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1 **Engineered Sorghum Bagasse Enables a Sustainable Biorefinery with *p*-**
2 **Hydroxybenzoic Acid-Based Deep Eutectic Solvent**
3
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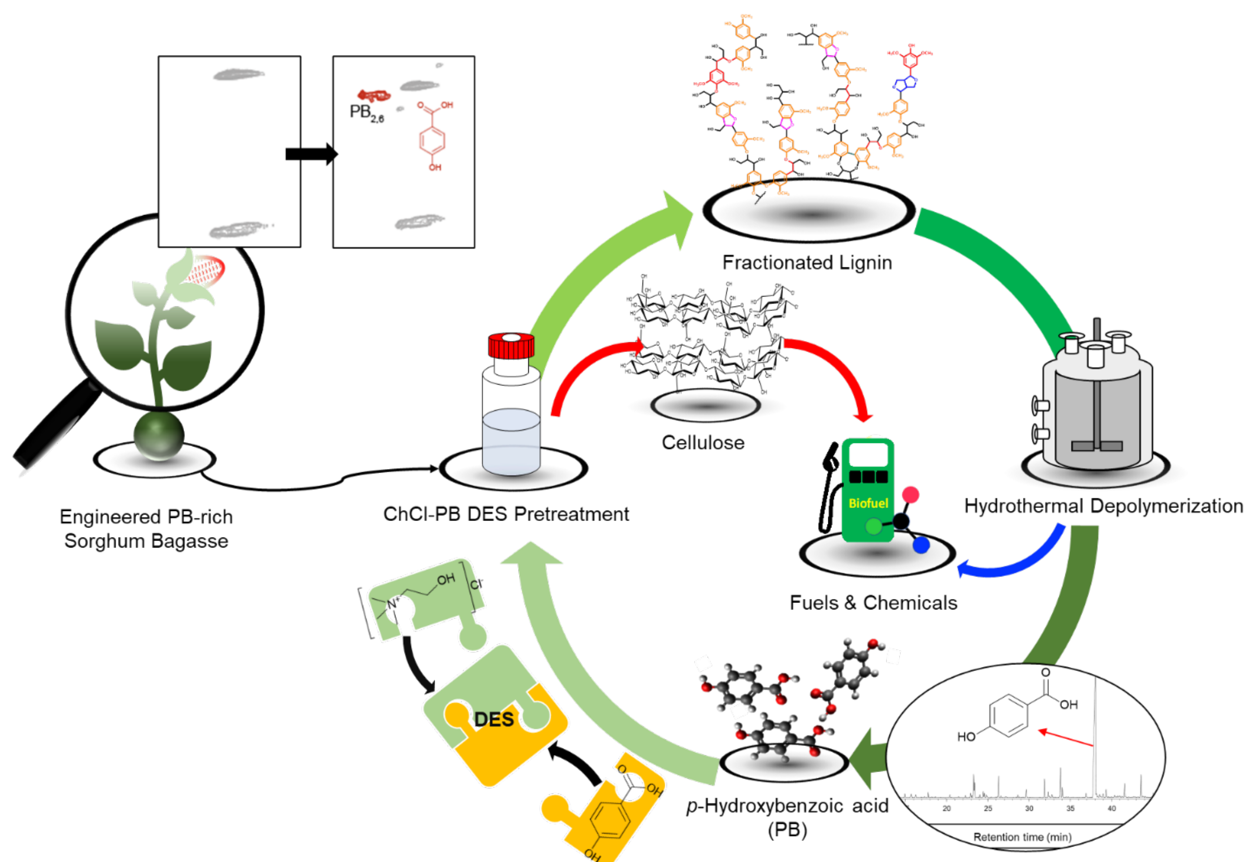


51
52 **Table of contents**: integrating lignin genetic modification and lignin-based deep eutectic solvents
53 enables a sustainable biorefinery. Engineered biomass released high fermentable sugars, and the
54 fractionated lignin could produce DES constituents through hydrothermal treatment.
55

56 ## Abstract

57 Integrating multidisciplinary research in plant genetic engineering and renewable deep eutectic
58 solvent can facilitate a sustainable and economic biorefinery. Herein, we leveraged a plant genetic
59 engineering approach to specifically incorporate C_6C_1 monomers into the lignin structure. By
60 expressing the bacterial *ubiC* gene in sorghum, *p*-hydroxybenzoic acid (PB)-rich lignin was
61 incorporated into the plant cell wall, while this monomer was completely absent in the lignin of
62 the wild-type (WT) biomass. A deep eutectic solvent (DES) was synthesized with choline chloride
63 (ChCl) and PB and applied to the pretreatment of the PB-rich mutant biomass for a sustainable
64 biorefinery. The release of fermentable sugars was significantly enhanced (~190% increase)
65 compared to untreated biomass by the DES pretreatment. In particular, the glucose released from
66 the pretreated mutant biomass was up to 12% higher than that from the pretreated WT biomass.

67 Lignin was effectively removed from the biomass with the preservation of more than half of the
68 β-O-4 linkages without condensed aromatic structures. Hydrogenolysis of the fractionated lignin
69 was conducted to demonstrate the potential of phenolic compound production. In addition, a
70 simple hydrothermal treatment could selectively extract PB from the same engineered lignin,
71 showing a possible circular biorefinery. These results suggest that the combination of PB-based
72 DES with engineered PB-rich biomass is a promising strategy to achieve a sustainable closed-loop
73 biorefinery.
74
75 **Key words:** Biorefinery, Lignin, Green Solvent, Lignin Depolymerization, Sustainable Process.
76

