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# 1 **Multimodal characterization of acid-pretreated poplar reveals spectral** 2 **and structural parameters strongly correlate with saccharification**

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4

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9

## 10 **Highlights:**

- 11 • Multiscale characterization of dilute acid pretreated poplar samples was performed
- 12 • A severity threshold effect (2.5-2.7) affecting lignin organization is identified.
- 13 • Hydrolysis can be predicted by autofluorescence and Raman spectroscopy
- 14 • Cellular sphericity is negatively correlated with hydrolysis yield
- 15 • Quantitative relations across scales highlight recalcitrance

16

## 17 **Abstract**

18 Lignocellulose biomass can be transformed into sustainable chemicals, materials and  
19 energy but its natural recalcitrance requires the use of pretreatment to enhance subsequent  
20 catalytic steps. Dilute acid pretreatment is one of the most common and efficient ones,  
21 however its impact has not yet been investigated simultaneously at nano- and cellular-  
22 scales. Poplar samples have been pretreated by dilute acid at different controlled severities,  
23 then characterized by combined structural and spectral techniques (scanning electron  
24 microscopy, confocal microscopy, autofluorescence, fluorescence lifetime, Raman).  
25 Results show that pretreatment favours lignin depolymerization until severity of 2.4-2.5

26 while at severity of 2.7 lignin seems to repolymerize as revealed by broadening of  
27 autofluorescence spectrum and strong decrease in fluorescence lifetime. Importantly, both  
28 nano-scale and cellular-scale markers can predict hydrolysis yield of pretreated samples,  
29 highlighting some connections in the multiscale recalcitrance of lignocellulose.

30

31 **Keywords:** Lignocellulose, Recalcitrance, Multiscale, Pretreatment, Hydrolysis

32

### 33 **1. Introduction**

34 The environmental effects of climate change and fast depletion of fossil resources (Trends,  
35 2017) have promoted the development of alternative energy sources and bio-based  
36 chemicals and polymers. Lignocellulosic biomass (LB) is an important feedstock to  
37 produce such sustainable products and can be a solution to increasing concerns over  
38 energy demand and climate change without compromising global food security (Yuan et  
39 al., 2018).

40 LB is mainly composed of cellulose and hemicelluloses which are carbohydrate polymers  
41 and of lignin which is a highly branched phenylpropanoid polymer. The different biomass  
42 feedstocks (mainly wood and grass biomass species) have significant differences in  
43 proportion of the main constituents (Zhao et al., 2012a). While cellulose and lignin are  
44 more abundant in woody biomass, grass biomass has higher proportion in hemicelluloses.  
45 LB includes dedicated energy crops growing on low-quality soil such as miscanthus or  
46 switchgrass. Also considered as LB resources are agricultural wastes such as cereal straw,  
47 bagasse and forest biomass such as forest and mill residues and woody crops such as  
48 poplar and pine.

49 Main issue in the transformation of LB is that it is naturally recalcitrant to deconstruction  
50 into bio-based products because of its complex structure and chemical composition (Zhao

51 et al., 2012b). Several factors underlying LB recalcitrance have been identified such as the  
52 content of lignin/hemicellulose/cellulose (Studer et al., 2011), cellulose crystallinity (Xu et  
53 al., 2019), the degree of cellulose polymerization (Hallac & Ragauskas, 2011) and pore  
54 size/density (Meng et al., 2015). To overcome the recalcitrance of lignocellulose, a  
55 pretreatment step is essential to facilitate LB enzymatic hydrolysis. The pretreatment  
56 changes the physical and/or chemical structure of LB and facilitates the conversion of  
57 polysaccharides into fermentable sugars by enzymes by increasing accessibility to  
58 enzymes (McCann & Carpita, 2015). A wide range of pretreatment methods have been  
59 developed which can be classified into physical (e.g. milling, irradiation (e.g. Gamma-  
60 ray)), chemical & physico-chemical methods (e.g. explosion (with steam), acid (sulfuric)),  
61 and biological (fungi and actinomycetes) methods. Pretreatment is an expensive step in LB  
62 conversion process and the choice of the pretreatment type depends on the biomass  
63 species, due to variations in the content and composition of lignin and hemicellulose  
64 among LB species.

65 Dilute Acid Pretreatment (DAP) is one of the most commonly used industrial methods of  
66 pretreatment due to its relatively low cost and its efficiency particularly with hardwoods  
67 (Silveira et al., 2015). The DAP is mostly performed using 0.4-2.0% (w/w) H<sub>2</sub>SO<sub>4</sub> at a  
68 temperature of 160-220°C (Cao et al., 2012). It solubilizes hemicelluloses, disrupts the  
69 lignin structure, and increases cellulose accessibility to enzymes (Santos et al., 2018)  
70 favoring subsequent enzymatic hydrolysis. The influence and efficiency of the DAP  
71 depends on the pretreatment temperature, acidity (pH) and residence time. An indication of  
72 pretreatment harshness can be represented by a single value called CSF (Combined  
73 Severity Factor) (Lee & Jeffries, 2011). Despite extensive studies investigating the  
74 influence of the DAP on LB physico-chemical structure and morphology, the effect of the  
75 DAP at cellular and tissular scale remains yet poorly explored.

76 In this article, we have selected poplar (*Populus nigra x deltoides*) as a model of hardwood  
77 to investigate the effect of DAP pretreatment on LB at multiple scales under different  
78 pretreatment severities. Indeed, poplar is the first woody plant to have its genome  
79 sequenced (Chang et al., 2016), it has a fast growth rate, an easy in vitro cultivation and  
80 vegetative propagation as well as extensive geographical distribution (Meng et al., 2017).  
81 Moreover, the possibility of performing genetic modifications in poplar turns it into one of  
82 the most promising species for reducing LB recalcitrance (Lee & Jeffries, 2011). In order  
83 to investigate the effect of DAP pretreatment on poplar, compositional, structural and  
84 topochemical changes of poplar samples during DAP and cellulose digestibility were  
85 measured. This multimodal analysis was carried out using different microscopy techniques  
86 including advanced fluorescence microscopy techniques, chemical composition analysis  
87 by confocal Raman microscopy, scanning electron microscopy, and enzymatic hydrolysis  
88 to monitor the changes of substrate properties. Correlation between chemical / structural  
89 information and digestibility was finally done to highlight the impacts of specific markers  
90 on recalcitrance.

