Increasing yield of nanocrystalline cellulose preparation process by a cellulase pretreatment

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Abstract

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

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

Keywords

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

1. Introduction

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

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

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

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

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

2. Materials and Methods

Raw material and enzymatic treatment

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

A cellulase preparation (named "C"), provided by Fungal Bioproducts® (Spain) and obtained from *Cerrena sp.* fungus was used for treatments. Activity as U/g from enzyme stock was 1700. Activity was expressed as CMCase units, that is to say, the amount of enzyme degrading 1 µmol of CMC (carboxymethilcellulose) per minute. Treatments with C were carried out according to three different conditions (Table 1). Two different reactors were used for treatments; treatment 1 was carried out in a cylindrical 4 L reactor with agitation produced by rotating blades at 30 rpm. Treatments 2 and 3 were performed in an Ahiba Easydye (Datacolor, USA) apparatus having 250 mL independent units with agitation consisting on upside-down inversions of these units at 20 oscillations per minute. When corresponding (Table 1), 50 mM acetate buffer was used to set the pH to 5 during enzymatic treatment. In all cases enzyme was deactivated after reaction by increasing temperature to 105 ºC for 15 min. Fibers were filtered using

a Nº2 filter and reaction liquor was passed through fibers 3 times in order to recover fines. No washing was performed after enzymatic treatments to avoid sample loss. Control fibers, as indicated on Table 1, in the three different cases were treated with the same conditions as enzyme-treated fibers but without enzyme addition. Control fibers were referred as "KC".

Nanocrystalline cellulose preparation

Nanocrystalline cellulose (NCC) was obtained by a controlled sulfuric acid hydrolysis, adapting to our conditions the protocol proposed by (Dong et al., 1998). Fibers were fluffed prior to hydrolysis, oven dried and cooled in a desiccator. Typically, 2.5 g of sample weighted immediately from desiccator were hydrolyzed with 62 % or 64 % (w/w) sulfuric acid for 45 min at 45 ºC with magnetic stirring and an acid-to-fibers ratio of 10:1 (*i.e.* 10 mL/1 g cellulose). Reaction was stopped by diluting the acid with chilled (4 ºC) distilled water in a 10-fold basis, also placing sample container on an ice bath. Samples were then centrifuged at 6000 rpm for 15 min and supernatant was discarded. Then, samples were re suspended in distilled water and centrifugation step was repeated, discarding supernatant. Small fractions of supernatants were saved for analysis. Samples were then sonicated for dispersion using a Hielscher UP100H ultrasonic processor at 100 % amplitude and 0.75 cycles for 20 min on an ice bath to prevent heating, which may cause desulfation (Dong et al., 1998). Re suspended samples were then dialyzed against distilled water using a 10 kDa Thermo Fischer dialysis membrane for three days. After dialysis sonication step was repeated. Finally, samples were filtered through Whatman ashless paper filters, Nº 40 (pore size 8 µm) or 41 (pore size 20-25 µm). The whole process of preparation was repeated three times for each sample in order to ensure repeatability. In text, when referring to NCC samples

will be noted as X_NCC (*e.g.* Initial or C1 indicate fiber samples, while C1_NCC or initial_NCC refer to the NCC isolated from these fibers).

Characterization

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

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

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

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

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

(Cusola et al., 2013) a 4 μL water drop was delivered to the sample surface. At least 8 measurements were made for each sample.

