

1 **Increasing yield of nanocrystalline cellulose preparation process by a**  
2 **cellulase pretreatment**

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17

18 **Abstract**

19 In this work the introduction of a cellulase treatment prior to NCC isolation was  
20 assessed. NCC was produced using sulfuric acid at two different concentrations (62 and  
21 64% wt.). The effect of pore size for filtration step was also assessed. The smaller acid  
22 dose led to yields up to 65-70% and average size up to 160 nm. It also produced

23 crystals with reduced sulfur content (0.6-1%). Cellulase pretreatment influenced NCC  
24 characteristics, as it increased overall yield a 12%, increased average particle size  
25 around 35 nm and reduced NCC sulfur content up to a 0.8%. We found that different  
26 conditions of enzymatic treatments led to quantitative differences on their effects on  
27 NCC. Acetate buffer used for enzymatic treatments was found to counteract effects of  
28 acid. The evidence presented in this work suggested that pretreating fibers with this  
29 cellulase represents a very interesting option to partially replace chemicals on NCC  
30 isolation.

### 31 **Keywords**

32 Nanocrystalline cellulose; Cellulase; Enzymatic treatment; Yield increase; Acid  
33 hydrolysis

### 34 **1. Introduction**

35 Nowadays, with the world facing an alarming situation of shortage of non-  
36 renewable resources such as coal, petroleum and natural gas, there is a growing interest  
37 in the use of renewable resources to fulfill the necessities of our society (Brito et al.,  
38 2012; Xu et al., 2013). In this scenario, cellulose, which is considered to be one of the  
39 most important renewable polymers on earth, offers a wide range of possibilities  
40 (Brinchi et al., 2013). Cellulose annual production is estimated to be over  $7.5 \times 10^{10}$   
41 tons in our planet (Habibi et al., 2010). Due to this availability, it has been used for  
42 centuries in the form of wood or plant fibers as an energy source, building materials,  
43 paper or clothing. Many of these uses continue nowadays, fact that is verified by the  
44 huge number of cellulose-based industries existing in the present day (paper, textiles,  
45 etc.). Although these long-known applications are still benefiting our society in the  
46 present, during the last decade cellulose has been receiving a new and growing interest

47 due to the understanding that fibers are built by smaller entities that could be extracted  
48 from them under proper conditions (Charreau et al., 2013). Exploring efficient ways to  
49 extract these smaller entities (crystalline regions) from fibers has attracted plenty of  
50 attention of authors during the last years, fact that can be observed in the growing  
51 number of patents related to this field published since year 2000 (Charreau et al., 2013).

52         The more extensively used method to obtain these crystalline regions consists of  
53 a controlled hydrolysis with sulfuric acid, basically due to the stability of the resulting  
54 suspensions (Abitbol et al., 2013; Habibi et al., 2010). During this reaction, amorphous  
55 domains are attacked preferentially, while crystalline regions present higher resistance  
56 to acid attack (Habibi et al., 2010). Microfibrils are then destructed at their “defects”,  
57 leading to the release of rod-like particles, the former crystalline regions, now  
58 nanocrystals. This differentiated susceptibility to acid attack is thought to be provoked  
59 by differences in the kinetics of hydrolysis between amorphous and crystalline domains,  
60 where the first ones are more rapidly accessible by acid and thereafter, hydrolyzed first  
61 (Habibi et al., 2010).

62         During the last decades, authors have untiringly studied ways to introduce  
63 biotechnology in the cellulose-related industry. Within these studies, among all the  
64 available options, enzymes have been preferentially chosen due to special features they  
65 present (high specificity, environmental friendliness, etc.). According to Brinchi et al.  
66 (Brinchi et al., 2013), limited literature has yet been published for ways to introduce  
67 enzymes in the preparation process of nanocrystalline cellulose. From the wide range of  
68 options of enzymatic activities existing on nature, cellulases (EC 3.2.1.4), have a special  
69 interest due to our objective of breaking down the hierarchical structure of cellulose  
70 (Garcia-Ubasart et al., 2013). Examples of this could be the application of treatments  
71 with cellulases to isolate crystalline regions from cellulose (Anderson et al., 2014; Chen

72 et al., 2012; Filson et al., 2009). In this work, however, effects of pre-treating a pure  
73 cellulosic source such as cotton linters with a cellulase, prior to acid hydrolysis were  
74 studied for the first time. The objective was to evaluate the consequences of this pre-  
75 treatment on the characteristics presented by final product and to make a first attempt to  
76 establish working parameters in order to partially replace, the use of harsh chemicals by  
77 these environmentally friendly catalysts.

78

## 79 **2. Materials and Methods**

### 80 **Raw material and enzymatic treatment**

81 Cotton linters (cellulose content  $97.7 \pm 0.3$  %) were used as cellulose source. They were  
82 provided by Celsur (Spain) and were refined for 90 minutes on a valley mill in order to  
83 reduce their average length. Obtained fibers were named as initial.

84 A cellulase preparation (named “C”), provided by Fungal Bioproducts® (Spain) and  
85 obtained from *Cerreña sp.* fungus was used for treatments. Activity as U/g from  
86 enzyme stock was 1700. Activity was expressed as CMC<sub>case</sub> units, that is to say, the  
87 amount of enzyme degrading 1  $\mu$ mol of CMC (carboxymethylcellulose) per minute.

88 Treatments with C were carried out according to three different conditions (Table 1).

89 Two different reactors were used for treatments; treatment 1 was carried out in a  
90 cylindrical 4 L reactor with agitation produced by rotating blades at 30 rpm. Treatments  
91 2 and 3 were performed in an Ahiba Easydye (Datacolor, USA) apparatus having 250  
92 mL independent units with agitation consisting on upside-down inversions of these units  
93 at 20 oscillations per minute. When corresponding (Table 1), 50 mM acetate buffer was  
94 used to set the pH to 5 during enzymatic treatment. In all cases enzyme was deactivated  
95 after reaction by increasing temperature to 105 °C for 15 min. Fibers were filtered using

96 a N°2 filter and reaction liquor was passed through fibers 3 times in order to recover  
97 fines. No washing was performed after enzymatic treatments to avoid sample loss.  
98 Control fibers, as indicated on Table 1, in the three different cases were treated with the  
99 same conditions as enzyme-treated fibers but without enzyme addition. Control fibers  
100 were referred as “KC”.