77 Introduction

78 Lignocellulosic biomass, composed of cellulose, hemicellulose, and lignin, is the most abundant
79 renewable carbon source on earth.[1] In recent decades, carbohydrates in biomass have been
80 successfully converted into value-added products through biological and chemical methods, such
81 as ethanol, furfural, and levulinic acid.[2] However, these biomass-derived products are still
82 economically challenging to compete with petroleum-based products. To overcome the cost barrier
83 in biomass conversion, lignin valorization has been proposed.[3] Lignin is the largest non-
84 carbohydrate component (20 – 30 wt%) in lignocellulosic biomass. It is a complex polymer of
85 phenylpropane units linked by ether and carbon-carbon bonds.[4] It has been considered as a critical
86 recalcitrance factor in biomass conversion. It can physically block the enzyme access to cellulose
87 and bind with enzymes non-productively, leading to low sugar yields during enzymatic hydrolysis
88 of biomass.[5] Various pretreatment methods have been developed for effective lignin removal;
89 however, the structural modification of lignin (e.g., condensation) sometimes disrupts its
90 valorization.[6] Due to this technical challenge, the lignin fraction is still underutilized.[3] Despite
91 this hindrance, lignin has great potential as a renewable source for the production of aromatic
92 platform chemicals that are currently produced from petroleum.[7] Several strategies have been
93 reported to extract a high yield of lignin from the biomass while preserving its intact structural
94 properties using γ-valerolactone, tetrahydrofuran, ionic liquids (ILs), and other solvent systems.[8]
95 In particular, ILs have also gained much attention due to their high solubility of biomass
96 components and low volatility.[8c] In addition to these advantages, a distinct feature of ILs is that
97 they are highly tunable, as changing the combinations of its anion and cation would render the
98 solvent designed properties.[9] Nevertheless, their commercial application is still hindered by the
99 complex synthesis procedure and high price.[10]
100 As a green designer solvent akin to ILs, deep eutectic solvents (DESs) have gained increasing
101 attention in recent years. DESs are mixtures composed of a hydrogen bond donor (HBD) and a
102 hydrogen bond acceptor (HBA). Simple physical mixing of the HBA and HBD at proper ratios
103 results in a significant depression of the melting point of the new mixture, leading to the formation
104 of a liquid at temperatures much lower than the melting points of its individual components.[11]
105 DESs share advantageous properties of ILs like low vapor pressure and high tunability, while they
106 can be synthesized with simpler methods and cheaper compounds.[12] In addition, DESs have

107 exhibited high lignin extraction capacity and selectivity during biomass pretreatment.[13] Thus far,
108 DESs have been extensively investigated for biomass processing and have achieved promising
109 results.[14]
110 The idea of using renewable solvents prepared from lignin-derived phenolic compounds to achieve
111 a sustainable bioeconomy is much acknowledged to Socha et al., who synthesized ILs with lignin
112 and hemicellulose-derived chemicals and applied them to biomass pretreatment.[15] Kim et al.
113 adapted the principle idea to DES, since compared with biomass-derived ILs, biomass-derived
114 DESs are easier to synthesize, which further reduces the cost.[16] They screened ten DESs
115 synthesized with lignin-derived phenolic compounds for switchgrass pretreatment and achieved
116 the highest lignin removal with ChCl-*p*-coumaric acid DES (ChCl-PCA). Chen et al. extended the
117 pretreatment temperature and time and resulted in increased delignification with ChCl-PCA.[17]
118 They also found that the addition of water intensified the pretreatment performance, resulting in
119 approximately complete enzymatic digestion (99.4% enzymatic digestibility). Recently, Huang et
120 al. reported a DES using guaiacol as the HBD with $AlCl_3$ as a catalyst.[18] Our group proposed to
121 use *p*-hydroxybenzoic acid (PB), an atypical aromatic unit found in certain hardwood lignins (e.g.,
122 poplar, willow, and aspen), as a DES component to pretreat hardwood and successfully delignified
123 poplar wood without additional catalyst.[19]
124 The concept of a sustainable biorefinery was highlighted by integrating DES pretreatment and
125 genetically modifying the model plant Arabidopsis in our recent study.[20] Here, a genetic
126 modification was applied to an actual crop, sorghum bagasse, in order to reduce the recalcitrance
127 of biomass. For further development of the integrated biorefinery strategy with this PB-rich
128 bagasse as feedstock, ChCl-PB DES was employed as the processing solvent. Naturally occurring
129 wild-type (WT) sorghum does not have PB in its lignin structure, while *in-planta* expression of
130 the *ubiC* gene from *E. Coli* encoding a chorismate pyruvate-lyase enables conversion of
131 endogenous chorismate into PB.[21] We show in this work that some of the PB overproduced in
132 sorghum engineered with *ubiC* is incorporated to the lignin structure. To the best of the authors'
133 knowledge, this is the first time such genetic modification is combined with biomass pretreatment
134 to achieve a closed-loop biorefinery. To investigate the impact of this genetic modification on
135 biorefinery, factors including chemical composition, cellulose accessibility, enzymatic
136 digestibility, and lignin structural properties before and after pretreatment were characterized.
137 Additionally, lignin was recovered after pretreatment, and PB was extracted from the lignin.
138 Results of this study could provide insights toward designing proper renewable DESs for biomass
139 pretreatment and lignin valorization and promote sustainable closed-loop biorefineries.
140
141 ## Results and Discussion
142 **Structural property of lignin in Eng-2 mutant sorghum bagasse.** To determine the effect of the
143 genetic modification on lignin, CEL was isolated from WT and Eng-2 mutant sorghum bagasse
144 and characterized by 2D HSQC NMR analysis. The NMR spectra of the two isolated CELs are
145 shown in Figure 1. The spectra were divided into aromatic regions (δ_C/δ_H 90-150/5.5-8.5 ppm) and
146 aliphatic regions (δ_C/δ_H 50-90/2.5-6.0 ppm). As shown in the aromatic regions of WT CEL, cross-

signals corresponding to typical lignin subunits including syringyl (S), guaiacyl (G), *p*-hydroxyphenyl (H), *p*-coumaric acid (PCA), and ferulates (FA) were observed at δ_C/δ_H 103.9/6.7 ppm ($S_{2,6}$), δ_C/δ_H 110.8/6.9 ppm (G_2), δ_C/δ_H 127.7/7.2 ppm ($H_{2,6}$), δ_C/δ_H 130.2/7.4 ppm ($PCA_{2,6}$), and δ_C/δ_H 110.8/7.3 ppm (FA_2), respectively.[22] Tricin (T) was also present in the NMR spectra, as indicated by peaks at δ_C/δ_H 93.9/6.6 ppm (T_8), δ_C/δ_H 98.5/6.2 ppm (T_6), and δ_C/δ_H 103.6/7.2 ppm ($T_{2',6'}$). The absence of cross-peak corresponding to *p*-hydroxybenzoic acid (PB) at δ_C/δ_H 131.4/7.67 ppm ($PB_{2,6}$) confirms that natural WT sorghum does not have this structure in its lignin, while the PB cross-peak was clearly detected in Eng-2 mutant CEL,[23] which by the semiquantitative analysis, accounted for 30% of the total lignin subunits (S + G + H). Other than the appearance of PB, the amount of lignin subunits and the S/G ratio of the WT and Eng-2 mutant CEL were similar (Supporting Information, Table S1).

