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1 Alkaline treatment combined with enzymatic hydrolysis for efficient cellulose nanofibrils production

2

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10 Abstract

11 Cellulose nanofibrils were efficiently produced from eucalyptus fibers using a combined NaOH and  
12 enzymatic treatment followed by a pilot scale grinding process. The structural changes of fibers were  
13 assessed after NaOH treatments at 5, 10 and 15 wt% concentrations. A progressive shift from a  
14 cellulose I to a cellulose II crystalline structure was observed with X-ray diffraction (XRD) and nuclear  
15 magnetic resonance (NMR). The further enzymatic hydrolysis was improved for the NaOH treated  
16 samples. The increase of crystallinity indices due to enzymatic hydrolysis was of + 4.7 %, + 3.5 %, and  
17 +10.3 % for samples treated with NaOH 5, 10 and 15 wt% respectively, and DP values were drastically  
18 reduced to 340, 190 and 166 respectively. A morphological analysis underlined an optimum with the  
19 combination of NaOH 10 wt% and enzymatic hydrolysis. This treatment followed by the grinding  
20 process resulted in CNF with a rigid structure, with diameters ranging from 10 to 20 nm and lengths  
21 between 150 and 350 nm. A multi-scale analysis enabled to study the impact of this combined  
22 treatment on CNF properties and energy consumption. A decrease in mechanical properties of  
23 nanopapers was observed for the combined treatment and NaOH treatment alone compared to  
24 enzymatic hydrolysis alone, with Young's modulus of 8.94, 4.84 and 11.21 GPa respectively.  
25 However, optical properties were improved, with transmittance values of 42.2, 15.4 and 7.1 %  
26 respectively. This new pretreatment can therefore lead to CNF with tunable properties depending on  
27 the application, with possible industrialization thanks to the reduction of energy needs.

28 Keywords

29 Cellulose nanofibrils, sodium hydroxide, enzymatic hydrolysis, fiber morphology, nanopapers.

30 Highlights

- 31 - alkaline treatments facilitated the further enzymatic hydrolysis of cellulose fibers.
- 32 - combined NaOH and enzymatic treatments easily led to a CNF network after grinding.
- 33 - mechanical properties of CNF were negatively affected by the combined treatments.
- 34 - optical properties of CNF were positively affected by the combined treatments.

35 1. Introduction

36 Cellulose is produced in nature by a wide variety of organisms, and is the main structural element in  
37 the cell wall of majority of plants. It composes a large portion of wood, cotton, hemp, linen, ramie,  
38 etc. This biopolymer is thus produced in considerable quantities each year, with an estimated annual  
39 production by terrestrial plants of  $50 \cdot 10^9$  tons. The total available cellulose is estimated to be more  
40 than  $10^{11}$  tons (Stevanovic, 2016), making it the most abundant polymer on Earth. Natural cellulose  
41 fibers, which are relevant for applications such as paper or textile, can be processed into nanoscale  
42 elements by the use of chemical, enzymatic and/or mechanical treatments (Nechyporchuk et al.,  
43 2016). The obtained nanocellulose, with rigid highly crystalline (cellulose nanocrystals, CNC) or  
44 flexible semi-crystalline structure (cellulose nanofibrils, CNF) exhibit interesting mechanical, optical,  
45 rheological and barrier properties. CNF, in particular, have been discovered in the 1980's (Turbak et  
46 al., 1983) and considered relevant for a wide range of applications, such as composite reinforcement  
47 (Miao & Hamad, 2013), food packaging (Azeredo et al., 2017) or barrier and active coatings (Ferrer et  
48 al., 2017; Spieser et al., 2020). They appear as a potential alternative to oil-based products, and could  
49 be a major actor of the bioeconomy, as attested by the several national and international  
50 development projects from the last decade.

51 The industrial production of CNF, however, still needs to overcome some issues. Despite the recent  
52 improvements concerning chemical pretreatments (Inamochi et al., 2017; Rol et al., 2018), enzymatic  
53 hydrolysis (Hu et al., 2018) or mechanical fibrillation (Rol et al., 2020a; Taheri & Samyn, 2016;  
54 Tsalagkas et al., 2018), the production volumes remain low, and the CNF price cannot compete with  
55 synthetic polymers (Assis et al., 2018). These issues can be explained on one hand by the limitations  
56 in CNF quality observed with pretreatments which are easy to be upscaled, such as enzymatic  
57 hydrolysis. The discovery of this pretreatment was a major breakthrough for CNF production  
58 (Henriksson et al., 2007; Pääkkö et al., 2007), but a plateau in CNF properties is often obtained by this  
59 mean (Nechyporchuk et al., 2015; Siqueira et al., 2010). On the other hand, the highly interesting  
60 properties obtained by chemical grafting such as TEMPO-oxidation (Saito & Isogai, 2006),  
61 carboxymethylation (Kaldéus et al., 2018) or phosphorylation (Rol et al., 2020b) are usually

62 associated with time-consuming processes, using in some cases toxic chemicals. There is therefore a  
63 need to develop and improve new pretreatments in terms of CNF properties and process ease, by  
64 using recyclable solvents and up scalable processes.

65 To this end, the use of aqueous sodium hydroxide (NaOH) as a pretreatment for CNF production has  
66 been proposed since the early 2010s. The use of alkaline solutions on native cellulosic fibers is not  
67 new, and was first reported more than one century ago in 1850 by John Mercer, who observed an  
68 improvement of the properties of cotton fibers treated with a NaOH solution. This patented  
69 treatment (Mercer, 1850), named mercerisation after its inventor, was proven to enhance fibers  
70 lustre, smoothness, dimensional stability and mechanical properties. Since then, the use of NaOH has  
71 expanded to the textile industry, the production of regenerated cellulose, or even the cellulose  
72 dissolution. Its action on cellulose is nowadays well documented, and consists in a conversion from  
73 the native cellulose I structure to the thermodynamically more stable cellulose II structure. This  
74 phenomenon takes place with a swelling of cellulose fibers by NaOH hydrates and the formation of  
75 alkali complexes. This results in a shift from a parallel crystalline conformation (cellulose I) to an  
76 antiparallel one (cellulose II) through translation diffusion mechanisms, although the physical state of  
77 cellulose during this reaction is still not fully understood (Budtova & Navard, 2016). The conversion  
78 to cellulose II is irreversible, and the study of its crystalline structure by X-ray and neutron diffraction  
79 highlighted a greater number of hydrogen bonds in this conformation (Pérez & Mazeau, 2005).

80 Alkaline treatment on previously individualized CNF was reported in 2011 (Abe & Yano, 2011)  
81 resulting in high-strength hydrogels compared to untreated cellulose. Dissolution of CNF in aqueous  
82 NaOH was also performed in 2015 (Yamane et al., 2015), leading to a stable cellulose solution in  
83 terms of viscosity. However, the use of NaOH treatment alone for CNF production was first reported  
84 by Wang et al., 2014. The process was composed of a mercerisation step in 17.5 wt% NaOH for 12  
85 hours, followed by several passes in a grinder and a homogenizer. The obtained CNF, with 15 - 100  
86 nm widths, had better thermal stability compared to untreated fibers, but lower mechanical  
87 properties (Young's modulus of 8.6 and 11.8 GPa respectively). Later, this treatment was adapted  
88 with simultaneous ball-milling (Abe, 2016, 2019) leading to stable hydrogels but poorly individualized  
89 CNF due to quick gelation. Under the form of films, the CNF exhibited lower mechanical properties  
90 compared to typical CNF films, but the continuous network formed and possibility to tune the  
91 properties with controlled crystalline structures were underlined. These studies were of great  
92 interest as they showed the possibility to obtain CNF with a simple NaOH treatment. The remaining  
93 issues were (i) the difficulty to individualize CNF due to the gelation during NaOH treatment and (ii)  
94 the medium quality of the resulting CNF.

95 The use of enzymatic hydrolysis of cellulose along with alkaline treatment has been explored  
96 recently. An enzymatic hydrolysis prior to dissolution in NaOH was proven to improve the dissolution  
97 kinetics and efficiency, due to DP decrease and deconstruction of fibers (Budtova & Navard, 2016; Le  
98 Moigne et al., 2010). Inversely, NaOH treatment prior to enzymatic hydrolysis was shown to have a  
99 positive effect. The shift to cellulose II was proven to increase cellulose accessibility for enzymes  
100 (Kobayashi et al., 2012; Ling et al., 2017), thus improving hydrolysis kinetics (Wada et al., 2010) and  
101 yield (Kuo & Lee, 2009; SriBala et al., 2016). An alkaline pretreatment prior to enzymatic hydrolysis  
102 was also proven to be efficient for CNF production from soybean straw (Martelli-Tosi et al., 2016). In  
103 this study, the alkaline pretreatment is coupled with a bleaching step using sodium chlorite or  
104 hydrogen peroxide. A laboratory scale mechanical treatment (Ultra Turrax) enabled the production  
105 of CNF with 8-12 nm widths as observed with transmission electron microscopy. However, according  
106 to the authors, the combination of alkaline and enzymatic treatments has not been clearly applied to  
107 a pilot scale CNF production process.

108 This study aims to investigate the relevance of a combined NaOH and enzymatic treatments for CNF  
109 production. To that end, three NaOH treatments at 5, 10 and 15 wt% were performed on refined  
110 kraft bleached eucalyptus fibers, followed by enzymatic hydrolysis with an endoglucanase. The term  
111 mercerisation was not used, as it refers to NaOH treatments under specific conditions (NaOH 18 - 32  
112 wt%, temperature 25 - 40 °C, short treatment time) that were not used here. The crystalline  
113 structure of cellulose was studied by X-ray diffraction and nuclear magnetic resonance, to assess the  
114 shift from cellulose I to cellulose II, and to quantify the improvement of enzymatic hydrolysis. Fiber  
115 morphology was investigated with scanning electron microscopy, MorFi analysis and degree of  
116 polymerization. Optimal pretreatment conditions were determined and the pretreatment was  
117 adapted to a pilot reactor. Cellulose nanofibrils were thereafter produced with a grinding treatment,  
118 and CNF properties were studied as suspensions and films, to assess the combined pretreatment  
119 efficiency compared to NaOH and enzymes alone.

