

1 **Revealing the mechanism of lignin re-polymerization inhibitor in**
2 **acidic pretreatment and its impact on enzymatic hydrolysis**

3 Authors:

4 Juan He¹, Caoxing Huang², Chenhuan Lai², Zhongkai Wang^{1*}, Liang Yuan¹, Arthur
5 Ragauskas^{3,4,5}, Youxian Yan¹, Qiang Yong^{2*}

6

7 Affiliation:

8 1: Biomass Molecular Engineering Center, School of Forestry and Landscape

9 Architecture, Anhui Agricultural University, Hefei, 230036, China

10 2: Co-Innovation Center for Efficient Processing and Utilization of Forest Resources,

11 College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037,

12 China

13 3: Department of chemical and Biomolecular Engineering, The University of

14 Tennessee, Knoxville, TN, 37996, USA

15 4: Department of Forestry, Wildlife and Fisheries, Center for Renewable Carbon, The

16 University of Tennessee Institution of Agriculture, Knoxville, TN, 37996, USA

17 5: Joint Institute for Biological Sciences, Bioscience Division, Oak Ridge National

18 Laboratory, Oak Ridge, TN, USA

19

20 Corresponding author:

21 * E-mail: swhx@njfu.com.cn (Q. Yong), Tel./fax: +86 25 85427471

22 * E-mail: wangzk6@ahau.edu.cn (Z. Wang)

23 **Abstract**

24 Phenolic additives, including 2-naphthol, 2-naphthol-7-sulfonate, and resorcinol were
25 evaluated for their impact on the lignin re-polymerization and subsequent hydrolysis
26 of bamboo residues. Low surface lignin concentration (65.68%) of the substrate and
27 high T_g value (185 °C) of lignin was observed for 2-naphthol-7-sulfonate assisted
28 pretreatment, leading to the most significant increase (14%) in enzymatic hydrolysis
29 efficiency of pretreated bamboo residues. Meanwhile, physicochemical properties of
30 lignin revealed that the introduced sulfonic acid groups increased the surface charge,
31 decreased the molecular weight, promoted the cleavage of β -O-4 linkages, and
32 prevented the re-condensation of lignin. Even though resorcinol-assisted pretreatment,
33 by contrast, was found to be an inhibitor for enzymatic hydrolysis. NMR analysis of
34 modified lignin showed that resorcinol led to a higher amount of non-condensed
35 phenolic OH groups and promoted the cleavage of β -O-4 linkages, acting as an
36 inhibitor in lignin re-polymerization during the dilute acidic pretreatment. In short, the
37 above findings present a detailed view of suppressing lignin re-polymerization for
38 boosting the utilization of bioresources with acidic pretreatment.

39 **Keywords:** Dilute acid pretreatment; Additives; Enzymatic hydrolysis; Lignin
40 reconstruction

41

43 **1. Introduction**

44 Lignocellulosic biomass is an abundant and sustainable resource, which is
45 gradually replacing depleted petrochemical resources to produce various bio-based
46 fuels, chemicals, and materials (Ragauskas et al., 2014). Bamboo residues, an
47 agricultural and forestry wastes residue, have the potential to be the feedstock for
48 biorefining operations (Huang et al., 2015). Among the methods of bioconversion,
49 enzymatic hydrolysis of lignocellulosic is considered to be a green and
50 environmentally acceptable method to gain small fermentable molecules, such as
51 glucose (Lai et al., 2018; Lin et al., 2019). However, the natural recalcitrant structure
52 of lignocellulose hinders accessibility of cellulase to cellulose, which results in low
53 efficient bioconversion (Zhao et al., 2021). Dilute acid (DA) pretreatment is well
54 known as an economical and operable method to deconstruct the compact
55 lignocellulose matrix, especially the effective removal of hemicellulose. After
56 pretreatment, the reactivity of cellulose in biomass residues is enhanced in
57 comparison to native biomass.

58 Nevertheless, lignin is hard to be removed with DA pretreatment, and the rate of
59 de-polymerization is slower than re-polymerization during the acid pretreatment,
60 which results in an increase in the molecule weight of lignin with respect to native
61 lignin (Cao et al., 2012; Araya et al., 2015; Bian et al., 2017). It's also reported that
62 the adsorption behavior between lignin and enzymes is enhanced with the increasing
63 molecule weight of lignin (Zhou et al., 2013; Pielhop et al., 2015). Therefore, it is
64 essential to alleviate the structural changes of lignin and provide a more suitable

65 condition for biomass with- acid pretreatment.

66 Native lignin is a three-dimensional amorphous polymer most often consisting of
67 monolignol monomers including syringyl units (S), guaiacyl units (G), and
68 *p*-hydroxyphenyl units (H). Ralph et al (2019) suggested that these monomers
69 radically couple and cross-coupled combinatorially to form the complex structure of
70 lignin. β -*O*-4 (40-65%), β -5 (10%), β - β (5%), β -1 (5%), 4-*O*-5 (5%) and 5-5 (5%)
71 couplings are considered to be the major inter-unit linkages (Adler., 1977; Ralph et al.,
72 2019; Boerjan et al., 2003; Kim et al., 2011). However, these lignin units or linkages
73 are not stable under hydrothermal or acidic pretreatment. Due to the lack of
74 methoxy groups at the C-5 position, G units are much easier to undergo
75 re-polymerization reaction than S units in autohydrolysis conditions (Wayman and
76 Chua., 1979). Trajano et al (2013) found a similar phenomenon for *P.trichocarpa* x *P.*
77 *deltoides* hydrolysates, there were more S units derivatives than G units under
78 flowthrough pretreatment. During the deconstruction of lignin units, the cleavage of
79 aryl-ether- β -*O*-4 bonds is considered to be the main de-polymerization reaction
80 (Araya et al., 2015). Li et al (2007) suggested that the increasing severity of steam
81 explosion (3~4.5) resulted in the decreasing amount of β -*O*-4 linkages (45%~10%).
82 Cao et al (2012) reported that with the time of dilute acid pretreatment extending to
83 26.8 min, the content of β -*O*-4 linkages in lignin decreased by ~53%. By contrast, β -5
84 and β - β bonds are always identified as condensed lignin linkages (Sun et al., 2016).
85 Cao et al (2012) suggested that after acidic pretreatment, the intensity of C_{α} -H, C_{β} -H,
86 and C_{γ} -H in HSQC spectra (β -*O*-4 bonds) have been significantly reduced, but the

87 correlation signal intensity of resinol (β - β) and phenlcoumaran (β -5) was observed to
88 be increased.

89 Researchers have suggested that lignin carbonium cations are formed in lignin
90 under acidic pretreatment, which is responsible for the depolymerization especially in
91 the cleavage of β -O-4 linkages (Sarkanen and Ludwig., 1971; Pielhop et al., 2015).
92 Santi et al (2021) employed a computational procedure to confirm that the acidolysis
93 of β -O-4 linkages follows three steps: (1) the protonation of the α -OH by H^+ ; (2) the
94 release of one molecular water; (3) the formation of a carbocation in $C\alpha$ position.
95 Moreover, the carbocation readily forms stable C-C bonds with lignin fragments
96 (Sarkanen and Ludwig., 1971; Pielhop et al., 2016). The newly formed C-C bonds
97 usually result in lignin re-condensation and enhance its hydrophobicity, which has
98 been shown to further increase undesirable non-productive binding with enzymes and
99 inhibit the efficiency of enzymatic hydrolysis (Sun et al., 2016).