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

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

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

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

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

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

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

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

Optical and SEM microscopy

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

Released oligosaccharides

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

3. Results and discussion

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

3.1.Cellulase treatments

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

In order to elucidate the effects of C on fibers and on final NCC, it was applied under 3 different conditions (Table 1). Regarding treatment 1, viscosity was highly reduced, suffering a reduction of about 500 mL/g (Table 2). This reduction was of course related to enzyme's cleavage effect of cellulose microfibrils, which leaded to a reduction of their average degree of polymerization. Also, this *cutting* effect of C modified fibers macroscopically, reducing average fiber length in ≈0.3 mm and increasing fines amount about a 2 % compared to KC1. Treatment 2, which was conducted at the same pH, temperature and enzyme dose as in treatment 1 but in a different reactor, produced different effects on fibers. These differences can then only be attributable to the reactor, where the smaller volume and the stronger mixing led to a different interaction between fibers and enzyme. This statement was supported by fiber length data as C2 fibers showed a lower value compared to C1 fibers. Fines amount, which were also produced as a result of enzyme action, also experienced a higher increase compared to control pulps (≈11 %) after treatment 2. One plausible explanation for these two contrasting evidences relays on the stronger mixing performed by Ahiba Easydye® reactor, which could have enhanced the physical separation of enzymatically pre-hydrolyzed fibers, leading to a higher fines amount and also to shorter fibers. Treatment 3 was carried out with the objective of studying the influence of enzymatic treatments in absence of buffer on NCC. Comparing to treatment 2, it produced the same effect on fibers in terms of fiber length and viscosity. Fiber width (Table 2) also showed the effects of C, as it was reduced a 9 % after treatments in the three cases, compared to control fibers. Finally, FTIR analysis provided evidence that C treatment increased average crystallinity of fibers. Obtained TCI values for KC and C fibers were 0.69 and 0.92, respectively, indicating an increase in fibers crystallinity. Generally, TCI is claimed to be proportional to cellulose crystallinity index (Široký 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 (≈23º) 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 70º, 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

concentration for NCC preparation, and another dose thought to be weaker (62 % wt.). All indicated samples were filtered through the Nº41 membrane.

Yield of NCC preparation process is a key aspect to be analyzed due to its evident impact on the economic cost of the whole process. Acid dose had a major impact on yield, as greater NCC yields about a 30-35 % bigger, were obtained for hydrolysis carried out with 62 % H_2SO_4 compared to 64 % (Figure 1). This evidence accorded with the results reported by several authors, where smaller acid concentrations resulted in higher yields due to weaker hydrolysis (Bondeson et al., 2006; Lu et al., 2013). Regarding enzymatic treatments, C1 and C2 fibers led to higher yields with both acid concentrations compared to crystals obtained after C3 fibers. Size of crystals, by its side, as measured by DLS did not provide a real value of their dimensions due to the rod-like structure they present (Fraschini et al., 2014). DLS uses laser scattering to measure radio (diameters) of particles and with this data calculates particles hydrodynamic diameters considering all elements to be spheres (Fraschini et al., 2014). Still, authors have used DLS data to establish comparisons between NCC samples, being aware that this value was not necessary representing the true physical size (Brito et al., 2012; Habibi et al., 2010). Hydrodynamic diameters in Figure 1a show that acid dose also had great influence on size. Bigger crystals seemed to be obtained with the weaker acid treatment (62 %) with values around 30-60 nm bigger compared to those obtained with 64 % acid. Regarding cellulase treatments, C1_NCC and C2_NCC again showed very similar values, yielding ≈35 nm bigger crystals with 62 % sulfuric acid compared to C3_NCC. With 64 % acid, differences were small. On Figure 1a it can be observed that both particle size and yield seem to be related, fact that will be further discussed in the following section. Sulfur content of nanowhiskers, in the form of 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_2SO_4 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_4^{2-} 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

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

3.3.Effects of enzymatic treatment and acid dose on NCC

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

3.3.1. NCC properties

For a proper understanding of C enzyme influence on NCC, results were compared with KC1_NCC, attributing further differences from initial pulp to buffer effect. Again, enzymatic pre-treatment on fibers increased NCC yield, especially with 62 % acid, with yields 7-8 % higher (Figure 2). Our hypothesis for explaining this behavior was related to a different interaction between acid and cellulose fibrils after being cleaved by C action. Reduction of cellulose chains length by enzyme (indicated by viscosity values) and reduction in fibers dimensions (Table 2) might have modified fiber-acid interaction. Crystalline regions are more difficult to be degraded by cellulases (Ahola et al., 2008), and according to previously mentioned crystallinity measurements,