120

## 121 2. Experimental section

### 122 2.1 Materials

123 Bleached Eucalyptus Kraft Pulp was purchased as dry pulp sheets from Cenibra, Brazil. Enzyme  
124 solution FiberCare<sup>®</sup> R (Novozyme, Denmark) with an endoglucanase activity of 4770 ECU/g was  
125 kindly supplied by the partner company Arjowiggins, France. Chemicals were used as received from  
126 suppliers: sodium hydroxide (NaOH 50 % in H<sub>2</sub>O, Sigma-Aldrich), acetic acid (CH<sub>3</sub>COOH ≥ 99.7 %,   
127 Sigma-Aldrich), sodium acetate trihydrate (CH<sub>3</sub>COONa · 3H<sub>2</sub>O ≥ 99.0 %, Sigma-Aldrich), hydrochloric

128 acid (HCl 37 %, Sigma-Aldrich), bis(ethylenediamine)copper(II) hydroxide solution  
129 ( $\text{Cu}(\text{H}_2\text{NCH}_2\text{CH}_2\text{NH}_2)_2(\text{OH})_2$  1.0 M in  $\text{H}_2\text{O}$ , Sigma-Aldrich), sulfuric acid ( $\text{H}_2\text{SO}_4$  95.0 – 98.0 %, Sigma-  
130 Aldrich), potassium iodide ( $\text{KI} \geq 99.0$  %, Sigma-Aldrich), potassium permanganate ( $\text{KMnO}_4 \geq 99.0$  %,   
131 Sigma-Aldrich), sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$  99 %, Sigma-Aldrich).

## 132 2.2 Cellulose alkaline and enzymatic treatment

### 133 2.2.1 Pulping and refining

134 Cellulose fibers were dispersed with deionized water into a 2 wt% suspension with a laboratory scale  
135 pulper for 10 minutes. Refining was performed with a Valley beater (Voith, Germany) until a  
136 fibrillation degree of 70 SR as measured with a Shopper Riegler tester (Paper Testing Association,  
137 France) following the ISO 5267-1 standard. The pulp was then filtered with a nylon sieve with a mesh  
138 size of 1  $\mu\text{m}$  until a concentration of 10 wt%.

### 139 2.2.2 NaOH treatment

140 Refined cellulose fibers were treated with three different sodium hydroxide solutions, with NaOH  
141 concentrations of 5, 10 and 15 wt% respectively. The reactions were performed under stirring at  
142 room temperature for 1 h, with a pulp solid content of 2 wt% (20 g pulp, 980 g NaOH solution). The  
143 NaOH solutions with various concentrations were prepared by diluting a NaOH 50 wt% solution with  
144 deionized water, considering the water present in the pulp. After the reaction, the suspensions were  
145 filtered once with a Buchner funnel using a nylon sieve with a mesh size of 1  $\mu\text{m}$ , and dispersed at 2  
146 wt% with deionized water. The residual sodium hydroxide was neutralized with a 0.5 M HCl solution.  
147 Filtration and neutralization steps were repeated several times until a stable pH of 7.0 was obtained.  
148 The 10 wt% NaOH treatment was adapted to 200 g of fibers, with the same protocol adapted to a 12  
149 L reactor. 100 g were recovered for direct fibrillation into the grinder, and 100 g were further treated  
150 with enzymes before fibrillation into the grinder.

### 151 2.2.3 Enzymatic hydrolysis

152 Enzymatic hydrolysis was performed on refined pulp as such, and after NaOH treatments at  
153 concentrations of 5, 10 and 15 wt% respectively. 10 g of pulp were introduced with a solid content of  
154 2 wt% in a reaction flask under mechanical agitation. A temperature of 50 °C was set with an oil bath,  
155 and a stable pH of 5.0 was obtained with an acetate buffer composed of acetic acid and sodium  
156 acetate trihydrate. The enzyme solution (FiberCare R cellulase with an activity of 4770 ECU/g of  
157 solution) was then poured into the reaction flask, with an enzyme concentration of 300 ECU/g of  
158 cellulose ( $6.29 \times 10^{-2}$  g of enzyme solution / g of cellulose). After 2 hours of reaction, the enzymatic  
159 hydrolysis was stopped by diluting the pulp suspension to 1 wt% with boiling deionized water during

160 5 minutes. The pulp was filtered with a Buchner funnel using a nylon sieve with a mesh size of 1  $\mu\text{m}$ .  
161 Additional washing and filtration steps were performed, in order to remove the residual salts and the  
162 sugars produced during the reaction. The pulp with a solid content of approximately 20 wt% was  
163 then carefully recovered from the nylon sieve and stored in fridge. The enzymatic hydrolysis on  
164 NaOH 10 wt% treated pulp was adapted to 100 g of fibers, with the same protocol adapted to a 12 L  
165 reactor, before fibrillation into the grinder.

#### 166 2.2.4 Process yields

167 The process yields were calculated for each reaction with the following equation:

$$168 \text{ yield (\%)} = \frac{m_f}{m_i} \times 100 \quad (1)$$

169 where  $m_i$  is the initial dry mass of fibers in grams and  $m_f$  the final dry mass of (nano)fibers in grams.  
170 For processes including several steps, *e.g.* coupled NaOH treatment and enzymatic hydrolysis, the  
171 initial mass corresponds to the dry mass of refined fibers before any reaction, and the final mass  
172 corresponds to the dry mass of fibers after both process steps.

### 173 2.3 CNF preparation

#### 174 2.3.1 Mechanical fibrillation processes

175 Two fibrillation processes were used for the deconstruction of fiber structure: (i) a mild and low  
176 shear laboratory scale mechanical fibrillation process (Ultra Turrax T-25 disperser, IKA, Germany) to  
177 assess the pretreatments efficiency on a small quantity of fibers, and (ii) a pilot scale ultra-fine  
178 friction grinder (Model MKZA6-2, Disk model MKG-C 80, Masuko Sangyo Co., Ltd., Japan) for an  
179 efficient high-quality CNF production. The Ultra Turrax mechanical treatment was performed on 5 g  
180 of pulp with a solid content of 2 wt%, with a rotation speed of 8,000 rpm and 10 min treatment time  
181 at room temperature. Samples before and after the mechanical treatment were recovered, in order  
182 to study its impact on fiber morphology and creation of CNF. The ultra-fine friction grinding  
183 treatment was performed on 100 g of pulp with a solid content of 2 wt%, with a rotation speed of  
184 1,500 rpm. The pressure between rotor and stator disks was determined with the apparatus arbitrary  
185 units. A progressive shift from graduation 0 (low pressure) to graduation 20 (high pressure) was  
186 achieved, for a total number of passes between 30 and 40. For the sample treated with both NaOH  
187 10 wt% and enzymes, the graduation 30 could be used without process issues. The specific energy  
188 consumption was calculated with the Equation 2:

$$189 \text{ Specific Energy Consumption (MWh/t)} = \frac{P_{\text{net}} \times t}{m_{\text{dry}}} \quad (2)$$

190 where  $P_{\text{net}}$  is the net power (MW) measured during the mechanical treatment,  $t$  the fibrillation time  
191 (h) and  $m_{\text{dry}}$  the dry mass of fibers (t). The net power and fibrillation time were measured for each  
192 graduation used, and the specific energy consumptions were summed to obtain the total energy  
193 consumption of the process. The energy consumption of the refining step (approx. 0.6 MWh/t) was  
194 considered. Samples were recovered at different stages of the process for further characterization.

### 195 2.3.2 Preparation of nanopapers

196 Nanopapers (60 g/m<sup>2</sup>) were prepared by filtration using a Rapid Köthen sheet former. 2 g of CNF  
197 were diluted to 0.5 wt% with deionized water and homogenized for 1 min with an Ultra Turrax T-25  
198 disperser at 8,000 rpm. The suspension was then filtered with the sheet former equipped with an  
199 additional nylon sieve with a mesh size of 1  $\mu\text{m}$ , under a controlled vacuum of – 500 mbar. After a  
200 complete removal of water, the CNF sheets were dried between two nylon sieves under vacuum at  
201 85 °C for 20 min. 2 nanopapers were made for each CNF suspension. The nanopapers were stored in  
202 a conditioned room at 23 °C and 50 %RH for 48 h before any characterization.

## 203 2.4 Cellulose fibers and nanofibrils characterization

### 204 2.4.1 MorFi analysis

205 Fiber morphology was analyzed with a MorFi LB-01 fiber analyzer (Techpap, France). 300 mg of pulp  
206 were diluted in 7 L of water and kept under constant recirculation in the image acquisition system.  
207 The fiber / fine limit was set to 200  $\mu\text{m}$  in length, and the analysis was carried out until 5,000 fibers  
208 were detected. The analysis was done twice on each sample, and average fiber length ( $\mu\text{m}$ ) and fine  
209 content (% in area) were determined.

### 210 2.4.2 Optical microscopy

211 Optical microscopy images were acquired with a microscope Axio Imager M1 (Carl Zeiss, Germany)  
212 equipped with an AxioCam MRc 5 digital camera. Before slide preparation, suspensions were diluted  
213 to 0.1 wt%. Magnifications of x50 and x100 were used, at least 8 images were taken for each  
214 magnification and the most representative were selected.

### 215 2.4.3 Scanning Electron Microscopy (SEM)

216 Fiber and CNF suspensions were diluted to 0.1 wt%, dried under vacuum on a carbon adhesive and  
217 coated with a 5 nm Au/Pd layer. For the study of fiber morphology, images were acquired with an  
218 ESEM Quanta 200 (FEI, Japan) in ETD mode. A working distance of 10 mm and an acceleration voltage  
219 of 10.0 kV were used. At least 15 images by sample were acquired, with magnifications between  $\times$   
220 100 and  $\times$  4,000, and the most representative ones were selected. For the study of CNF morphology,



221 images were acquired with a FEI Quanta 250 equipped with a field emission gun in ETD mode. A  
222 working distance of 8 mm and an acceleration voltage of 2.50 kV were used. At least 10 images by  
223 sample were acquired, with magnifications between  $\times 5,000$  and  $\times 40,000$ , and the most  
224 representative ones were selected.

