

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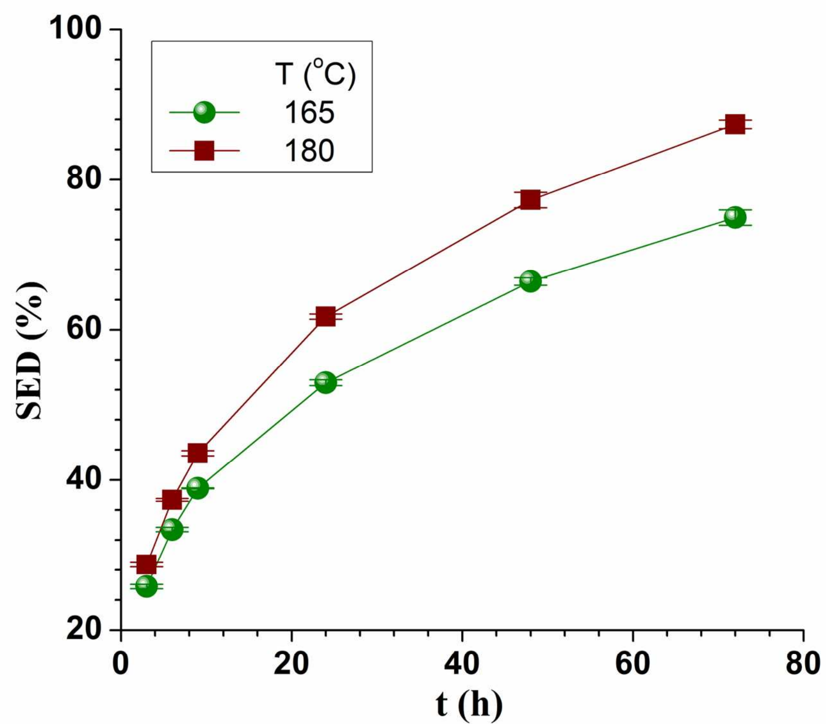

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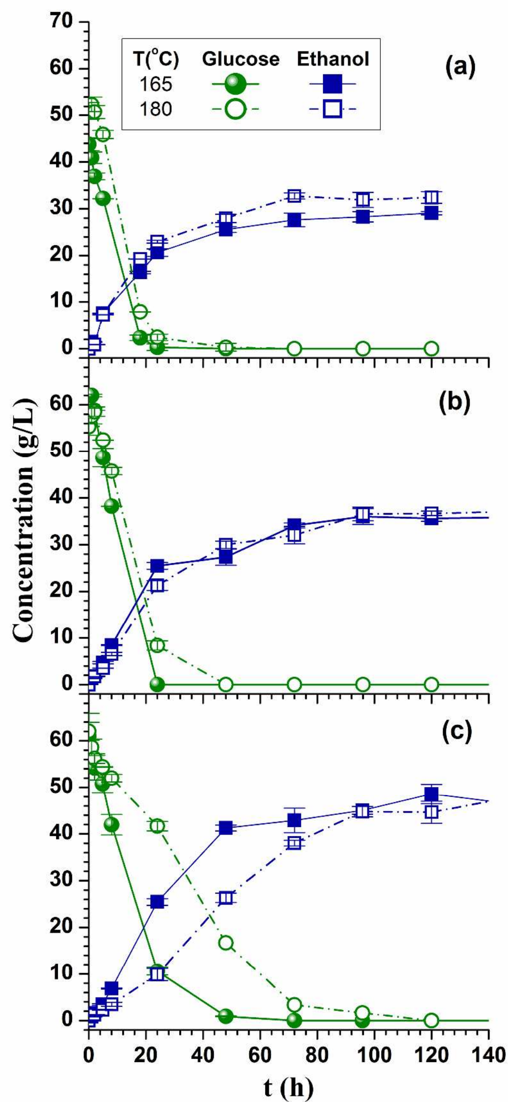

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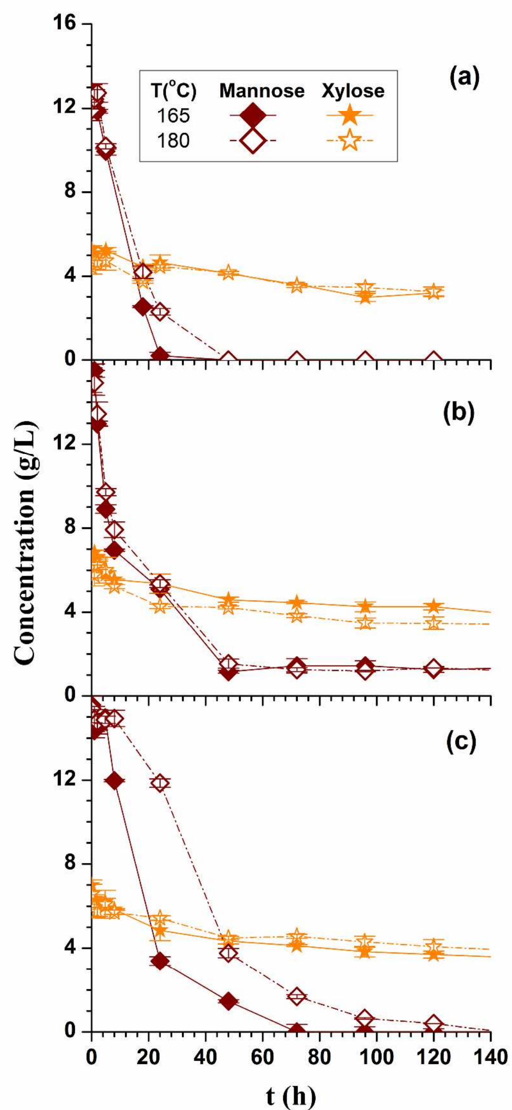