100 Therefore, suppression of lignin re-condensation during its acidolysis is
101 necessary to enhance enzymatic saccharification of biomass. Research studies have
102 employed 2-naphthol and 2-naphthol-7-sulfonate in acidic pretreatment of hardwoods
103 or softwoods (Chu et al., 2021; Lai et al., 2018; Sheng et al., 2021). In the current
104 work, phenolic additives such as 2-naphthol, 2-naphthol-7-sulfonate were also applied
105 in the dilute acid pretreatment of bamboo residues. Resorcinol was investigated as an
106 alternative additive, under conditions, as it was suggested to be a re-polymerization
107 bridge than a blocking agent. The chemical composition and surface properties of
108 pretreated bamboo residues were analyzed to elucidate the impact of different

109 additives on the enzymatic hydrolysis of cellulose. Moreover, the structural
110 information of residual lignin from pretreated bamboo residues was characterized by
111 DLS, GPC, NMR, and DSC methods to correlate lignin structure with enzymatic
112 hydrolysis of cellulose.

113 **2. Materials and methods**

114 *2.1. Materials*

115 Bamboo residues (*Phyllostachys heterocycla*) was provided by He Qi Cang Bamboo
116 Processing Factory (Fujian, China). The air-dried samples were milled to a mesh size
117 of 20-80. The chemical components of bamboo residues were as follows (% dry
118 weight basis): 40.4% glucan, 24.3% xylan, and 31.6% total lignin. 2-naphthol,
119 2-naphthol-7-sulfonate, and resorcinol were purchased from Sinopharm Chemical
120 Reagent Co., Ltd. (Shanghai, China). Cellulase cocktail (L-100) was kindly provided
121 by You tell Biochemical Co., Ltd. (China) with a filter paper activity of 188.54 FPU/g.
122 β -glucoside with an activity of 237.52 U/g was purchased from Sigma-Aldrich
123 (Shanghai, China).

124 *2.2. Dilute acid pretreatment*

125 The pretreatment experiments were carried out in a rotary cooking system with ten
126 1.25 L autoclave bombs and an oil bath for heating. Specifically, 40 g dried bamboo
127 residues were cooked with 0.2% (w/w) sulfuric acid at a solid-liquid ratio of 1:10. The
128 amount of 2-naphthol, 2-naphthol-7-sulfonate, and resorcinol were added to the
129 biomass slurry and chosen according to the pre-experiments and Pielhop's study
130 (2016): 8%, 4%, and 2%, respectively (wt%, based on the dried weight of biomass).

131 The reaction mixture was pretreated at 170 °C for 30 min. After pretreatment, the
132 reactors were cooled in a cold water bath before the solids were separated from the
133 slurry and washed with DI water until the effluent was pH neutral. The obtained
134 substrates pretreated with the addition of 2-naphthol, 2-naphthol-7-sulfonate and
135 resorcinol were assigned as DA-N, DA-NS, and DA-R, respectively. The dilute acid
136 pretreatment of bamboo residues without phenolic additives was also carried out as a
137 control, and the product was defined as DA.

138 *2.3. Compositional analysis of pretreated bamboo residues*

139 The chemical compositional analysis of pretreatment bamboo residues was
140 determined according to the National Renewable Energy Laboratory (NREL) analysis
141 method (Sluiter et al., 2008). An HPLC system quantified sugar content in the
142 supernatants, using an Aminex HPX-87H column (300×7.8 mm) and a refractive
143 index (RI) detector. A 5 mM H₂SO₄ solution was used as the mobile phase and flowed
144 at a rate of 0.6 ml/min. All reported components of samples reported in this study
145 were tested in duplicate.

146 *2.4. Enzymatic hydrolysis of pretreated bamboo residues*

147 Enzymatic hydrolysis of samples was performed in a sodium citrate buffer (50 mM,
148 pH 4.8) at 50 °C with shaking at 150 rpm for 120 h. The concentration of substrate
149 was 2% (w/v) with cellulase and β-glucoside loadings of 40 FPU/g and 20 U/g,
150 respectively. Samples were respectively taken from the hydrolysis system at 3, 24, 48,
151 72, and 120 h. HPLC analysis was used to determine the concentration of glucose
152 with the same conditions as detailed in Section 2.3.

153 *2.5. Hydrophobicity test*

154 Hydrophobicity tests were performed on the bamboo residues recovered from
155 dilute acid pretreatment to measure their initial water contact angle. The pretreated
156 bamboo residues with or without cooking additives were milled to a mesh size of
157 20-80 and dried at 40 °C overnight before testing. The dried samples were pressed at a
158 pressure of 25 MPa for 1 min to form a disk. A deionized water droplet (13µL) was
159 employed for measuring contact angle on the sample's surface with Attension Theta
160 Lite (KSV Instruments, Helsinki, Finland). The average value of contact angle was
161 obtained by measuring 3 times within 5 sec.

162 *2.6. Zeta potential analysis*

163 The zeta potentials of bamboo residues at pH 4.8 were measured with DelsaMax PRO
164 (Beckman Coulter, America). The concentration of samples was 1 mg/ml and
165 ultrasonically dispersed in citrate buffer solution (pH 4.8) by ultrasonication for 60
166 min. After that, the suspension was left to stand for 60 min before using the
167 supernatant for testing. All zeta potential measurements were performed 5 times in
168 each analysis to ensure experimental repeatability and the averages are reported in this
169 work.

170 *2.7. X-ray photoelectron spectroscopy analysis*

171 The atomic composition of samples was quantified on the X-ray photoelectron
172 spectrometer (ThermoFisher, USA) equipped with a monochromatic Al K α X-ray
173 source, using a pass energy of 30.0 eV. The analyzed spot size was 400 µm and at
174 least scan 5 times on each sample. The surface lignin concentration of these four

175 samples was calculated as follows (Liu et al., 2017):

$$176 \quad \text{Surface lignin concentration} = \frac{O/C_{\text{sample}} - O/C_{\text{carbohydrates}}}{O/C_{\text{lignin}} - O/C_{\text{carbohydrates}}} \times 100\%$$

177 Where $O/C_{\text{carbohydrates}}$ is 0.83, O/C_{lignin} is 0.33.

178 *2.8. Lignin extraction and characterization*

179 *2.8.1. Isolation and purification of residual lignin*

180 Residual lignin samples were isolated from the four pretreated bamboo residues with
181 or without additives by ball milling and dioxane extraction, according to a classical
182 isolation method developed by Björkman (1956). Specific experimental procedures
183 were according to our previous work (He et al., 2020a).

184 *2.8.2. Zeta potential analysis*

185 The zeta potentials of lignin samples were measured at pH 4.8 as in section 2.6.

186 *2.8.3. ^{31}P and 2D NMR spectroscopic analysis*

187 ^{31}P and 2D nuclear magnetic resonance (NMR) analyses of the four isolated lignin
188 samples were acquired using Bruker AVANCE 600 MHz spectrometer. For ^{31}P NMR
189 analysis, all samples (40 mg) were separately dissolved in the stock solution of
190 pyridine/ CDCl_3 (v/v=1.6/1), then 200 μL of
191 *endo*-N-hydroxy-5-norbornene-2,3-dicarboximide (e-NHI) solution (9.23 mg/mL
192 acting as internal standard) and 50 μL of chromium (III) acetylacetonate solution (5.6
193 mg/mL acting as relaxation reagent) were added. Finally, 100 μL of 2-chloro-4, 4, 5,5
194 -tetramethyl-1, 2, 3-dioxaphospholane (serving as phosphitylating reagent) was added
195 to derivatize lignin samples. For 2D NMR analysis, all samples were separately
196 dissolved in 0.5 mL DMSO-d_6 . The acquisition parameters were as follows: 1024 data

197 points in the F_2 (^1H) dimension with 53 ms acquisition time, and 256 data points in the
198 F_1 (^{13}C) dimension with 5.14 ms. A coupling constant $^1J_{\text{C-H}}$ of 147 Hz was used.