91

## 92 **2. Material and methods**

### 93 **2.1 Sample preparation**

94 Poplars (*Populus nigra x deltoides*) of about 2 years old were collected in Estrées-Mons,  
95 France. The collected poplar dried stems were cut into fragments of 0.4 cm wide, 2 cm  
96 long, 0.2 cm thickness using a razor blade. Poplar fragments were pretreated in a batch  
97 mode using mineralization bombs equipped with Teflon cups. 500 mg of poplar samples  
98 were presoaked in 2% (v/v) sulfuric acid (solution at a ratio of 1:30) and incubated in an  
99 oil bath at 170°C for 10, 15 and 20 minutes corresponding to three combined severity  
100 factor (CSF) of 2.4, 2.5 and 2.7, respectively. **These conditions were chosen because a**

101 **lower temperature did not provide significant modifications of the tissue architecture**  
102 **while higher temperature strongly damaged the tissues so that microscopy analysis**  
103 **was no longer possible.** The CSF integrates the reaction time, temperature and acid  
104 concentration into a single value and represents the pretreatment harshness. The CSF is  
105 computed using the formula  $CSF = \log_{10} (t \cdot \exp[(T_H - T_R)/14.75] - pH)$  as previously  
106 defined (Chum et al., 1990), where  $t$  is the reaction time in minutes,  $T_H$  is the temperature  
107 in °C,  $T_R$  is a reference temperature (typically 100°C) and pH is the acidity of the aqueous  
108 solution. Following DAP pretreatment of the samples, the Teflon cups were immersed in  
109 an ice bath for 5 min. The pretreated fragments were then washed three times with a 50%  
110 ethanol solution and three times with deionized water until the pH of the wash reached 7.0  
111 to stop the acid hydrolysis. The samples were dried for 24 hrs at room temperature. To  
112 acquire microscopy images, the dried fragments were sectioned in the transverse plane  
113 from the xylem using a sliding microtome 40µm thickness. **All sections used for**  
114 **microscopic analysis were made 0.5 cm thickness starting from the edge to be sure to**  
115 **have a homogeneous effect of the pretreatment.**

116 To perform chemical composition analysis and enzymatic saccharification, the dried  
117 samples were milled into particles with 80 µm diameter (granulometry measurement).  
118 Untreated fragments were cut using a sliding microtome into sections of 40 µm thickness  
119 or milled into 80 µm diameter particles for imaging, chemical composition and enzymatic  
120 saccharification analysis.

121

## 122 **2.2 Chemical composition and enzymatic saccharification**

123 The chemical composition including moisture, ash, lignin, and carbohydrates' contents of  
124 untreated and pretreated poplar samples was determined using the methods already  
125 described (Herbaut et al., 2018). The enzymatic hydrolysis assays were performed on both

126 untreated and pretreated samples using a commercial cellulase preparation Cellic® CTec2  
127 (Novozymes A/S Bagsværd, Denmark), selected for its hydrolysis efficiency with a  
128 cellulase activity of 195 FPU/mL measured by the filter paper method. Enzymatic  
129 saccharification of poplar samples (2 % w/v) was carried out in 10 mL acetate buffer  
130 (0.05M, pH 5) for 72 hrs at 50°C and 200 rpm containing 0.02 % sodium azide and the  
131 Cellic® CTec2 cocktail with a final enzyme concentration of 20 FPU/g of dry matter.  
132 The reaction mixtures (buffer and sodium azide) were pre-incubated for 30 min at 50°C  
133 and 200 rpm. The enzymatic hydrolysis was then initiated by adding the enzymatic  
134 cocktail. During the enzymatic hydrolysis, the samples were kept at 50°C for 72 hrs.  
135 Aliquots were taken at different timepoints: 0, 0.5, 1, 2, 4, 6, 8, 24, 32, 56 and 72 hrs to  
136 compute hydrolysis kinetics (Auxenfans et al., 2017a). The concentration of glucose  
137 released from enzymatic hydrolysis in the supernatant was determined using a high-  
138 performance anionic exchange chromatography (HPAEC-PAD, Dionex) to calculate the  
139 cellulose conversion from the untreated and pretreated poplar, as follows:

140 Cellulose conversion (%)

141 = Amount of released glucose/Amount of cellulose in samples before hydrolysis x 100

142 All experiments and analysis were carried out three times (biological triplicate).

143

### 144 **2.3 Scanning electron microscopy (SEM)**

145 To investigate and compare the surface morphology changes between untreated and  
146 pretreated samples, the samples were observed using a binocular microscope (Stemi 2000-  
147 C, Zeiss, Germany), then imaged by scanning electron microscopy (SEM) using an  
148 environmental tabletop electron microscope Hitachi TM-1000 (Japan) in low-vacuum  
149 mode. SEM pictures of untreated and pretreated poplar sections were acquired at, 600x and  
150 3000x magnifications.

151

## 152 **2.4 Confocal Raman microscopy (CRM)**

153 The untreated and pretreated poplar sections were pre-incubated in water for 30 min and  
154 then placed on a quartz slide with a drop of ultrapure water and sealed with a cover slip  
155 (0.17 mm thickness) to avoid evaporation during the Raman signal detection. Raman  
156 spectra were acquired with a microspectrometer LabRam ARAMIS (Horiba Jobin-Yvon,  
157 Villeneuve d'Ascq, France). The laser source was a diode at 785 nm to avoid fluorescence  
158 (Zeng et al., 2016), with 13mW power on sample. The microspectrometer was coupled to a  
159 microscope BX41 (Olympus, France) equipped with a motorized (x, y) stage. All  
160 measurements were recorded using a 100x water immersion objective (LUMPlanFI, NA =  
161 1.0, Olympus, France). The laser and the light scattered by sample were collected through  
162 the same objective. A confocal pinhole rejects signals from out-of-focus regions of the  
163 sample. A multichannel charge-coupled-channel device (1024x256 pixels) was used to  
164 detect the Raman Stokes signal dispersed by a holographic grating (1200 lines/mm)  
165 (Gierlinger et al., 2012; Chen et al., 2016a). Raman spectra were recorded with LabSpec 5  
166 software (Horiba, Jobin Yvon, Villeneuve d'Ascq, France) in the specific spectral range  
167 950-1700  $\text{cm}^{-1}$ . Three samples for each condition of pretreatment were analysed and for  
168 each sample ten spectra were recorded on different regions of cell wall (cell corner (CC),  
169 compound middle lamella (CML) and secondary wall (SW)). These spectra were averaged  
170 for each sample. Then, a pre-treatment of Raman spectra was performed with a homemade  
171 interface in Matlab were smoothed using Savitzky-Golay function (5 points, 3<sup>rd</sup>  
172 polynomial order) and baseline corrected (3<sup>rd</sup> polynomial order). Bands were assigned  
173 according to the literature are summarized in Table 1.

174

## 175 **2.5 Laser Scanning Confocal Microscopy for 3D imaging**

### 176 **2.5.1 3D image acquisition**

177 Poplar cross sections were incubated in 0.05 M acetate buffer at pH 5 for 30 min prior to  
178 mounting in the same medium on a microscope slide and covered with a cover slip (0.17  
179 mm thickness). Using a confocal laser scanning microscope (Leica TCS SP8, Germany)  
180 equipped with 63× oil-immersion objective (NA = 1.4), z-stacks (0.3µm) of both untreated  
181 and pretreated poplar samples were acquired at scan speed of 400 Hz. A 405 nm laser (4%  
182 intensity) was used for imaging cell wall sample autofluorescence by detecting  
183 fluorescence emission on the 415-700 nm range using the HyD detector in counting mode.  
184 The z-stacks were acquired with a resolution of 512 x 512 pixels. The microscope  
185 parameters and image resolution were optimized to avoid sample photo-bleaching.  
186 For each pretreatment condition (CSF = 2.4, 2.5, 2.7) as well as untreated samples, z-  
187 stacks on three different randomly selected areas of three different samples were acquired.