### 101 **Nanocrystalline cellulose preparation**

102 Nanocrystalline cellulose (NCC) was obtained by a controlled sulfuric acid hydrolysis,  
103 adapting to our conditions the protocol proposed by (Dong et al., 1998). Fibers were  
104 fluffed prior to hydrolysis, oven dried and cooled in a desiccator. Typically, 2.5 g of  
105 sample weighted immediately from desiccator were hydrolyzed with 62 % or 64 %  
106 (w/w) sulfuric acid for 45 min at 45 °C with magnetic stirring and an acid-to-fibers ratio  
107 of 10:1 (*i.e.* 10 mL/1 g cellulose). Reaction was stopped by diluting the acid with chilled  
108 (4 °C) distilled water in a 10-fold basis, also placing sample container on an ice bath.  
109 Samples were then centrifuged at 6000 rpm for 15 min and supernatant was discarded.  
110 Then, samples were re suspended in distilled water and centrifugation step was  
111 repeated, discarding supernatant. Small fractions of supernatants were saved for  
112 analysis. Samples were then sonicated for dispersion using a Hielscher UP100H  
113 ultrasonic processor at 100 % amplitude and 0.75 cycles for 20 min on an ice bath to  
114 prevent heating, which may cause desulfation (Dong et al., 1998). Re suspended  
115 samples were then dialyzed against distilled water using a 10 kDa Thermo Fischer  
116 dialysis membrane for three days. After dialysis sonication step was repeated. Finally,  
117 samples were filtered through Whatman ashless paper filters, N° 40 (pore size 8 µm) or  
118 41 (pore size 20-25 µm). The whole process of preparation was repeated three times for  
119 each sample in order to ensure repeatability. In text, when referring to NCC samples

120 will be noted as X\_NCC (*e.g.* Initial or C1 indicate fiber samples, while C1\_NCC or  
121 initial\_NCC refer to the NCC isolated from these fibers).

## 122 **Characterization**

123 Initial, enzymatically treated and control fibers were characterized in terms of fiber  
124 length, viscosity, zeta potential, cationic demand, free hydroxyl content and water  
125 contact angle. NCC samples were characterized in terms of yield, sulfur content,  
126 particle size, surface charge, zeta potential, water contact angle and viscosity. NCC  
127 films were prepared by evaporation of aqueous phase of NCC suspensions (water) in an  
128 air circulating oven at 60°C.

129 Fibers length, width and % of fines were measured using Kajaani fiber analyzer (FS300,  
130 Metso automation, Finland) according to ISO 16065-1 and viscosity of fibers and NCC  
131 suspensions was determined according to ISO 5351:2010.

132 Zeta potential of fibers was determined according to Cadena et al. (Cadena et al., 2009)  
133 using a Müttek zeta potential equipment (SZP-06, Müttek, Germany), while  
134 electrophoretic mobility of aqueous NCC suspensions was determined using Malvern  
135 Zetamaster (ZEM, Malvern instruments, UK) from which data was averaged over 6  
136 measurements. All samples were analyzed at room temperature.

137 Free hydroxyl content of fibers was determined according to the protocol described by  
138 Genung et al (Genung, 1950).

139 Water contact angle of fibers and NCC film samples was measured by using a  
140 Dataphysics OCA15EC contact angle goniophotometer (Dataphysics, USA), using an  
141 image capture ratio of 25 frames/s. Following the procedure described by Cusola et al.

142 (Cusola et al., 2013) a 4  $\mu$ L water drop was delivered to the sample surface. At least 8  
143 measurements were made for each sample.

144 Surface charge of suspensions of fibers and NCC was determined using Müttek particle  
145 charge detector (PCD03PH, Müttek, Germany). Suspensions were titrated using 0,001N  
146 Poly-Dadmac (cationic poly-electrolyte). Surface charge density was calculated  
147 according to the following formula:

$$Surface\ charge\ \left(\frac{meq}{g}\right) = \frac{VxC}{wt}$$

148 Where V and C are the volume and the concentration of the titration agent (poly-  
149 dadmac), respectively, and wt is the weight of the NCC sample.

150 Yield of NCC isolation process was determined drying 20-25 mL of the suspension and  
151 determining the mass after evaporation. Total solids in suspension were calculated and  
152 yield was expressed as % of initial fiber mass. Values were given as average of three  
153 independent determinations for each sample.

154 Sulfur content of NCC was determined according to a procedure proposed by (Abitbol  
155 et al., 2013). Briefly, a small sample of suspension was titrated using 1.25 mM NaOH  
156 recording conductivity values. The equivalence point corresponded to the amount of  
157 NaOH necessary to neutralize all the sulfate groups attached to crystals surface. Results  
158 were calculated as % of mass of atomic sulfur over NCC mass. Values are given as  
159 average of three independent measurements for each sample.

160 Particle size of NCC samples was determined using a particle size analyzer (DLS 135,  
161 Cordouan Technologies, France). Size distribution was determined with dynamic laser  
162 scattering (DLS) at room T° (25°C). Aqueous suspensions were placed directly in the

163 measuring cell and laser power was adjusted for counting around 2000 particles per  
164 minute.

165 Cellulose fibers FTIR spectra were recorded at room temperature using an ATR-FTIR  
166 spectrophotometer (Spectrum 100, Perkin Elmer, USA). FTIR spectral analysis were  
167 conducted within the wavenumber range of 600-4000  $\text{cm}^{-1}$ . A total of 64 scans were run  
168 to collect each spectrum at a  $1\text{cm}^{-1}$  resolution. Total crystallinity index (TCI), proposed  
169 by Nelson and O`Connor (Nelson and O`Connor, 1964), was estimated from the ratio  
170 between the absorption peaks at  $1370\text{cm}^{-1}$  and  $2900\text{cm}^{-1}$  bands.

