A novel enzymatic approach to nanocrystalline cellulose preparation

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Abstract

In this work, conditions for an enzymatic pretreatment prior to NCC isolation from cotton linter were assessed. Different cellulase doses and reaction times were studied within an experimental design and NCC were obtained. At optimal enzymatic conditions (20U, 2h), a total yield greater than 80% was achieved and the necessary enzymatic treatment time was reduced 90%. Different intensities of enzymatic treatments led to proportional decreases in fiber length and viscosity and also were inversely proportional to the amount of released oligosaccharides. These differences within fibers lead to quantitative differences in NCC: increase in acid hydrolysis yield, reduction of NCC surface charge and crystallinity increase. Benefits produced by enzymatic treatments did not have influence over other NCC characteristics such as their sulfur content (≈1%), size (≈200 nm), zeta potential (≈ -50 mV) or degree of polymerization (≈200). Evidence presented in this work would reduce the use of harsh sulfuric acid generating a cleaner stream of profitable oligosaccharides.

Keywords: Nanocrystalline cellulose; Cellulase; Optimization; Yield increase; fiber length

1. Introduction

Research in nanocrystalline cellulose (NCC), a material also named cellulose nanocrystals, started some years ago (Rånby, 1951; Revol, Bradford, Giasson, Marchessault, & Gray, 1992) and has generated a huge interest in recent years due to the promising features this material holds (Habibi, Lucia, & Rojas, 2010; Sun et al., 2014; Trache, Hussin, Haafiz, & Thakur, 2017). Typically, it consists on a rigid rod-like monocrystalline cellulose domain with dimensions among 1-100 nm in width and up to several hundred nanometers in length (Lin & Dufresne, 2014). Also, they are produced from cellulose fibers, a very abundant raw material (Zhu et al., 2016). NCC has a high degree of crystal structure, a high aspect ratio (length-to-diameter, up to 300), a large surface area (above 150 m^2 g^{-1}), a very high elastic moduli, (estimated to be over 130-150 GPa) and a low thermal expansion coefficient (6 ppm K^{-1}) (Tanaka, Saito, Ishii, & Isogai, 2014). This material finds many potential applications in diverse fields such as an additive for composite materials (Moon, Martini, Nairn, Simonsen, & Youngblood, 2011), optical applications (Lin, Huang, & Dufresne, 2012), or diverse uses in biomedicine (Lin & Dufresne, 2014), to name a few.

Biotechnology has been used for several applications in cellulose industry, such as biobleaching, biorefining, or even pulp quality upgrades (Beltramino, Valls, Vidal, & Roncero, 2015; Beltramino, 2016; Garcia-Ubasart, Torres, Vila, Pastor, & Vidal, 2013; Quintana, Valls, Vidal, & Blanca Roncero, 2013; Valls & Roncero, 2009). Generally, the use of enzymes as a green technology allows reducing the pollution generated by traditional chemical processes, providing a solution for an enormous social concern. Cellulases, enzymes degrading cellulose include three different enzymatic activities (Teixeira et al., 2015). Endoglucanases (E.C. 3.2.1.4) catalyze the hydrolysis of the 1, 4-glycosidic linkages of the amorphous regions of cellulose. In nature, they hydrolyze cellulose in synergy with cellobiohydrolases (E.C. 3.2.1.91), which act upon the reducing and non-reducing ends of cellulose chains. Finally, β-glucosidases (E.C. 3.2.1.21), catalyze the hydrolysis of cellobiose into glucose. Generally, this enzymatic cellulose degrading activity is capable of participating into NCC preparation, fact that is

reflected in some examples of authors successfully introducing enzymes (cellulases) into nanocellulose preparation process (Anderson et al., 2014; Teixeira et al., 2015; Zhang, Xue, Zhang, & Zhao, 2012). The first proposal of the concept of using enzymes for producing cellulose nanomaterials was stated by Zhu, Sabo, & Luo, 2011. Furthermore, enzymatic preparation of NCC has been related with an improved quality of final product compared to pure chemical processes (George, Ramana, Bawa, & Siddaramaiah, 2011).

One of the main drawbacks associated with NCC preparation is the low yield presented by the typical acid hydrolysis with sulfuric acid used for its preparation (Chen et al., 2015). Considering this evidence, a previous work from our group demonstrated that a cellulase pretreatment on cotton linters could increase the yield of NCC as well as to influence other characteristics of them (Beltramino, Roncero, Vidal, Torres, & Valls, 2015). Optimizations via factorial designs have been widely used in literature for optimizing enzymatic and chemical treatments for diverse applications (Bondeson, Mathew, & Oksman, 2006; Fillat & Roncero, 2009, 2010; Valls & Roncero, 2009). In a previous study, conditions of sulfuric acid hydrolysis in order to maximize NCC yield from cellulase-pretreated fibers were optimized using a factorial design (Beltramino, Roncero, Torres, Vidal, & Valls, 2016). Maximal yield was achieved with 25 minutes of hydrolysis at 47ºC and using 62% wt. H_2SO_4. In the light of the results formerly obtained, this work intended to find the best conditions for obtaining the maximum profit of enzyme action. For this, conditions for the enzymatic pretreatment were optimized before and after obtaining NCC within a 2^2 complete factorial design. The main objective was to maximize the yield of the whole enzymatic and chemical process. We focused into the assessment of quantitative effects of these pretreatments of different intensity and their relations in both cellulose fibers and NCC. The purpose of this study was to find the best conditions for the enzymatic pretreatment providing the highest NCC yield in combination with optimal conditions established in a previously reported work (Beltramino, Roncero, Torres, Vidal, & Valls, 2016).

2. Materials and methods

2.1. Cellulose source and enzyme

Cotton linters provided by Celsur (Spain) were used as a raw material for experiments. Composition of fibers was: glucans content (cellulose) 97.7% ± 0.3; xylans content 2% ± 0.2; Rhamnans 0.2% ± 0.15; acetyl groups 0.1% ± 0.1. Fibers, as received from provider, were beated in a valley mill for 90 minutes for reducing average length. Obtained fibers were named as "initial". A commercial cellulase preparation (named "C"), provided by Fungal Bioproducts (Spain) and obtained from *Cerrena sp.* fungus was used for treatments. Previous works demonstrate that it is not a mono-component enzyme (Beltramino, Valls, Vidal, & Roncero, 2015; Beltramino, 2016). Activity as U g^{-1} from enzyme stock was 1700 and was expressed as CMCase units *i.e.* the amount of enzyme degrading 1 µmol of CMC (carboxymethilcellulose) per minute.