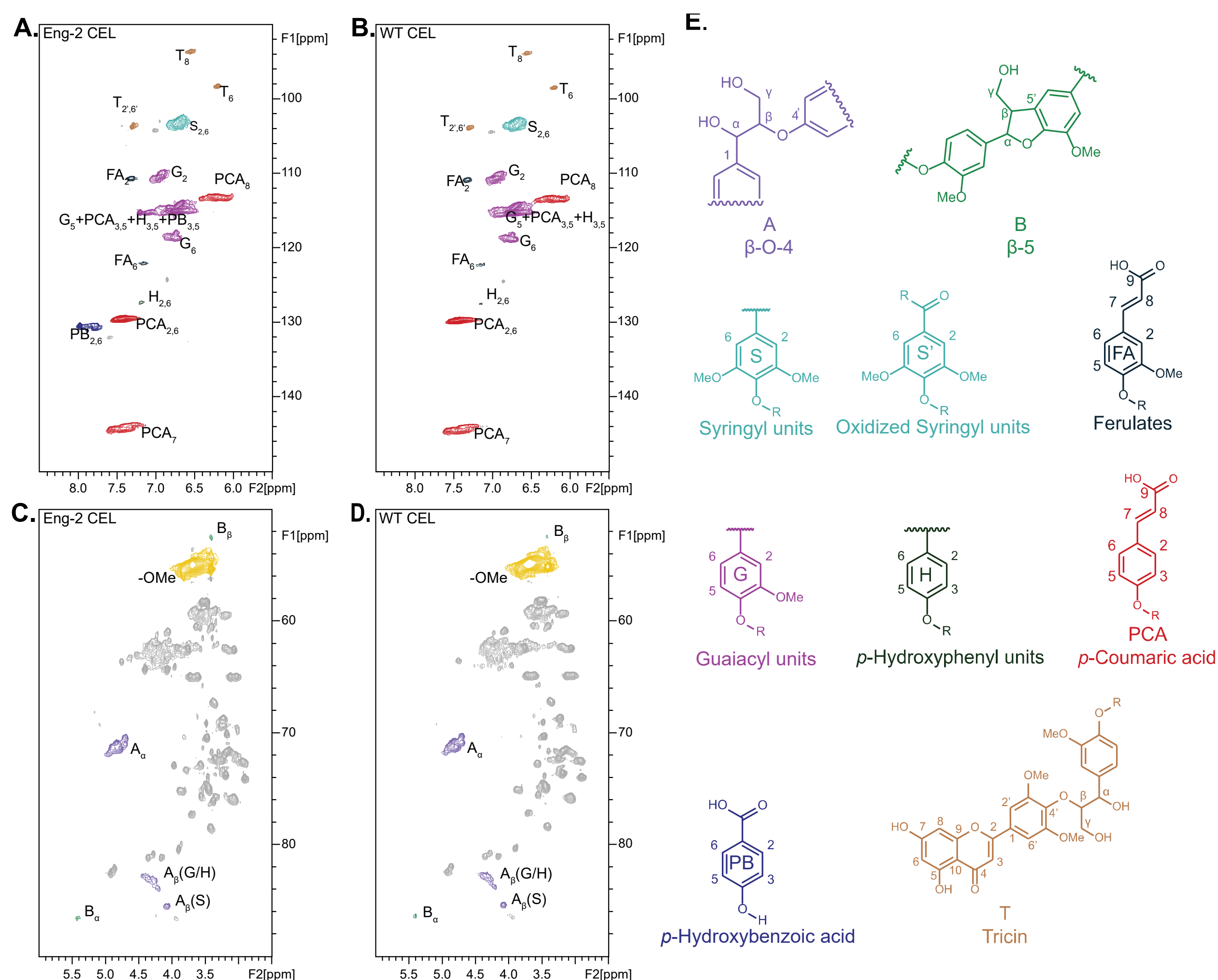
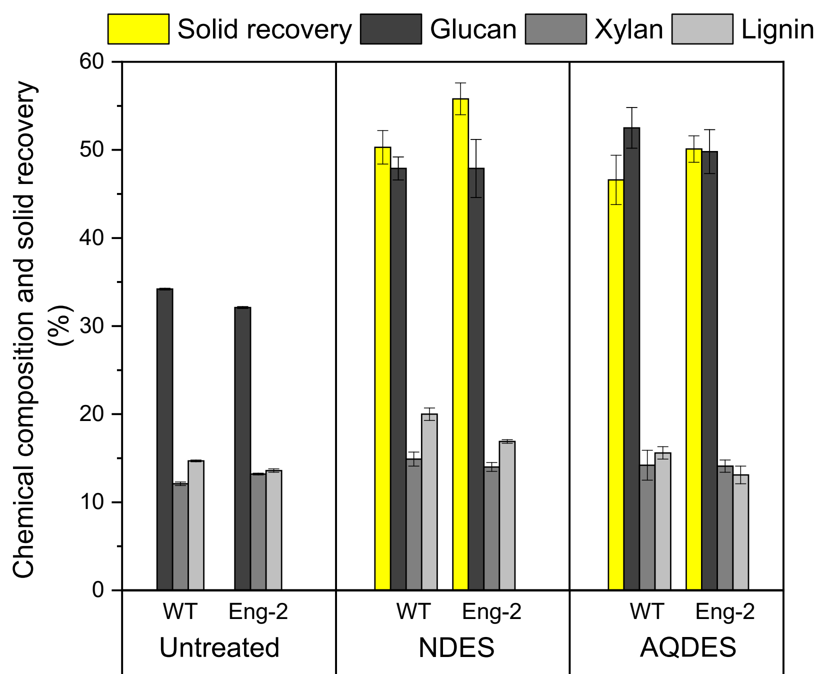


Figure 1. 2D HSQC NMR spectra of cellulolytic enzyme lignin (CEL) isolated from WT and Eng-2 mutant sorghum bagasse. **A:** aromatic regions of CEL from Eng-2 mutant; **B:** aromatic regions of CEL from WT; **C:** aliphatic regions of CEL from Eng-2 mutant; **D:** aliphatic regions of WT; **E:** Structures of detected lignin subunits and interunit linkages.