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

Regarding crystals size, differences were only significant for samples at 62% acid, as C1_NCC crystals were 25-30 nm larger than KC1_NCC (Table 4). If yield is plotted against sample size (Figure 3) a linear correlation seemed to be found linking both parameters. This linear relation suggested that the increase in yield, meaning an increase in obtained NCC mass, was related to an increase in average particle size, which ultimately implied a higher mass per crystal unit. This finding supports our hypothesis for the mechanism behind the increase in yield after C treatment, which consisted in a facilitated interaction between cellulose and acid, reducing cellulose sample loss, yielding bigger crystals and a larger NCC mass.

Comparison between C_NCC and KC1_NCC also confirmed that C treatment affected the incorporation of sulfate groups onto crystals (Figure 4). C1_NCC sulfur content was smaller (0.1 % sulfur) than KC1_NCC. If compared to initial_NCC,

enzymatic effects were bigger. This might be related to the difference in free OH- groups available in fibers after C treatment (Table 2). It is well known that sulfate groups incorporation occurs by an esterification with free OH- present in cellulose (Habibi et al., 2010). However, this reaction is not equally allowed to occur in all free OH- positions from glucose residues, as OH- reactivity depends on its carbon position (Gu et al., 2013). A modification in OH- distribution on cellulose surface may have been the cause for this reduction in SO_4^{2-} incorporation onto NCC. Sulfurs content was also influenced by acetate (Figure 4). Sulfur contents 1% higher were obtained on initial_NCC hydrolyzed with 64 % acid and 0.4 % higher for 62 % acid compared to KC1_NCC. Supporting the observed section 3.2, acetate presence also affected the incorporation of sulfate moieties to NCC.

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

acetate buffer influence was also observable on cationic demands (Figure 4) as KC_NCC showed smaller charges compared to initial_ NCC.

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

Surfaces hydrophobicity as WCA (water contact angle) was determined. WCA values for NCC dried films are given in Table 4. All samples had WCA values around 50 º, indicating that samples were hydrophilic. This hydrophilicity was caused by 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 º to ≈50 º. 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^{st} vs 2^{nd} washing). We also observed that larger sugar amounts
430 were released after sulfuric acid hydrolysis from the fibers treated with C in comparison

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

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

4. Conclusions

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

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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 ºC			
pH	5 (50 mM acetate buffer)	✓	✓	
	5 (adjusted with H_2SO_4)			✓
Reactor	4L blade stirred reactor	✓		
	250mL Ahiba Easydye®		✓	✓
Agitation	Rotating blades - 30 rpm	✓		
	Oscillating - 20 rpm		✓	✓
Control fibers	No enzyme addition	KC1	KC2	KC3

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_2SO_4 62%			H_2SO_4 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

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

Sulfuric acid (% wt)	Filter nº (Whatman®)	Enzymatic treatment	Size (nm)	Zeta potential (mV)	WCA (º)
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

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

	Oligosaccharide concentration (mg/mL)							
	Washing waters						Final suspensions	
Sample	C1 64 %		KC1 64 %		Initial 62 %	Initial 64 %	C1 64 %	KC1 64 %
Washing	1st	2nd	1st	2nd	1st	1st	-	-
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

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

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_2SO_4.

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_2SO_4. 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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Figure 1