199 *2.8.4. Thermal property analysis*

200 Differential scanning calorimetry (DSC) was performed on the lignin samples using a
201 DSC 200 F3 Maia[®] Instrument (NETZSCH Instruments) under a nitrogen atmosphere.
202 Samples (1-2mg) were equilibrated at room temperature and heated to 200 °C at the
203 rate of 50 °C/min. Then samples were melted at 200 °C for 3 min to remove thermal
204 history and then rapidly cooled down to -50 °C. Finally, samples were heated again to
205 250 °C at a heating rate of 10 °C/min to obtain the glass transition temperature (T_g) of
206 lignins.

207 **3. Results and discussion**

208 *3.1. Effects of additives on the chemical composition and enzymatic digestibility of* 209 *dilute acid pretreated bamboo residues*

210 Dilute acid pretreatment of bamboo residues was performed without or with the
211 addition of 2-naphthol, 2-naphthol-7-sulfonate, and resorcinol. The pretreated
212 products were defined as DA, DA-N, DA-NS, and DA-R in this work, respectively.
213 The detailed chemical composition of the pretreated substrate is shown in Table 1.
214 The delignification of DA was decreased from 34.4% to 15.5% and 8.3% with the
215 addition of resorcinol and 2-naphthol, respectively. Pielhop et al (2015) suggested that
216 during an autohydrolysis pretreatment with resorcinol and 2-naphthol, the removal of
217 lignin in spruce wood was not obvious, indicating that aromatic additives like
218 resorcinol and 2-naphthol would participate in the re-polymerization of lignin

219 fragment and inhibit the removal of lignin under autohydrolysis conditions. While
220 2-naphthol-7-sulfonate slightly promoted the delignification of bamboo residues from
221 34.4% to 35.8%, this effect was attributed to the increased water-solubility of lignin
222 modified with a sulfonic acid group (Lai et al., 2018).

223 Insert Table 1 here

224 To investigate the effects of dilute acid pretreatment with 2-naphthol,
225 2-naphthol-7-sulfonate, and resorcinol on enzymatic hydrolysis, enzymatic
226 digestibility of four pretreated bamboo residues (DA, DA-N, DA-NS, and DA-R) was
227 evaluated (Fig. 1.). After 120 h digestion, the glucose yield of DA was promoted from
228 33.59% to 37.63% for DA-N and 47.31% for DA-NS. Lai et al (2018) reported that
229 additives like 2-naphthol and 2-naphthol-7-sulfonate could decrease the adsorption
230 ratio of cellulase on acid pretreated wood sawdust. In addition, 2-naphthol-7-sulfonate
231 could increase the negative zeta potential and electrostatic repulsion force of substrate
232 (Table 2), suggesting that it could improve the efficiency of enzymatic hydrolysis
233 better than 2-naphthol. It is worth mentioning that the substrate pretreated with 8%
234 w/w (relative to biomass load) of 2-naphthol exhibited higher enzymatic hydrolysis
235 efficiency as compared to DA, even though its delignification ratio was much lower
236 as discussed earlier. The structure of residual lignin played a significant role during
237 the biomass enzymatic hydrolysis, and proper modification of lignin could achieve
238 higher efficiency of enzymatic hydrolysis. Fig.1. also shows that the 120 h glucose
239 yield of DA was decreased from 33.57% to 22.75% by adding 2% w/w (relative to
240 biomass load) of resorcinol, suggesting that additives like resorcinol with multiple

241 reactive sites for lignin substitutions could inhibit the enzymatic hydrolysis of
242 pretreated samples.

243 Insert Figure 1 and Table 2 here

244 *3.2. Effect of additives on the surface properties of dilute acid pretreated bamboo*
245 *residues*

246 The surface properties of pretreated substrate play an important role in enzymatic
247 hydrolysis. In this work, the surface chemical composition (Fig.2, Table S1, and
248 Fig.S3), Zeta potential, and hydrophobicity (Table 2) of pretreated bamboo residues
249 with different additives were characterized and compared. Fig.2 presented the XPS
250 C1s spectra for surface information of the pretreated substrates. It showed that
251 C2(C-OH, C-O-C) tend to be the predominant linkage in DA (31.49), DA-NS (32.16),
252 and DA-R (31.03) samples. However, DA-N has a slightly higher content of C1 (C-C,
253 C-H, C=C) linkage (31.17) than C2 linkage (30.24), indicating that there were less
254 exposed cellulose of DA-N than others (Nzokou and Kamdem, 2005; Ma et al., 2015).
255 As calculated in Table S1, DA-N has the highest surface lignin concentration
256 (74.95%), followed by DA-R (69.97%), DA (66.82%), and DA-NS (65.68%). This
257 phenomenon suggested that the high content of phenolic additives like 2-naphthol
258 (8wt%, based on dried biomass) might not promote lignin removal during the acid
259 pretreatment of bamboo residues. However, blocking more active sites of
260 depolymerized lignin fraction, can decrease the repolymerization reaction of lignin
261 and increase the efficiency of enzymatic hydrolysis. Theoretically, a higher O/C
262 reflects a higher cellulose or hemicellulose content, while a lower one suggests a

263 higher content of lignin (Ma et al., 2015). The O/C data in Table S1 confirmed that
264 with 2-naphthol or resorcinol additives, more lignin would be deposited on the surface
265 of pretreated residues (DA-N, DA-R). Besides, the S2p XPS spectra for all substrates
266 were examined and the relative sulfur concentration was calculated (Fig.S3). It shows
267 that only DA-NS present oxidized sulfur ca. 168 eV sulfur (as sulfonic groups)
268 (Mena-Durán et al., 2018), and the atomic concentration is low at 0.25%. This result
269 suggested that additives like 2-naphthol-7-sulfonate could be modified on the residual
270 substrate, which lead to the change in surface properties. And combined with its
271 reaction pathway (Fig.S4), it probably grafts on residual lignin.

272 Insert Fig.2 here

273 The hydrophobicity properties of samples were compared by water contact angle
274 (θ) measurement. All substrates showed a hydrophilic surface due to their small static
275 water contact angle θ ($<90^\circ$) (Table 2). As compared to DA, DA-NS has the most
276 obvious drop from 77.0° to 72.8° , while the zeta potential value was dropped from
277 -4.7 to -7.1 . Furthermore, the zeta potential of residual lignin is summarized in Table
278 3. It represents a similar tendency that 2-naphthol-7-sulfonate modified lignin has the
279 lowest zeta potential (-30.9). Therefore, DA-NS has a lower hydrophobic interaction
280 and higher electrostatic repulsion with enzymes. Combined with the data from Table
281 2, the zeta potential of samples seems to be a more crucial role in improving the
282 efficiency of enzymatic hydrolysis than hydrophobicity.