165 In the aliphatic regions, cross-peaks correlating to methoxy groups (OMe), β-aryl ether (β-O-4)
166 and phenylcoumaran (β-5) linkages were identified for both WT and Eng-2 CELs. It can be
167 observed that the β-O-4 linkage is the most abundant interunit linkage in both lignins. The contents
168 of interunit linkages of the two CELs were similar. Besides, the molecular weights of the two
169 CELs were also similar to each other (M_W of WT CEL and Eng-2 CEL were 10,367 g/mol and
170 10,498 g/mol, respectively) (Supporting information, Figure S1, Table S2). Based on the results
171 of 2D HSQC NMR and molecular weights, it can be inferred that expression of the *ubiC* gene from
172 *E. Coli* in the sorghum leads to the incorporation of PB in the lignin structure, while other lignin
173 subunits and interunit linkages were not significantly altered.
174
175 **DES pretreatment.** Several previous studies have shown that adding extra components, such as
176 water, to the DES improves the DES pretreatment performance.[17, 24] It appears that water
177 decreases the viscosity of DES, which facilitates better solubilization capacity.[25] However, there
178 is an upper limit to the extent of water addition, where above this limit the mixture became a water
179 solution rather than a DES.[26] To enhance the pretreatment performance of the PB-based DES, a
180 water-containing DES was formed and tested for the pretreatment. The PB-based DES (neat DES,
181 NDES) was composed of ChCl and PB at a molar ratio of 3:2, while the water-containing DES
182 (aqueous DES, AQDES) consisted of ChCl, PB and water at a molar ratio of 3:2:5.[26] All the
183 pretreatments were conducted at 120 °C for 3 h. After pretreatment, the efficiency of NDES and
184 AQDES were determined in terms of delignification and xylan removal. Figure 2 shows the
185 chemical composition of glucan, xylan and lignin of untreated and pretreated biomass, and the
186 solid recovery yields after pretreatment. A detailed mass balance calculation of the three major
187 components for all four pretreatment processes (based on 1 kg initial biomass) is presented in the
188 Supporting Information, Figure S2. Chemical compositions of WT and Eng-2 biomass were
189 similar, with WT having slightly higher glucan (34.2% in WT, 32.1% in Eng-2) and lignin (14.7%
190 in WT, 13.6% in Eng-2) contents, and Eng-2 mutant having marginally higher xylan content (12.1%
191 in WT, 13.2% in Eng-2). These results suggest that expressing the bacterial *ubiC* gene *in-planta*
192 did not result in notable changes in the biomass composition. Previous studies have shown that
193 incorporating C_6C_1 monomers like hydroxybenzaldehydes into lignin structures would not alter
194 lignin and carbohydrate content in the cell wall,[27] which is in good agreement with our
195 observations. The composition of pretreated biomass shows that the delignification yield of WT
196 biomass was 31.6% and 50.4% when pretreated by NDES and AQDES, respectively, and those of
197 the Eng-2 mutant were similar (30.8% and 51.7% from NDES and AQDES, respectively). In
198 comparison, the xylan removal from the Eng-2 mutant was higher than that from WT, regardless
199 of the DES used.
200 AQDES showed higher delignification than NDES, which is in accordance with previous
201 observations.[17, 28] Chen et al. reported that the addition of water to ChCl-*p*-coumaric acid DES
202 increased the removal of lignin and hemicellulose, while increasing the water content did not
203 further improve the removal.[17] Kumar et al. found that ChCl-lactic acid with 5% (w/v) water had
204 higher lignin extraction than neat ChCl-lactic acid DES, while no difference in cellulose or xylan

205 solubility was observed.[28] Since DESs are formed by hydrogen bonding between HBA and HBD,
206 and water possesses a strong hydrogen bonding capacity, it can be inferred that the addition of
207 water results in competing hydrogen bonding between the DES constituents.[29] As a result, the
208 hydrogen bonding between the two constituents is weakened, and the viscosity is decreased, as the
209 high viscosity of DES is mostly attributed to the extensive hydrogen bonding between the
210 components.[24b] Lower viscosity facilitates better mass transfer, which explains the better
211 pretreatment performance of AQDES compared with NDES. However, the exact intermolecular
212 interactions between water and DES constituents are still unclear, and in-depth studies are required.
213



214
215 **Figure 2.** Chemical compositions of WT and Eng-2 mutant sorghum bagasse before and after
216 pretreatment with NDES and AQDES.
217
218 **Cellulose accessibility and enzymatic hydrolysis.** Cellulose accessibility, defined as the amount
219 of cellulose surface area that is accessible to enzymes, is a valid parameter to predict the
220 effectiveness of biomass pretreatment.[30] Since the high molecular weight fraction of the direct
221 orange (DO) dye has a similar diameter to typical cellulase (~5.1 nm), the amount of DO dye
222 adsorbed by the substrate can serve as an indicator of the accessible surface area of cellulose to
223 cellulase.[31] In this study, a modified Simon's staining method was applied to the WT and Eng-2
224 sorghum bagasse to access the cellulose accessibility. A Langmuir adsorption isotherm of DO dye
225 was obtained (Figure 3A), and the results expressed as maximum mg DO dye adsorbed per g of
226 biomass are shown in Figure 3B. The maximum amount of DO dye adsorbed by untreated sorghum
227 bagasse were 21.2 and 30.0 mg/g biomass for WT and Eng-2 mutant, respectively. These values
228 increased to 65.1 mg/g biomass (WT) and 75.1 mg/g biomass (Eng-2 mutant) after NDES
229 pretreatment and further increased to 71.4 mg/g biomass (WT) and 76.0 mg/g biomass (Eng-2

mutant) after AQDES pretreatment. It can be speculated that regardless of the pretreatment, the Eng-2 mutant had higher cellulose accessibility than WT, suggesting that the Eng-2 mutant is more susceptible to enzymatic degradation, despite having a similar chemical composition with the WT biomass. Both NDES and AQDES pretreatments significantly increased the cellulose accessibility compared to the untreated biomass. Though NDES pretreatment resulted in ~20% lower delignification than AQDES pretreatment, the resulting cellulose accessibility of NDES pretreatment was only 6.3 mg/g biomass and 0.9 mg/g biomass lower than those of AQDES pretreatment on WT and Eng-2 mutant, respectively. This observation indicates that both DES pretreatments disrupted the lignin structure and reduced its inhibitive effect on the enzyme to similar levels, while AQDES exhibited higher delignification due to its greater lignin solubility.[32]