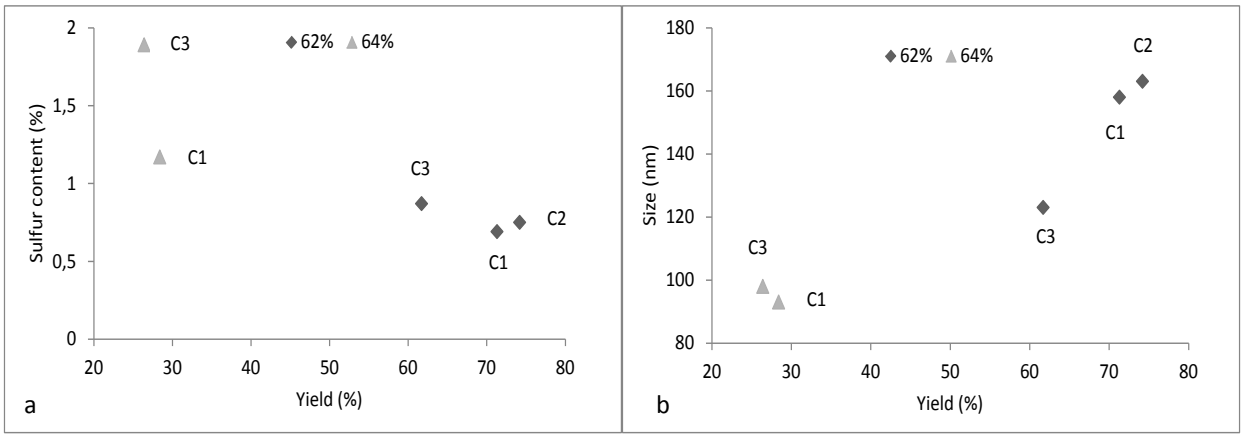

Sulfur content (%)
2
1,5
1
0,5
0
20
30
40
50
60
70
80
Yield (%)
C3
62%
64%
C1
C3
C2
C1
a
Size (nm)
180
160
140
120
100
80
20
30
40
50
60
70
80
Yield (%)
62%
64%
C2
C1
C3
C3
C1
b

Figure 2

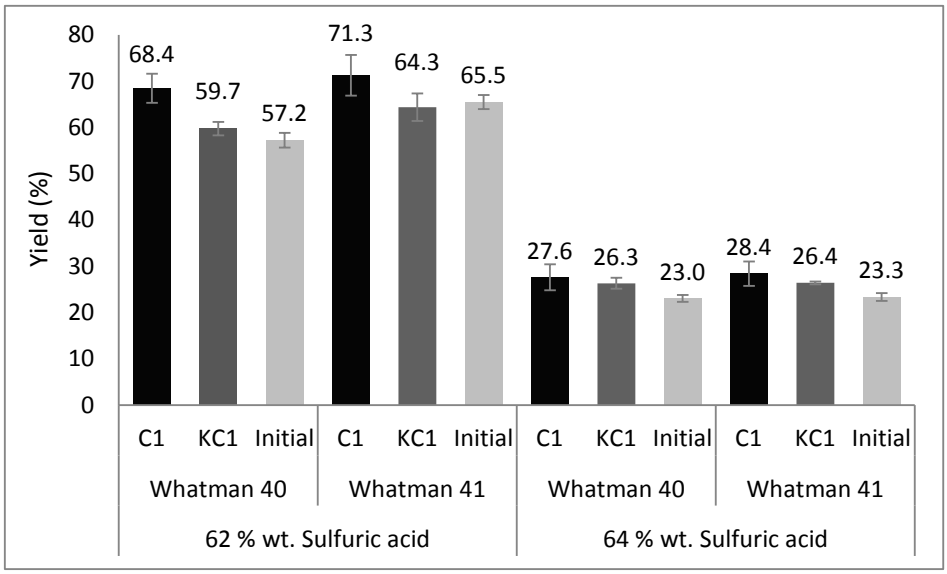

Yield (%)
80
70
60
50
40
30
20
10
0
68.4
59.7
57.2
71.3
64.3
65.5
27.6
26.3
23.0
28.4
26.4
23.3
C1
KC1
Initial
C1
KC1
Initial
C1
KC1
Initial
C1
KC1
Initial
Whatman 40
Whatman 41
Whatman 40
Whatman 41
62 % wt. Sulfuric acid
64 % wt. Sulfuric acid