2.2. Enzymatic treatments

Enzymatic treatments were held using cellulase C on an Ahiba Easydye (Datacolor, USA) apparatus having independent 250 mL vessels with agitation consisting on upside-down inversions at 20 oscillations per minute. Treatments were performed at 55ºC, 5% consistency and pH 5 maintained with a 50 mM sodium acetate buffer solution on distilled water. Enzyme dose and reaction time were variables chosen in accordance to an experimental design (Table 1). After reactions a liquor sample was recovered for residual enzymatic activity determination and enzyme was deactivated by heating samples to 105ºC during 15 min. Fibers were then filtered using a filter with pore size Nº2 and reaction liquor was passed through fibers 3 times in order to recover fines. No washing was performed after treatments in order to avoid sample loss and samples of reaction liquor were saved for sugar content analysis. A control for enzymatic treatments was also performed on fibers, applying the same conditions as for treatments during 2h, but with no enzyme addition.

2.2.1. Experimental design

Enzymatic treatments were applied in accordance to a 2^2 statistical factorial plan involving two levels and two variables plus three repetitions in the central point, which required a total of 7 experiences (Table 1). Variables were: X1(enzyme dose), varied within 2 – 20 U g^{-1} odp (oven-dried pulp) range and X2 (reaction time) varied within 2 – 24 h. These independent variables were coded as -1 or +1; both for direct comparison of coefficients and to better understand the effect of each variable on the responses. The results of the three repetitions at the central point and their variance were used in combination with the variance of the saturated model to calculate Snedecor's F-value in order to determine whether the variance was homogeneous or heterogeneous. Since the variance was homogeneous in all cases, a linear model was constructed, its significant terms identified and potential curvature detected. Two additional points were required for solving quadratic terms confounding. Linear multiple regression technique was applied by using an Microsoft Excel spreadsheet to implement the stepwise backward regression method and discard all terms with a probability (p-value) less than 0.05.

Table 1. Experiences of the statistical plan with their conditions

Y	X1	X2	Cellulase dose (U g^{-1} odp)	Enzymatic treatment time (h)
Y1	-1	-1	2	2
Y2	1	-1	20	2
Y3	-1	1	2	24
Y4	1	1	20	24
Y5	0	0	11	13
Y6	0	0	11	13
Y7	0	0	11	13
Y8	1	0	20	13
Y9	0	-1	11	2

2.3. Nanocrystalline cellulose preparation

Nanocrystalline cellulose (NCC) was obtained from initial, control and enzymatically pretreated fibers by a controlled sulfuric acid hydrolysis, using the protocol proposed by Dong et al., 1998. Fibers were fluffed prior to hydrolysis, oven dried and cooled in a desiccator. Typically, 1.5 g of

148 sample weighted immediately from desiccator was hydrolyzed with 62 % (w/w) sulfuric acid
149 for 25 min at 47 ºC with an acid-to-fibers ratio of 10:1 (*i.e.* 10 mL g^{-1} cellulose), optimal
150 hydrolysis conditions described in a previous work (Beltramino, Roncero, Torres, Vidal, &
151 Valls, 2016). In all cases, hydrolysis reaction was stopped by diluting the acid with chilled (4ºC)
152 distilled water in a 10-fold basis, and also cooling samples immediately on an ice bath. Samples
153 were then centrifuged at 6000 rpm for 15m and supernatant was discarded. Samples were re
154 suspended in distilled water and centrifugation step was repeated, discarding supernatant.
155 Samples were then sonicated to disperse them using a Hielscher UP100H ultrasonic processor at
156 100% amplitude and 0.75 cycles for 20 min on an ice bath to prevent heating which may cause
157 desulfation (Dong et al., 1998). Re suspended samples were then dialyzed against distilled water
158 using a 10kDa Thermo Fischer dialysis membrane until pH 3. Final samples were filtered
159 through Whatman ashless paper filters, Nº 41 (pore size 20-25 µm).

160 **2.4. Samples characterization**

161 **2.4.1. Cellulose fibers**

162 Enzymatic treatment yield was calculated by determining the solid residue (treated fibers) after
163 treatments and was indicated as % of recovered fibers mass. Initial and enzymatically treated
164 fibers were characterized in terms of viscosity and fiber length according to ISO 5351:2010, and
165 TAPPI Standard T271, respectively.

166 Infrared spectra of fibers samples were recorded at room temperature using a Perkin Elmer
167 Spectrum 100 ATR-FTIR spectrophotometer. Fourier transformed infrared spectroscopy (FTIR)
168 spectral analysis was conducted within the wavenumber range of 600-4000 cm^{-1}. A total of 64
169 scans were run to collect each spectrum at a 1cm^{-1} resolution. Total crystallinity index (TCI) as
170 proposed by Nelson and O`Connor (Nelson & O'Connor, 1964) was estimated from the ratio
171 between the absorption peaks at 1370 cm^{-1} and 2900 cm^{-1}, respectively.

172 **2.4.2. Enzymatic treatment effluents**

Released reducing sugars on enzymatic reaction effluents were analyzed using a 1100 Agilent HPLC instrument (Agilent technologies, USA) furnished with a BIO RAD Aminex HPX-42A ion-exchange column. Residual enzymatic activity on effluents was determined using an adapted version of Somogyi-Nelson method to determine reducing sugar concentrations on a solution (Spiro, 1966).

2.4.3. Nanocrystalline cellulose

Yield NCC isolation by acid hydrolysis was determined drying 25 mL of the suspension and determining the mass after evaporation at 60ºC in an air circulating oven. Solids content was 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. (Abitbol, Kloser, & Gray, 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 (Z average) as well as polydispersity index (PDI) were determined using a DL135 particle size analyzer (Cordouan Technologies, France). Size distribution was determined with dynamic light scattering (DLS) at room temperature (25ºC). Aqueous suspensions were placed directly in the measuring cell and laser power was adjusted for counting around 2000 particles per minute.

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 (Cadena, García, Vidal, & Torres, 2009) :

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

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.

Zeta potential (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.