283 SEM images of four samples pretreated with or without phenolic additives are
284 shown in Fig.S2. Based on previous studies (He et al., 2018; He et al., 2020a;

285 Donohoe et al., 2008), both lignin and pseudo-lignin can evolve from the initial state
286 to spherical droplets and be deposited on the biomass surface under dilute acid
287 pretreatment. As shown in Fig.S2, the deposited droplet size ranges from 30 nm to 2
288 μm . With 2-naphthol and resorcinol additives (Fig.S2.A and B), droplets of bigger
289 size than the control one were observed (Fig.S2.D), which indicated that both
290 2-naphthol and resorcinol could promote the accumulation of lignin or pseudo-lignin
291 droplets. Besides, Fig.S2.C showed fewer droplets than the control one, which
292 suggested that 2-naphthol-7-sulfonate could hinder lignin or pseudo-lignin droplets
293 generation. As mentioned before, 2-naphthol exhibited a positive impact on the
294 enzymatic hydrolysis of substrate, which means that lignin droplets aren't the
295 determining factor in enzymatic hydrolysis and lignin structural information needs to
296 be elucidated for clarification.

297 *3.3. Structural changes in lignin during the acidic treatment*

298 As is well reported, re-polymerization of lignin readily occurs via lignin
299 side-chain carbonium ions formed during dilute acid pretreatment, presenting an
300 additional obstacle for the enzymatic hydrolysis of cellulose (Li et al., 2007; Lai et al.,
301 2018; Huang et al., 2022). The addition of carbocation scavengers is a direct and
302 effective method for suppressing undesired lignin re-polymerization reactions. To
303 have a careful and impartial observation of lignin structure and find suitable
304 scavengers, additives like 2-naphthol, 2-naphthol-7-sulfonate, and resorcinol were
305 applied in acid pretreatment. And relevant residual lignin (MWL) was extracted from
306 the pretreated bamboo residues, respectively.

329 non-condensed phenolic OH groups in DA-MWL from 1.57 to 3.62 (DA-N-MWL),
330 1.81 (DA-NS-MWL), and 1.92 mmol/g (DA-R-MWL), respectively. Combined with
331 the structure information in Fig.S2, these results suggested that lignin is fragmented
332 and modified with additives under the condition employed (Wen et al., 2014). It is
333 interesting to find that 2-naphthol and 2-naphthol-7-sulfonate could concurrently
334 increase the condensed OH groups of lignin (DA-MWL) from 1.56 to 2.04 and 2.00
335 g/mol, respectively. This suggested that part of naphthol or its derivatives could
336 modify lignin fragments on its C5 position and yield the original hydroxyl groups to
337 be classified as C5-substituted phenolic OH by ^{31}P NMR (Fig.S4.) (Balashin and
338 Capanema, 2015; Meng et al., 2019). Therefore, biomass with high content of
339 guaiacyl (G) or *p*-hydroxyphenyl units (H) is much suitable for adding naphthol to
340 prevent lignin re-polymerization under acidic pretreatment. Table 4 also showed that
341 the content of aliphatic OH of DA-MWL has been increased from 2.30 mmol/g to
342 4.00 mmol/g by adding 2-naphthol, but decreased to 1.34 mmol/g by adding
343 2-naphthol-7-sulfonate. Based on our previous work, pseudo-lignin could combine
344 with lignin during acidic pretreatment and increase the content of functional groups in
345 residual lignin (He et al., 2020b). As shown in Fig S1, 2-naphthol-7-sulfonate could
346 remove the spherical droplets that deposited on the surface of the substrate, which
347 would result in the decrease in aliphatic OH of DA-NS-MWL. Besides, in this work, a
348 higher amount of 2-naphthol was added in acidic pretreatment of bamboo residues
349 than 2-naphthol-7-sulfonate, and it would probably cause the low modification in the
350 lignin side chain.

351 Insert Table 4 here

352 **2D HSQC spectra.** HSQC NMR spectra has been applied to reveal structural
353 changes about lignins with or without additives, and the original lignin substructures
354 were assigned by comparison with the published literature (Wen et al., 2014; Huang
355 et al., 2016). The C-H correlations in the aromatic region (δ_C/δ_H 90-150/5.5-8.5 ppm)
356 of the spectrum showed typical grass lignin correlations from the residual
357 *p*-Hydroxyphenyl (H) units (Fig.S5).

358 In the aliphatic side chain region (δ_C/δ_H 50-90/2.5-6.0 ppm), the prominent
359 correlating signals are methoxy groups (OMe, δ_C/δ_H 55.6/3.73 ppm), β -*O*-4 aryl ether
360 linkages (A, A', and A''), resinol substructure (β - β linkages, B) and phenylcoumaran
361 substructure (β -5, C) as given in Fig.S4. The number of the inter-unit linkages per 100
362 aromatic units (Ar) were identified by their cross-peaks at δ_C/δ_H of 71.8/4.84 (A α),
363 84.8/4.64 (B α) and 86.8/5.45 ppm (C α), respectively. Based on our previous work, the
364 β -*O*-4 linkages of lignin in raw bamboo residues was around 39 per 100 Ar (Huang et
365 al., 2015), which was decreased to 16.7 per 100 Ar after DA pretreatment (Table 5),
366 meaning that around 60% of β -*O*-4 linkages had been ruptured. Meanwhile, the β -*O*-4
367 linkages in DA-N-MWL, DA-R-MWL, and DA-NS-MWL were decreased
368 significantly to 15.0, 13.1, and 2.2 per 100 Ar, respectively. These results suggested
369 that the phenolic additives facilitated lignin depolymerization. Gierer ~~et al~~ (1985) also
370 suggested that nucleophilic groups could attack the β -carbon atom of the side chain in
371 lignin and promote the delignification process during pulping and bleaching.

372 In harsh acidic or alkaline conditions, the C-O-C linkages such as β -*O*-4

373 linkages could be broken easily and leave reactive lignin-derived intermediates prone
374 to irreversible C-C condensation, which increases the amount of recalcitrant and high
375 molecular weight lignin products (Aelst et al., 2020). The number of β - β linkages
376 decreased from 1.8 per 100 Ar (DA-MWL) to 1.5 (DA-N-MWL), 1.3 (DA-R-MWL),
377 and 0.7 (DA-NS-MWL) per 100 Ar respectively. Meanwhile, the β -5 linkages in
378 lignin also decreased from 6.0 per 100 Ar (DA-MWL) to 4.3 (DA-N-MWL), 2.1
379 (DA-NS-MWL), and 1.2 (DA-R-MWL) per 100 Ar respectively. These results
380 indicate that nucleophilic reagents' addition during acidic pretreatment suppressed
381 lignin re-polymerization reactions, and 2-naphthol-7-sulfonate did the most
382 remarkable actions. Gierer et al (1985) suggested that O, S, and SO₃ in a chemical
383 pulping liquor were nucleophilic and could introduce various polaric groups in lignin,
384 which suggests that 2-naphthol-7-sulfonate has higher nucleophilic ability and decrease
385 the degree of re-polymerization more obviously than 2-naphthol.