### 171 **Optical and SEM microscopy**

172 Images of fibers were obtained using an Olympus BH-2 optical microscope connected  
173 to a digital camera. Images of films were obtained using a scanning electron microscope  
174 (SEM) (JSM-6400, JEOL, Japan). NCC films were first coated with a very thin layer  
175 (14 nm thick) of gold–palladium in a sputter coater SCD005 in order to obtain a  
176 conductive surface, and images were obtained at 15 kV.

### 177 **Released oligosaccharides**

178 Dissolved Oligosaccharides were identified and quantified in centrifuges supernatants  
179 of NCC preparation process and liquid phase of final suspensions using a HPLC  
180 instrument (1100, Agilent technologies, USA) furnished with a BIO RAD Aminex  
181 HPX-42A ion-exchange column. Samples were first filtered and their pH was set to 7  
182 using HCl or NaOH. Operating conditions were: 0.35 ml/min flow, column temperature  
183  $65\text{ }^{\circ}\text{C}$  and the mobile phase was MQ water. Data was collected by refractive index  
184 detector (RID). Identification and quantification of compounds was done by  
185 interpolation into calibration curves run from standards.



186        **3. Results and discussion**

187            The aim of this work was to evaluate the possibility of introducing enzymes  
188 (biotechnology) into the nanowhisker isolation process, which has been principally  
189 chemical up to these days (Brinchi et al., 2013; Charreau et al., 2013). Examples of  
190 cellulase application for NCC (cellulose nanocrystals) or MFC (microfibrillar cellulose)  
191 isolation available in literature generally include a first chemical step in which cellulose  
192 is treated in order to weaken the structure and then a second step in which it is  
193 enzymatically treated to finally isolate NCC or MFC (Anderson et al., 2014; Chen et al.,  
194 2012; Filson et al., 2009). The idea now was to facilitate the acid hydrolysis step by pre-  
195 weakening cellulose structure with a cellulase. So, to our knowledge sulfuric acid  
196 hydrolysis as a NCC isolation method was now performed on cellulase pre-treated  
197 fibers for the first time.

198        **3.1. Cellulase treatments**

199            In literature, studied cellulases could produce a fibrillation effect on fiber  
200 surface, which does not necessary imply a reduction in average length or viscosity  
201 (Garcia-Ubasart et al., 2013). However, previously reported results treating dissolving-  
202 grade pulps (Quintana et al., 2015a, 2015b) , indicated that this particular cellulase  
203 (“C”) produced a shortening effect in fiber length, compared to the fibrillation effect  
204 produced by another cellulase on the same study. Being the action of isolating cellulose  
205 crystalline regions a deconstruction process which takes advantage of the hierarchical  
206 structure of this material (Brinchi et al., 2013; Habibi et al., 2010), it was likely that a  
207 cellulase could take part on it. Treatments with this enzyme were performed for a long  
208 period (24 h) in order to ensure the noticeability of their effects.

209 In order to elucidate the effects of C on fibers and on final NCC, it was applied  
210 under 3 different conditions (Table 1). Regarding treatment 1, viscosity was highly  
211 reduced, suffering a reduction of about 500 mL/g (Table 2). This reduction was of  
212 course related to enzyme's cleavage effect of cellulose microfibrils, which led to a  
213 reduction of their average degree of polymerization. Also, this *cutting* effect of C  
214 modified fibers macroscopically, reducing average fiber length in  $\approx 0.3$  mm and  
215 increasing fines amount about a 2 % compared to KC1. Treatment 2, which was  
216 conducted at the same pH, temperature and enzyme dose as in treatment 1 but in a  
217 different reactor, produced different effects on fibers. These differences can then only  
218 be attributable to the reactor, where the smaller volume and the stronger mixing led to a  
219 different interaction between fibers and enzyme. This statement was supported by fiber  
220 length data as C2 fibers showed a lower value compared to C1 fibers. Fines amount,  
221 which were also produced as a result of enzyme action, also experienced a higher  
222 increase compared to control pulps ( $\approx 11$  %) after treatment 2. One plausible explanation  
223 for these two contrasting evidences relays on the stronger mixing performed by Ahiba  
224 Easydye® reactor, which could have enhanced the physical separation of enzymatically  
225 pre-hydrolyzed fibers, leading to a higher fines amount and also to shorter fibers.  
226 Treatment 3 was carried out with the objective of studying the influence of enzymatic  
227 treatments in absence of buffer on NCC. Comparing to treatment 2, it produced the  
228 same effect on fibers in terms of fiber length and viscosity. Fiber width (Table 2) also  
229 showed the effects of C, as it was reduced a 9 % after treatments in the three cases,  
230 compared to control fibers. Finally, FTIR analysis provided evidence that C treatment  
231 increased average crystallinity of fibers. Obtained TCI values for KC and C fibers were  
232 0.69 and 0.92, respectively, indicating an increase in fibers crystallinity. Generally, TCI  
233 is claimed to be proportional to cellulose crystallinity index (Šíroky et al., 2010), and

234 therefore this increase suggested that enzyme attacked preferentially the amorphous  
235 regions of cellulose.

236           Regarding surface characteristics (Table 2), fibers resulted chemically modified  
237 after cellulase treatment. It can be seen how the amount of free hydroxyl groups on  
238 cellulose fibrils increased a 9 % after cellulase treatment. Probably, after cutting  
239 cellulose chains, OH- groups that were hidden were then exposed, increasing the total  
240 number of free (accessible) hydroxyl groups in relation to cellulose mass. It is important  
241 to remark as well that these newly accessible OH- could be in more than carbon position  
242 of each glucose residue and not all of them are equally reactable (Gu et al., 2013).  
243 Cationic demand of fibers was very low and no differences were observable between  
244 samples. Zeta potential values showed the effect of buffer, as both C and KC treatments  
245 had smaller values than initial fibers. This difference in zeta potential was thought to be  
246 caused by a modification of ionic distribution around cellulose fibers caused by ions  
247 provided by buffer. Contact angle was not affected by treatments, and observed value  
248 ( $\approx 23^\circ$ ) indicated that fibers were hydrophilic. A reported contact angle value for initial  
249 cotton linters by another author (Morais et al., 2013) indicated values around  $70^\circ$ , very  
250 different from those reported on Table 2. Finally, yield (as % of recovered dried fibers)  
251 was calculated after treatment 1, obtaining a value of 90.2 % after C1 and 97.8 % after  
252 KC1.