188

### 189 **2.5.2 Image analysis and 3D segmentation of confocal images**

190 Acquired z-stacks were processed using an automated 3D segmentation and quantification  
191 pipeline. The original z-stacks were then saved in tif files with customized tags which  
192 guaranteed that the metadata (e.g. voxel dimensions, file type) were saved properly. The  
193 images were then denoised using Alternating Sequential Filter (ASF) and Gaussian filters  
194 (Willis et al., 2016; Michelin et al., 2016). To segment the filtered z-stack in order to  
195 identify individual cell walls, the z-stack was first segmented using a 3D watershed  
196 algorithm whose seeds were determined using the h-minima operator which computed  
197 local minima regions in the denoised z-stacks. The 3D watershed algorithm provided a 3D  
198 image in which voxels (volumetric pixel) of the same cell were labeled by a unique integer  
199 as cell identifier. Thresholding was then used to compute cell walls by replacing the voxels  
200 of the segmented images with background value (fixed to 1) if the intensity of the

201 corresponding voxels on filtered z-stack was below a global threshold. The segmentations  
202 were visually inspected for segmentation error (over-segmentation, under-segmentation,  
203 missed cell, or shape error) and the segmentation parameters were subsequently optimized  
204 (Willis et al., 2016).

205

### 206 **2.5.3 Quantification of cellular scale structural parameters**

207 Following 3D segmentation of z-stacks, individual cell wall volumes were computed by  
208 counting the number of voxels which had the same label and multiplying this number by  
209 the voxel volume which was approximately  $0.039\mu m^3$ . Cell wall surface area was  
210 estimated from triangular meshes of cell wall surfaces obtained using the marching cubes  
211 algorithm from the Visualization Toolkit (VTK) (Willis et al., 2016; Schroeder et al.,  
212 2004). Then volume,  $V$ , and surface area,  $A$ , were computed to determine sphericity

213  $\Psi = \frac{6\pi^{1/3}V^{2/3}}{A}$ , a dimensionless cellular scale parameter, for each individual cell.

214

## 215 **2.6 Laser Scanning Confocal Microscopy for spectral imaging**

### 216 **2.6.1 Autofluorescence**

217 Spectral images of untreated and pretreated poplar samples were acquired using laser  
218 scanning microscope LSM 710 NLO Zeiss (Zeiss SAS, Germany) coupled with a  
219 Chameleon TiSa accordable 80 MHz pulsed laser (COHERENT, USA). Samples sections  
220 ( $40\mu m$ ) were mounted in 0.5 M acetate buffer at pH 7 and their excitation was performed  
221 using a biphoton excitation at 750 nm. Spectral images were acquired using spectral  
222 detector (32 channel simultaneously) of the microscope between 420 and 722 nm using a  
223 20x objective (NA = 0.8). Fluorescence images of untreated and pretreated samples were  
224 colored based on their spectrum (each of the 32 channels was represented by its  
225 corresponding color from blue to red).

226

## 227 **2.6.2 Fluorescence lifetime measurement and analysis**

228 The fluorescence lifetime imaging (FLIM) measurements were performed on untreated  
229 and pretreated poplar sections between 455 and 655 nm using a spectral time-correlated  
230 single photon counting (TCSPC) detector from Becker and Hickl (Becker & Hickl, Berlin,  
231 Germany). Data were analyzed using a multi-exponential decay model with 2 components  
232 using SPCImage software (Becker & Hickl, Berlin, Germany). Measurement of average  
233 fluorescence lifetime was made by applying regions of interest (ROIs) to cell wall regions  
234 such as cell corner (CC), compound middle lamella (CML) and secondary wall (SW) for  
235 untreated and pretreated poplar using 10 replicate measurements on each of the 3 regions.  
236 ROI data were summed to provide estimates of distributions in these regions.

237

## 238 **2.7 Data and statistical analysis**

239 All the experiments were carried out in triplicate, and the results were expressed as means  
240  $\pm$  standard deviations. Analysis of variance (ANOVA) was performed on the obtained data  
241 followed by a Tukey's post hoc test for comparison between the untreated and pretreated  
242 poplar. *p*-value for statistical difference was set to 0.05. Statistical analyses were  
243 performed using the SigmaPlot 12.0 software (Systat Software, Chicago, USA). Kruskal-  
244 Wallis test from SciPy library was performed on computed cellular sphericity values from  
245 untreated and pretreated datasets.

246

## 247 **3. Results and Discussion**

### 248 **3.1 DAP changes LB chemical composition and improves enzymatic saccharification**

249 The chemical composition of biomass samples is a significant factor that affects the  
250 sequential enzymatic hydrolysis. In order to better understand the changes induced by the

251 different degree of severity of the DAP, the chemical composition of both untreated and  
252 pretreated samples was analysed. Cellulose, hemicelluloses, lignin, moisture and ash  
253 content were determined on a dry weight basis (Fig. 1). The untreated poplar contained ca.  
254  $40\% \pm 0.4$  cellulose,  $21\% \pm 0.2$  hemicelluloses with xylose as the major component (also  
255 comprising arabinose, rhamnose, mannose and uronic acids) and 26% lignin, which is  
256 consistent with reports from the literature (Herbaut et al., 2018; Esteghlalian et al., 1997).  
257 As expected, hemicelluloses solubilisation increased significantly with pretreatment  
258 severity. About 70% of the hemicellulose fraction was removed from the pretreated  
259 substrate under the most severe pretreatment conditions (CSF 2.7). The decrease in  
260 hemicellulose content, proportional to the pretreatment severity, was due to its degradation  
261 into other chemicals under higher temperature and acid conditions. The lignin content  
262 gradually increased with increasing CSF: this result is due to the concomitant loss of  
263 hemicellulose and the formation of condensed lignin products (Sannigrahi et al., 2011).  
264 This could also be due to the formation of pseudo-lignin by the combination of  
265 carbohydrates and lignin degradation products (Sannigrahi et al., 2011). Many studies  
266 reported similar changes in carbohydrates and lignin content after DAP (Herbaut et al.,  
267 2018; Chen et al., 2016b).

268 In order to evaluate the efficiency of DAP on cellulose digestibility, the same samples used  
269 for the chemical composition analysis were subjected to a 72 hrs enzymatic hydrolysis  
270 using commercial cellulase cocktail Cellic CTec 2 with a loading of 20 FPU/g of dry  
271 matter. The effect of the different severity of DAP on poplar digestibility was estimated by  
272 determining the percentage of cellulose conversion over 72 hrs (Fig. 2). A remarkable  
273 increase in the efficiency of cellulose conversion was observed when DAP severity was  
274 increased, which confirms that pretreatment reaction time is a factor affecting the  
275 effectiveness of pretreatment (An et al., 2019). Indeed, the cellulose conversion of

276 untreated poplar was about  $11\% \pm 0.11$  after 72 hrs, then reached  $17\% \pm 0.41$  for  
277 pretreated poplar at CSF 2.4,  $28\% \pm 0.77$  (more than 2-fold) for pretreated poplar at CSF  
278 2.5 and  $58\% \pm 2.17$  (5-fold) for pretreated poplar at CSF 2.7, which is in agreement with  
279 results reported in the literature (Meng et al., 2016; Linde et al., 2008; Chen et al., 2018).  
280 Overall, results confirm that DAP increases the efficiency of enzymes by improving their  
281 accessibility, likely due to hemicellulose removal and lignin reorganization.