NCC degree of polymerization (DP) was determined using a modified version of ISO 5351:2010, using 0.2-0.3 g of dried NCC suspensions as samples. The degree of polymerization was calculated from the intrinsic viscosity [η], using the equation of (SCAN-CM 15:88): $DP^{0.085}=1.1\ [\eta]$.

FTIR spectra of dried NCC films were recorded following the same procedure as for fibers. TCI was also calculated from spectra.

3. Results and discussion

The effects produced by the enzyme were analyzed and optimized before and after obtaining the NCC in order to evaluate if quantitative differences in enzymatic effects on fibers led to proportional differences in NCC. This kind of optimization had not been performed before.

3.1. Modelling enzymatic treatment response on fibers

Due to the degrading nature of cellulase action, a loss of cellulose mass is associated to these enzymatic treatments, fact that must be taken into account when considering process yield. In the same direction, cellulase action strongly reduced average fiber length. For studying this, values of enzymatic treatment yield and fiber length were found to fit Equation 1 and 2, respectively. As shown by equations, both responses were affected by both individual variables and also by the quadratic term of reaction time, being it the most influential one. Data predicted by models showed that enzymatic yield and fiber length suffered a great variation from 2 hours to ≈11 hours, in which a yield loss of ≈10 points (Figure 1A) and a ≈1 mm reduction of fiber length (Figure 1B) were produced. On the other hand, enzyme dose had a smaller influence than reaction time in both parameters, particularly in enzymatic treatment yield. At 2 h of treatment,

the reduction in fiber length produced by increasing enzyme dose did not produce a noticeable loss in fiber mass.

$$\text{Enzymatic treatment yield (\%)} = 88.4 - 1.4\,X1 - 3.8\,X2 - 1.6\,X1X2 + 5.7\,X2^2 \quad R^2 = 0.93$$

Equation 1

$$\text{Fiber length (mm)} = 0.71 - 0.25\,X1 - 0.33\,X2 + 0.48\,X2^2 \quad R^2 = 0.95$$

Equation 2

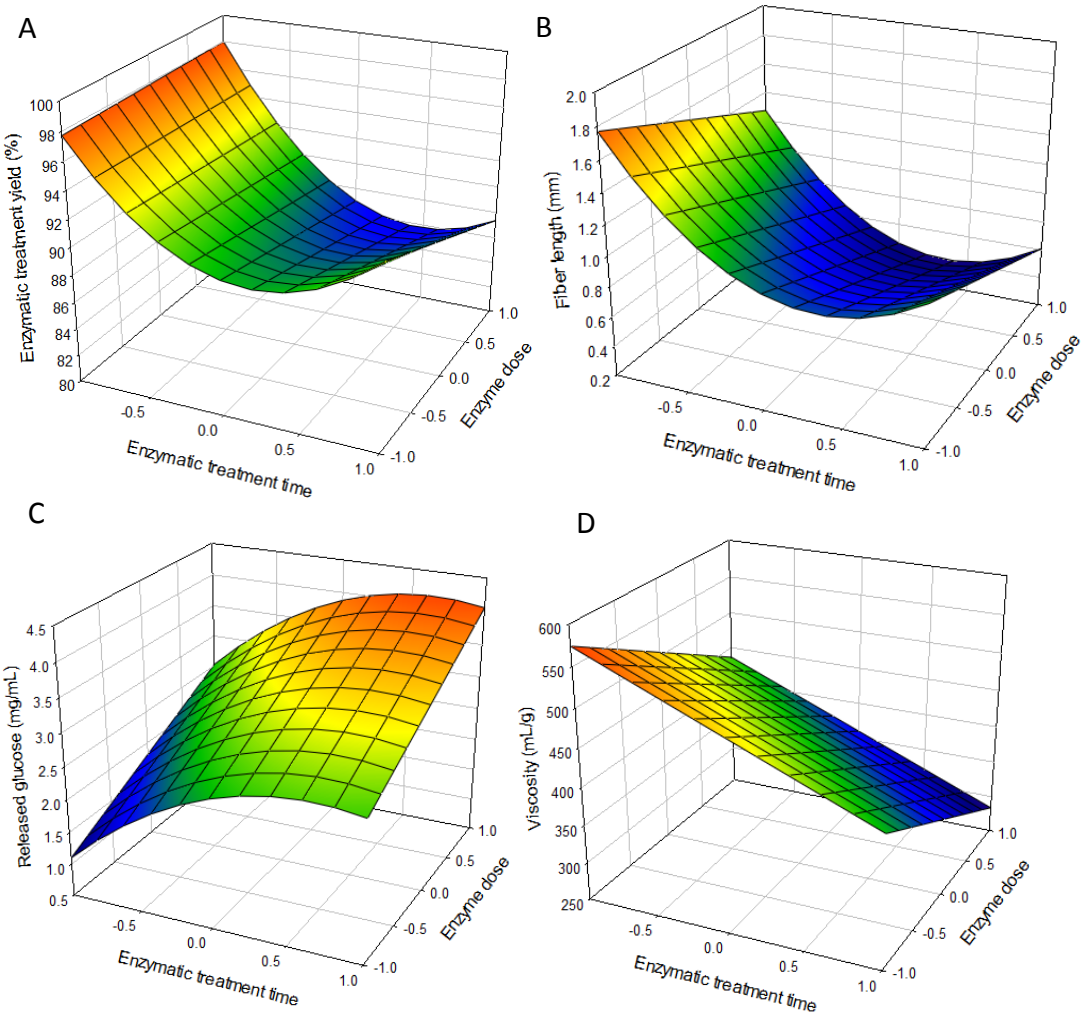

Figure 1: Models relating enzymatic treatment yield (A), fiber length (B), total released glucose (C) and fiber viscosity (D) to enzyme dose and enzymatic treatment time.

In order to fully understand the effects of enzymatic treatments in fiber length, the distribution among different measures was studied and illustrated in Figure 2A. Comparing samples at 2 h

237 of treatment, increase in enzyme from 2 to 20 U g^{-1} odp dose slightly reduced the amount of
238 fibers above 1.2 mm while it increased the amount of the ones below this length. In turn,
239 reaction time produced a major effect, as previously observed, strongly reducing the presence of
240 fibers longer than 1.2 mm and thereafter increasing the presence of shorter ones. The action
241 pattern of enzyme in the reduction of fiber length seemed to be the same for increases in
242 enzyme dose or reaction time. However, the magnitude of the effects of the increase in the
243 former was much smaller than the one of the latter. This fact could explain that no loss in
244 cellulose mass was associated to increases in enzyme dose although a small reduction in length
245 was observed.