386 It's reported that under auto-hydrolysis conditions (170°C, 30 min) a small
387 amount of resorcinol (0.125 mole/C₉ unit) could act as a bridge in lignin
388 re-polymerization, but a large excess (7.73 mole/C₉ unit) of it would behave like a
389 blocking agent (Lora and Wayman., 1980). Pielhop et al (2016) also suggested that at
390 the severity of logR₀=5.4, resorcinol would act as a crossing agent for lignin
391 fragments when the amount of it was 0.205 mol/mol lignin C₉ unit. Somehow,
392 resorcinol in the current study (~0.11 mole/C₉ units) worked more like an inhibitor
393 for lignin re-polymerization during the acid pretreatment process since we observed
394 around 30% β - β linkage and 80% β -5 linkage decrease. Even though,

395 resorcinol-modified bamboo residues did show a decrease in its efficiency of
396 enzymatic hydrolysis.

397 Overall, three additives present as efficient inhibitors in lignin re-polymerization
398 reactions, which low down the content of β -O-4 aryl ether linkages and obstruct the
399 formation of C-C bonds. 2-naphthol-7-sulfonate tends to be the remarkable blocking
400 agent during dilute acid pretreatment and leads to the highest delignification of
401 DA-NS (35.8%). Surprisingly, the content of inter-linkages was lower in
402 DA-N-MWL and DA-R-MWL than DA-MWL, but DA (34.4%) have a higher degree
403 of delignification than DA-R (15.5%) and DA-N (8.3%). On one hand, the structural
404 information of residual lignin confirmed that both 2-naphthol and resorcinol would
405 replace part of de-polymerized lignin fragments then graft on residual lignin, which
406 inhibit the removal of lignin. Although 2-naphthol-7-sulfonate similarly modifies
407 lignin, the existence of the sulfonic acid group could increase the hydrophilicity of
408 lignin fragments and promote the removal of lignin. On the other hand, in this work,
409 the amount of 2-naphthol (8%, based on dried raw materials) is higher than resorcinol
410 (2%, based on dried raw materials), which results in a higher degree of delignification
411 of DA-R than DA-N.

412 Insert Table 5 here

413 **Thermo-Mechanical Properties.** When the pretreatment temperature is higher
414 than T_g of lignin, lignin will coalescence, migrate, and redeposit on biomass cell wall
415 as shown in SEM images from Fig.S1. In this work, DSC was performed to determine
416 the T_g of lignin samples. Fig.S6 shows that the T_g value of Original-MWL,

417 DA-MWL, DA-N-MWL, DA-NS-MWL, and DA-R-MWL are 154, 164, 125, 185,
418 and 165 °C, respectively. Lower T_g in DA-N-MWL than DA-MWL suggests that
419 lignin is easier to be fluidized and extruded to the surface of biomass after 2-naphthol
420 modification (Fig.S1.). However, the modification with 2-naphthol-7-sulfonate led to
421 a much higher T_g of DA-NS-MWL than DA-MWL, which is reasonable after the
422 introduction of sulfonate groups. Moreover, combined with the data in Table 3 and
423 Table 4, it's indicated that T_g of lignin has a negative correlation with its' molecular
424 weight but no obvious relationship with the amount of non-condensed phenolic OH
425 groups.

426 *3.4. The correlation between modified lignin and enzymatic hydrolysis*

427 Preliminary studies verified that lignin could be modified with three additives
428 (2-naphthol, 2-naphthol-7-sulfonate, and resorcinol) during dilute acid pretreatment.
429 And in this part, the correlation between modified lignin and enzymatic hydrolysis
430 will be discussed. It's interesting to find that the sulfonic acid group plays a more
431 significant role during the depolymerization and re-polymerization reactions of lignin
432 than naphthol rings. Even the amounts of 2-naphthol (8%, based on dried raw
433 materials) is higher than 2-naphthol-7-sulfonate (4%, based on dried raw materials),
434 the content of lignin inter-linkages in DA-NS-MWL is much lower than in
435 DA-N-MWL (Table 5), which lead to higher delignification of DA-NS (35.8%) than
436 DA-N (8.3%). Besides, the introduction of the sulfonic acid group in lignin
437 (DA-NS-MWL) results in less unfavored lignin-enzyme interactions (Table 2, Table 3,
438 and Fig.S1) than DA-N-MWL. Therefore, compared to the naphthol ring, additives

439 with the sulfonic acid group could give a more obvious enhancement in cellulose
440 hydrolysis.

441 The non-productive adsorption driven by lignin was widely considered to be the
442 major mechanism to govern the efficiency of enzymatic hydrolysis. It's related to the
443 content, distribution, and flexibility of phenolic hydroxyl (Yang and Pan, 2016). As
444 shown in Table 4, the non-condensed phenolic hydroxyl content in DA-R-MWL (1.92
445 mmol/g) is higher than DA-MWL (1.27 mmol/g), which indicated that DA-R-MWL
446 would be easier to form hydrogen bonds with cellulase and increase the degree of
447 non-productive adsorption. Although DA-N-MWL has a higher content in
448 non-condensed phenolic hydroxyl (3.62 mmol/g) than DA-R-MWL, the 120 h sugars
449 yields of DA-N (37.63%) is higher than DA-R (22.75%). This phenomenon is
450 probably due to the different structure of additives, which indicates that hydroxyl in
451 benzene rings is much easier to inactive cellulase than hydroxyl in naphthol rings.

452 **4. Conclusions**

453 Pretreatment with nucleophile additives like 2-naphthol and 2-naphthol-7-sulfonate
454 has a positive influence on the enzymatic hydrolysis of bamboo residues. The surface
455 properties of the pretreated samples suggested that the introduced sulfonic acid group
456 increased the surface charge density and hydrophilicity while decreasing the lignin
457 content. Structural analysis of the residual lignin indicated that sulfonic acid
458 modification could promote lignin's depolymerization to have a lower molecular
459 weight and degree of re-condensation, which maximizes the cellulose hydrolysis
460 yields. Although resorcinol represented inhibition in the enzymatic hydrolysis, the

461 structural information of modified lignin indicated that it reacted like an inhibitor
462 rather than a crossing agent in lignin re-polymerization in this work. Overall, this
463 study provides a deep insight into the nucleophile modification in lignin and its
464 possible relationship with cellulose saccharification of acid pretreated substrates. In
465 future work, more suitable additives could be developed to overcome the bamboo
466 residues' recalcitrance in a process to remove or modify lignin, then improve the
467 efficiency of lignocellulosic biorefinery.

468 **Conflicts of interest**

469 There are no conflicts to declare.

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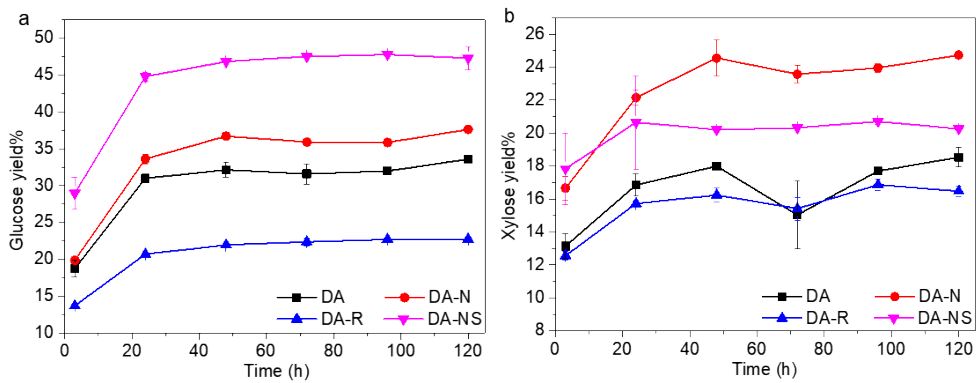
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609 **Figure Captions**