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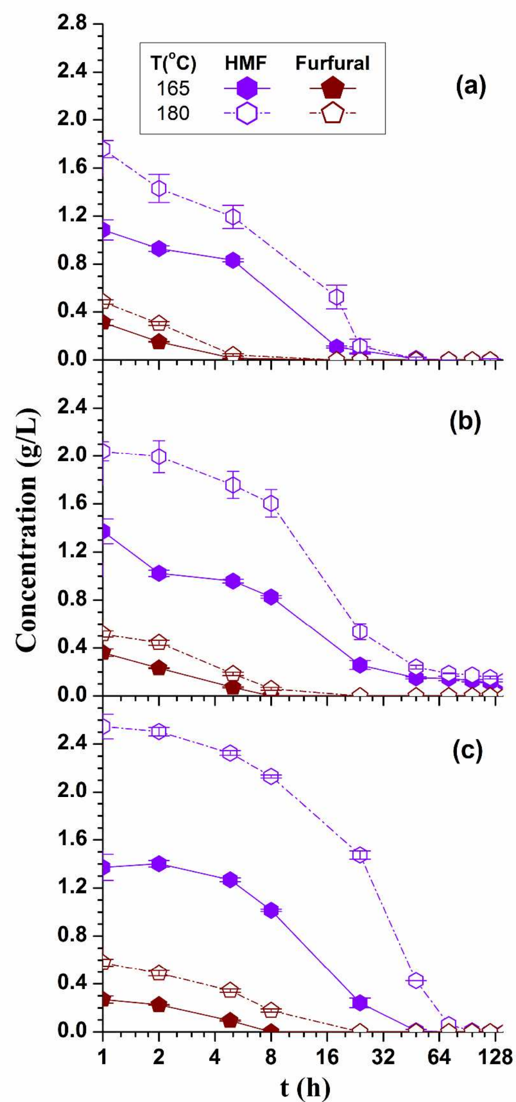

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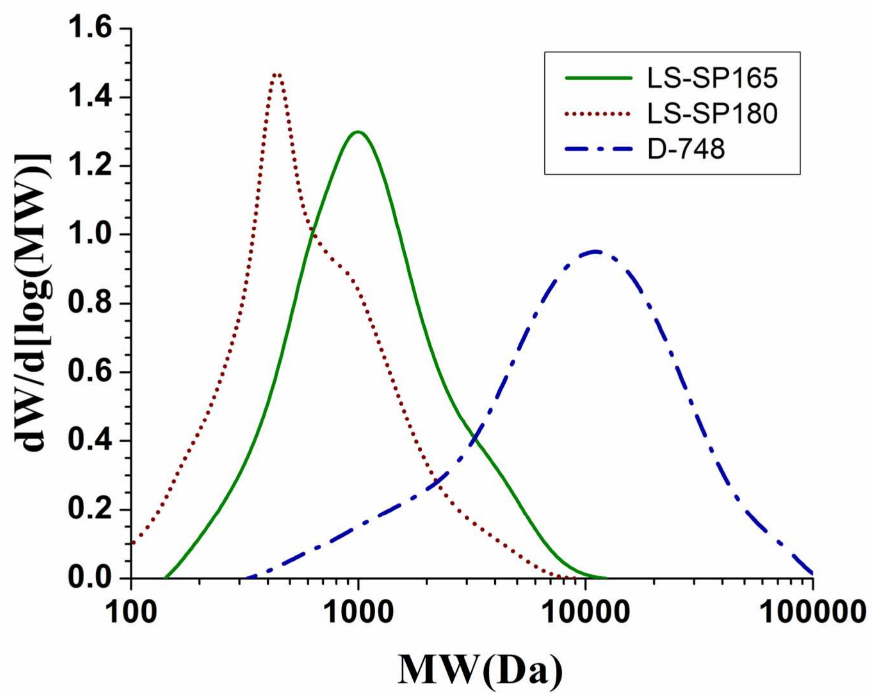