#### 225 2.4.4. Transmission Electron Microscopy (TEM)

226 Transmission electron microscopy images were acquired on the supernatant of CNF suspensions at  
227 0.1 wt% after 24 hours of settling, to avoid the presence of micrometric fragments during sample  
228 preparation. Droplets of dilute CNF suspension were deposited onto glow-discharged carbon-coated  
229 TEM grids. After a few minutes, the liquid in excess was blotted with filter paper and, prior to drying,  
230 the preparation was negatively stained with 2 wt% uranyl acetate. The stain in excess was blotted  
231 and the specimen allowed to dry. Images were recorded with a JEOL JEM-2100-Plus microscope  
232 operating at 200 kV equipped with a Gatan Rio 16 digital camera. Images were further analyzed with  
233 the software Fiji – ImageJ for the determination of CNF length and diameter, at least 50  
234 measurements were performed.

#### 235 2.4.5 Chemical composition

236 The lignin content was determined with the micro-kappa number following the TAPPI T 236 om-13  
237 standard. 2 g of sample were diluted in 150 mL of deionized water, and 20 mL of sulfuric acid 4 N  
238 were added. 20 mL of potassium permanganate 0.05 N were added and the suspension was allowed  
239 to react for 10 min at a temperature of 25 °C, controlled with a water bath. 10 mL of potassium  
240 iodide 1 M were then added to stop the reaction, and the remaining permanganate was titrated with  
241 sodium thiosulfate 0.05 N. The micro-kappa number was calculated using the following formula:

$$242 \text{ Micro-kappa} = \left[ \frac{(V_1 - V_2) \times 0.05}{0.1} \right] \times \frac{D}{m} \quad (3)$$

243 where  $V_1$  is the added volume of sodium thiosulfate for the blank test without pulp,  $V_2$  is the added  
244 volume of sodium thiosulfate for the test with pulp,  $m$  is the dry mass of pulp, and  $D$  a correction  
245 coefficient. The lignin content was calculated with the formula:

$$246 \text{ Lignin content (\%)} = 0.147 \times \text{micro-kappa} \quad (4)$$

247 The cellulose and hemicellulose contents were adapted from the TAPPI T 203 cm-99 standard. 2 g of  
248 oven dried pulp were dispersed in 100.0 mL of NaOH 17.5 % in a 500 mL flask, at a temperature of 25  
249 °C controlled with a water bath. After 30 min of reaction, 100.0 mL of deionized water were added  
250 and the suspension was allowed to react for another 30 min. The pulp was then recovered and

251 washed several times with deionized water in a Buchner funnel using a 1  $\mu\text{m}$  nylon sieve. The  $\alpha$ -  
252 cellulose content was calculated with the following formula:

$$253 \quad \alpha\text{-cellulose content (\%)} = \frac{m_1}{m_0} \times 100 \quad (5)$$

254 where  $m_0$  is the initial mass of pulp and  $m_1$  the mass of pulp after treatment. The estimated  
255 hemicellulose content was then calculated by subtraction, knowing the  $\alpha$ -cellulose and lignin  
256 contents in the pulp. The analyses were duplicated and the average values were calculated.

#### 257 2.4.6 Degree of polymerization

258 Degree of polymerization (DP) was determined following the ISO 5351:2010 standard. Fibers were  
259 dissolved in a bis(ethylenediamine)copper(II) hydroxide solution, and intrinsic viscosity  $[\eta]$  in mL/g  
260 was measured with a capillary viscometer. The viscosity average  $\text{DP}_v$  was calculated using the Mark-  
261 Houwink-Sakurada equation:

$$262 \quad \text{DP}_v^{0.905} = 0.75 \times [\eta] \quad (6)$$

263 An average DP value was calculated from triplicates.

#### 264 2.4.7 X-ray diffraction

265 Prior to X-ray diffraction and nuclear magnetic resonance, fibrous samples were milled into powder  
266 form by cryocrushing. 1 g of sample was cryocrushed for 2 minutes with 2 zirconium balls in a 20 mL  
267 chamber cooled with liquid nitrogen, with an oscillation frequency of 30 Hz. An amorphous reference  
268 was produced by cryocrushing raw fibers during 20 min under similar conditions. Powders were  
269 deposited on a zero-background Si substrate, and X-ray diffraction (XRD) was performed using a  
270 diffractometer X'Pert Pro MPD (PANalytical, Netherlands) equipped with a Bragg-Brentano geometry.  
271 A copper anode ( $K\alpha \lambda = 1.5419 \text{ \AA}$ ) was used, with the angle  $2\theta$  ranging from  $6^\circ$  to  $60^\circ$  with a  $0.05^\circ$   
272 interval. The crystallinity index was calculated by amorphous subtraction with the following formula:

$$273 \quad \text{CI (\%)} = \frac{A_c}{A_c + A_a} \times 100 \quad (7)$$

274 where  $A_c$  corresponds to the crystalline area of the XRD pattern, and  $A_a$  to the amorphous area of the  
275 XRD pattern (amorphous reference). The crystalline area is therefore obtained by subtracting the  
276 area of the amorphous reference to the area of the sample's total XRD pattern.

#### 277 2.4.8 Nuclear magnetic resonance

278 Solid-state  $^{13}\text{C}$  Nuclear Magnetic Resonance ( $^{13}\text{C}$  NMR) was performed on a spectrometer Avance 500  
279 (Bruker, USA) using cross-polarization, high power proton decoupling and magic angle spinning (CP-

280 MAS). Spectra were acquired at 25 °C, with a 4 mm probe operating at 125.78 MHz for <sup>13</sup>C and  
281 500.18 MHz for <sup>1</sup>H. Samples were placed in a 4 mm ZrO<sub>2</sub> rotor, and the acquisition was made with a  
282 number of scans of 40,000, relaxation time of 2.0 seconds, CP time of 2.0 ms, 12 kHz MAS and 300  
283 ppm spectral width. The crystallinity index was calculated using equation (7) with A<sub>c</sub> being the area of  
284 crystalline contribution of carbon C<sub>4</sub> (93.0 - 86.2 ppm), and A<sub>a</sub> being the area of disordered  
285 contribution of carbon C<sub>4</sub> (86.2 – 80.0 ppm).

#### 286 2.4.9 Turbidity

287 Suspensions were diluted to 0.1 wt% with deionized water and homogenized for 1 min with an Ultra  
288 Turrax T-25 disperser at 8,000 rpm. Turbidity values in Nephelometric Turbidity Units (NTU) were  
289 measured with a turbidity meter AL-250 (Aqualytic, Germany). The test measures the scattered light  
290 at 90° from an incident light (λ = 860 nm), which is related to the size and shape of the cellulosic  
291 fibrous elements of the suspension. At least 9 measurements were done for each sample and the  
292 average value was calculated.

#### 293 2.4.10 Transmittance

294 The transmittance of CNF nanopapers (in % of the incident light) was measured at a wavelength of  
295 550 nm with a UV-spectrophotometer (Shimadzu Manufacturing, USA) in photometric mode. 5  
296 measurements were done on each nanopaper, 2 nanopapers were analyzed for each sample leading  
297 to 10 measurements in total, and the average value was calculated.

#### 298 2.4.11 Tear resistance

299 Tear resistance of nanopapers was measured with a tear tester equipped with a 4000 mN pendulum  
300 (Noviprofibre, France) **in a conditioned room (23 °C and 50 %RH)**. Samples of 50 × 65 mm<sup>2</sup> were pre-  
301 cut, and the force (in mN) to complete the propagation of the cut was measured. 3 measurements  
302 were done on each nanopaper, 2 nanopapers were analyzed for each sample leading to 6  
303 measurements in total, and the average value was calculated.

#### 304 2.4.12 Tensile test

305 Tensile properties of nanopapers were measured **in a conditioned room (23 °C and 50 %RH)** with a  
306 dual column testing system 5965 of 50 kN maximum force (Instron, USA), following the NF Q03-004  
307 standard. Samples with dimensions 15 × 100 mm<sup>2</sup> were tested with a **tensile** speed of 10 mm/min.  
308 The Young's modulus of each sample was calculated using the thickness values of the nanopaper as  
309 measured for porosity. 3 measurements were done on each nanopaper, 2 nanopapers were analyzed  
310 for each sample leading to 6 measurements in total, and the average value was calculated.

#### 311 2.4.13 Porosity

312 The basis weight of each nanopaper was calculated from its mass after 48 h in a conditioned room.  
313 Thickness was measured with a Lhomargy micrometer at 8 different positions on the nanopaper, and  
314 an average value was calculated. The porosity of the nanopaper was then calculated using the  
315 following formula:

$$316 \text{ Porosity (\%)} = 1 - \frac{\text{BW}}{e \times \rho_c} \times 100 \quad (8)$$

317 where BW is the basis weight (kg/m<sup>2</sup>), e the thickness (m) and  $\rho_c$  the density of cellulose (1540  
318 kg/m<sup>3</sup>).