### 253           **3.2.Effect of conditions of cellulase treatment on NCC preparation**

254           In order to further understand the effects of this enzyme on NCC preparation and  
255 final characteristics, NCC were prepared after fibers obtained in treatments C 1, 2 and 3.  
256 Two different acid doses were used for trials. One dose (64 % wt.) which was chosen  
257 based on evidence obtained from previous work of our group and considered a standard

258 concentration for NCC preparation, and another dose thought to be weaker (62 % wt.).  
259 All indicated samples were filtered through the N°41 membrane.

260 Yield of NCC preparation process is a key aspect to be analyzed due to its  
261 evident impact on the economic cost of the whole process. Acid dose had a major  
262 impact on yield, as greater NCC yields about a 30-35 % bigger, were obtained for  
263 hydrolysis carried out with 62 % H<sub>2</sub>SO<sub>4</sub> compared to 64 % (Figure 1). This evidence  
264 accorded with the results reported by several authors, where smaller acid concentrations  
265 resulted in higher yields due to weaker hydrolysis (Bondeson et al., 2006; Lu et al.,  
266 2013). Regarding enzymatic treatments, C1 and C2 fibers led to higher yields with both  
267 acid concentrations compared to crystals obtained after C3 fibers. Size of crystals, by its  
268 side, as measured by DLS did not provide a real value of their dimensions due to the  
269 rod-like structure they present (Fraschini et al., 2014). DLS uses laser scattering to  
270 measure radio (diameters) of particles and with this data calculates particles  
271 hydrodynamic diameters considering all elements to be spheres (Fraschini et al., 2014).  
272 Still, authors have used DLS data to establish comparisons between NCC samples,  
273 being aware that this value was not necessary representing the true physical size (Brito  
274 et al., 2012; Habibi et al., 2010). Hydrodynamic diameters in Figure 1a show that acid  
275 dose also had great influence on size. Bigger crystals seemed to be obtained with the  
276 weaker acid treatment (62 %) with values around 30-60 nm bigger compared to those  
277 obtained with 64 % acid. Regarding cellulase treatments, C1\_NCC and C2\_NCC again  
278 showed very similar values, yielding ≈35 nm bigger crystals with 62 % sulfuric acid  
279 compared to C3\_NCC. With 64 % acid, differences were small. On Figure 1a it can be  
280 observed that both particle size and yield seem to be related, fact that will be further  
281 discussed in the following section. Sulfur content of nanowhiskers, in the form of  
282 sulfate groups attached to their surface determines their behavior in several aspects.

283 Among other aspects, their presence is crucial for the stabilization of crystal  
284 suspensions in water, (Abitbol et al., 2013; Habibi et al., 2010). In spite of this, they  
285 compromise nanowhiskers thermostability (Gu et al., 2013; Roman and Winter, 2004).  
286 Thus, an optimal sulfur content value considering advantages and inconvenients should  
287 be found. Regarding acid dose, sulfur content (Figure 1b) showed bigger values for  
288 NCC obtained with H<sub>2</sub>SO<sub>4</sub> at the higher concentration (64 % wt.) compared to the other  
289 (62 % wt.), with differences among 0.5-0.8% sulfur. This evidence has already been  
290 reported and is explained by the fact that SO<sub>4</sub><sup>2-</sup> groups are incorporated to crystals by an  
291 esterification reaction (Habibi 2010; Abitbol et al. 2013). As water is a reaction product  
292 in esterifications, having it in smaller amounts (as happens for the acid at 64 %)   
293 enhances its occurring compared to smaller acid concentrations, where water content is  
294 higher (Roman and Winter, 2004). Among samples, C3\_NCC presented the highest  
295 sulfur content for both acid concentrations studied, with higher differences between  
296 C1\_NCC and C3\_NCC at 64 % sulfuric acid (Figure 1). In this case, 0.7 % higher sulfur  
297 content was measured in C3\_NCC. Data on Figure 1 suggested that acetate buffer  
298 affected NCC isolation process, fact that arises from the comparison of C3 NCC with  
299 C2 NCC, as the lack on buffer on the former treatment is the only difference between  
300 these samples. In this direction, buffer presence seemed to increase yield and particle  
301 size and reduce sulfur content.

302 Table 3 indicates schematically the different effects of cellulase treatments on  
303 NCC properties related to initial\_ NCC. At both acid doses, data reflected an: Increase  
304 in yield, decrease in sulfur content and increase in average size as a consequence of  
305 cellulase treatments. A further study will be discussed in the following section for  
306 properly studying this evidence. Data on Table 3 remarked the fact that C effects were  
307 in all cases more visible at the lower acid concentration (62 % wt.). Also, it can be

308 observed how cellulase treatments in buffer presence provided better results than in its  
309 absence (C3). Additionally, with 62 % acid, C3 fibers performed worse yielding NCC  
310 than initial fibers did, providing evidence that treatment 3 was not useful for NCC  
311 production. Finally, Treatments 1 and 2 provided very similar results on NCC.  
312 However, the reactor used for treatment 1 offered better conditions for a treatment  
313 intended for its industrial application. Therefore, the influence of C1 treatment on NCC  
314 will be more thoroughly analyzed.

### 315 **3.3.Effects of enzymatic treatment and acid dose on NCC**

316 As no agreement seems to exist in bibliography regarding pore size for the  
317 filtration step in nanowhiskey preparation process, we used two different filter papers  
318 with two different pore sizes to study their influence (Figure 2 and Figure 3). Yield was  
319 found to be affected by the used filter, with yields between 3-8 % bigger obtained with  
320 Whatman 41 filter compared to Whatman 40. Particle size was also affected in the same  
321 direction. The rest of properties did not show any affectation.