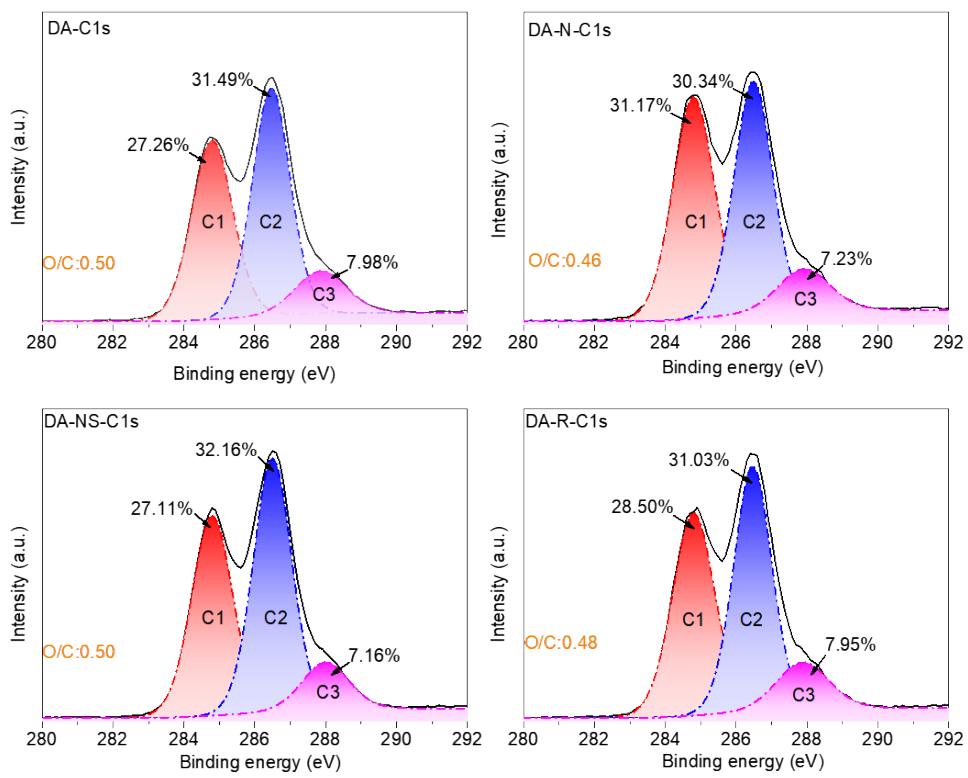
610 **Figure. 1.** Effect of cooking additives on the enzymatic digestibility of acid pretreated
611 bamboo residues.

612 **Figure. 2.** The high-resolution XPS C1s spectra of substrates after dilute acid (DA)
613 pretreatment assisted with or without various additives: 2-naphthol (N),
614 2-naphthol-7-sulfonate (NS), and resorcinol (R).



616

617 **Fig. 1.**



619

620 **Fig. 2.**

621

623 **Table 1** Composition analysis of dilute acid pretreated bamboo residues with different
 624 additives

Biomass ^a	Modification (%)	Composition (%)			Delignification (%)	Removal of xylan (%)
		Glucan (%)	Xylan (%)	Lignin (%)		
Raw Materials	-	40.4 ± 0.0	24.3 ± 0.3	31.6 ± 0.2	-	-
DA	0	62.3 ± 0.8	13.8 ± 0.2	38.0 ± 0.5	34.4	68.9
DA-N	8	54.3 ± 0.4	10.9 ± 0.2	42.5 ± 0.2	8.3	69.4
DA-NS	4	64.6 ± 0.3	11.4 ± 0.1	38.5 ± 0.5	35.8	75.1
DA-R	2	57.3 ± 0.0	11.2 ± 1.5	40.7 ± 0.2	15.5	69.6

625 ^a DA, DA-N, DA-NS, DA-R: bamboo residues dilute acid pretreated with no additive, 2-naphthol,
 626 2-naphthol-7-sulfonate, resorcinol, respectively.

628 **Table 2** Hydrophobicity and Zeta potential of dilute acid pretreated materials with
629 different additives

	DA	DA-N	DA-NS	DA-R
Hydrophobicity	77.0±3.2	75.3±1.6	72.8±1.5	73.3±4.6
Zeta potential (mV)	-4.7±0.4	-4.6±0.3	-7.1±0.2	-5.4±0.7

630

632 **Table 3** Zeta potential and molecular weight of various lignin samples

	DA-MWL	DA-N-MWL	DA-NS-MWL	DA-R-MWL
Zeta potential (mV)	-19.0 ± 0.6	-17.0 ± 0.7	-30.9 ± 1.9	-19.1 ± 0.5
Mw (g/mol)	9080	9121	5682	8953
Mn (g/mol)	3204	3746	2256	3096
PDI	2.83	2.43	2.52	2.89

634 **Table 4** Functional group contents of various lignin samples by quantitative ³¹P NMR
635 analysis

Sample	OH content (mmol/g)				
	Aliphatic OH	Condensed phenolic OH	Noncondensed Phenolic OH	Total Phenolic OH	COOH
DA-MWL	2.30	1.56	1.57	3.13	0.35
DA-N-MWL	4.00	2.04	3.62	5.66	0.30
DA-NS-MWL	1.34	2.00	1.81	3.81	0.45
DA-R-MWL	2.27	1.50	1.92	3.42	0.37

637 **Table 5** Quantity of lignin substructures per 100 phenylpropane units based on the
638 integration of 2D HSQC NMR spectra of lignin samples isolated from various
639 pretreated materials

Lignin	β-O-4	β-β	β-5	S/G
DA-MWL	16.7	1.8	6.0	1.6
DA-N-MWL	15.0	1.5	4.3	1.3
DA-NS-MWL	2.2	0.7	2.1	2.3
DA-R-MWL	13.1	1.3	1.2	2.1

640

Supporting information

642

643 **Figure Captions**

644 **Figure. S1.** SEM image of dilute acid pretreated bamboo residues with cooking
645 additives. A) DA-N, B) DA-R, C) DA-NS, D) DA.