#### 319 2.4.14 Quality index

320 As a tool to compare CNF suspensions together, an equation regrouping 5 test values was used,  
321 adapted from the publication of Desmaisons et al., 2017. The obtained value named quality index is  
322 representative of the global quality of CNF suspensions in terms of optical and mechanical  
323 properties. The adapted equation used for quality index calculation was:

$$324 \text{ QI} = 2 \times \text{turbidity mark} + 1 \times \text{transmittance mark} + 2 \times \text{tear resistance mark} + 2 \times \\ 325 \text{ Young's modulus mark} + 2 \times \text{porosity mark} + 1 \quad (9)$$

326 where marks are calculated from raw test values as indicated in the original publication. The resulting  
327 equation including the raw test values was therefore:

$$328 \text{ QI} = -0.02 \times x_1 + 1.65 \times \ln(x_2) - 7.18 \times \ln(x_3) - 0.07 \times x_4^2 + 2.54 \times x_4 - 0.32 \times x_5 + 89.96 \quad (10)$$

329 where  $x_1$  is the turbidity (NTU),  $x_2$  is the transmittance (%),  $x_3$  is the tear resistance (mN),  $x_4$  is the  
330 Young's modulus (GPa), and  $x_5$  is the porosity (%). Nanosized fraction and optical microscopy values  
331 were not used for the quality index calculation as compared with the original publication, due to the  
332 high standard deviations, poor repeatability of the tests and inaccurate values due to the presence of  
333 salts during centrifugation. Higher factors are associated with mechanical properties, leading to a  
334 possible difference of  $\pm 3$  in the quality index values compared to the values obtained with the  
335 original calculation. However, the two values remain very close and comparable, with a reduced  
336 standard deviation.

337

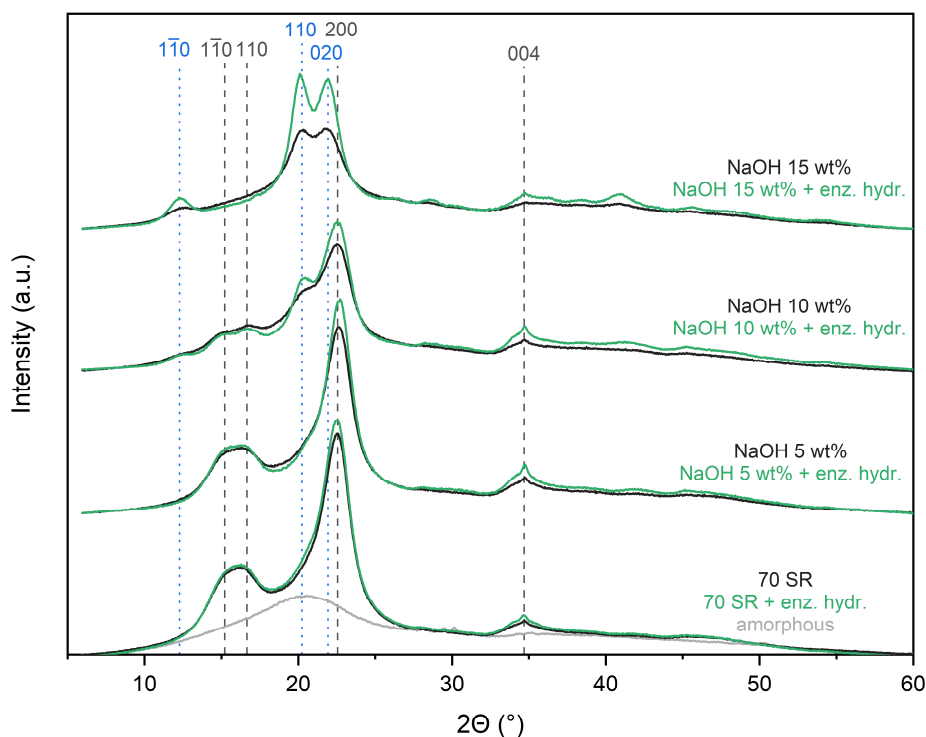
### 338 3. Results and discussion

#### 339 3.1 Influence of NaOH and enzymatic treatments on cellulose crystalline structure

340 The crystalline structure of cellulosic fibers was first assessed with X-ray diffraction (XRD), before and  
341 after NaOH treatments at various concentrations (Fig. 1). The diffraction pattern of 70 SR refined  
342 fibers is typical of cellulose I $\beta$  crystalline structure with diffraction peaks at  $2\theta$  angles of 15.2°, 16.7°,  
343 22.6° and 34.7°, corresponding to the (1 $\bar{1}0$ ), (110), (200) and (004) lattice planes respectively (French,  
344 2014; Park et al., 2010). The treatment of cellulose fibers with aqueous NaOH lead to a shift from  
345 cellulose I to cellulose II crystalline structures, which is a well-documented phenomenon in the  
346 literature (Abe, 2016; Budtova & Navard, 2016; Lee et al., 2018; SriBala et al., 2016; Wang et al.,  
347 2014). Diffraction peaks can be observed at  $2\theta$  angles of 12.3°, 20.2° and 21.9°, corresponding to the  
348 (1 $\bar{1}0$ ), (110) and (020) lattice planes of cellulose II respectively (French, 2014; Gong et al., 2017; Sèbe  
349 et al., 2012). As expected, the intensities of cellulose II peaks increase when NaOH concentration  
350 increases, indicating a higher cellulose II content. The sample treated with NaOH 5 wt% exhibits a  
351 cellulose I structure with a shoulder at  $2\theta = 20.2^\circ$ . The shift from cellulose I to cellulose II is therefore  
352 limited for this sample, which is standard in this range of concentration and temperature (Abe &  
353 Yano, 2011; Liu & Hu, 2008). The sample treated with NaOH 15 wt%, however, has undergone an  
354 almost complete shift to cellulose II. The NaOH 10 wt% treatment results in the presence of both  
355 cellulose I and II structures in the sample, with a predominance of cellulose I (Abe & Yano, 2012).

356 Crystallinity indices (CI) were calculated with an amorphous reference with an area-based method, as  
357 the peak height method tends to lead to overestimated values and is not suitable for cellulose II  
358 (Ahvenainen et al., 2016; Park et al., 2010). However, it is worth noting that the impact of  
359 cryocrushing step on molecular structure before analysis is not negligible. The CI obtained here from  
360 the powder form are therefore used only comparatively, and are significantly underestimated  
361 compared to the literature. It also should be noted that the CI is representative of the total  
362 crystallinity of the sample, irrespective of its cellulose I and cellulose II content.

363 As described in Table 1, refined fibers have a CI of 40.7 % prior to any treatment. The following NaOH  
364 treatments lead to a decrease of CI to 30.2 %, 22.4 % and 18.0 % for 5, 10 and 15 wt% respectively.  
365 The impact on crystallinity is more important when NaOH concentration increases, which is due to  
366 disruption of the cellulose structure by NaOH hydrates during its conversion into cellulose II form  
367 (Lee et al., 2018; Liu & Hu, 2008). The formation of the cellulose II crystalline structure is only partial,  
368 and fibers are left in a swollen, globally less organized state.



369  
 370 Fig. 1. X-ray diffraction patterns of eucalyptus fibers refined at 70 SR as such and after NaOH  
 371 treatments at several wt%, with and without additional enzymatic hydrolysis step. Characteristic  
 372 peaks and the associated lattice planes are indicated for Cellulose I (grey dashed lines) and Cellulose  
 373 II (blue dotted lines) respectively. The amorphous reference is used for the calculation of crystallinity  
 374 index when superimposed with the chosen pattern, as shown on the graph.

375 The following enzymatic hydrolysis step results in a global increase of the diffraction peaks intensities  
 376 for all samples (Fig. 1). The crystallinity indices are higher after enzymatic hydrolysis as shown in  
 377 Table 1, shifting from 40.7 to 45.7 % for refined fibers without NaOH treatment. This effect has been  
 378 widely reported and explained as a preferential action of endoglucanase on less organized cellulose  
 379 parts (Bansal et al., 2009; Nechyporchuk et al., 2015), although a decrease of CI due to enzymatic  
 380 hydrolysis is also possible (Le Moigne et al., 2010). The same trend is observed with NaOH treated  
 381 samples, with the most significant increase for the sample treated with NaOH 15 wt%, which has a CI  
 382 of 18.0 and 28.3 % before and after enzymatic hydrolysis respectively. Samples treated with NaOH 5  
 383 wt% and 10 wt% exhibit smaller increases of CI with the use of enzymes, from 30.2 to 34.9 % and  
 384 from 22.4 to 25.9 % respectively.

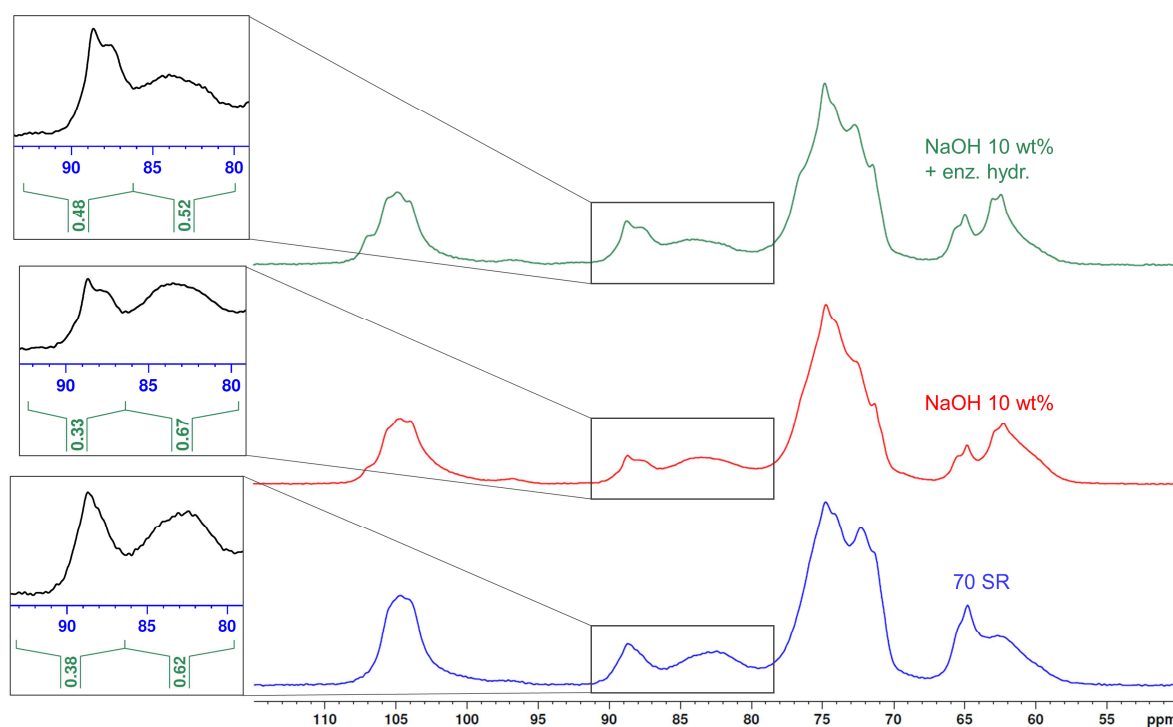
385 The enhancement of the efficiency of enzymatic hydrolysis for sample treated with NaOH 15 wt% is  
 386 due to two concurrent factors. On one hand, the hydrolysis rate tends to increase when the CI  
 387 decreases (SriBala et al., 2016). Enzymatic hydrolysis is thus facilitated for samples with a high