#### 322 **3.3.1. NCC properties**

323 For a proper understanding of C enzyme influence on NCC, results were  
324 compared with KC1\_NCC, attributing further differences from initial pulp to buffer  
325 effect. Again, enzymatic pre-treatment on fibers increased NCC yield, especially with  
326 62 % acid, with yields 7-8 % higher (Figure 2). Our hypothesis for explaining this  
327 behavior was related to a different interaction between acid and cellulose fibrils after  
328 being cleaved by C action. Reduction of cellulose chains length by enzyme (indicated  
329 by viscosity values) and reduction in fibers dimensions (Table 2) might have modified  
330 fiber-acid interaction. Crystalline regions are more difficult to be degraded by cellulases  
331 (Ahola et al., 2008), and according to previously mentioned crystallinity measurements,

332 amorphous regions seem to have been selectively degraded by C1 treatment, improving  
333 accessibility of acid to crystalline regions. We understand that this improvement in  
334 accessibility reduced the existence of obstacles, reducing the need of degrading  
335 cellulose mass to reach crystalline regions. This evidence was also supported by % of  
336 free OH-, which indicated that C fibers had a bigger number of OH- groups exposed  
337 compared to KC and initial fibers (Table 2), meaning a larger fraction of surface of  
338 fibrils was exposed, increasing accessibility to acid on fibers internal structure. In order  
339 to have a more realistic idea of the gain in yield provided by C treatment, the yield of  
340 the enzymatic treatment on fibers must be considered, as cellulolytic activities imply  
341 some cellulose mass conversion to oligosaccharides. After taking this into  
342 consideration, only treatments with 62 % sulfuric acid provided higher yields comparing  
343 C and KC treatments, as the gain in yield produced by cellulase was smaller with 64 %  
344 sulfuric acid.

345       Regarding crystals size, differences were only significant for samples at 62%  
346 acid, as C1\_NCC crystals were 25-30 nm larger than KC1\_NCC (Table 4). If yield is  
347 plotted against sample size (Figure 3) a linear correlation seemed to be found linking  
348 both parameters. This linear relation suggested that the increase in yield, meaning an  
349 increase in obtained NCC mass, was related to an increase in average particle size,  
350 which ultimately implied a higher mass per crystal unit. This finding supports our  
351 hypothesis for the mechanism behind the increase in yield after C treatment, which  
352 consisted in a facilitated interaction between cellulose and acid, reducing cellulose  
353 sample loss, yielding bigger crystals and a larger NCC mass.

354       Comparison between C\_NCC and KC1\_NCC also confirmed that C treatment  
355 affected the incorporation of sulfate groups onto crystals (Figure 4). C1\_NCC sulfur  
356 content was smaller (0.1 % sulfur) than KC1\_NCC. If compared to initial\_NCC,

357 enzymatic effects were bigger. This might be related to the difference in free OH-  
358 groups available in fibers after C treatment (Table 2). It is well known that sulfate  
359 groups incorporation occurs by an esterification with free OH- present in cellulose  
360 (Habibi et al., 2010). However, this reaction is not equally allowed to occur in all free  
361 OH- positions from glucose residues, as OH- reactivity depends on its carbon position  
362 (Gu et al., 2013). A modification in OH- distribution on cellulose surface may have  
363 been the cause for this reduction in  $\text{SO}_4^{2-}$  incorporation onto NCC. Sulfurs content was  
364 also influenced by acetate (Figure 4). Sulfur contents 1% higher were obtained on  
365 initial\_NCC hydrolyzed with 64 % acid and 0.4 % higher for 62 % acid compared to  
366 KC1\_NCC. Supporting the observed section 3.2, acetate presence also affected the  
367 incorporation of sulfate moieties to NCC.

368 For a deeper understanding of cellulase effects, crystals were characterized in  
369 additional terms. Surface charge (cationic demand) of NCC is important as electric  
370 charge of NCC affects their applicability (Lin and Dufresne, 2014). Firstly, big  
371 differences between NCC and original fibers were found. Charges about 100 times  
372 bigger were observed for NCC (per mass unit) compared to fibers, probably  
373 responsibility of the negatively charged sulfate groups attached to surface (Beck et al.,  
374 2011), not originally present on fibers. Cationic demand values of NCC suspensions, as  
375 indicated in Figure 4 follow a similar tendency as that observed for sulfur content,  
376 providing evidence that both parameters were related. As a regard of acid dose, NCC  
377 obtained from 64 % acid showed greater surface charges than those observed for 62 %  
378 acid, a similar behavior as that observed for sulfate groups. This parameter failed to  
379 show differences between C\_NCC and KC\_NCC samples, though, suggesting it was  
380 less sensitive than conductimetric titration used for sulfur content determination. Lastly,



381 acetate buffer influence was also observable on cationic demands (Figure 4) as  
382 KC\_NCC showed smaller charges compared to initial\_NCC.

383           Electrophoretic mobility of particles, expressed as zeta potential is a good  
384 indicator of colloidal stability of suspensions (Boluk et al., 2011; Teixeira et al., 2010).  
385 It has been reported that NCC colloidal stability provided by sulfate groups facilitates  
386 their further applicability, as well as it permits a broader range of uses (Lin and  
387 Dufresne, 2014). Among samples, in all cases zeta potential values were around -50 mV  
388 (Table 4), which is considered to be a value indicating high stability (Hornig and  
389 Heinze, 2008). Values were very similar to reported by other authors for similar  
390 samples (Teixeira et al., 2010). Furthermore, they indicated higher stabilities on final  
391 suspensions compared to those reported by Filson et al. (Filson et al., 2009), who  
392 reported values around -31 mV. This difference remarked the utility of using both  
393 enzymatic treatment and also sulfuric hydrolysis. In this way, benefits of enzymatic  
394 treatment and also the benefit in suspension stability provided by sulfate groups  
395 attached to NCC surface were obtained. Acid dose and enzymatic treatment influence  
396 was observed on zeta potential. 1-3 mV less-negative values for 62 % acid NCC  
397 compared to 64 % were obtained. Also, 3-5 mV smaller values were observed for  
398 C\_NCC samples compared to KC\_NCC and initial\_NCC, fact that was observable only  
399 for 64 % acid. Considering zeta potential is both influenced by colloidal particle size  
400 and electrical charge (Hornig and Heinze, 2008), these small differences could also be  
401 caused by the already stated existing difference in size between samples.