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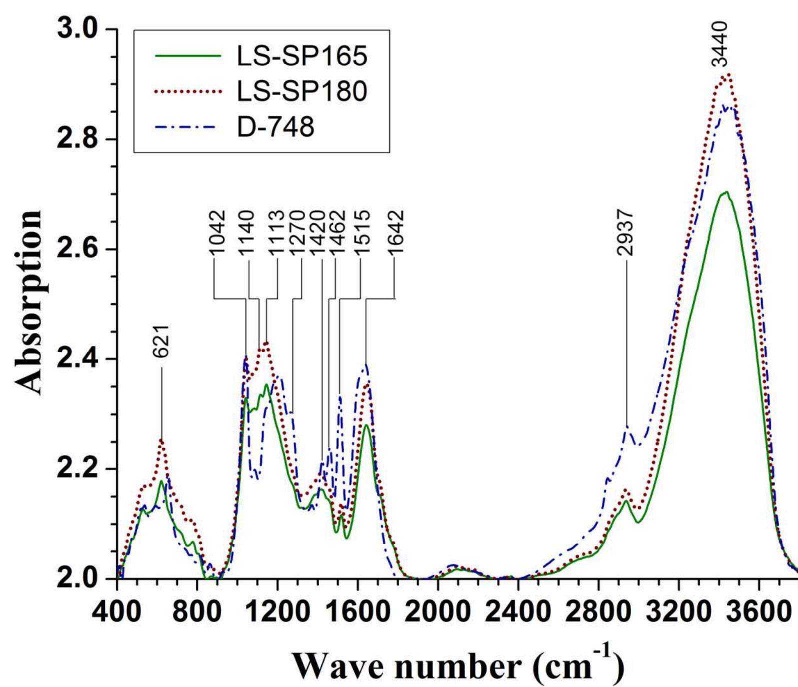

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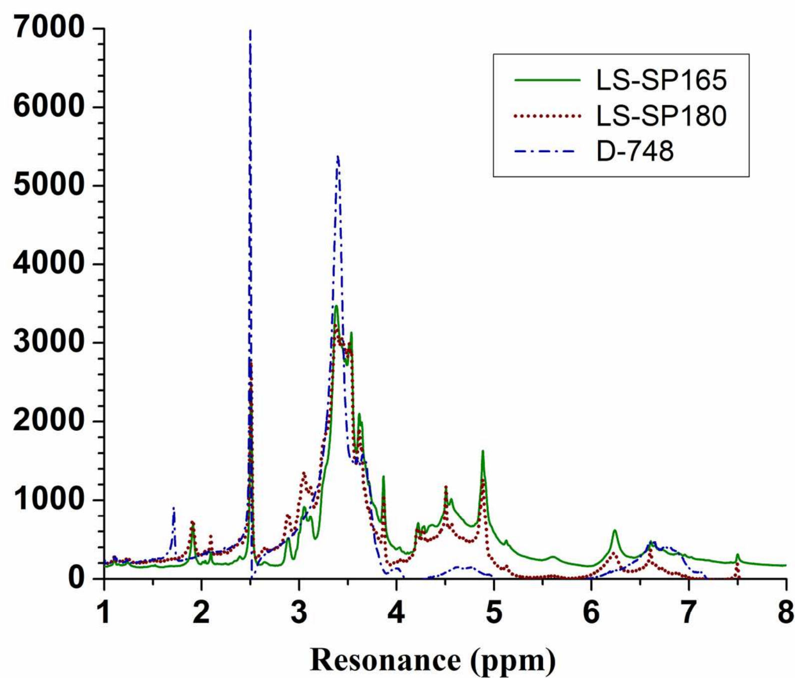

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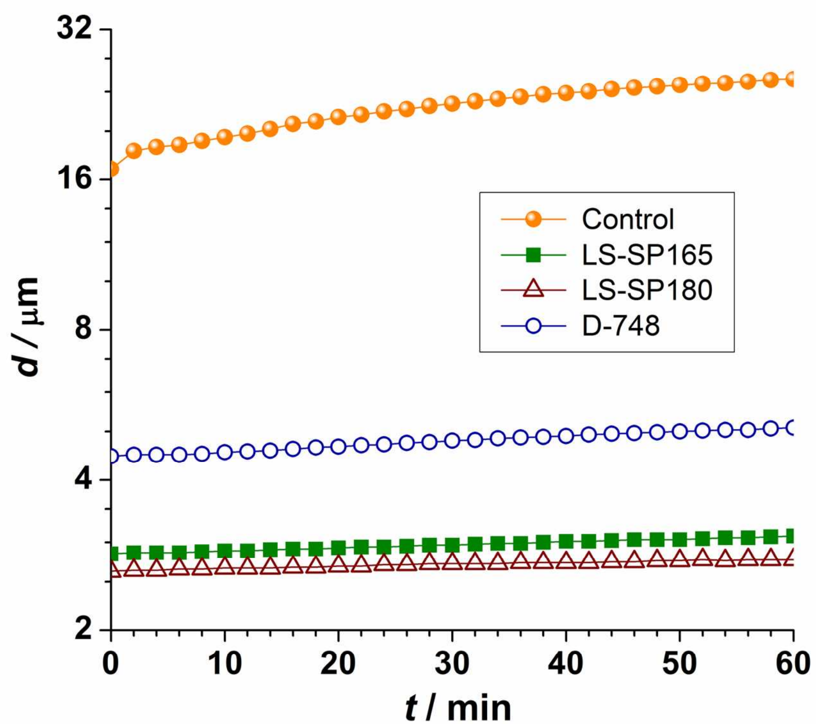

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