388 content of amorphous cellulose, due to a better accessibility of the cellulose chains. On the other  
389 hand, it has been reported that enzymatic hydrolysis is accelerated by the conversion from cellulose I  
390 to cellulose II (SriBala et al., 2016; Wada et al., 2010). The cellulose I structure consists in sheets  
391 composed of parallel cellulose chains held by hydrogen bonds. These sheets are stacked together by  
392 hydrophobic interactions such as van der Waals forces, without any intersheet hydrogen bond. The  
393 cellulose II structure, however, consists in sheets composed of antiparallel cellulose chains, resulting  
394 in a greater number of hydrogen bonds. These sheets are stacked together by weaker hydrophobic  
395 interactions, as well as intersheets hydrogen bonds (Pérez & Mazeau, 2005). The partial cleavage of  
396 these hydrogen bonds in water, as well as the weaker hydrophobic interactions could explain the  
397 faster hydrolysis rate for cellulose II compared to cellulose I. However, while the NaOH 10 wt%  
398 sample contains a larger proportion of cellulose II compared to the NaOH 5 wt% sample, the  
399 enzymatic hydrolysis step has a comparable effect on the CI of both samples (+ 3.5 and + 4.7 %  
400 respectively). For the NaOH 10 wt% sample, the enzymatic hydrolysis is also associated with a  
401 decrease of the disordered cellulose content, as shown by the decrease of the minimum at  $2\theta =$   
402  $17.5^\circ$ . This decrease influences the calculation of CI with the area-based method. The crystallinity of  
403 this sample was therefore further studied by CP-MAS nuclear magnetic resonance (NMR).

404  $^{13}\text{C}$  NMR spectra were acquired for untreated 70 SR refined fibers, and fibers treated with NaOH 10  
405 wt%, before and after enzymatic hydrolysis (Fig. 2). The spectrum of untreated 70 SR sample is  
406 characteristic of the cellulose I structure. The observed peaks can be assigned to the different  
407 carbons of the anhydroglucose unit, with the contribution of  $\text{C}_1$  (104.7 ppm),  $\text{C}_2$  (71.4 ppm),  $\text{C}_3$  (74.9  
408 ppm),  $\text{C}_4$  (82.4 ppm for disordered cellulose, 88.9 ppm for crystalline cellulose),  $\text{C}_5$  (72.2 ppm) and  $\text{C}_6$   
409 (62.5 and 64.9 ppm) (Foster et al., 2018; Kamide et al., 1984; Kono et al., 2004). The spectrum of the  
410 NaOH 10 wt% treated sample confirms the presence of cellulose II, while the remaining cellulose I  
411 peaks attest that the crystalline conversion is only partial. The intensity of cellulose I peaks decreased  
412 for the contributions of  $\text{C}_1$ ,  $\text{C}_4$  and  $\text{C}_6$ . Characteristic peaks of cellulose II can be observed at 107.2  
413 ppm, 87.9 ppm and 62.2 ppm, associated with  $\text{C}_1$ ,  $\text{C}_4$  and  $\text{C}_6$ , respectively (Dinand et al., 2002; Hesse  
414 & Jäger, 2005). Further enzymatic hydrolysis does not lead to a change in the crystalline structure,  
415 but to a magnification of the peaks corresponding to the contribution of crystalline cellulose, at 87.9  
416 ppm and 88.9 ppm for cellulose II and I respectively.

417 The clear separation of crystalline and disordered contributions for  $\text{C}_4$  allows the calculation of a  
418 NMR crystallinity index (Table 1) with an area-based method (Foster et al., 2018; Park et al., 2010). A  
419 CI of 38 % is obtained for refined fibers, which is comparable to the value obtained with XRD (40.7  
420 %). Here, the two techniques lead to similar values, the NMR value being slightly lower as specified in  
421 several other publications (Foster et al., 2018; Park et al., 2010). The NaOH 10 wt% treatment leads

422 to a decrease of the CI to 33 %. This trend is consistent with the XRD results and confirms the  
 423 disruption of the cellulose structure during its crystalline conversion. The preferential action of  
 424 endoglucanase on disordered cellulose is attested by the significant increase of CI after enzymatic  
 425 hydrolysis, from 33 to 48 %. The CI values obtained with NMR for the NaOH 10 wt% samples, which  
 426 contain both cellulose I and II, are significantly higher compared to the CI obtained with XRD. The  
 427 disagreement between data deduced from NMR and those calculated from XRD could be explained  
 428 by the unsuitability of NMR analysis concerning the amorphous zone of cellulose II. It was reported  
 429 that the disordered C<sub>4</sub> of cellulose II have a lower contribution compared to cellulose I (Kono et al.,  
 430 2004; Sèbe et al., 2012) which leads to overestimated crystallinity indices.



431  
 432 Fig. 2. Solid-state <sup>13</sup>C CP-MAS NMR spectra of eucalyptus fibers refined at 70 SR as such, after NaOH  
 433 10 wt% treatment and after NaOH 10 wt% treatment followed by enzymatic hydrolysis. C<sub>4</sub> crystalline  
 434 (93.0 – 86.2 ppm) and disordered contributions (86.2 – 80.0 ppm) are used for the calculation of  
 435 crystallinity index.

436 The degree of polymerization (DP) and process yield were studied for each treatment (Table 1). The  
 437 enzymatic treatment alone on refined pulp leads to a major reduction of DP, from 963 to 430. A  
 438 comparable decrease is observed for NaOH treatments, due to cellulose surface peeling and alkaline  
 439 hydrolysis (Mozdyniewicz et al., 2013). The influence of NaOH concentration on DP is low, but is  
 440 correlated to the reaction yields, the higher the concentration the lower the yield. **The chemical**  
 441 **composition of the NaOH 10 wt% treated sample underlines that the decrease of yield (85.4 %)**



442 comes from the solubilization of a large part of hemicelluloses. The hemicellulose content shifts from  
 443 16.2 % for the untreated pulp to 1.8 % after treatment. Most of hemicelluloses are indeed soluble in  
 444 NaOH-water based solvents (Budtova & Navard, 2016), and the decrease of yield with increasing  
 445 NaOH concentration is linked to the increasing amount of solubilized hemicelluloses. The yield after  
 446 NaOH 15 wt% treatment (78.2 %) is lower than the initial cellulose content in the pulp (83.4 %),  
 447 which shows that a fraction of cellulose is removed during the treatment. This result supports the  
 448 assertion of a facilitated cellulose degradation with increasing NaOH concentrations (SriBala et al.,  
 449 2016). The NaOH 10 wt% treatment is also associated with a decrease in the lignin content from 0.40  
 450 to 0.27 %. The effect of following enzymatic hydrolysis is highly correlated to the NaOH  
 451 concentration during alkaline treatment. After enzymatic hydrolysis, the DP shifts from 434 to 340  
 452 for NaOH 5 wt%, from 501 to 190 for NaOH 10 wt% and from 401 to 166 for NaOH 15 wt%. The  
 453 efficiency of enzymes is therefore clearly improved for these samples, attesting a better cellulose  
 454 accessibility due to higher disordered cellulose content and cellulose II structure. The hemicellulose  
 455 content after NaOH 10 wt% and enzymatic treatment is below 1 %, and the lignin content decreases  
 456 to 0.25 %. This treatment leads therefore to a purification of the pulp. Although all yield values are  
 457 higher than 70 %, these values are significantly lower than for enzymatic hydrolysis of untreated  
 458 sample (92.3 %). The improvement of enzymatic hydrolysis is therefore irremediably associated with  
 459 a higher loss of matter during process.