402           Surfaces hydrophobicity as WCA (water contact angle) was determined. WCA  
403 values for NCC dried films are given in Table 4. All samples had WCA values around  
404 50 °, indicating that samples were hydrophilic. This hydrophilicity was caused by  
405 sulfate groups esterified on crystals surface (Anderson et al., 2014; Morais et al., 2013).

406 In comparison with WCA of starting fibers, NCC films showed to be more hydrophobic  
407 than the original fibers, taking WCA from 23 ° to ≈50 °. The explanation for this could  
408 be found in the fact that crystalline regions are less accessible for water than amorphous  
409 regions, and NCC are lacking on the former. Also, the chiral ordering of NCC on films  
410 could have helped to difficult interaction with water. These results differ from those  
411 reported by Morais et al. (Morais et al., 2013) who observed a reduction in  
412 hydrophobicity after isolating crystalline regions. Regarding enzyme effect, a certain  
413 tendency of increased NCC hydrophilicity was observed for C1\_NCC compared to  
414 KC\_NCC and initial\_NCC (Table 4).

415 Finally, viscosity was measured for NCC suspensions obtained with 62% acid  
416 and filter 41 of KC and C, obtaining values of 45 and 40 mL/g, respectively. These  
417 values provided evidence of the high scission cellulose chains were submitted to during  
418 NCC preparation, as they were considerably smaller to those observed on fibers.

### 419 **3.3.2. Assessing the effects of cellulase and reaction conditions: SEM and HPLC** 420

421 For a better understanding of enzyme and acid action over cotton linters, content  
422 in oligosaccharides was determined in centrifuges supernatants (washing waters during  
423 NCC preparation) and also in liquid phase of NCC suspensions. The intention was to  
424 elucidate the effects of the used hydrolase (C) and also the effects of acid hydrolysis in  
425 relation to the generation of short oligosaccharides. These short oligosaccharides could  
426 then be used as a feedstock for the production of other compounds, such as bioethanol  
427 (Filson and Dawson-Andoh, 2009). Results are given on Table 5. As it could be  
428 expected, sugar concentration of samples decreased with centrifuge washings  
429 (comparing C/KC 64 1<sup>st</sup> vs 2<sup>nd</sup> washing). We also observed that larger sugar amounts  
430 were released after sulfuric acid hydrolysis from the fibers treated with C in comparison

431 with KC. This finding indirectly supported the evidence observed in NCC  
432 characteristics, as C enzymatic treatment modified sulfuric acid effect over cellulose  
433 fibers during NCC isolation.

434         Between the different oligosaccharides, it seemed that the shorter species were  
435 produced in a greater quantity than the longer forms ( $C1 \geq C6$ ), with concentrations  
436 about 10 times higher of glucose compared to the amount of cellopentaose and  
437 cellohexose produced (Table 5). Similar work reported by other authors (Filson and  
438 Dawson-Andoh, 2009) found only glucose and cellobiose as hydrolysis products, being  
439 glucose the main released sugar, as in our case. Among samples, fewer sugars were  
440 released with lower acid concentrations (initial 62 % vs. initial 64 %, Table 5). Lower  
441 sugar concentrations were found on washing waters of the hydrolysis of C treated fibers  
442 compared to initial. This was consistent with the higher NCC yields obtained from C  
443 treated fibers compared to initial\_NCC. Finally, it was evidenced that remaining  
444 dissolved sugars on samples were removed from suspensions during dialysis, as can be  
445 noticed comparing 2<sup>o</sup> washing samples with the two columns on the right on Table 5.

#### 446 **4. Conclusions**

447         As a result of cellulase action, yields up to 12 % greater and crystals with bigger  
448 dimensions, with values up to 35 nm higher were obtained. Also, its action reduced the  
449 incorporation of sulfate moieties onto crystals surface, yielding NCC with contents up  
450 to 0.8 % lower but maintaining suspensions stability (zeta potential). Data obtained in  
451 this study showed that cellulase treatment could be a promising first step for partially  
452 replacing the use of harsh chemicals and save energy during the isolation of cellulose  
453 nanocrystals.

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## 455 **5. Acknowledgments**

456 Authors are grateful to Spanish "Ministerio de Economía y Competitividad" for their  
457 support in this work under the BIOSURFACEL CTQ2012-34109 (funding also from the  
458 "Fondo Europeo de Desarrollo Regional, FEDER") and BIOPAP $\mu$ FLUID CTQ2013-  
459 48995-C2-1-R projects and a FPI grant (BES-2011-046674). Special thanks are also due  
460 to the consolidated research group AGAUR 2014 SGR 534 with Universitat de  
461 Barcelona (UB). We are also grateful to Celsur and Fungal Bioproducts for supplying  
462 cotton linters and enzymes, respectively.

463

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		Treatment 1 (C1)	Treatment 2 (C2)	Treatment 3 (C3)
Enzyme dose	10 U/g odp			
Treatment time	24 h	✓	✓	✓
Temperature	55 °C			
pH	5 (50 mM acetate buffer)	✓	✓	
	5 (adjusted with H <sub>2</sub> SO <sub>4</sub> )			✓
Reactor	4L blade stirred reactor	✓		
	250mL Ahiba Easydye®		✓	✓
Agitation	Rotating blades - 30 rpm	✓		
	Oscillating - 20 rpm		✓	✓
Control fibers	No enzyme addition	KC1	KC2	KC3

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**Table 1: Enzymatic treatment conditions**