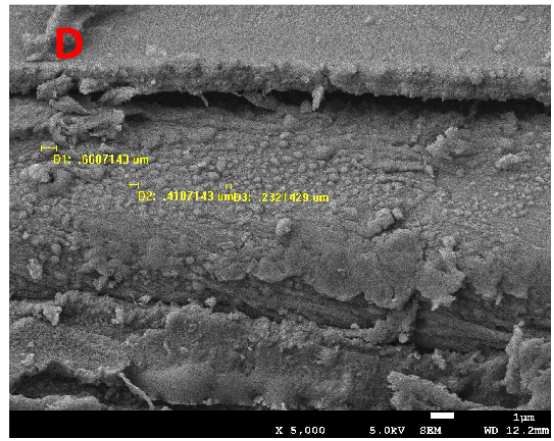
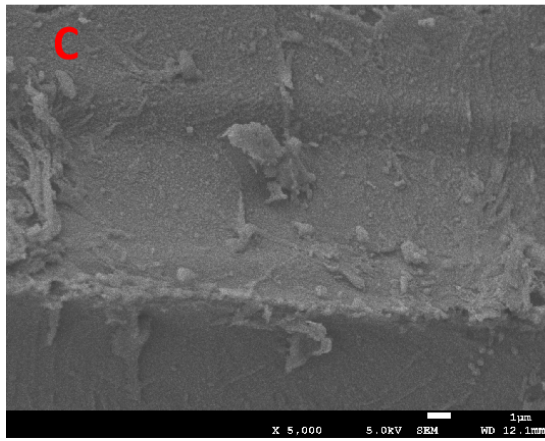
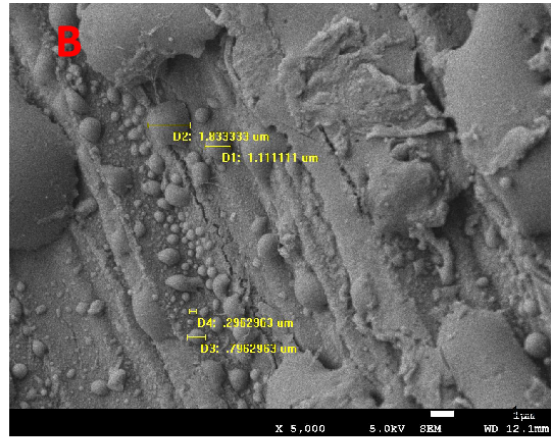
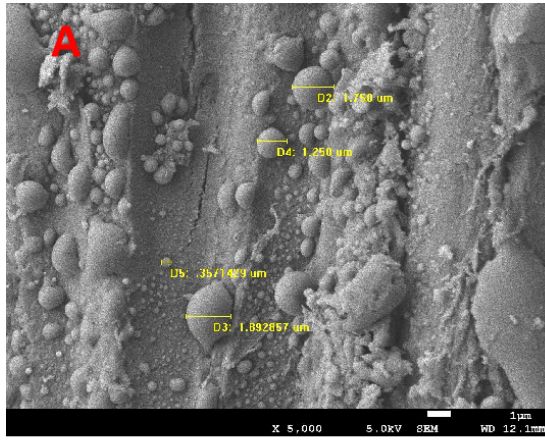
646 **Figure. S2.** Chemical structure of three additives. a) 2-naphthol
647 (144.1 g/mol); b) 2-naphthol-7-sulfonate (246.2 g/mol); c) resorcinol (110.1 g/mol).

648 **Figure. S3.** The high-resolution XPS S2p spectra of substrates after dilute acid (DA)
649 pretreatment assisted with or without various additives: 2-naphthol (N),
650 2-naphthol-7-sulfonate (NS), and resorcinol (R).

651 **Figure. S4.** Scheme for reactions of lignin in acidic condition with 2-naphthol.(1) the
652 de-polymerisation/re-polymerisation of lignin alone (just the cleavage of β -O-4 bond)
653 (Pielhop et al., 2015); (2) 2-naphthol working as a carbocation scavenger (Pielhop et
654 al., 2015); (3) lignin fragment modified with 2-naphthol;

655 **Figure. S5.** Short-range 2D NMR (HSQC) spectra of the isolated lignins with or
656 without additives. Top: side-chain region at δ_C/δ_H 50-90/2.5-6.0 ppm; bottom:
657 aromatic region at δ_C/δ_H 90-160/5.5-8.5 ppm.

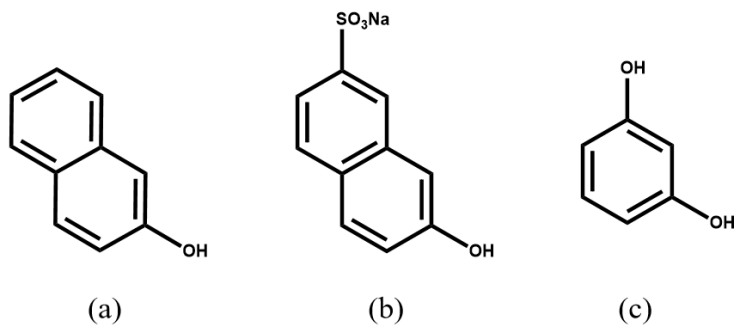
658 **Figure. S6.** DSC heat flow curves for lignin samples.



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Fig.S1.

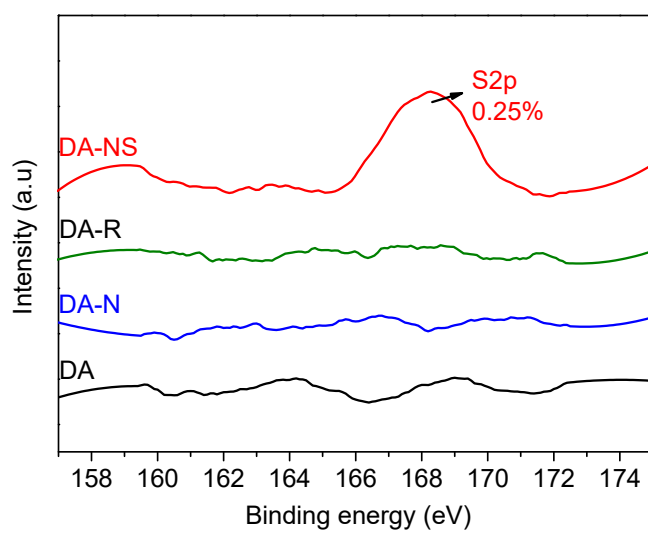


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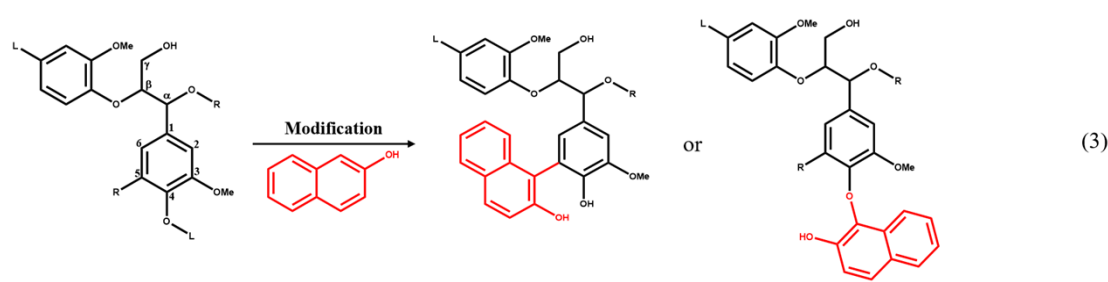
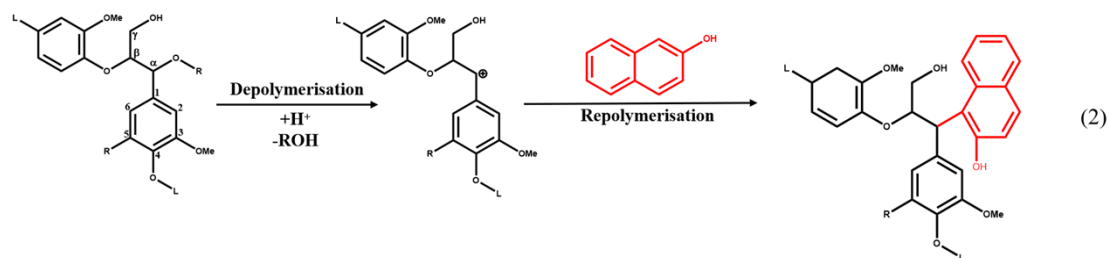
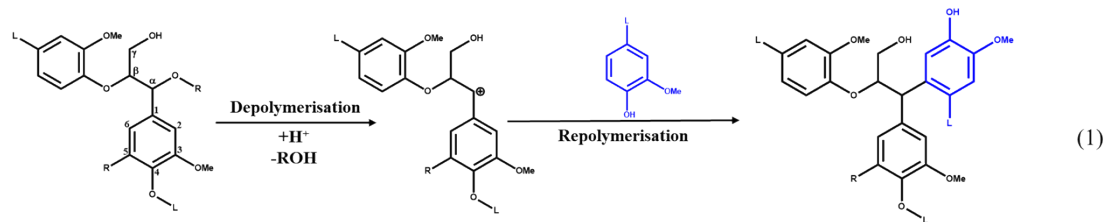
Fig.S2.