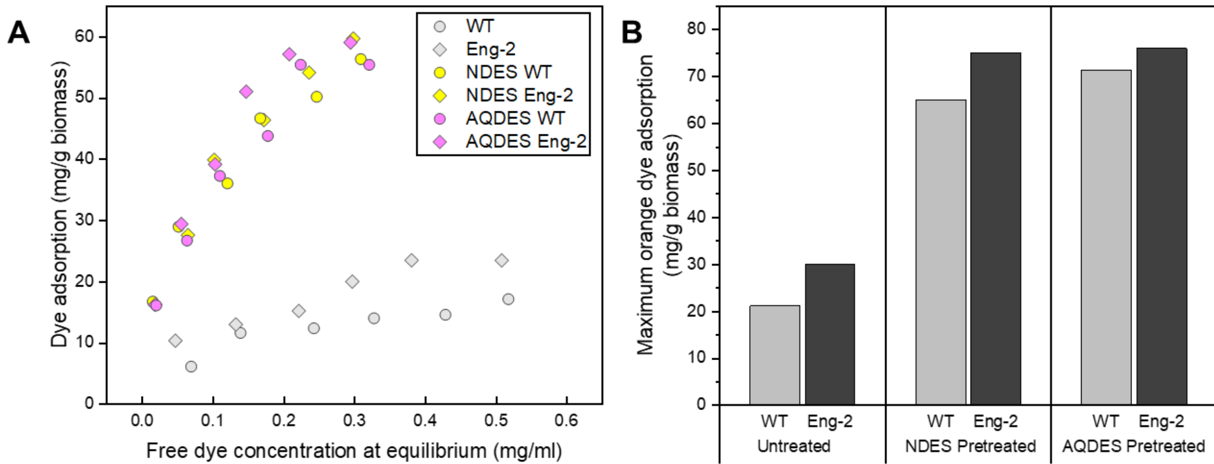


Figure 3. Cellulose accessibility measured by modified Simon's Stain method. **A:** direct dye (DO) adsorption isotherm curves. **B:** maximum amount of DO dye adsorbed (mg/g biomass) by untreated, NDES pretreated and AQDES pretreated WT and Eng-2 sorghum bagasse.

To evaluate the effect of genetic modification and different DES pretreatment on the conversion of sorghum bagasse to fermentable sugars, both untreated and pretreated WT and Eng-2 mutant biomass were subjected to enzymatic hydrolysis. Figure 4 presents the yields of sugars released from WT and Eng-2 mutant before and after NDES and AQDES pretreatment. Total sugar yields ranged from 126.6 to 367.8 mg/g biomass for glucose and from 24.9 to 106.1 mg/g biomass for xylose. As shown in the figure, Eng-2 mutant always had higher glucose release than WT (7.1% higher for untreated, 4.8% higher for NDES pretreated, 12.3% higher for AQDES pretreated). Yields of glucose released after NDES and AQDES pretreatment were approximately three times higher than those from the untreated biomass, while the difference between NDES pretreatment and AQDES pretreatment was less than 30 mg/g biomass. These observations are well in line with the cellulose accessibility. No significant difference was observed between the xylose release from WT and Eng-2 mutant. Pretreatment with both DESs resulted in more than threefold enhancement of xylose yield, and AQDES pretreatment showed ~8 mg/g biomass higher xylose yield compared with NDES pretreatment. Moreover, other studies showed that incorporating a higher amount of

p-coumarate esters into lignin led to a lignin structure with a higher frequency of free phenolic groups that was easier to solubilize under alkaline conditions.[33] Our work clearly demonstrates that the Eng-2 mutant with PB-rich lignin is less recalcitrant to enzymatic hydrolysis. However, more comprehensive research is needed to understand the impact of PB accumulation in the cell wall, such as determining the type of monolignols being acylated and whether PB alters lignin properties or lignin-polysaccharide crosslinks.

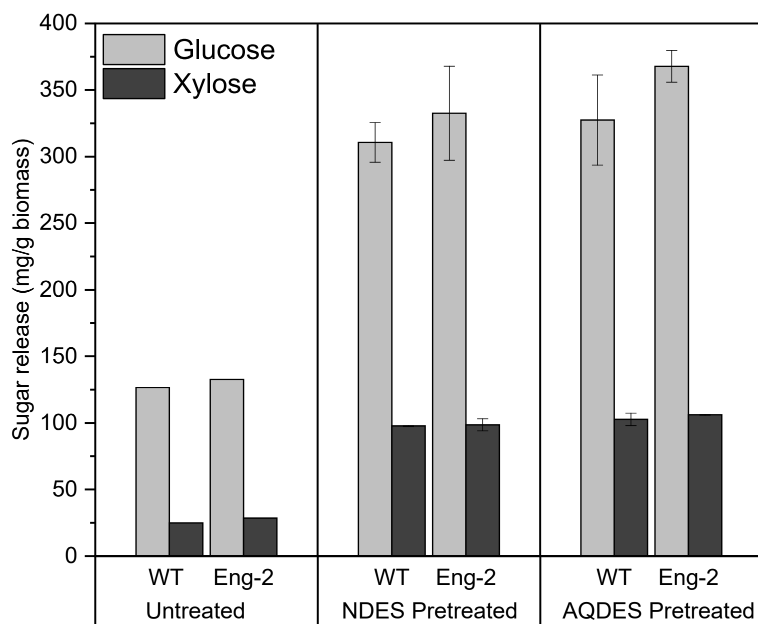
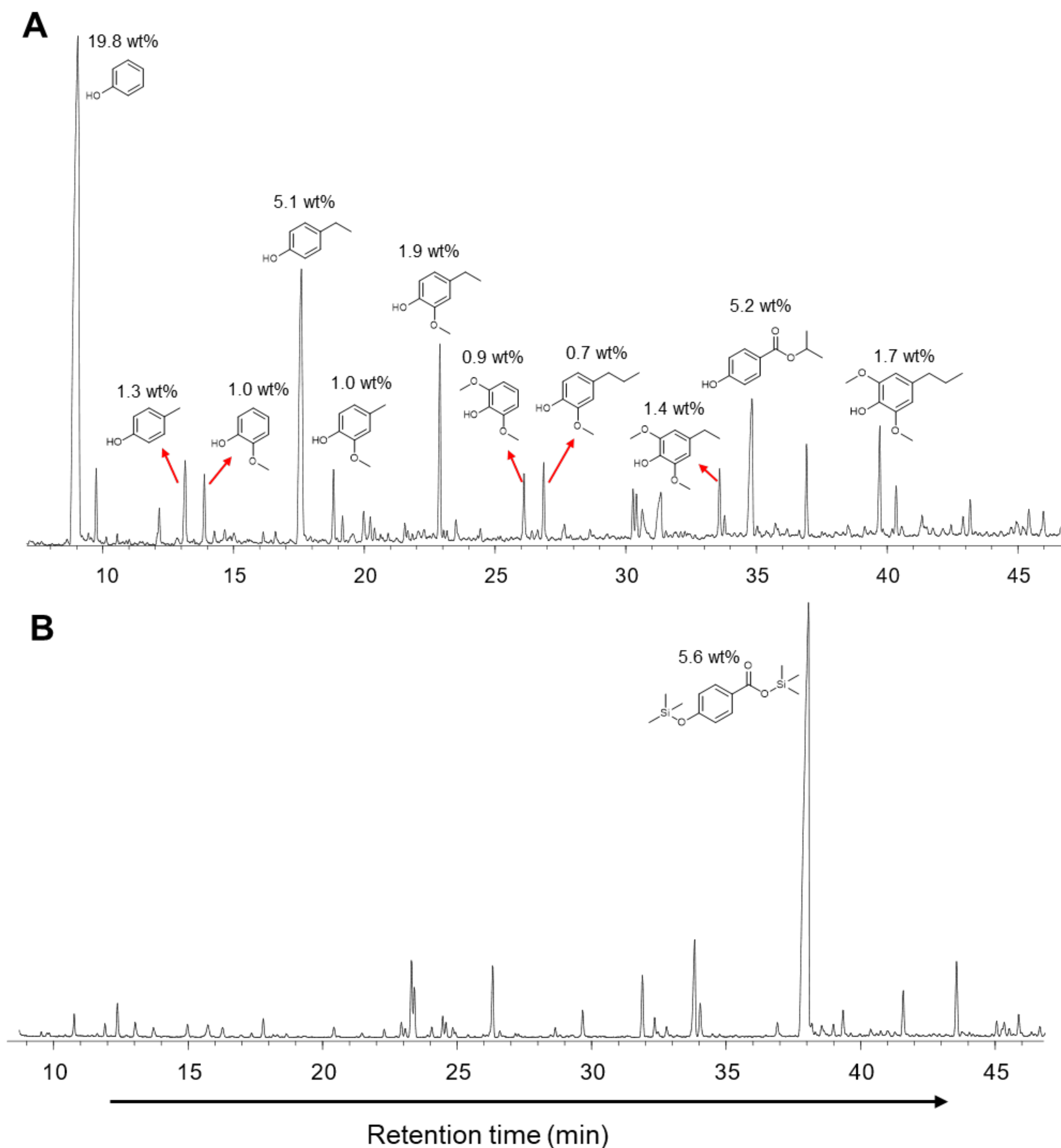