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	Fiber sample						
	Initial	C1	KC1	C2	KC2	C3	KC3
Fiber length (mm)	1.95 ± 0.1	1.51 ± 0.02	1.82 ± 0.04	0.52 ± 0.1	1.64 ± 0.07	0.55 ± 0.2	1.67 ± 0.03
Fiber width (µm)	22 ± 0.2	20.2 ± 0.2	22.1 ± 0.1	20.1 ± 0.2	22 ± 0.1	20 ± 0.4	22 ± 0.2
Fines (%)	39.7 ± 0.7	43.5 ± 0.4	41.2 ± 0.1	52.9 ± 0.6	42.1 ± 0.4	54.6 ± 1.2	44.2 ± 0.1
Viscosity (mL/g)	777 ± 37	256 ± 17	737 ± 20	346 ± 13	790 ± 24	369 ± 24	775 ± 15
Free OH- (%)	5.58 ± 0.02	6.22 ± 0.13	5.71 ± 0.03	-	-	-	-
Surface charge (µeq/g)	1.8 ± 0.64	1.3 ± 0.32	1.34 ± 0.1	-	-	-	-
Zeta potential (mV)	-123.9 ± 15.3	-74.1 ± 12.2	-53.2 ± 10.6	-	-	-	-
Contact angle (°)	23 ± 1	24 ± 1	23 ± 2	-	-	-	-

**Table 2: Fibers characteristics of initial, enzymatically treated (C) and control (KC) samples.**

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	H <sub>2</sub> SO <sub>4</sub> 62%			H <sub>2</sub> SO <sub>4</sub> 64%	
	C1_NCC	C2_NCC	C3_NCC	C1_NCC	C3_NCC
Increase in yield (% yield)	5.8	8.7	-3.8	5.1	3.1
Decrease in sulfur content (% S)	0.43	0.37	0.25	1.14	0.42
Increase in size (nm)	31	36	-4	14	19

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**Table 3: Effects of enzymatic treatments as differences in NCC characteristics between each indicated NCC sample and NCC from initial fibers. Samples were filtered using Whatman 41 membrane**

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Sulfuric acid (% wt)	Filter n° (Whatman®)	Enzymatic treatment	Size (nm)	Zeta potential (mV)	WCA (°)
62%	40	C1	147 ± 7	-53.7 ± 1.1	41 ± 5
		KC1	125 ± 2	-53.6 ± 1.1	50 ± 5
		Initial	115 ± 4	-55.5 ± 0.6	47 ± 3
	41	C1	158 ± 5	-53.5 ± 1.3	39 ± 4
		KC1	128 ± 3	-54.8 ± 1	50 ± 2
		Initial	127 ± 5	-55.9 ± 0.8	48 ± 4
64%	40	C1	90 ± 2	-50.1 ± 1	44 ± 4
		KC1	88 ± 1	-52.7 ± 1.2	51 ± 7
		Initial	85 ± 3	-54.5 ± 1.6	54 ± 3
	41	C1	93 ± 3	-49.6 ± 1.3	43 ± 3
		KC1	92 ± 4	-54.6 ± 1.2	48 ± 2
		Initial	79 ± 4	-56.5 ± 1.1	52 ± 3

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**Table 4: Average size, electrophoretic mobility, and water contact angle of NCC samples**

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Oligosaccharide concentration (mg/mL)								
Sample	Washing waters						Final suspensions	
	C1 64 %		KC1 64 %		Initial 62 %	Initial 64 %	C1 64 %	KC1 64 %
Washing	1 <sup>st</sup>	2 <sup>nd</sup>	1 <sup>st</sup>	2 <sup>nd</sup>	1 <sup>st</sup>	1 <sup>st</sup>	-	-
C6	0.055	0.015	0.048	0.000	0.044	0.073	0.000	0.000
C5	0.074	0.018	0.065	0.009	0.056	0.098	0.000	0.000
C4	0.126	0.024	0.108	0.009	0.080	0.163	0.000	0.000
C3	0.189	0.035	0.157	0.013	0.091	0.224	0.000	0.000
C2	0.301	0.077	0.246	0.028	0.118	0.302	Traces	0.000
C1	0.722	0.170	0.570	0.060	0.268	0.665	Traces	0.000

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632 **Table 5: Oligosaccharide concentration after centrifuges (washings) and in liquid phase of final suspensions.**  
633 **Cellulose source and acid concentration is indicated for each sample. C1, C2, C3, C4, C5 and C6 stand for**  
634 **glucose, cellobiose, cellotriose, celotetraose, cellopentaose and cellohexose, respectively**

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649 **Figure Captions**

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651 Figure 1: Size (a) and sulfur content (b) of NCC samples expressed as a function of  
652 yield for 62% and 64% H<sub>2</sub>SO<sub>4</sub>.

653 Figure 2: Yield of NCC preparation process for the three different fiber samples at the  
654 different studied conditions.

655 Figure 3: NCC average size vs yield. Circles indicate data with 62% and 64% wt.  
656 H<sub>2</sub>SO<sub>4</sub>. All samples were obtained from Initial, C1 and KC1 fibers.

657 Figure 4: Sulfur content of NCC, as % elemental sulfur (bars, left axis). Surface charge  
658 of NCC as cationic demand of suspensions (points, right axis).

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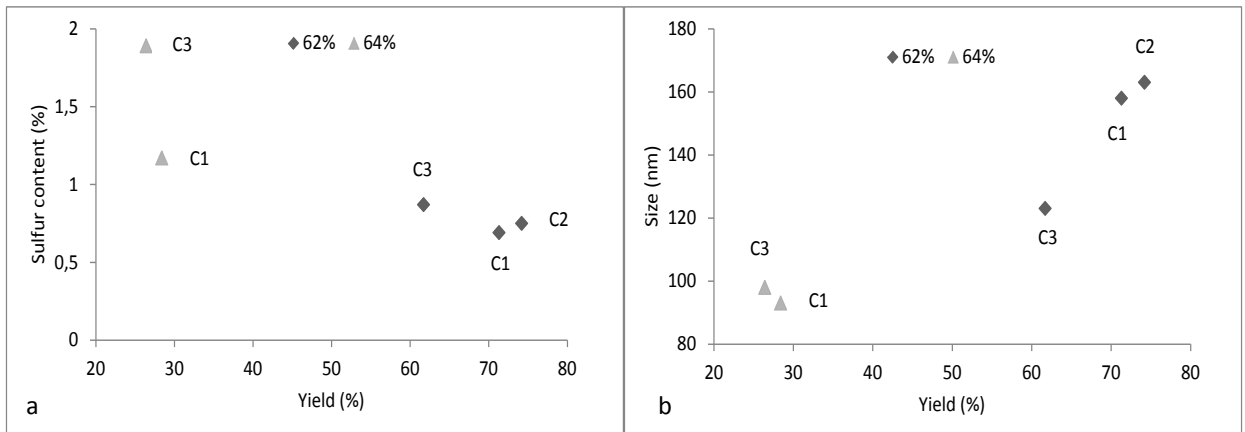
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670 **Figure 1**