282

### 283 **3.2 SEM images reveal effect of DAP on cellular morphology**

284 To better understand the significant enhancement of poplar samples enzymatic digestibility  
285 after pretreatment, the morphology of the untreated and pretreated samples was  
286 investigated by SEM. Initially, the most apparent effect of the DAP observed by binocular  
287 microscope was the color change of samples from yellow to dark brown which was more  
288 pronounced in samples pretreated at high severity . Previous studies have reported that this  
289 color change originated from chemical degradation of carbohydrates, lignin and wood  
290 extractives (Negro et al., 2003). The cell walls of untreated poplar observed by SEM had a  
291 rigid and highly ordered structure. The compound middle lamella (CML) and the  
292 secondary wall (S2) kept close together. This rigid structure is recognized as a structural  
293 organization limiting the accessibility of enzymes to cellulose (Mansfield et al., 1999). The  
294 pretreated samples showed fiber deformation especially for the pretreated samples at CSF  
295 2.5 and CSF 2.7. Poplar samples pretreated at the highest severity even showed fiber  
296 separation (loss of the fibrous network) and formation of cracks emphasized by the  
297 detachment between CML and S2. This structural change could be related to the  
298 solubilisation of the hemicellulose fraction which is considered as the adhesive between  
299 cell wall sublayers (Ling et al., 2015).

300

### 301 **3.3 Raman microscopy shows DAP induced topochemical modifications**

302 Confocal Raman microscopy provides information about the concentration and the spatial  
303 distribution of chemical components in cell walls (Gierlinger & Schwanninger, 2006). In  
304 this study, changes in the spatial distribution of polymers in the cell walls of pretreated  
305 poplar were investigated in situ. The average Raman spectra of untreated and pretreated  
306 poplar were collected from various regions: cell corner (CC), compound middle lamella  
307 (CML), and secondary wall (SW).

308 The spectral range from 950 to 1700  $\text{cm}^{-1}$  includes bands from the wood components such  
309 as cellulose, hemicelluloses and lignin. Band assignment for poplar is shown in Table 1.  
310 Typical bands of lignin are in the region between 1500  $\text{cm}^{-1}$  and 1700  $\text{cm}^{-1}$  with specific  
311 peaks at 1604  $\text{cm}^{-1}$  and 1660  $\text{cm}^{-1}$ . The bands at 1036  $\text{cm}^{-1}$ , 1095  $\text{cm}^{-1}$  and 1123  $\text{cm}^{-1}$  are  
312 attributed to carbohydrates.

313 For untreated poplar, the spectra from different cell wall regions presented different  
314 features. Lignin bands at 1604  $\text{cm}^{-1}$ , 1275  $\text{cm}^{-1}$  and 1331  $\text{cm}^{-1}$  were higher in the spectra of  
315 CC and CML than those in SW, which in accordance with the higher content in lignin of  
316 CC. Contrary, the peaks at 1095  $\text{cm}^{-1}$ , 1123  $\text{cm}^{-1}$  and 1150  $\text{cm}^{-1}$  were more pronounced in  
317 the SW than in CC and CML, indicating that SW mainly contains polysaccharides.

318 Previous studies reported similar results (Chen et al., 2016a; Chen et al., 2018; Gierlinger  
319 & Schwanninger, 2006).

320 DAP caused considerable changes in the band intensity for the majority of Raman peaks,  
321 also depending on cell wall regions. The intensity of the band at 1604  $\text{cm}^{-1}$ , which is  
322 related to lignin, increased significantly for CC and CML after DAP especially for the  
323 pretreated samples at CSF 2.5 and CSF 2.7. It can be explained by the fact that lignin was  
324 more exposed after the removal of hemicelluloses during the DAP. It may also result from  
325 the dissolved lignin redeposited onto cell wall surfaces upon cooling after DAP. The

326 increase of the lignin band intensity for the SW was less important which can be explained  
327 by the low lignin content in this region. The peak at  $1660\text{ cm}^{-1}$ , which is indicative of  
328 coniferyl alcohol and aldehyde (lignin-CAA) (Ma et al., 2013), has completely  
329 disappeared from Raman spectra of pretreated poplar at CSF 2.5 and CSF 2.7 particularly  
330 in the CC and CML. One possible explanation is that high severity DAP removes lignin-  
331 CAA structures. Previously, it has been reported that lignin is cross-linked with  
332 hemicelluloses that embed cellulose fibres and lignin-CAA associates with hydroxyl  
333 groups of cellulose and hemicelluloses via hydrogen bonding within cell walls. The  
334 removal of hemicelluloses during DAP may disrupt the cross-linked structures. This  
335 observation is consistent with the results from a study of the impact of alkali pretreatment  
336 on poplar cell walls (Ji et al., 2014). However, a significant decrease in the bands at  $992\text{ cm}^{-1}$ ,  
337  $1095\text{ cm}^{-1}$  and  $1123\text{ cm}^{-1}$  was noted, more important in the SW than that in the CML  
338 and CC, indicating significant removal of the polysaccharides from the SW region after  
339 DAP and correlated with DAP severity. This topochemical analysis reveals that  
340 modifications in chemical composition after DAP are heterogeneous throughout the poplar  
341 cell walls and stronger with higher DAP severity.

342

### 343 **3.4 DAP impacts cell wall autofluorescence and fluorescence lifetime**

344 In wood, lignin is the predominant fluorophore exhibiting autofluorescence under UV light  
345 excitation (Donaldson & Radotic, 2013). Fig. 3A-D shows the autofluorescence of  
346 untreated and DAP poplar samples. While the spectral confocal image of untreated poplar  
347 (Fig. 3A) has a homogenous blue color distribution along the cell wall, this color turns to  
348 blue-greenish for pretreated poplar with CSF 2.5 (Fig. 3C) and to green-yellowish for  
349 pretreated poplar with CSF 2.7 (Fig. 3D). Changes in autofluorescence are likely to reflect  
350 modification of the lignin chemistry during DAP. Indeed, several factors related to lignin

351 are recognized as influencing fluorescence, such as content in lignin and in carbonyl  
352 groups, lignin condensation and cross-linkages between lignin and other cell wall  
353 components (Auxenfans et al., 2017b). Spectral data were analysed for all the untreated  
354 and DAP samples in different cell wall regions like for Raman measurements. The  
355 untreated poplar has a narrow emission band centred on the blue spectral range (maximum  
356 478 nm) (Fig. 3E), with the CC and CMC spectra being close to each other and higher than  
357 SW spectrum. With the increase of DAP severity, the emission maximum expands in the  
358 green and yellow range (Fig. 3F-H) together with an increase in the fluorescence intensity  
359 and a distinct separation of the spectra corresponding to the cell wall regions. This result  
360 suggests that the DAP changed strongly the natural lignin arrangement, by solubilizing  
361 lignin molecules that interact with hemicellulose oligomers to form new fluorescent  
362 compounds emitting in the blue, green and yellow spectra. These results are in agreement  
363 with those previously published (Coletta et al., 2013; Li et al., 2007) and show how  
364 autofluorescence can reveal changes in lignin composition and arrangement after  
365 pretreatment.