Figure 4. Sugar release yield from WT and Eng-2 mutant sorghum bagasse before and after pretreatment with NDES and AQDES.

PB recycling and valorization of the recovered DES lignin. We have demonstrated that incorporating PB into the sorghum lignin structure reduced biomass recalcitrance and proved that the DES synthesized with PB is effective in pretreating the mutant biomass. To achieve a potential closed-loop biorefinery with combined transgenic biomass and the lignin-based DES, lignin was recovered from AQDES pretreated Eng-2 mutant (AQDES lignin) and processed to produce PB. We first examined the lignin structure and composition of subunits by 2D HSQC NMR. As shown in Figure S3 and Table S3 in the Supporting Information, after AQDES pretreatment, many of the contours in the aliphatic regions correlating to carbohydrates are weakened or disappeared compared with the spectra of CEL, leaving a lignin fraction with relatively high purity. Major lignin structures were well-preserved; even all cross-peaks corresponding to tricin were clearly observed in the NMR spectra of fractionated lignin.[34] More than half of the β-O-4 linkages, which was the linkage most susceptible to breakage,[35] were preserved after the pretreatment (53% of the abundance in native lignin (CEL)). In addition, no condensed S or G units were observed in the fractionated lignin.[36] The above observations can be attributed to the mild pretreatment conditions enabled by the aqueous ChCl-PB DES, as compared to other DES pretreatments that require harsher conditions.[14c, 16-17] The PB content was significantly enriched in the fractionated lignin

287 after the AQDES pretreatment, suggesting that PB from the DES also precipitated during lignin
288 recovery. To investigate the PB precipitation, we recovered lignin from the AQDES pretreated
289 WT sorghum bagasse and investigated its structure with 2D HSQC NMR as well. As shown in
290 Figure S4 and Table S3, PB was detected in the AQDES WT lignin, confirming that PB in the
291 DES was partially precipitated during the lignin recovery process. GPC analysis shows that the
292 molecular weight of AQDES lignin was markedly lower than the CEL isolated from Eng-2 mutant
293 (Supporting Information, Figure S1, Table S2), with the weight-average molecular weight (M_w)
294 dropping from ~10,500 g/mol of the CEL to less than 3,000 g/mol of the AQDES lignin.
295 Polydispersity index (PDI) also decreased from 3.4 (Eng-2 CEL) to 1.6 (AQDES lignin). These
296 results indicate that the lignin was already depolymerized during the pretreatment and had a more
297 uniformed size distribution, which is supported by the partial cleavage of β-O-4 linkages observed
298 by 2D HSQC NMR. Taken these results together, lignin with no condensation, preservation of half
299 of the β-O-4 linkage, relatively uniform molecular weight, and more importantly, high PB content
300 was produced from AQDES pretreatment of Eng-2 mutant, which is appealing for phenolic
301 monomer production through depolymerization.
302 To assess the depolymerization potential, AQDES lignin was subjected to hydrogenolysis
303 treatment. After the hydrogenolysis, lignin monomers were identified and quantified by gas
304 chromatography, as shown in Figure 5A. Total phenolic monomer yield reached 42.2 wt%, with
305 phenol, 4-ethylphenol, 4-ethylguaiacol and isopropyl-4-hydroxybenzoic acid being the major
306 products (Figure 5A). The average molecular weight of liquid products from lignin hydrogenolysis
307 was 423 g/mol. The molecular weight distribution shows several distinct peaks at 153, 286, and
308 495 g/mol (Figure S5, Supporting Information). Considering the molar mass of the initial material,
309 lignin depolymerization resulted in a significant decrease in molecular weight of the products,
310 possibly by the cleavage of lignin linkages (e.g., C-O cleavage). It is noted that *p*-coumaric acid
311 and ferulic acid observed from the original feedstock were not detected after lignin
312 depolymerization. It is likely that those compounds were reduced to alkylphenols, including 4-
313 propylphenol and 2-methoxyl-4-propylphenol, under the hydrogenolysis conditions.[37] It is also
314 possible that some monomers were from the residual ChCl-PB DES in the lignin. The high
315 monomer yield clearly demonstrates that the AQDES lignin is a promising substrate for upgrading.
316 However, it is impractical to use such a mixture of monomers to supply DES synthesis due to
317 difficulties in separation, and the requirement of hydrogen gas and expensive catalysts. To further
318 verify a closed-loop biorefinery by supplying PB from the product for DES synthesis, another
319 depolymerization test was carried out by using simple hydrothermal treatment without additional
320 catalyst. Trajano et al. reported that by adjusting the reaction conditions, PB could be obtained as
321 the major product from hydrothermal treatment of CEL isolated from poplar.[38] We adapted their
322 condition for PB extraction (180 °C, 60 min) from AQDES lignin and analyzed the products by
323 gas chromatography. Interestingly, as shown in Figure 5B, PB was the dominant compound in the
324 resulting mixture, accounting for 5.6 wt% of the initial substrate. Compounds other than PB have
325 a low amount and were possibly from residual carbohydrates. In the section above, we found that
326 part of PB in the AQDES lignin was from the DES. Based on the 2D HSQC NMR spectra of lignin