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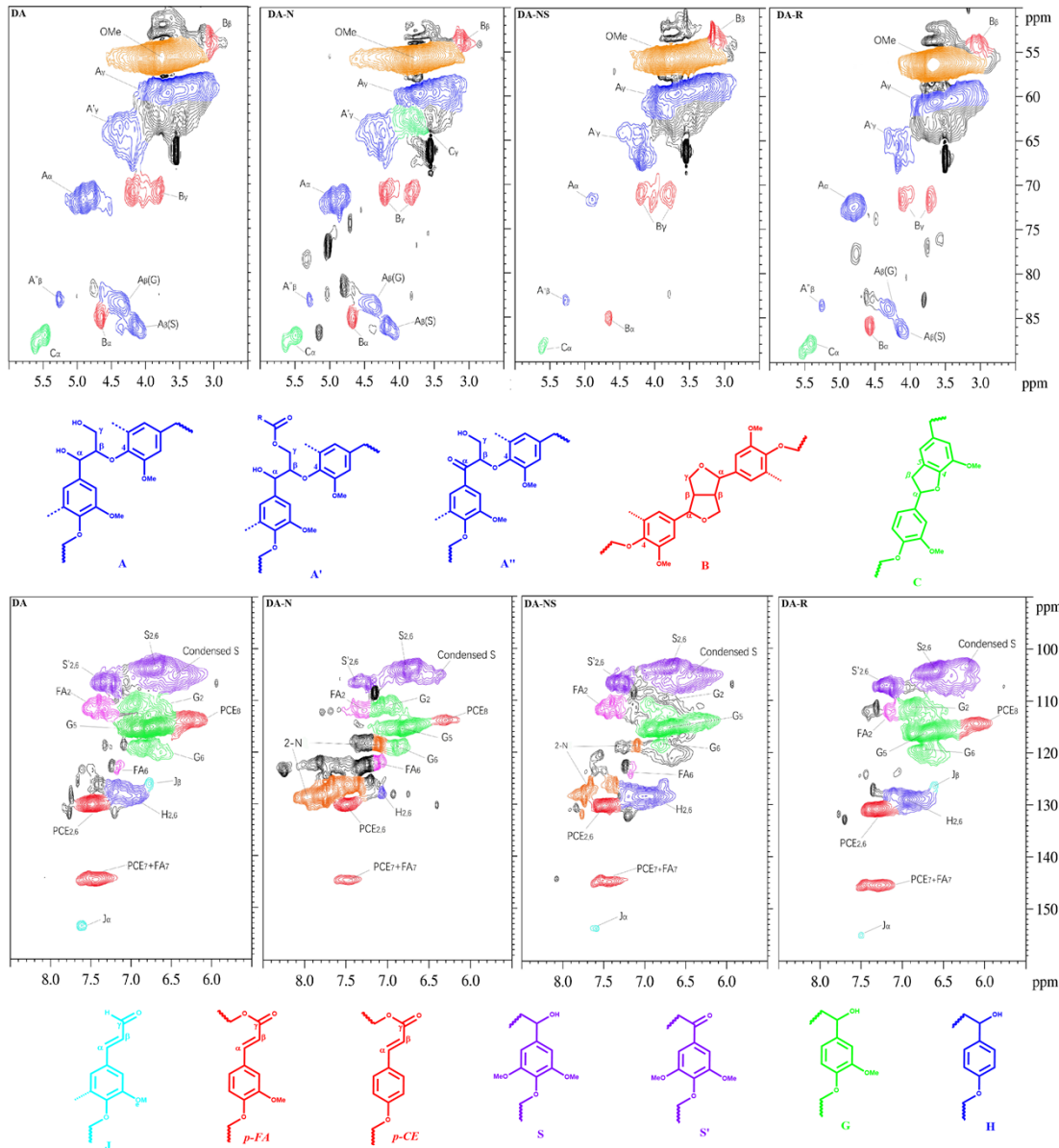
668 **Fig. S3.**



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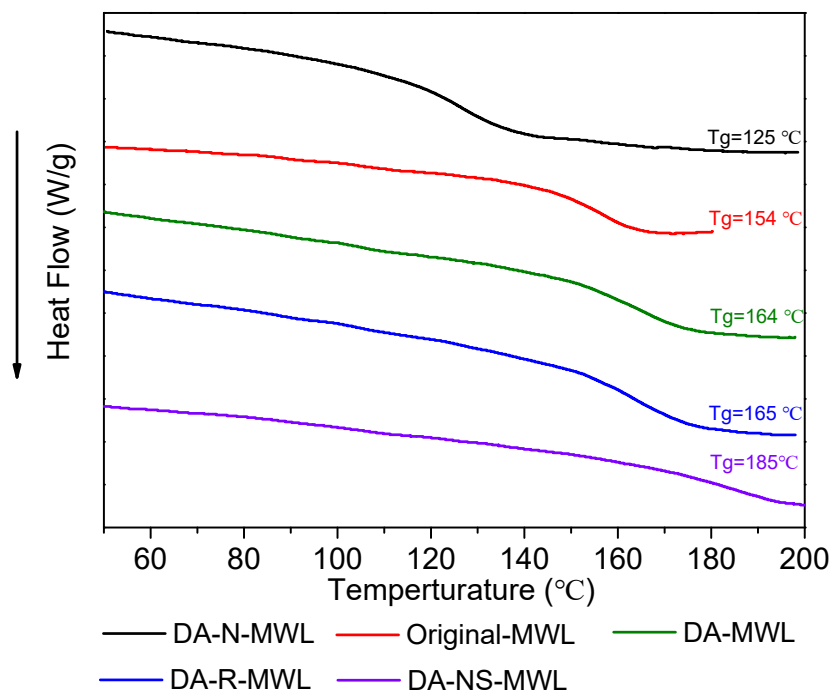
672

673 **Fig.S4.**



675
676

Fig.S5.



678

679 **Fig.S6.**

681 **Table S1** Surface chemical composition of dilute acid pretreated bamboo residues
682 with different additives

Sample	C1s(%)	C1^a(%)	C2^a(%)	C3^a(%)	O1s(%)	O/C	Surface lignin concentration (%)
DA	66.73	27.26	31.49	7.98	33.09	0.50	66.82
DA-N	68.64	31.17	30.24	7.23	31.25	0.46	74.95
DA-NS	66.43	27.11	32.16	7.16	33.32	0.50	65.68
DA-R	67.48	28.50	31.03	7.95	32.40	0.48	69.97

683