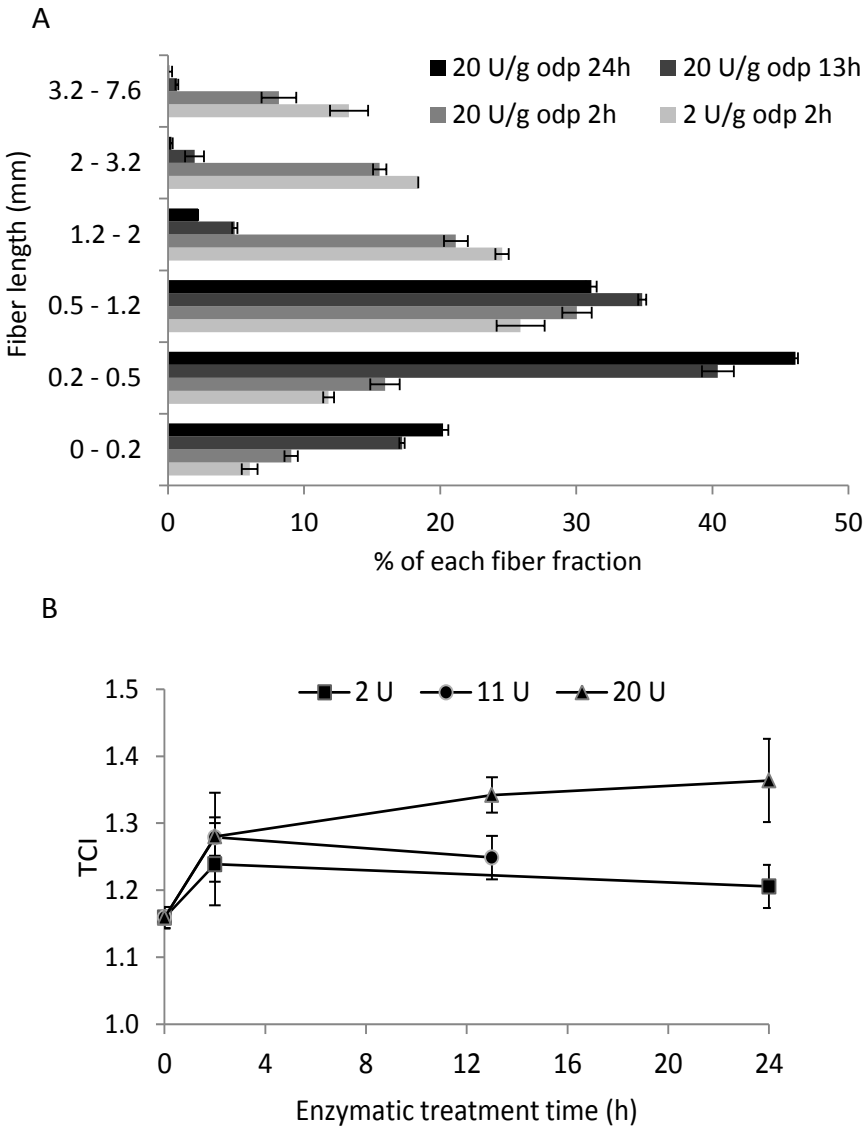

Figure 2: Fiber length distribution of samples after enzymatic treatments (A). Total crystallinity index (TCI) of fibers during enzymatic treatments (B)

Oligosaccharides released as a consequence of enzymatic treatments were expressed as glucose equivalents after the molar addition of each oligosaccharide multiplied by their number of glucose units. These values fitted Equation 3. For this response a similar effect to that of yield and fiber length was observed (Figure 1C). A large increase in glucose concentration was observed from 2 hours to ≈11 hours of treatment, up to ≈4 mg mL^{-1}, observing stabilization after this period. In this case, enzyme dose had a linear effect, smaller than that of reaction time and independent of it, increasing sugar concentration all along enzymatic treatment. On the other hand, fibers viscosity values fitted Equation 4. In it, the quadratic term of reaction time was not

found to affect the response and a linear surface was obtained (Figure 1D). Viscosity decreased as enzymatic treatment intensity increased with a minimum value obtained at the point of the most intensive enzymatic conditions, *i.e.* 20 U g^{-1} odp and 24 hours, accounting for a 50% reduction of viscosity.

$$\text{Released glucose (mg mL}^{-1}) = 3.17 + 0.71\ X1 + 0.77\ X2 - 0.59\ X2^2 \quad R^2 = 0.93$$

Equation 3

$$\text{Viscosity (mL g}^{-1}) = 429 - 69\ X1 - 76\ X2 \quad R^2 = 0.93$$

Equation 4

Enzymatic treatments also increased cellulose crystallinity, expressed as total crystallinity index (TCI). Data indicated that fibers TCI (Figure 2B) increased as a consequence of higher enzyme doses, while reaction time did not seem to produce any effect after 2h. Generally, this crystallinity increase indicates that a higher amount of crystalline cellulose was present on fibers after enzymatic treatments. The explanation of this might be found in the reduction in amorphous cellulose regions caused by cellulase preferential attack on them. This preference is due to the larger accessibility presented by β-1,4 glycosidic bonds in these domains (Tąta et al., 2015).