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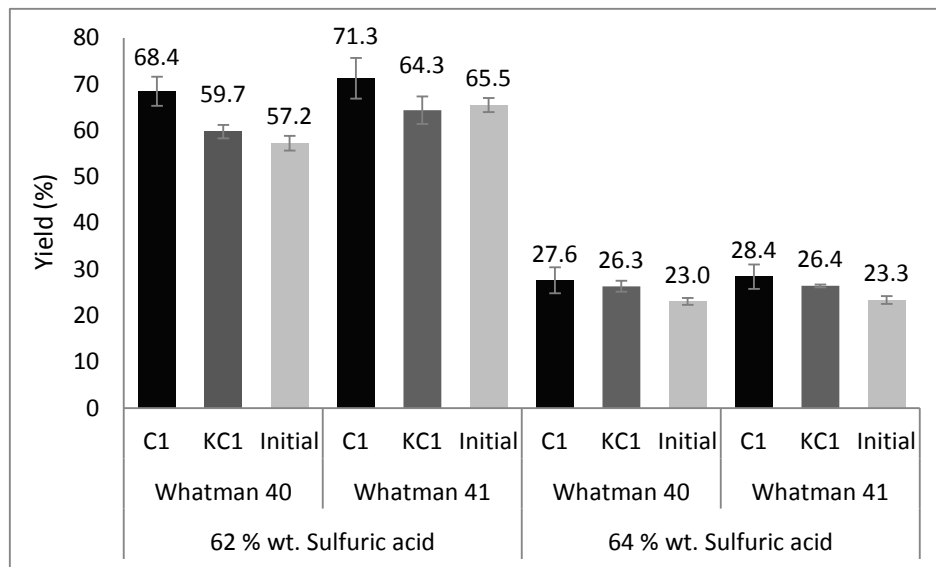
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692 **Figure 2**

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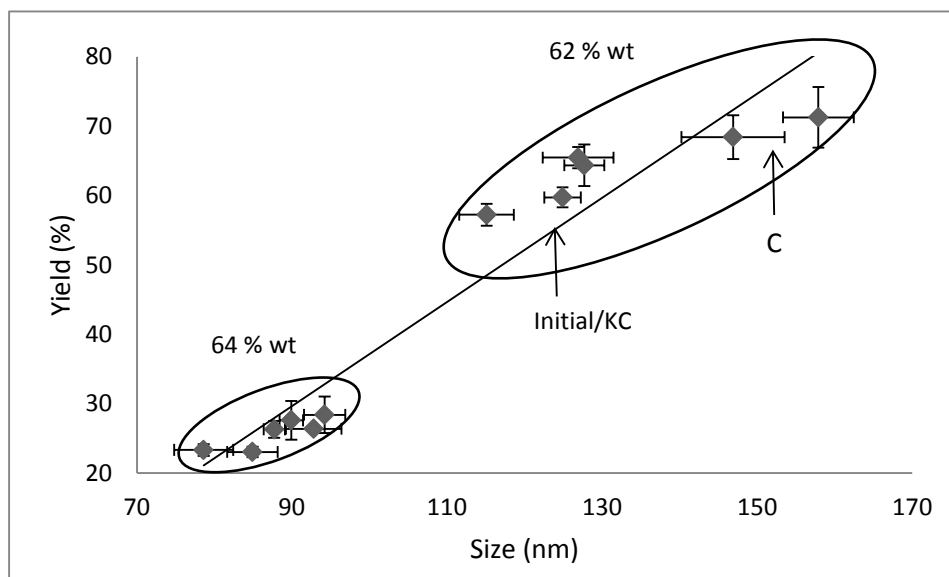
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712 **Figure 3**

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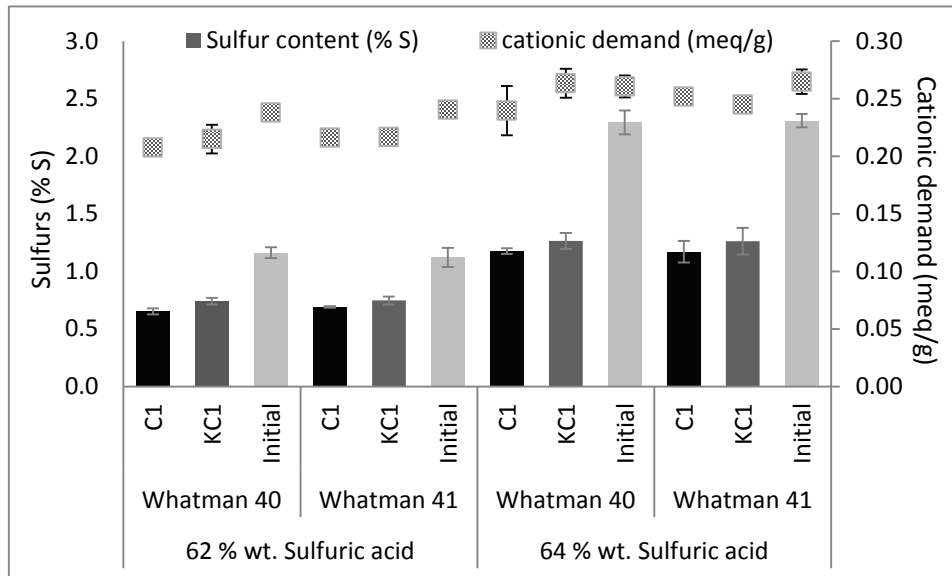
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726 **Figure 4**

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