460 Table 1

461 Structural properties of fibers and process yields after the different pretreatments.

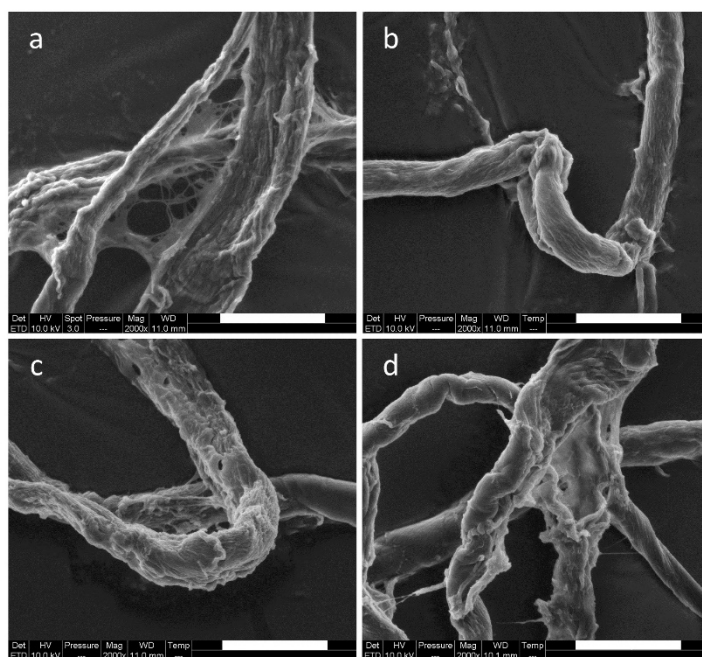
	70 SR	70 SR + enz. hydr.	NaOH 5 wt%	NaOH 10 wt%	NaOH 15 wt%	NaOH 5 wt% + enz. hydr.	NaOH 10 wt% + enz. hydr.	NaOH 15 wt% + enz. hydr.
CI (%) - XRD	40.7	45.7	30.2	22.4	18.0	34.9	25.9	28.3
CI (%) - NMR	38	-	-	33	-	-	48	-
DP	963 ± 2	430 ± 1	434 ± 14	501 ± 4	401 ± 6	340 ± 2	190 ± 1	166 ± 2
Cellulose (%)	83.4	-	-	97.9	-	-	98.8	-
Hemicellulose (%)	16.2 *	-	-	1.8 *	-	-	< 1 *	-
Lignin (%)	0.40	-	-	0.27	-	-	0.25	-
Yield (%)	-	92.3	100.0	85.4	78.2	82.7	73.3	72.0

462 \* estimated values

463

464 3.2 Morphological changes after NaOH and enzymatic treatments

465 The morphology of cellulose fibers after NaOH treatments was studied by scanning electron  
466 microscopy (Fig. 3). Before alkaline treatment, eucalyptus fibers have a relatively flat shape with  
467 fibrillated surfaces due to refining. A network of micrometric elements can be seen on Fig. 3a, which  
468 is characteristic of extensive refining and leads to an improvement of the fibers mechanical  
469 properties. NaOH treatments all lead to a global swelling of fibers with a relative smoothing of fiber  
470 surface (Nam & Condon, 2014). This effect was clearly visible for NaOH 10 and 15 wt% treatments  
471 (Fig. 3c and Fig. 3d respectively) with important curvature of fibers and partial destruction of the  
472 fiber structure. The irregularities of fiber surfaces seem to be due to the aggregation of micrometric  
473 elements and the apparition of kinks (Fig. 3b).

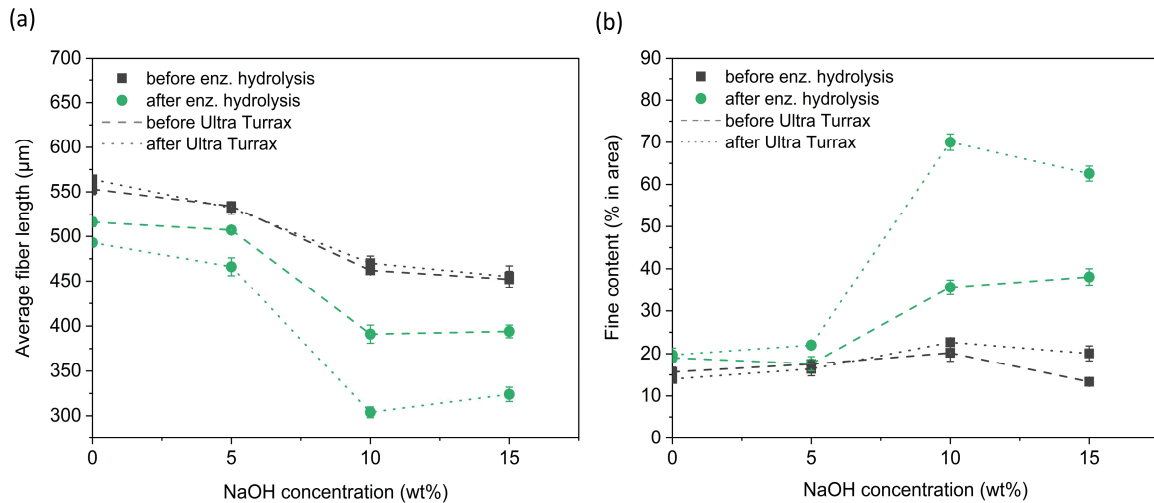


474  
475 Fig. 3. SEM images of eucalyptus fibers refined at 70 SR as such (a), after NaOH 5 wt% treatment (b),  
476 after NaOH 10 wt% treatment (c) and after NaOH 15 wt% treatment (d). Scale bar is 20  $\mu\text{m}$ .

477 The morphological changes were further studied by MorFi analysis (Fig. 4). Average fiber length and  
478 fine content (elements with length < 200  $\mu\text{m}$ ) were determined after each treatment, associated or  
479 not with Ultra Turrax fibrillation. This mild mechanical treatment compared to literature  
480 (Nechporchuk et al., 2016) was chosen because of the small available quantity of cellulose fibers  
481 after each treatment (5g). It is used as a first step for fiber deconstruction towards the production of  
482 CNF, and enables to comparatively assess the efficiency of each treatment. The objective of this mild  
483 mechanical fibrillation was therefore to determine an optimum in the pretreatment conditions, to  
484 further adapt these conditions to the pilot scale grinding process.

485 The NaOH treatments alone have little impact on both fiber length and fine content, independently  
486 of NaOH concentration. Average fiber length shifts from 553 to 452  $\mu\text{m}$  after NaOH 15 wt%  
487 treatment, although major structural changes and DP reduction have been noticed (Table 1). This  
488 attests that these structural changes have little influence on micrometric scale. Additionally, alkaline  
489 treatments do not seem to weaken the fibers enough for an efficient fibrillation, as attested by the  
490 weak impact of further Ultra Turrax treatment on fiber morphology. This low fibrillation efficiency is  
491 consistent with previous studies and could be caused by interdigitation of the cellulose nanofibrils in  
492 the cell wall (Wang et al., 2014).

493 The additional enzymatic hydrolysis step results in important changes in fiber morphology. Its effect  
494 on untreated pulp is already noticeable, with a reduction in fiber length to 516  $\mu\text{m}$  and an increase of  
495 fine content from 15.7 to 19.0 %. This effect is enhanced on NaOH treated samples, leading to  
496 average fiber lengths of 507, 391 and 394  $\mu\text{m}$  for fibers treated with NaOH 5, 10 and 15 wt%  
497 respectively. The creation of fine elements is not noticeable for NaOH 5 wt%, but is observed for  
498 NaOH 10 and 15 wt%, with fine contents of 35.6 and 38.0 % respectively. These results support the  
499 fact that the structural changes due to enzymatic hydrolysis have an impact on a micrometric scale.  
500 Here, the important DP reduction is correlated with a decrease in the fiber length, and a  
501 destructuration of the cell wall leading to fine elements. Interestingly, the subsequent Ultra Turrax  
502 step was found to be more efficient for combinations of NaOH and enzymatic treatments. After  
503 enzymatic hydrolysis and Ultra Turrax treatment, the samples treated with NaOH 5, 10 and 15 wt%  
504 exhibit fiber lengths of 466, 304 and 324  $\mu\text{m}$ , and fine content of 22.0, 70.0 and 62.6 % respectively.  
505 An optimum is reached with the NaOH 10 wt% sample which has the lowest average fiber length and  
506 highest fine content. On one hand, the low fiber lengths demonstrate that these fibers are efficiently  
507 weakened and can be easily cut with a mechanical process. On the other hand, the creation of fine  
508 elements indicates the creation of cellulose microfibrils, and probable nanofibrils which cannot be  
509 detected with the apparatus. **This optimum indicates the positive impact of the conversion from  
510 cellulose I to cellulose II for enzymatic hydrolysis and further deconstruction with a mechanical  
511 process, which is more effective with NaOH 10 wt% (partial conversion) than with NaOH 5 wt% (small  
512 conversion). However, the total conversion with NaOH 15 wt% seems to lead to fiber aggregation,  
513 which has a negative impact on their deconstruction and the creation of CNF.**



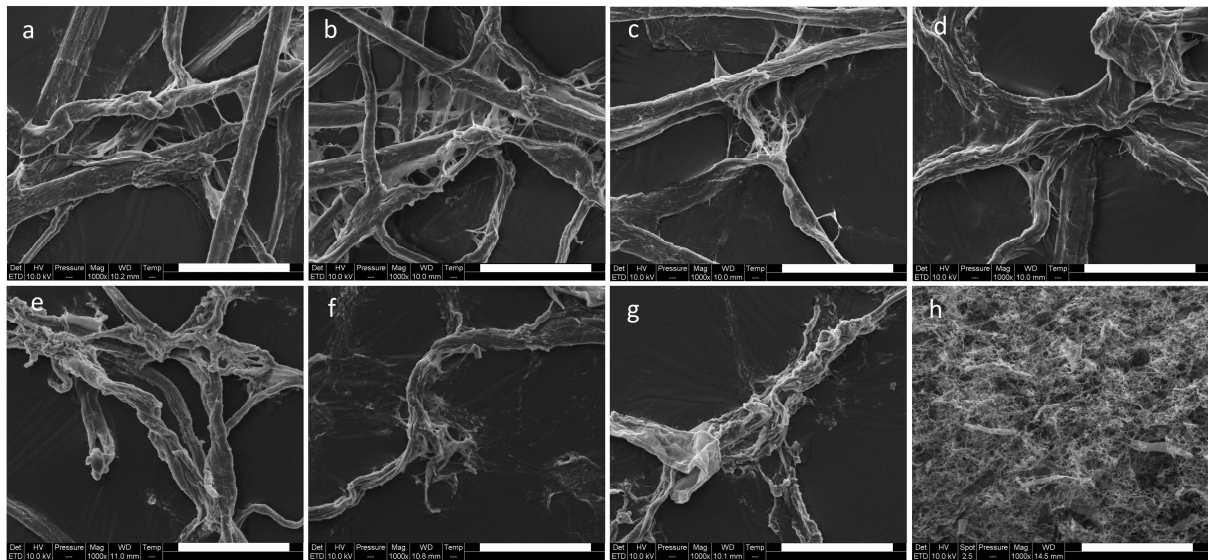
514

515 Fig. 4. Average fiber length (a) and fine content (b) obtained with MorFi analyzer, as functions of  
 516 NaOH concentration during treatment. The influence of enzymatic hydrolysis and Ultra Turrax steps  
 517 is studied, alone and in combinations. Dashed lines are guides to the eye.