366 Fluorophore lifetime is the average time between excitation of a fluorophore and emission  
367 of a fluorescence photon. Accordingly, fluorescence lifetime imaging microscopy (FLIM)  
368 is a technique to determine the spatial distribution of excited state lifetimes in microscopic  
369 samples (Van Munster & Gadella, 2005). In complement to autofluorescence, FLIM can  
370 advantageously be related to chemical properties of lignin (Auxenfans et al., 2017b ;  
371 Chabbert et al., 2018), since a short lifetime reflects high conjugation between  
372 fluorophores and vice-versa. Lifetime fluorescence variation could be visualized in the  
373 corresponding lifetime color-coded images of the poplar cell walls (Fig. 3I-L), from green  
374 to yellow, meaning that lifetime was decreased with increased DAP severity. At the cell  
375 wall level, fluorescence lifetime in SW appeared always slightly longer than in CML and

376 CC for all poplar samples considered (Fig. 4M-P). This can be explained by the  
377 differences in the chemical structure of lignin and its molecular environment in these  
378 regions, suggesting that both CC and CML regions contain short lifetime component  
379 (condensed lignin: *p*-hydroxyphenyl units) and SW regions contain both short and long  
380 lifetime components (loosely packed lignin) (Donaldson & Radotic, 2013; Zeng et al.,  
381 2015). Untreated poplar fluorescence lifetime was about  $813 \pm 25.22$  ps, then increased  
382 significantly to  $885 \pm 29.17$  ps for severity of 2.4;  $p$ -value  $< 0.05$  ( $p$ -value = 0.045) and  
383 even reached  $966 \pm 34.87$  ps for severity of 2.5 ( $p$ -value = 0.01), suggesting DAP  
384 increased the content of loosely-packed lignin by depolymerising dense lignin regions (Li  
385 et al., 2007). In contrast, DAP with the highest severity of 2.7 led to a drop of poplar  
386 fluorescence lifetime by 40% to  $585 \pm 30.49$  ps ( $p$ -value  $< 0.001$ ), suggesting the existence  
387 of a threshold effect around CSF 2.5-2.7. Indeed, it seems the population of denser lignin  
388 has suddenly extended in all considered cell wall regions because of the extraction of the  
389 loosely packed lignin during the solubilisation of hemicellulose and the formation of  
390 pseudo-lignin. Such harsh conditions have probably generated lignin degradation products  
391 which have been able to repolymerize as highly conjugated polymers with shortened  
392 fluorescence lifetime (Sannigrahi et al., 2011; Trajano et al., 2013).

393 Overall, fluorescence analysis reveals that DAP strongly alters lignin itself and its  
394 environment mainly due to partial lignin depolymerization and hemicellulose  
395 solubilisation on the range of CSF 2.4-2.5, while higher CSF of 2.7 generates lignin  
396 chemical modifications such as lignin condensation and creation of new inter-linkages  
397 between fluorophores.

398

399 **3.5 DAP induces significant shape changes at cellular scale**

400 Images obtained using scanning electron microscopy indicated structural changes at  
401 cellular scale on pretreated samples compared to untreated samples. However, the  
402 drawbacks of SEM imaging including artifacts potentially introduced by sample  
403 preparation (drying) and limited spatial dimensions (2D) have led to a mostly qualitative  
404 conclusions so far (Herbaut et al., 2018; Chen et al., 2018). To quantitatively investigate  
405 the effect of pretreatment at cellular scale and study its correlation with acquired spectral  
406 and chemical data, an original method combining 3D confocal acquisition and 3D  
407 segmentation and quantification was developed (see Materials and Methods) and the  
408 morphological parameters including cell volumes, surface areas and subsequently cellular  
409 sphericity, a dimensionless cellular scale structural parameter, were computed. The mean  
410 sphericity values were  $0.62 \pm 0.19$ ,  $0.54 \pm 0.21$ ,  $0.52 \pm 0.19$ ,  $0.43 \pm 0.11$  for untreated, and  
411 pretreated samples at 2.4 CSF, 2.5 CSF, 2.7 CSF respectively. The quantifications showed  
412 that cellular sphericity gradually decreased as the DAP pretreatment severity increased  
413 (Kruskal–Wallis test,  $p$ -value =  $7e-109$ ,) (Fig. 4), with the most striking cell shape  
414 alteration at 2.7 CSF. The decreasing trend in cell sphericity with increasing pretreatment  
415 severity confirms the morphological changes observed in SEM images and can be  
416 explained by the fact that the pretreatment modifies the compact structure of cell walls  
417 through dissolution of hemicelluloses and solubilisation and redistribution of lignin  
418 therefore decreases cell compactness measured by sphericity. Moreover, sphericity as a  
419 function of the computed cell volume over computed surface area, decreases by increasing  
420 surface area which has been previously reported (Torr et al., 2016; Zhang et al., 2018). Our  
421 results show that measurements of cell sphericity can provide quantitative useful  
422 information about the DAP severity and consequently predict hydrolysis yield. The results  
423 also highlight the importance of multi-scale approaches involving cellular and tissular

424 scale in thoroughly investigating LB recalcitrance and shed light on parameters behind LB  
425 recalcitrance at scales yet little explored.

426

### 427 **3.6 Correlation analysis of measured chemical, spectral and structural parameters** 428 **reveals quantitative relationships across scales**

429 Given the different properties determined for the untreated and DAP poplar samples,  
430 results clearly show strong correlations between DAP severity and modifications in  
431 chemical composition (in particular lignin) together with structural modifications, in  
432 different cell wall regions. Importantly, a threshold effect has been identified as severity  
433 was increased from CSF 2.5 to 2.7, revealing that an increase of only 5 minutes of DAP  
434 has dramatic consequences on polymer organization as revealed by spectral analysis.  
435 Consequently, these data can be useful to understand the correlation between related  
436 factors and saccharification potential. Indeed, lignocellulose chemical and structural  
437 complexity limit the establishment of easy and low-cost transformation processes, and  
438 many studies have searched to find some factors that could help predicting recalcitrance  
439 (Auxenfans et al., 2017b; Chabbert et al., 2018; Huang et al., 2017; Paës et al., 2019). To  
440 understand relationships between parameters, we conducted a detailed correlation analysis  
441 between quantified parameters from **autofluorescence, FLIM, Raman and structural**  
442 **analysis and hydrolysis yield at 72 hrs (Fig. 5).**

443 Among spectral factors, fluorescence lifetime appears as a strong predictor of hydrolysis  
444 (correlation values ca. 0.70-0.75), while autofluorescence and Raman data showed much  
445 stronger correlations (0.95-1) and can thus be considered as excellent predictors of  
446 hydrolysis. Regarding autofluorescence, its capacity to predict hydrolysis was previously  
447 demonstrated on different biomass species and different pretreatment types (Herbaut et al.,  
448 2018; Auxenfans et al., 2017a), which strengthens its potential universality. This means

449 that autofluorescence, more than fluorescence lifetime, reveals accessibility of enzymes to  
450 polysaccharides, probably because it is related to both structure and composition of lignin.  
451 Nonetheless, lifetime analysis is also relevant to understand the chemical modifications of  
452 lignin during pretreatment. Raman data are also a good compromise to predict hydrolysis  
453 by using specific ratio between lignin and cellulose-related bands, which is directly related  
454 to polymer accessibility.