3.2. Enhancing enzymatic effects on nanocrystalline cellulose

3.2.1. Modelling enzymatic treatment response on nanocrystalline cellulose

Low yields traditionally attributed to NCC isolation raised interest in the study of ways for its increase, in order to increment the industrial feasibility of this process (Chen et al., 2015; Fan & Li, 2012). NCC yield values from sulfuric acid hydrolysis fitted the model indicated in Equation 5, showing that it was positively influenced by both independent variables studied in this work. Cellulase pretreatment increased the yield of sulfuric acid hydrolysis up to a 90%, with a larger effect produced by reaction time (Figure 3A). NCC yield model revealed a linear inverse correlation to the model presented by fibers viscosity. The minimal and maximal values of NCC yield were shown by 2 U g^{-1} odp, 2 h and 20 U g^{-1} odp, 24 h samples, respectively. These

samples also showed the maximal and minimal fiber viscosity and fiber length values, respectively. This suggested that a higher depolimerization and shortening of fibers by cellulase was the cause for the increase in the yield of NCC hydrolysis.

$$\text{NCC yield (\%)} = 84.4 + 2.6\ X1 + 3.3\ X2 \quad R^2 = 0.97. \qquad \textbf{Equation 5}$$

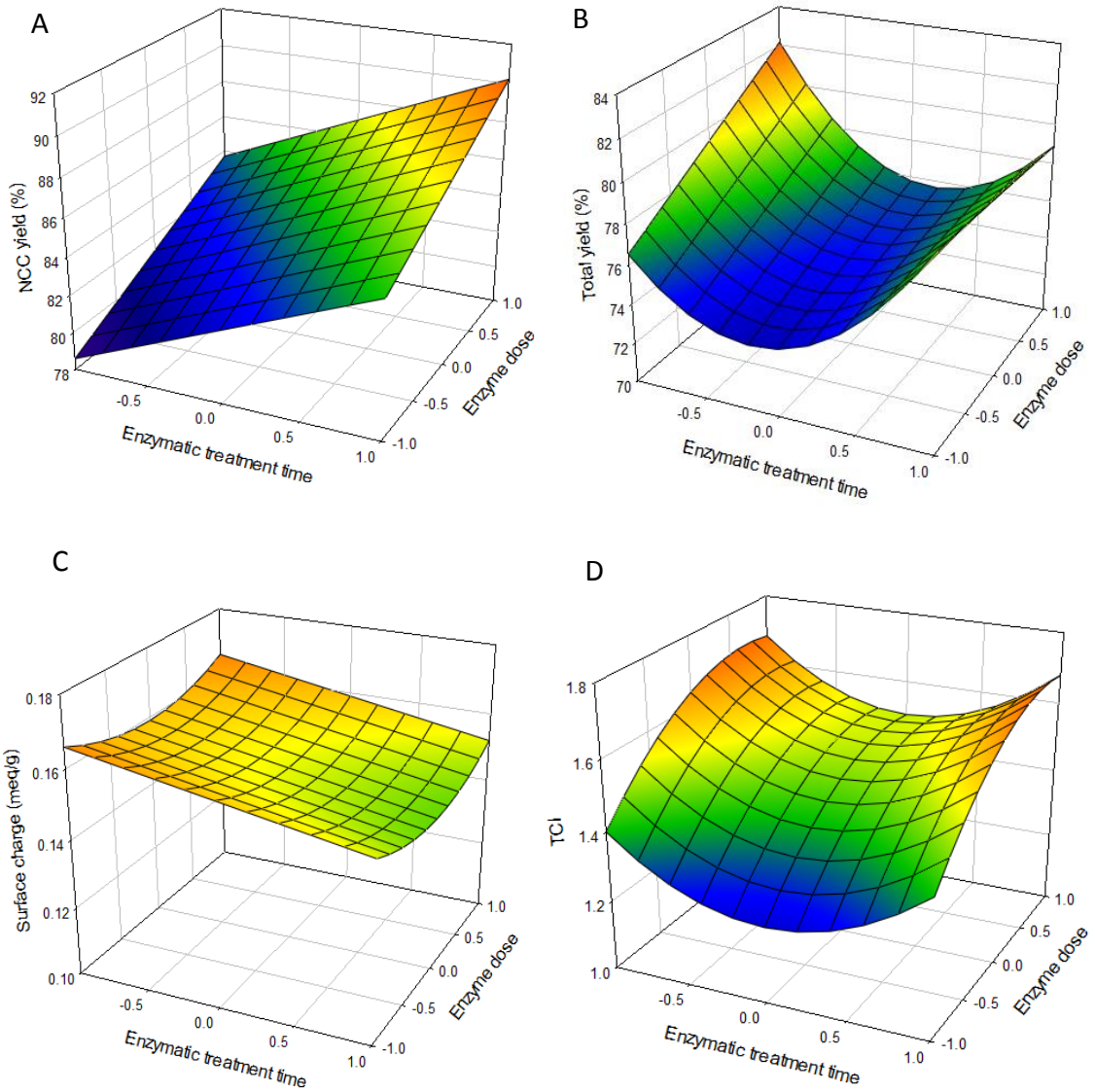

Figure 3: Model relating: NCC hydrolysis yield (A), Total yield (B), NCC surface charge (C) and NCC total crystallinity index (TCI) (D) to enzyme dose and enzymatic treatment time.

As stated in introduction and considering evidence previously exposed, calculation of total yield, as combined enzymatic and acid hydrolysis yields becomes crucial for acknowledging a

real value of the outcome of the NCC isolation process. A compromise solution between the gain in the NCC yield and the loss of fibers mass both due to enzymatic pretreatment must be found. Total yield values were found to fit Equation 6. In this equation, compared to the model expressed in Equation 5, individual influence of enzyme dose decreased. On the other hand, treatment time influenced only in the quadratic term and double-interacting with the enzyme dose. Total yield (Figure 3B) had a minimum value at around 11 h of treatment, coinciding with the point of stabilization of enzyme effect on fibers, showing higher values with shorter and longer times. This was explained by yields of both enzymatic and sulfuric acid hydrolysis (Figure 1A and Figure 3A). At short reaction times the loss in cellulose mass by enzymatic treatments was small, while with extended treatments, cellulose mass loss was compensated by higher gains in NCC hydrolysis yield.

$$\text{Total yield } (\%) = 74.6 + 1.2\ X1 - 1.6\ X1X2 + 4.7\ X2^2 \quad R^2 = 0.98$$

Equation 6

Surface charge of NCC was found to fit Equation 7. As can be observed, it was negatively influenced by enzymatic reaction time and positively by the quadratic term of enzyme dose. It was observed that surface charge of NCC was slightly reduced with longer enzymatic pretreatments (Figure 3C), while enzyme dose produced no significant affectation. This charge reduction was in accordance with previous observations (Beltramino, Roncero, Torres, Vidal, & Valls, 2016; Beltramino, Roncero, Vidal, Torres, & Valls, 2015) where enzymatic effects seemed to reduce this parameter.