518 The morphological analysis was completed by SEM images at different stages of the process (Fig. 5).  
 519 The impact of each step (NaOH treatment, enzymatic hydrolysis, Ultra Turrax treatment) was  
 520 determined, alone and in combinations. Enzymatic hydrolysis leads to fiber cutting and creation of  
 521 fine elements for both untreated and NaOH treated samples (Fig. 5b and Fig. 5f respectively). This  
 522 effect is more visible on the NaOH treated sample, as the initial fine elements from refined pulp are  
 523 aggregated during alkaline treatment. The effect of Ultra Turrax treatment alone (Fig. 5c and Fig. 5g)  
 524 is comparable for untreated and treated fibers, and leads to a destruction of the fiber structure in  
 525 localized positions. The fibrous structure is clearly attacked for the NaOH sample, which exhibits an  
 526 aggregation of cellulose fibers and microfibrils.

527 A major difference was observed in the morphology of untreated and NaOH treated fibers after  
 528 enzymatic hydrolysis and Ultra Turrax fibrillation. Fibers without NaOH treatment, after further  
 529 enzymatic and Ultra Turrax treatments, exhibit a destroyed structure and fiber aggregation, but only  
 530 few micrometric elements (Fig. 5d). In contrast, sample with coupled NaOH and enzymatic  
 531 treatment, followed by Ultra Turrax treatment, exhibits a network structure of micro- and  
 532 nanometric elements (Fig. 5h). The sample is relatively homogeneous, with several residual fiber  
 533 fragments. Remarkably, this conversion to a micro- nanometric network with the mild mechanical  
 534 treatment was observed for the coupled NaOH and enzymatic treatment only, and not for the NaOH  
 535 treatment alone (Fig. 5g) or enzymatic treatment alone (Fig. 5c). This combination seems, therefore,  
 536 to overcome the fibrillation issue encountered with NaOH treatments (Abe, 2016; Wang et al., 2014),  
 537 and to facilitate the fibrillation of enzymatically treated fibers. This encouraging result was observed

538 for the NaOH 10 wt% only, and was not as effective for the NaOH 5 and 15 wt% samples (results not  
539 shown here). This confirms that the partial conversion to a cellulose II structure (NaOH 10 wt%) is  
540 more efficient than a small (NaOH 5 wt%) or a total conversion (NaOH 15 wt%) for CNF production.  
541 This effect could be due to the positive impact of fiber disruption during crystalline conversion as  
542 shown with XRD and NMR, while limiting the fiber aggregation observed for high NaOH  
543 concentrations.



544  
545 Fig. 5. SEM images of eucalyptus fibers refined at 70 SR (a – d) and after NaOH 10 wt% treatment (e –  
546 h), without further treatment (a, e), after enzymatic hydrolysis alone (b, f), after Ultra Turrax  
547 treatment alone (c, g) and after coupled enzymatic hydrolysis and Ultra Turrax treatment (d, h)  
548 respectively. Scale bar is 50  $\mu\text{m}$ .

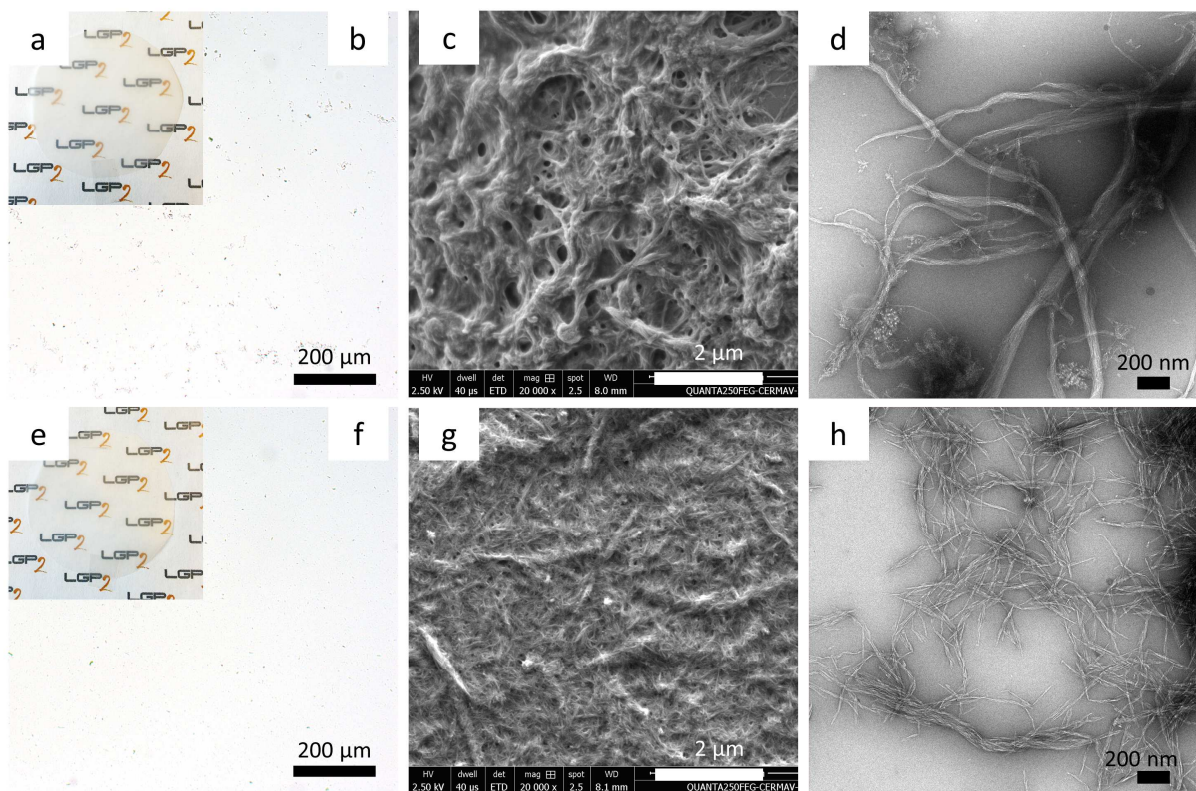
549

### 550 3.3 Cellulose nanofibrils properties

551 Based on the morphological study of fibers, NaOH 10 wt% pretreatment on one hand, and coupled  
552 NaOH 10 wt% and enzymatic pretreatment on the other hand, were used for CNF production using  
553 an ultra-fine friction grinder. The morphology of the obtained CNF was studied at different scales  
554 using optical microscopy, scanning and transmission electron microscopy (Fig. 6).

555 Both treatments lead to an efficient fibrillation into micro- and nano-scale elements, with few  
556 residual fragments visible by optical microscopy (Fig. 6b and Fig. 6f). SEM images reveal however  
557 major differences in the CNF structures. The NaOH treatment alone leads to an aggregation of CNF in  
558 a matrix of disordered cellulose (Fig. 6c). The analysis of TEM images (Fig. 6d) enable to determine  
559 that these CNF are composed of bundles of elementary fibrils, with widths between 30 - 100 nm and

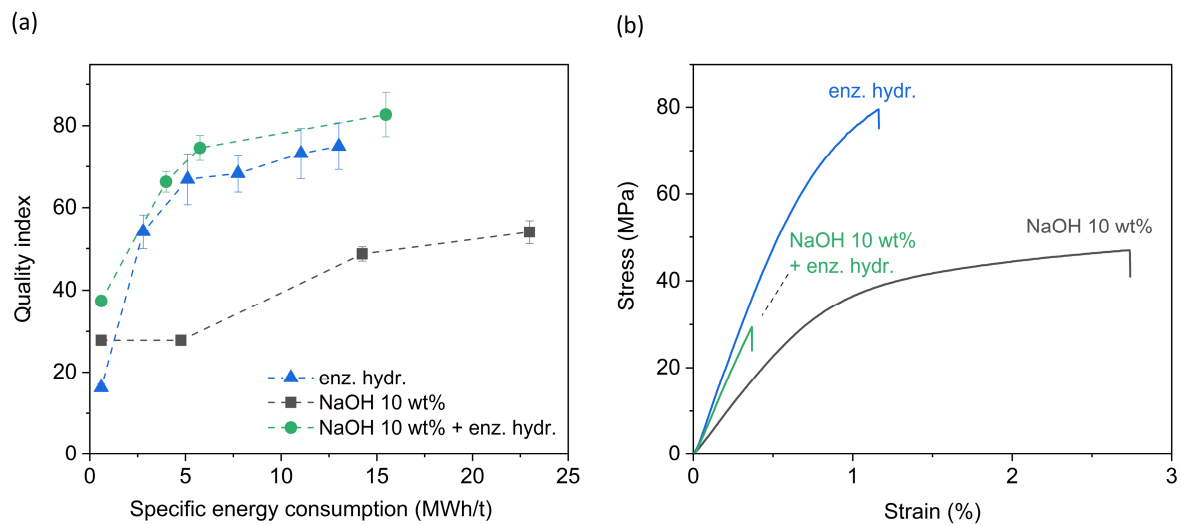
560 lengths superior to 1  $\mu\text{m}$ . These observations are consistent with previous findings on the difficulty to  
561 individualize cellulose II CNF after an alkaline treatment (Abe, 2016; Wang et al., 2014). In contrast, the  
562 combined NaOH and enzymatic treatment leads to well individualized CNF, with few micrometric  
563 fiber fragments visible on SEM images (Fig. 6g). Further observations with TEM (Fig. 6h) reveal CNF  
564 composed of only few elementary fibrils, with widths between 10 - 20 nm and lengths between 150  
565 - 350 nm, close to the dimensions of cellulose nanocrystals (Nechyporchuk et al., 2016). This low  
566 aspect ratio, associated with the low DP values (Table 1), is an indicator of the preferential action of  
567 the enzymes on the disordered cellulose structure produced during the crystalline conversion from  
568 cellulose I to cellulose II. The resulting CNF, with rigid structures, exhibit a high transmittance in the  
569 form of nanopaper (Fig. 6e).