455 Furthermore, cellular scale morphological parameter, namely sphericity is identified which  
456 exhibits a strong negative correlation with hydrolysis yield ( $-0.96$ ,  $p$ -value  $< 0.05$ ). This  
457 demonstrates that a structural factor can be measured at cellular scale correlated with DAP  
458 severity can predict saccharification. Even though, it was previously demonstrated that  
459 nanoscale structural features (porosity, cellulose accessible surface for example) were  
460 related to hydrolysis (Herbaut et al., 2018; Zhang et al., 2018; Kruyeniski et al., 2019), it is  
461 the first time to our knowledge that a cellular scale feature like sphericity can also predict  
462 hydrolysis. This reveals some un-commonly studied connections between scales:  
463 hydrolysis by enzymes occurs at the nano-scale, but cell shape might be a strong marker of  
464 polymer organization.

#### 465 **4. Conclusions**

466 Multiscale analysis of dilute-acid pretreated poplar samples revealed that several markers  
467 could help understanding the effect of pretreatment on polymer composition and  
468 organization and subsequently hydrolysis. In particular, autofluorescence appears as a  
469 marker previously identified, thus likely to be considered as pivotal. Importantly, the  
470 results show that cellular scale structural parameters such as cellular sphericity, seem also  
471 to be strongly correlated with hydrolysis which should be further studied using other

472 biomass species and pretreatments, including investigating other structural markers to  
473 assay their universality.

474

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481

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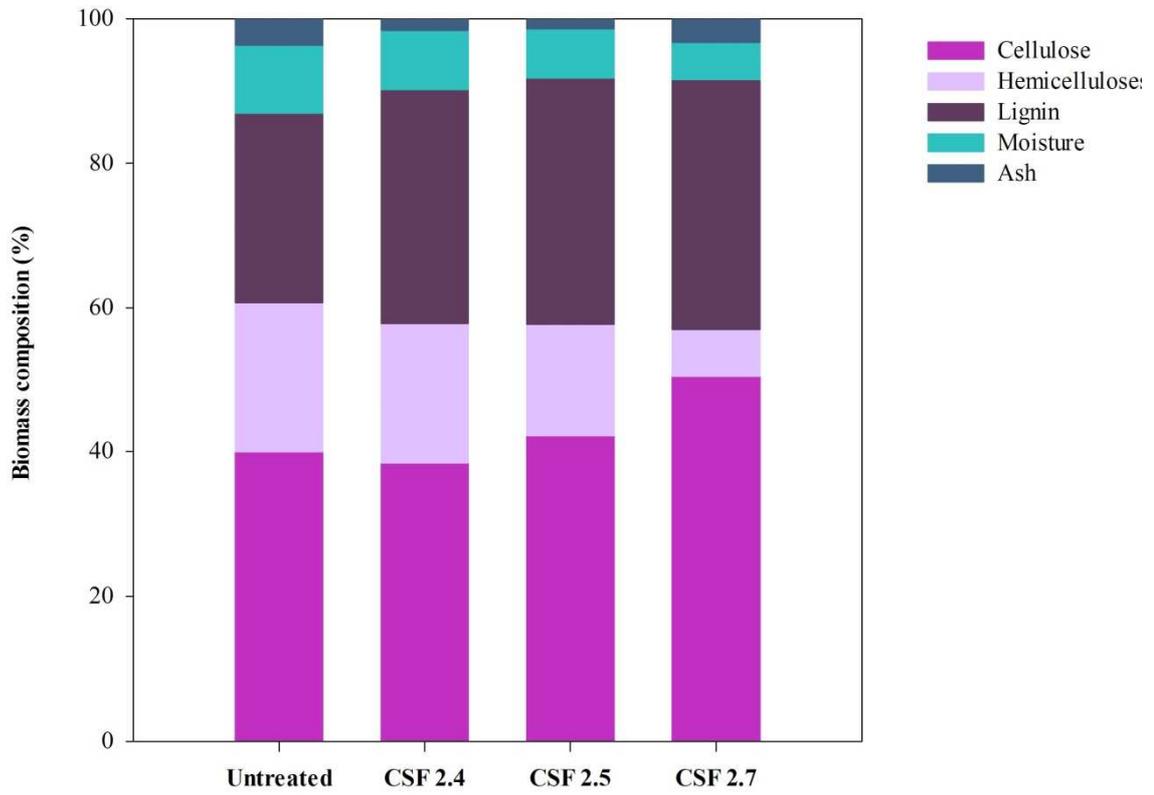
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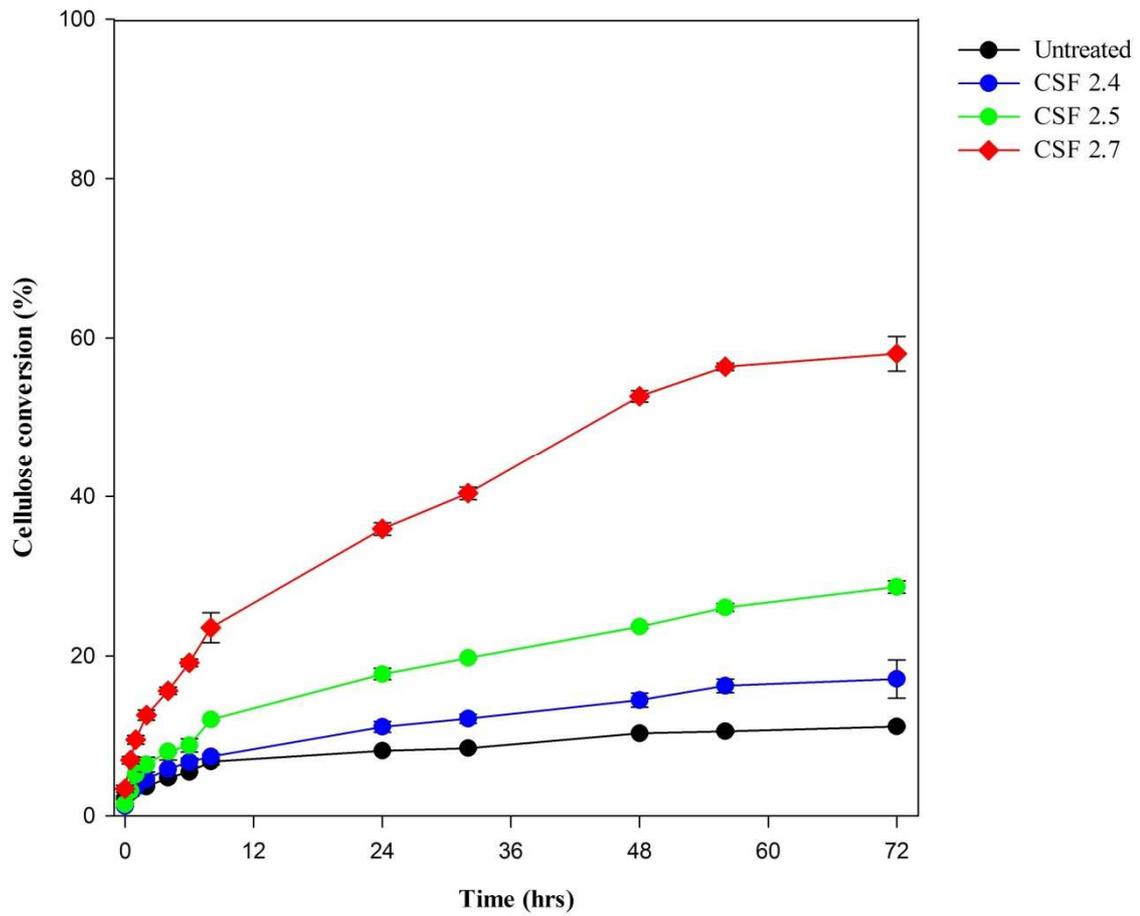
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- 633