$$\text{Surface charge } (\text{meq g}^{-1}) = 0.152 - 0.007\ X2 + 0.007\ X1^2 \quad R^2 = 0.79$$

Equation 7

Crystallinity values of NCC, as TCI fitted Equation 8. TCI of NCC was affected by enzyme dose linearly and by quadratic terms of both variables. In Figure 3D it can be observed how enzymatic pretreatment on fibers led to NCC with a higher crystallinity. Data shows that TCI was majorly increased by enzyme dose with values tending to stabilize after a ≈10 U g^{-1} odp dose. However, no significant effect was found to be produced by enzymatic reaction time, a

similar behavior to that observed in TCI of fibers. Also, it is important to remark that the optimal point of the process concerning total yield (20 U g^{-1} odp, 2h) corresponded to NCC with the higher crystallinity, providing further evidence of the quality increase produced by this enzymatic-aided process.

$$TCI = 1.46 + 0.14\,X1 - 0.1\,X1^2 + 0.18\,X2^2 \quad R^2 = 0.99$$

Equation 8

The observation of former data also foregrounded the fact that quantitative differences in enzymatic treatment intensity led to quantitative differences in NCC features. This statement is well illustrated in Figure 4A, where it can be observed how NCC hydrolysis yield is linearly correlated to fibers viscosity (inverse correlation) and also to total released glucose (as glucose equivalents). Also, with the aim of further illustrating this, Figure 4B correlates chain scission number (CSN), *i.e.* the average number of cuts produced in cellulose chains with the increase in yield derived from enzymatic action. The correlation between both parameters indicated again that a higher number of cuts, *i.e.* a stronger enzymatic action, corresponded to a greater increase in yield. Finally, the reduction of NCC electrical charge produced by the enzyme is well illustrated in Figure 4C, where larger NCC yields (*i.e.* larger enzymatic effects) led to smaller values of surface charge, agreeing with data exposed in Figure 3C.

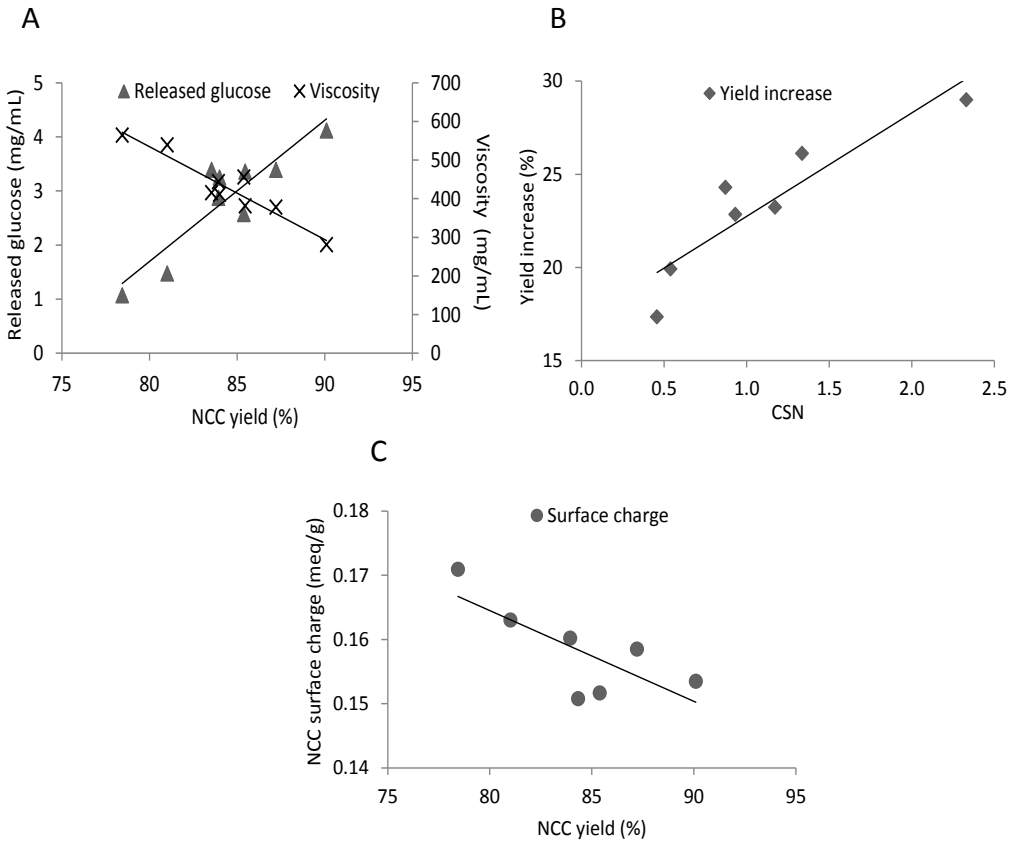

Figure 4: Cellulase quantitative effects. Total released sugars (as glucose equivalents) and fibers viscosity expressed in front of NCC yield (A), NCC yield increase expressed versus chain scission number (CSN), both calculated from initial fibers (B) and NCC surface charge expressed versus NCC yield (C).

3.2.2. Studying enzymatic reaction effluents

Enzymatic hydrolysis rate, calculated dividing the total glucose equivalents produced during each enzymatic treatment by the total duration of the treatment (in minutes), is illustrated in Figure 5A in front of the hydrolysis yield obtained from each sample. For all enzymatic doses, highest hydrolysis rates were found at 2 h, with higher values at a higher dose. From this point, extending treatment up to 24 h time seemed to reduce hydrolysis rate. This reduction was possibly due to the increase in oligosaccharides concentration on reaction media, compounds which are known to be capable of act as cellulase inhibitors (Nguyen, Neo, & Yang, 2015). Interestingly, the maximal hydrolysis rate, *i.e.*, the point of maximal hydrolytic efficiency, was found at 2 h of treatment and with 20 U g^{-1} odp. This point was also found to be the optimal for cellulase application as it offered the highest total yield, showing a correlation between

efficiency of the entire process and of enzymatic catalysis. Furthermore, in order to validate these results, residual activity (as % of initial dose) was measured. After 24 hours of treatment, 2 U g^{-1} odp and 20 U g^{-1}odp samples showed activity conservation values of 55% ± 10 and 24% ± 4, confirming that the enzyme was still active after 24 h and thereafter validating data shown in Figure 5A.