residue after hydrothermal treatment (Figure S6 and Table S4, Supporting Information), it is clear that the proposed hydrothermal treatment effectively removed PB from the lignin, evidenced by the drastic decrease of PB abundance. Although further optimization is required to maximize PB yield, it is soundly demonstrated that a simple hydrothermal treatment is effective for PB production from lignin isolated after ChCl-PB DES pretreatment of mutant sorghum bagasse. Additionally, our previous study showed that the ChCl-PB DES could be recovered from the liquid after pretreatment and reused for the next pretreatment.[19] Taken together, these results show a potential of a sustainable biorefinery achievable by the closed-loop production of lignin-based DES.



338

339 **Figure 5. A:** Gas chromatogram of products from hydrogenolysis of lignin recovered from Eng-2 340 mutant pretreated by AQDES and their yields; **B:** Gas chromatogram of products from 341 hydrothermal depolymerization of lignin recovered from Eng-2 mutant pretreated by AQDES. 342 *Note.* The yield was calculated based on initial substrate weight.

343

344 Conclusions

345 Expression of the *ubiC* gene from *E. Coli* in sorghum successfully resulted in the incorporation of
346 PB into the lignin structure without significant changes in its other properties. Compared with non-
347 PB containing WT biomass, the mutant showed higher fermentable sugar release when pretreated
348 by DES synthesized with PB. The addition of a small amount of water to the DES significantly
349 improved the delignification, which facilitated not only fermentable sugar production but also
350 lignin valorization. After the pretreatment, more than half of the β-O-4 linkages in the native lignin
351 were preserved in the lignin recovered from aqueous DES pretreatment. Additionally, the lignin
352 had a narrow molecular size distribution and no condensation, which allowed for effective
353 valorization *via* hydrogenolysis (42.2 wt% of the initial substrate). Moreover, simple hydrothermal
354 treatment of the fractionated lignin resulted in a product with PB as the dominant aromatic
355 compound, which is promising for using the fractionated lignin to supply DES synthesis. Despite
356 the encouraging results, future works are still required to understand the different forms of PB in
357 the mutant biomass, interactions between water and DES, and to optimize the PB yield from
358 hydrothermal treatment. Taken together, pretreatment of strategically engineered biomass using
359 lignin-based DES is a promising approach towards a sustainable biorefinery.
360

361 Experimental Section

362 **Biomass feedstock and chemicals.** Sorghum (*Sorghum bicolor* L., variety Tx430) bagasse
363 samples from previously characterized wild-type (WT) and transgenic line *pRUBI2:ubiC*
364 (thereafter named Eng-2) in the T1 generation were supplied by the Joint BioEnergy Institute.[21]
365 The bagasse was from stems and leaves of fully mature senesced plants. The samples were dried
366 at 50 °C for five days, Wiley-milled using a 2-mm mesh, and further screened to 12 – 20 mesh for
367 this study. Chemicals including choline chloride (ChCl), *p*-hydroxybenzoic acid (PB), ethanol,
368 dioxane, pyridine, acetic anhydride, dimethyl sulfoxide-d_6 (DMSO-d_6), and tetrahydrofuran were
369 purchased from Millipore Sigma (St. Louis, MO), VWR (Radnor, PA), and Fisher Scientific™
370 (Fair Lawn, NJ). The chemicals were used as received without further purification. Cellulase
371 enzyme cocktail (Accelerase® 1500) was provided by DuPont Industrial Biosciences (Palo Alto,
372 CA). The BCA protein content of the enzyme was 82 mg/mL, as reported previously.[39]
373

374 **DES synthesis.** The ChCl-PB DES was prepared by mixing ChCl and PB at molar ratios of 3:2,
375 2:1, 1:1 and 2:3. The mixture was heated at 90 °C with continuous stirring. Among the tested molar
376 ratios, a clear and homogeneous solvent was formed only at 3:2, as shown in Figure S7 (Supporting
377 Information). Therefore, the molar ratio of 3:2 was used for the following tests. For aqueous DESs
378 formation, deionized (DI) water was added to the formed DES at a molar ratio of 3:2:5 (ChCl :
379 PB : water) at 90 °C with continuous stirring until the mixture became homogenous.
380

381 **DES pretreatment.** For the pretreatment of WT and engineered biomass, 10 wt% of biomass was
382 loaded to the DES at the test temperature and allowed to react at 120 °C for 3 h. The mixture was
383 continuously stirred at 300 rpm. Each experiment was conducted in duplicate. Once the
384 pretreatment was completed, the mixture of solid residue and DES was washed with a solution of