# 1 Figures



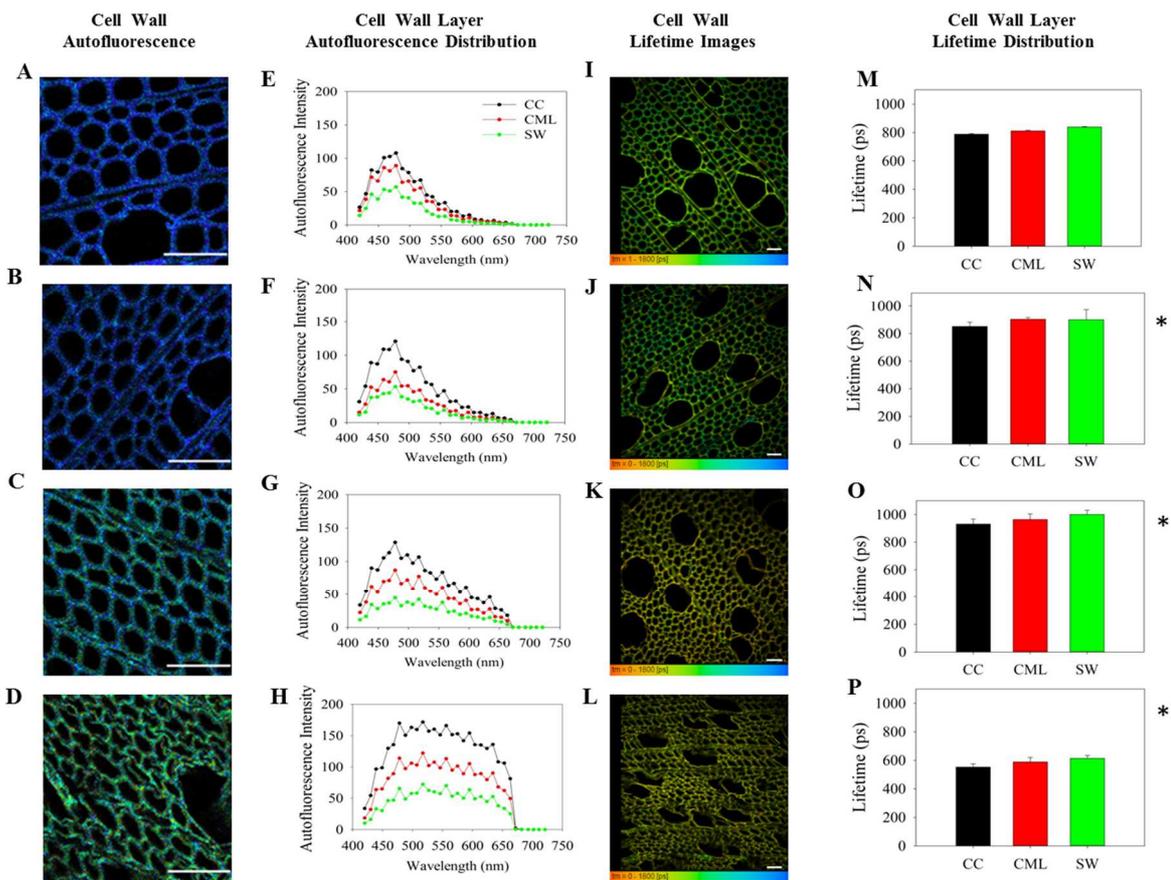
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3 Fig. 1. Chemical composition of untreated and pretreated poplar samples. Contents are  
4 expressed as weight percentages (%w/w) of the dry biomass amount.



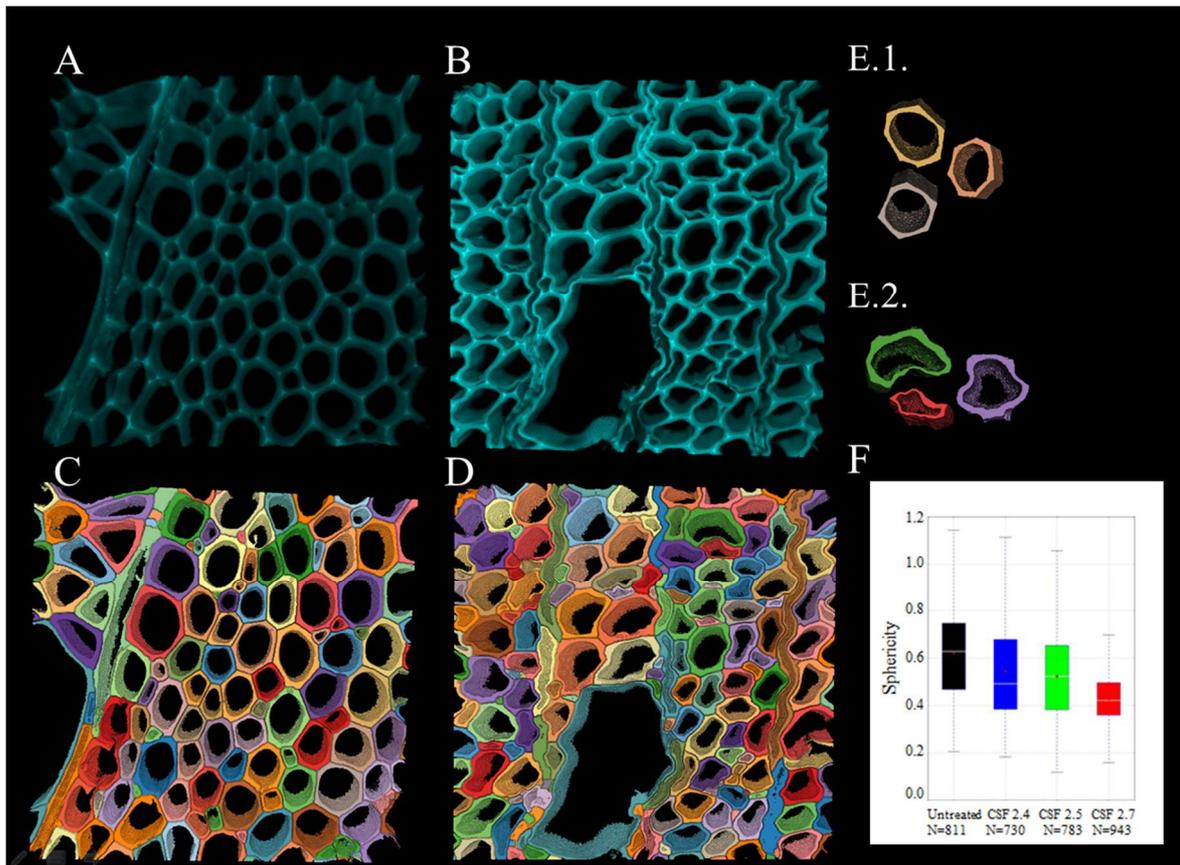
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6 Fig. 2. Cellulose conversion during enzymatic hydrolysis over 72 hrs.