570  
571 Fig. 6. Images of the resulting CNF as films and in suspension after the NaOH 10 wt% treatment  
572 followed by the grinding process (a-d) or the NaOH 10 wt% and enzymatic treatment followed by the  
573 grinding process (e-h). The associated specific energy consumptions are 23.0 and 15.5 MWh/t,  
574 respectively. Nanopapers (a, e) have a basis weight of 60 g/m<sup>2</sup>, optical microscopy images (b, f) were  
575 acquired on 0.1 wt% suspensions, SEM images (c, g) were acquired on 0.1 wt% suspensions after  
576 vacuum drying and TEM images (d, h) were acquired on the supernatant of 0.1 wt% suspensions  
577 after 24h of settling.

578 The optical and mechanical properties of CNF were further assessed using a multi-scale quality index  
579 (Fig. 7a). The properties of CNF for each pretreatment after the highest number of passes through  
580 the grinder are detailed in Table 2. A previous study using enzymatic hydrolysis alone with similar  
581 grinding treatment was used as a reference (Desmaisons et al., 2017). For enzymatic hydrolysis  
582 alone, the quality index undergoes a fast increase with the increase of specific energy consumption,  
583 as the cellulose fibers start being deconstructed in the grinding process. As expected, this reflects a  
584 good efficiency of this pretreatment (Henriksson et al., 2007; Pääkkö et al., 2007) and an effective  
585 fragilisation of the fibers. The gradual decrease of the size of cellulose fibrous elements leads to  
586 higher energy needs for their deconstruction, resulting in a slower increase rate of the CNF  
587 properties (quality index shifting from 66.9 to 73.2 for energy consumptions of 5.1 and 11.0 MWh/t  
588 respectively). The final quality index for enzymatic treatment alone is 75.0, associated with a specific  
589 energy consumption of 13.0 MWh/t. The NaOH 10 wt% alone leads to a completely different  
590 behavior in the mechanical process. The fibrillation efficiency is drastically reduced, as attested by  
591 the slow increase of quality index with the increase of specific energy consumption. Although the  
592 suspension gradually exhibited a high viscosity typical of CNF suspensions, the associated optical and  
593 mechanical properties remain lower than the reference (Table 2). The final quality index for this  
594 treatment is 54.0, associated with a specific energy consumption of 23.0 MWh/t. Interestingly, the  
595 combined NaOH 10 wt% and enzymatic treatment leads to higher quality indices compared to  
596 reference for a same energy consumption. The starting quality index before grinding process (energy  
597 of 0.6 MWh/t due to refining) is higher (37.4 compared to 16.2 for reference). The increase rate of  
598 the CNF properties is comparable, leading to a final quality index of 82.7, associated with an energy  
599 consumption of 15.5 MWh/t. This places this sample among the high quality CNF that can be found  
600 commercially (Desmaisons et al., 2017).

601



602

603 Fig. 7. Quality index as a function of specific energy consumption (a) and typical **tensile** curves (b) for  
604 NaOH 10 wt% treatment alone and combined NaOH 10 wt% and enzymatic treatment, compared to  
605 a reference treatment (enzymatic hydrolysis alone, values extracted from Desmaisons et al., 2017).  
606 The **tensile** curves refer to the CNF with the highest number of passes through the grinding process.  
607 The specific energy consumption of the refining step (0.6 MWh/t) is taken into account. Dashed lines  
608 are guides to the eye.

609 The **tensile** curves of CNF nanopapers underline the drastically different behavior of each sample  
610 (Fig. 7b). For each pretreatment, the sample with the highest energy consumption was studied, and  
611 the typical **tensile** curves are presented here. Enzymatic hydrolysis alone leads to nanopapers with an  
612 average tensile strength of 78 MPa, average strain at break of 1.27 %, and Young's modulus of 11.21  
613 GPa. **The Young's modulus value is in the average that can be found in the literature, as values**  
614 **between 8 and 15 GPa are often obtained with an enzymatic pretreatment (Benítez & Walther, 2017;**  
615 **Nechporchuk et al., 2016).** However, the strain and strength values are in the low average, as they  
616 are usually between 2 - 10 % and 100 - 200 MPa, respectively (Benítez & Walther, 2017). This is  
617 possibly due to the important enzyme dosage during enzymatic hydrolysis. NaOH 10 wt% treatment  
618 alone leads to a higher strain value (2.92 %), but lower stiffness (4.84 GPa) and tensile strength (50.2  
619 MPa). The decrease of mechanical properties with the use of NaOH has been reported previously  
620 (Abe, 2019; Wang et al., 2014). This trend is due to several concomitant factors, namely (i) the  
621 **removal of hemicelluloses (Table 1), which is expected to decrease the elastic modulus and tensile**  
622 **strength (Carvalho et al., 2019),** (ii) the lower elastic modulus of cellulose II compared to cellulose I  
623 (Nishino et al., 1995), and (iii) CNF aggregation during alkaline treatment (Abe & Yano, 2011). The  
624 combined NaOH 10 wt% and enzymatic treatment results in an increase of Young's modulus (8.94  
625 GPa) compared to NaOH alone, likely due to the partial removal of disordered cellulose during



enzymatic hydrolysis. However, both strength and strain at break are drastically reduced, with values of 30 MPa and 0.47 % respectively. This brittle behavior can be linked to the morphology of the associated CNF, which exhibit a rigid structure of relatively low aspect ratio, and do not allow any plastic deformation to occur.

Table 2

**Optical and mechanical properties of cellulose nanofibrils in suspension or as nanopapers, and their associated quality index.**

	Turbidity (NTU)	Transmittance (%)	Tear resistance (mN)	Young's modulus (GPa)	Porosity (%)	Quality index
Enz. hydr.	240 ± 48	7.1 ± 0.06	31 ± 9	11.21 ± 0.41	29.7 ± 2.5	75.0 ± 5.1
NaOH 10 wt%	761 ± 37	15.4 ± 1.17	27 ± 4	4.84 ± 0.20	38.5 ± 1.8	54.0 ± 2.8
NaOH 10 wt% + enz. hydr.	314 ± 13	42.2 ± 0.86	9 ± 3	8.94 ± 0.24	27.2 ± 9.5	82.7 ± 5.4

Optical and mechanical properties of CNF as suspensions or nanopapers are displayed in Table 2. The values presented here refer to the CNF with the highest number of passes through the grinding process. The associated specific energy consumptions are 13.0, 23.0 and 15.5 MWh/t for enzymatic treatment (reference, values extracted from Desmaisons et al., 2017), NaOH 10 wt% treatment and combination of NaOH 10 wt% and enzymatic treatments, respectively. The CNF aggregation caused by NaOH 10 wt% treatment alone is attested by the high turbidity (761 NTU) and porosity of the nanopaper (38.5 %). The tear resistance, however, is comparable to the reference (27 and 31 mN respectively), and the nanopapers exhibit higher transmittance (15.4 and 7.1 % respectively). The combination of NaOH 10 wt% and enzymatic treatment leads to nanopapers with similar porosity compared to reference, but lower tear resistance (9 mN) and drastically higher transmittance (42.2 %). These two properties confirm the presence of small elements with homogeneous distribution in the material. The high transmittance value could open the door to the use of this nanopaper in the packaging industry, or as a substrate for printed electronics for example, where both high transmittance and heat resistance are needed (Hoeng et al., 2016). In suspension, the turbidity is slightly higher for the combined treatment (314 NTU) compared to reference (240 NTU). The resulting CNF obtained with these various pretreatments exhibit therefore a wide range of properties, which can be favoured depending on the application.

4. Conclusions

652 This study confirms the positive impact of a combined NaOH and enzymatic treatment for CNF  
653 production. The structural properties of cellulose after NaOH treatments at various concentrations  
654 were assessed by XRD and <sup>13</sup>C NMR. A clear shift from a cellulose I to a cellulose II crystalline  
655 structure was observed, with a coexistence of both allomorphs for NaOH 10 wt% treated sample. The  
656 alkaline treatments were proven to improve further enzymatic hydrolysis, due to the preferential  
657 action of endoglucanase on disordered cellulose, and a faster hydrolysis rate of cellulose II compared  
658 to cellulose I as shown by DP and yield decrease. **The final process yields were lower for the  
659 combined pretreatments compared to NaOH and enzymes alone, while remaining suitable for a large  
660 scale CNF production. The decrease of yields was found to be due to the solubilization of a large part  
661 of hemicelluloses during alkaline treatment, as well as a fraction of cellulose during both treatments.**  
662 The morphological study by SEM and MorFi enabled to underline the positive influence of combined  
663 NaOH and enzymatic treatment on further fibrillation by a mechanical treatment. The difficulty of  
664 CNF individualization, which has been reported for CNF produced with NaOH treatment alone, was  
665 here partially overcome. An optimum was found for a NaOH concentration of 10 wt%, suggesting a  
666 positive influence of the coexistence of cellulose I and II for further enzymatic hydrolysis and fiber  
667 degradation to CNF. The production of CNF with a pilot scale grinder confirmed the positive influence  
668 of the combined treatment compared to NaOH and enzymatic treatments alone, with an increase in  
669 the quality index and a decrease of energy consumption. This combined treatment results in well  
670 individualized CNF, with rigid structures and relatively low aspect ratio. On one hand, the study of  
671 their mechanical properties underlined an important decrease of tensile strength and strain for the  
672 combined treatment under the form of nanopaper, while maintaining a stiffness comparable to the  
673 reference. On the other hand, the optical properties were found to be substantially improved. This  
674 new pretreatment could therefore be used for CNF production with tunable properties for various  
675 applications, **the high transparency being an asset for their use in the packaging industry, or for high-  
676 added value applications such as substrates for printed electronics. Finally, this pretreatment could  
677 be suitable for industrial production thanks to the decrease of energy needs.**

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