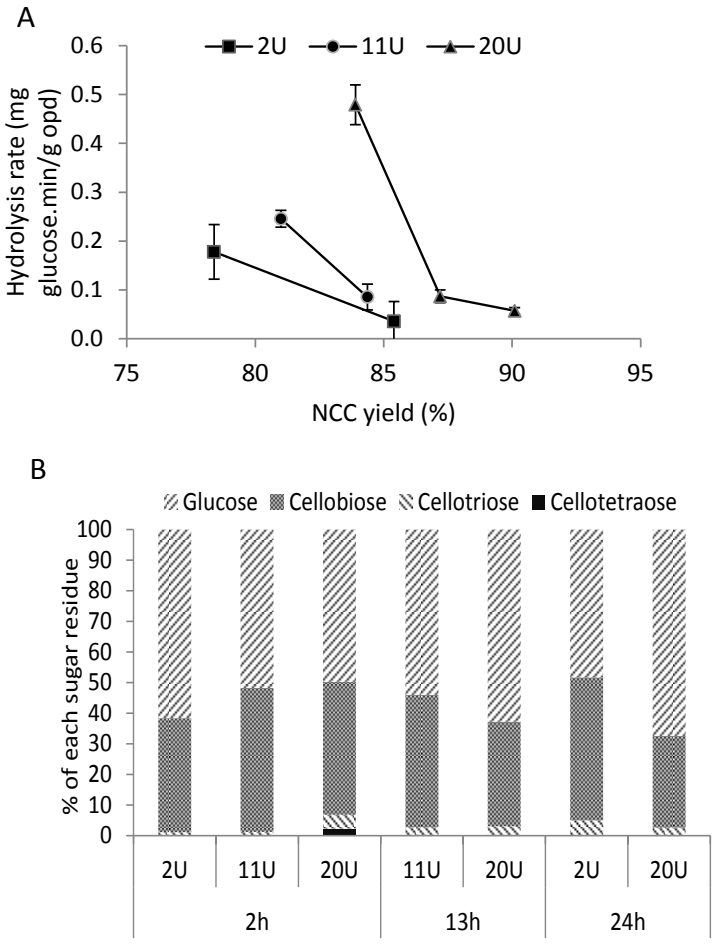

Figure 5: Enzymatic hydrolysis rate, as mg glucose released per minute as a consequence of enzymatic treatments expressed in front NCC hydrolysis yield (A). Proportion of each oligosaccharide released during enzymatic hydrolysis (B).

Concerning the different sugar species found in effluents (Figure 5B), in the first place, a small amount of xylose was found. This presence was product of a xylanolytic activity present on cellulase preparation and proceeded of the hydrolysis of the small amount of xylans initially

present on fibers. In the second place, concerning glucose-oligosaccharides, glucose was found to be the main released sugar, followed by cellobiose and cellotriose, in a decreasing amount. Differences in enzyme dose led to a variation of glucose-oligosaccharides. Generally, higher enzymatic doses led to the release of longer oligosaccharides within two hours of treatment, fact well illustrated by the finding of cellotetraose only in one sample. Meanwhile, reaction time seemed to tend to reestablish the original proportions among oligosaccharides, *i.e.* ≈67% glucose, ≈30% cellobiose and ≈3% cellotriose.

3.2.3. NCC sulfur content, size and stability

NCC surface charge is responsibility of charged sulfate moieties introduced on their surface during sulfuric acid hydrolysis (Abitbol et al., 2013; Peyre et al., 2015). Sulfur content data of NCC (Table 2) failed to show quantitative reductions produced by cellulase pretreatment, as observed for surface charge. Nevertheless, it could be observed that compared to initial fibers, enzymatic pretreatment on fibers led to NCC with lower sulfur content. In addition, sulfate groups on NCC are known to increase the thermodegradability of the material (Roman & Winter, 2004) making the reduction produced by cellulase a positive modification for NCC quality.

Another NCC parameter, suspension stability, is critical in the preparation of nanocomposites (Filson, Dawson-Andoh, & Schwegler-Berry, 2009). This stability is indicated by the absolute value of zeta potential (electrophoretic mobility) of suspensions and is promoted by the negative charge of sulfate groups on crystals surface (Peyre et al., 2015). Table 2 shows zeta potential values, being all them among -50 mV indicating high suspension stability, which seemed to be maintained regardless of the enzymatic treatment performed. A similar behavior was shown by PDI, as all suspensions showed a narrow particle size distribution.

Concerning NCC dimensions, it was observed that different intensities of enzymatic pretreatment did not produce any modification in average particle size of resulting NCC (Table 2). With this, it was highlighted that the benefits of cellulase pretreatment did not result in

deleterious modifications in the morphology of NCC. Also, this fact was already observed (Beltramino, Roncero, Torres, Vidal, & Valls, 2016) when cellulase pretreatment produced no affectation on NCC size with 62% wt. sulfuric acid. However, in a different study, a slight size increase in NCC was produced by enzymatic pretreatment (Beltramino, Roncero, Vidal, Torres, & Valls, 2015). This evidence remarks again the fact that the effects of enzymatic pretreatment are largely dependent on the acid hydrolysis conditions, which were modified within these studies.

Finally, the degree of polymerization (DP) of cellulose chains in NCC was calculated from viscosity values (Table 2). DP of NCC did not seem to be modified by enzymatic treatments, observing in all cases that cellulose chains were formed by ≈200 glucose units. These values were similar to those reported by other authors for NCC obtained via sulfuric acid hydrolysis (Satyamurthy, Jain, Balasubramanya, & Vigneshwaran, 2011).

Table 2: NCC sulfur content, electrophoretic mobility, polydispersity index (PDI), average size and degree of polymerization (DP).