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Fig. 3. Fluorescence properties of untreated and pretreated poplar. (A, B, C and D) spectral confocal images of the cell wall cell corner (CC) in black, compound middle lamella (CML) in red, and secondary wall (SW) in green for untreated and pretreated poplar samples illustrating the autofluorescence of lignin generated by 750 nm bi-photon excitation, emission measured from 420 nm (blue) to 722 nm (red), scale bars 20 $\mu$ m. (E, F, G and H) Fluorescence spectra of the cell wall for untreated and pretreated poplar samples. (I, J, K and L) Fluorescence lifetime images and (M, N, O and P) lifetime measurements for untreated and pretreated poplar samples, scale bars 20 $\mu$ m. Fluorescence lifetime values are averaged from measurement on three different samples and three different areas from the same sample. Error bars indicate standard deviation. Asterisks indicate statistically significant difference between pretreated and untreated poplar samples.



21

22 Fig. 4. Effect of DAP on poplar samples at cellular scale. (A) 3D projection of confocal  
 23 image of untreated poplar section. (B) 3D projection of confocal image of pretreated  
 24 poplar section at CSF 2.7. (C) Rendering of 3D segmentation of the untreated sample  
 25 confocal stacks. (D) Rendering of 3D segmentation of the pretreated sample confocal  
 26 stack. (E.1.) Randomly selected cells showing effect of the segmented untreated sample  
 27 and (E.2.) and the pretreated sample, showing effect of DAP on individual cell shapes. (F)  
 28 Distribution of cellular sphericity of untreated and pretreated samples with different  
 29 severity conditions. The boxes represent the interquartile range and the white lines in the  
 30 boxes represent the medians. The whiskers show the range of values from the highest to  
 31 the lowest excluding outliers (not shown). N is the number of segmented cells.

32

|     |                       |                          |                           |                          |                               |                                |                               |                               |                                |                               |            |                          |                           |                          |
|-----|-----------------------|--------------------------|---------------------------|--------------------------|-------------------------------|--------------------------------|-------------------------------|-------------------------------|--------------------------------|-------------------------------|------------|--------------------------|---------------------------|--------------------------|
| Y72 | -0.75                 | -0.68                    | -0.73                     | -0.68                    | 0.96                          | 0.8                            | 0.8                           | 0.99                          | 0.98                           | 0.98                          | -0.96      | 0.97                     | 0.96                      | 0.94                     |
|     | Fluorescence Lifetime | Fluorescence Lifetime CC | Fluorescence Lifetime CML | Fluorescence Lifetime SW | Autofluorescence at 478 nm CC | Autofluorescence at 478 nm CML | Autofluorescence at 478 nm SW | Autofluorescence at 595 nm CC | Autofluorescence at 595 nm CML | Autofluorescence at 595 nm SW | Sphericity | Ration Raman 1604/992 CC | Ration Raman 1604/992 CML | Ration Raman 1604/992 SW |

33

34 Fig. 5 Pearson's correlation coefficients between quantified spectral, structural factors and  
 35 hydrolysis yield ( $p$ -value  $< 0.05$ ).

## 1 Tables

2 Table 1. Raman bands and their assignment to lignin and carbohydrate components

3 (cellulose, glucomannan and xylan) according to the literature. str: stretching

| Wavenumbers (cm <sup>-1</sup> ) | Components                        | Assignments   |
|---------------------------------|-----------------------------------|---|
| 992                             | Cellulose                         | Heavy atom (CC and CO) str.   |
| 1036                            | Cellulose                         | HCC and HCO bending   |
| 1095                            | Cellulose<br>Glucomannan<br>Xylan | Heavy atom (CC and CO) str.   |
| 1123                            | Cellulose<br>Glucomannan<br>Xylan | Heavy atom (CC and CO) str.   |
| 1150                            | Cellulose                         | Heavy atom (CC and CO) str.<br>plus HCC and HCO bending                             |
| 1275                            | Lignin                            | Aryl-O of aryl OH and aryl<br>O-CH <sub>3</sub> ; guaiacyl ring (with<br>C=O group) |
| 1331                            | Lignin                            | HCC and HCO bending   |
| 1376                            | Cellulose                         | HCC, HCO, and HOC bending   |
| 1604                            | Lignin                            | aryl ring str., sym.  |
| 1660                            | Lignin-CAA                        | ring conjugated C=C str. of<br>coniferyl alcohol; C=O str. of<br>coniferaldehyde    |

4

# Graphical abstract

## Poplar samples

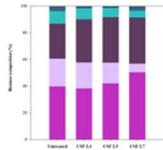


Untreated

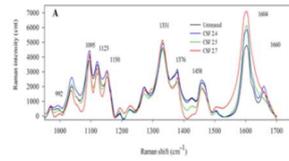
CSF 2.4

CSF 2.5

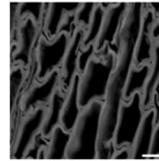
CSF 2.7



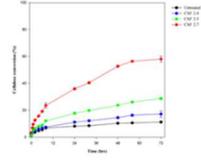
Chemical composition



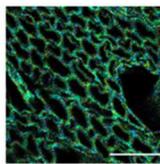
Topochemical analysis



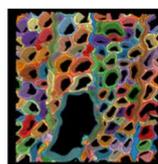
Morphological analysis



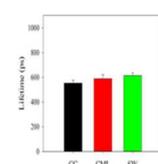
Enzymatic degradability



Spectral analysis



3D analysis



Fluorescence lifetime analysis