385 ethanol and water (1:1, v/v) until the filtrate was clear and colorless. After washing, the solid
386 residue was stored at 5 °C until further tests. The liquid fraction was collected and used to recover
387 lignin. In a typical run of lignin recovery, the volume of the liquid fraction was firstly reduced by
388 rotary evaporation at temperatures not higher than 40 °C, then stored at 5 °C overnight for lignin
389 precipitation. The precipitated lignin was recovered by centrifugation followed by freeze-drying.
390
391 **Isolation of cellulolytic enzyme lignin (CEL).** CEL was isolated from both WT and Eng-2 mutant
392 sorghum bagasse following the procedure described in a previous study.[40] The biomass was firstly
393 ball-milled for 2.5 h, and then hydrolyzed using CTec2® cellulase in sodium acetate buffer solution
394 with pH 4.8 at 50 °C for 48 h. After the hydrolysis, the solid residue was recovered by
395 centrifugation and hydrolyzed again under the same conditions with a fresh buffer solution and
396 enzyme. Then the solid residue was washed with DI water and extracted twice with a mixture of
397 dioxane and water (96%, v/v) at room temperature for 48 h. Solid and liquid fractions were
398 separated by centrifugation, and the supernatants were combined. CEL was recovered by rotary
399 evaporation of the combined supernatant and freeze-drying.
400
401 **Lignin molecular weights measurement.** The weight-average molecular weight (M_w), number-
402 average molecular weight (M_n), and the polydispersity index (PDI) of the CEL and lignin
403 recovered from the pretreated biomass were analyzed by gel permeation chromatography (GPC).
404 Prior to GPC analysis, lignin samples were acetylated using a mixture of acetic anhydride and
405 pyridine (1:1, v/v) at room temperature with continuous stirring for 48 h. Acetylated lignin samples
406 were recovered by rotary evaporation and dissolved in tetrahydrofuran (THF). Tests were
407 performed using a Waters 2489 GPC system equipped with a UV detector (270 nm) and three
408 Waters Styragel columns (HR0.5, HR3, and HR4e). THF was used as the mobile phase with a flow
409 rate of 1.0 mL/min. Calibration was conducted using polystyrene standards with different
410 molecular weights.
411
412 **Lignin structural analysis.** Structural features of the CEL and lignin recovered from pretreated
413 biomass were characterized using two-dimensional heteronuclear single quantum coherence (2D
414 HSQC) nuclear magnetic resonance (NMR). DMSO-d_6 was used to dissolve the lignin samples.
415 The 2D HSQC spectra were acquired with a Bruker AVANCE III HD 800 MHz NMR equipped
416 with a TCI cryoprobe.
417
418 **Compositional analysis of biomass.** The chemical composition of untreated and pretreated WT
419 and Eng-2 mutant sorghum bagasse was conducted according to the NREL procedure.[41] About
420 300 mg of biomass was loaded to 3 mL of 72% (w/w) sulfuric acid and hydrolyzed at 30 °C in a
421 water bath for 1 h. Upon the completion of the first hydrolysis, the solution was diluted to 4%
422 sulfuric acid and further hydrolyzed at 121 °C in an autoclave for 1 h. After the two-step hydrolysis,
423 solid and liquid fractions were separated by vacuum filtration. The solid fraction was dried at 105 °C
424 overnight and deashed at 575 °C for 24 h to gravimetrically determine the acid-insoluble lignin

425 content. The liquid fraction was analyzed by high-performance anion-exchange chromatography
426 with pulsed amperometric detection (HPAEC-PAD) and UV-vis spectrophotometer for sugar and
427 acid-soluble lignin determination, respectively. A series of carbohydrate standards were used for
428 the calibration curves, and fucose was used as an internal standard.
429
430 **Cellulose accessibility analysis.** Cellulose accessibility of untreated and pretreated WT and Eng-
431 2 mutant sorghum bagasse was measured by a modified Simons' Stain method, which has been
432 previously reported.[42] An Amicon ultrafiltration apparatus (Amicon Inc., Beverly, MA) was used
433 to remove the low molecular weight fraction of the direct Orange (DO) dye by filtering 1% solution
434 of DO dye through a 100 K membrane under 200 kPa nitrogen gas. For the analysis, ~100 mg wet
435 biomass samples were loaded into six centrifuge tubes containing 1.0 mL of phosphate-buffered
436 saline solution. Then, each tube was loaded with different amounts of DO dye solution and
437 incubated at 70 °C with continuous stirring at 200 rpm for 6 h. After adsorption, the dye
438 concentration was measured *via* a Lambda 35 UV-vis spectrophotometer at 455 nm, which
439 represents the wavelength of maximum absorbance for DO dye. Finally, the maximum amount of
440 dye adsorbed by the biomass was calculated following the Langmuir adsorption equation.
441
442 **Enzymatic hydrolysis.** Enzymatic hydrolysis of untreated and pretreated WT and Eng-2 mutant
443 sorghum bagasse was carried out by loading 200 mg of wet biomass sample into 125 mL
444 Erlenmeyer flasks containing 50 mM citrate buffer solution (pH 4.8). Accellerase® cellulase was
445 used for hydrolysis as described in a previous study.[39] Enzymatic hydrolysis was conducted at
446 50 °C with continuous stirring at 150 rpm for 72 h. Upon completion of the enzymatic hydrolysis,
447 an aliquot was taken to monitor the sugar release. After quenching the hydrolysis reaction by
448 incubating the aliquot in a boiling water bath, the aliquot was centrifuged at 10,000 rpm for 5 min.
449 The supernatant was recovered, diluted, and analyzed by HPAEC-PAD for the monomeric sugar
450 quantification.
451
452 **Lignin depolymerization.** The hydrothermal reaction of fractionated lignin was conducted to
453 recover PB. For the test, 200 mg of lignin sample was placed in a 50 mL Parr reactor (Parr
454 Instrument Company, IL, USA), and 25 mL of deionized water was then added. The reactor was
455 completely sealed and mounted on a heater. The reactor was purged and pressurized to 300 psi
456 with He. The mixture was heated to 180 °C and maintained for 1 h with continuous stirring at 300
457 rpm. After the reaction, the reactor was immediately removed from the heater and quenched in an
458 ice bath. The resulting solution was evaporated under reduced pressure. The final products were
459 silylated by adding 1.0 mL of dichloromethane and 1.0 mL of N,O-Bis(trimethylsilyl)acetamide
460 at 50 °C, which was injected into gas chromatography. Identification of the reaction products was
461 conducted using an Agilent 7820A GC equipped with a 5975 mass spectrometry detector. The
462 capillary column used was an Agilent HP-5MS (30 m × 0.25 mm × 0.25 μm). Injection temperature
463 was set to 270 °C, and oven temperature was programmed to hold at 70 °C for 5 min, ramp to 300 °C
464 at 3 °C/min, and then hold for 5 min at the final temperature.

For the hydrogenolysis reaction, 200 mg of lignin samples and 20 mg of Ru/C (5% Ru on activated carbon) were placed in the same batch reactor. Then, 25 mL of isopropyl alcohol, a hydrogen-donor solvent, was added to the reactor. After purging and pressuring to 300 psi with He, the reactor was heated and maintained at 300 °C for 1 h. Once the reaction was completed, the resulting solution was filtered and the solvent was completely evaporated. The final products were dissolved in 1 mL of acetone and analyzed by GC-MS under the same analysis conditions described above.

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