		Sulfur content (% S)	Zeta Potential (mV)	PDI	Z average (nm)	DP
Initial		1.21 ± 0.03	-47.2 ± 0.6	0.18 ± 0.04	205 ± 4	183 ± 17
Control 2h		1.12 ± 0.06	-49.1 ± 0.7	0.19 ± 0.03	184 ± 19	200 ± 25
2 U	2 h	1.22 ± 0.02	-46.7 ± 0.7	0.19 ± 0.01	191 ± 25	188 ± 6
	24 h	1.12 ± 0.04	-48.2 ± 0.4	0.18 ± 0.03	206 ± 7	193 ± 17
11 U	2 h	0.99 ± 0.01	-49.3 ± 0.7	0.18 ± 0.04	199 ± 4	193 ± 12
	13 h	1.03 ± 0.04	-49.4 ± 0.5	0.19 ± 0.01	206 ± 5	179 ± 12
	13 h	0.92 ± 0.01	-48.7 ± 0.4	0.17 ± 0.02	204 ± 13	173 ± 21
	13 h	0.87 ± 0.01	-48.5 ± 0.6	0.19 ± 0.02	209 ± 24	210 ± 12
20 U	2 h	0.99 ± 0.05	-48.9 ± 0.7	0.20 ± 0. 01	206 ± 10	208 ± 26
	13 h	1.03 ± 0.03	-48.9 ± 0.6	0.19 ± 0.02	195 ± 9	203 ± 14
	24 h	1.05 ± 0.01	-48.2 ± 0.7	0.19 ± 0.02	183 ± 14	198 ± 41

3.3. Optimal point and models verification

As stated in previous sections, the objective of this work was to find the optimal conditions for enzymatically pretreating fibers in order to produce the largest possible total NCC yield. Thus, the optimal point of the cellulase combined with acid hydrolysis was found at: 20 U g^{-1} odp

418 cellulase dose and 2 h of treatment, producing a ≈82 % total yield, 21 points higher than that of
419 NCC obtained from initial fibers. This total yield was similar to that reported by Tang et al.,
420 (2013) using a non-conventional preparation procedure obtaining NCC esterified with acetic
421 acid. Also, it was noticeably bigger than other optimal values reported using sulfuric acid
422 hydrolysis (Chen et al., 2015; Fan & Li, 2012). Moreover, compared to a previous study
423 (Beltramino, Roncero, Torres, Vidal, & Valls, 2016) this optimization allowed reducing in a
424 90% the required enzymatic treatment time, although the enzyme dose was duplicated. In
425 addition, if increasing enzyme dose resulted unaffordable, a total yield of ≈79% was obtained
426 using a ≈11 U g^{-1} odp dose and 2 hours of treatment, representing a loss of 3 points in total yield
427 but a smaller enzyme dose. This strong reduction would increase the industrial feasibility of this
428 greener process, as industry is usually reluctant to long treatments. Accordingly, enzyme
429 showed the largest hydrolysis rate *i.e.* the one using more efficiently its potential, at 20 U g^{-1}
430 odp and at 2 hours of treatment, conditions defined as optimal. In other words, this optimization
431 meant a reduction of the hydrolysis of biomass mediated by sulfuric acid in benefit of an
432 efficient enzymatic catalysis. Furthermore, sugars present on effluents as a result of NCC
433 manufacture could be used as a feedstock, for example, for bioethanol conversion (Brinchi,
434 Cotana, Fortunati, & Kenny, 2013). In this case, enzymatic hydrolysis effluents permit an easier
435 usage than those produced by sulfuric acid hydrolysis, due to the absence of sulfuric acid in
436 them, highlighting another benefit of the proposed enzymatic-assisted process.

437 Finally, with the aim of verifying the obtained models, new samples were prepared using the
438 optimal cellulase conditions plus another sample with a 20 U g^{-1} odp dose and 24 h of treatment,
439 which led to a total yield of ≈79% and thereafter was also interesting. Table 3 shows data
440 obtained from these new samples and also the predicted data by models. As can be observed,
441 new values were in all cases in accordance with those predicted by models or similar to previous
442 experimental data.

443

Table 3: Characterization of samples for models verification. New experimental values and those predicted by models are indicated. *When no model was found fitting data, previous experimental data is indicated.

	20U 2h		20U 24h	
Fibers	Predicted*	Observed	Predicted*	Observed
Enzymatic treatment yield (%)	98	96.3	87.4	85.8
Fiber length (mm)	1.27	1.28 ± 0.04	0.61	0.48 ± 0.02
Viscosity (mL g^{-1})	436.1	457 ± 28	283.7	296 ± 12
Released glucose (mg mL^{-1})	2.52	2.62 ± 0.09	4.06	4.3 ± 0.19
TCI*	1.28 ± 0.03	1.25 ± 0.02	1.36 ± 0.06	1.30 ± 0.06
NCC				
NCC yield (%)	83.7	84.5 ± 0.8	90.2	89.8 ± 0.8
Total yield (%)	82.2	81.4	78.9	77.1
Surface charge (meq g^{-1})	0.166	0.172 ± 0.005	0.152	0.156 ± 0.004
TCI	1.68	1.61 ± 0.1	1.68	1.65 ± 0.16
Sulfur content (% S)*	0.99 ± 0.05	1.1 ± 0.09	1.05 ± 0.01	0.94 ± 0.05
Z average (nm)*	206 ± 10	186 ± 11	183 ± 14	204 ± 8
Z potential (mV)*	-48.9 ± 0.7	-49.6 ± 0.5	-48.2 ± 0.7	-50.7 ± 0.8
PDI*	0.2 ± 0.1	0.21 ± 0.02	0.19 ± 0.02	0.2 ± 0.02
DP*	208 ± 26	198 ± 18	198 ± 41	203 50

4. Conclusions

Evidence presented in this work allowed finding the optimal enzymatic conditions for NCC isolation in combination with optimal sulfuric acid hydrolysis ones from a previous work (25 minutes of hydrolysis at 47ºC and 62% wt. H_2SO_4). Now, an enzyme dose of 20 U g^{-1} odp and 2h of hydrolysis allowed reaching a total NCC yield of ≈82%. This outcome was found to be 12 percentage points higher to that of NCC from control fibers and 21 percentage points higher than that of NCC obtained from initial ones. Also, this optimization reduced the necessary enzymatic treatment time in a 90% (from 24h to 2h) compared to former studies, boosting the industrial feasibility of this greener technology. Furthermore, enzymatic pretreatment showed to increase NCC crystallinity and to slightly reduce their surface charge, not affecting other characteristics. We found that quantitative differences in enzymatic effects on fibers led to proportional differences in NCC. The use of optimal enzymatic conditions would permit to reduce the use of harsh corrosive sulfuric acid for NCC production while generating a more easily exploitable stream of oligosaccharide-rich effluents.

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