Figure 3

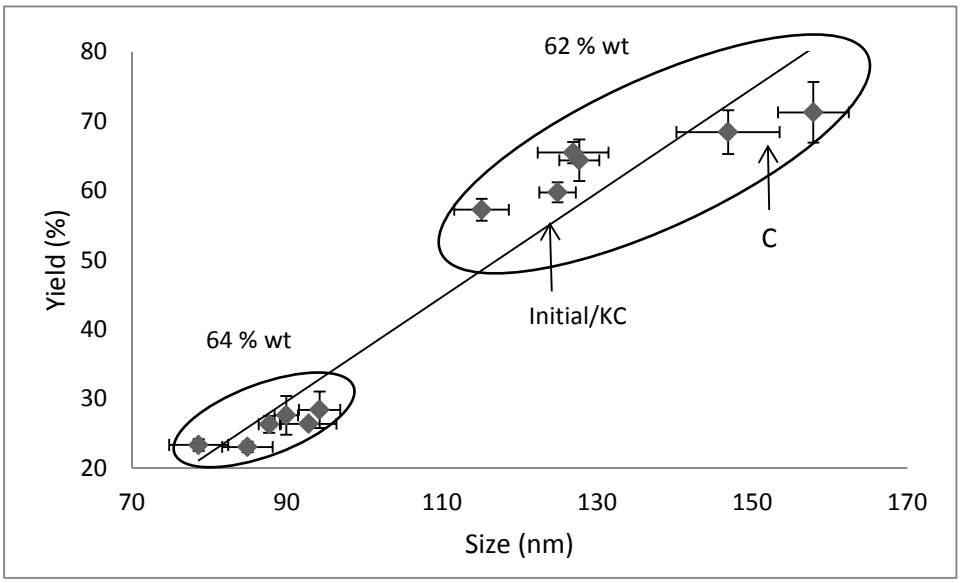
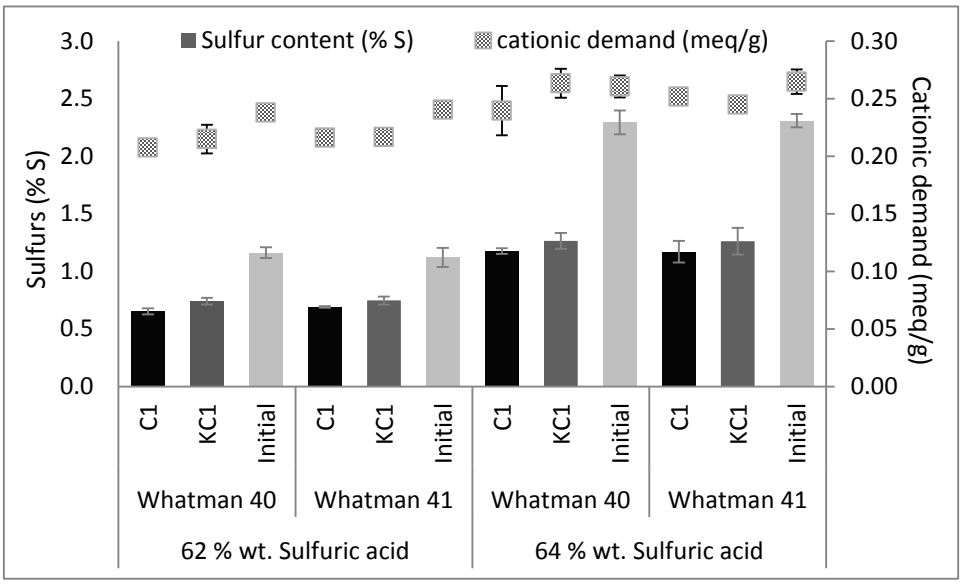

80
70
60
50
40
30
20
Yield (%)
70
90
110
130
150
170
Size (nm)
62 % wt
64 % wt
Initial/KC
C

Figure 4


Sulfur content (% S)
cationic demand (meq/g)
Sulfurs (% S)
Cationic demand (meq/g)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C1
KC1
Initial
Whatman 40
Whatman 41
62 % wt. Sulfuric acid
64 % wt. Sulfuric acid