Enhancing cellulose nanofibrillation of eucalyptus Kraft pulp by combining enzymatic and mechanical pretreatments

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ABSTRACT

Nanofibrillated cellulose (NFC) extracted from biomass has potential applications in material science and biomedical engineering. In this study, NFC was obtained from bleached eucalyptus Kraft pulp (BEKP) using two commercial enzyme cocktails with cellulolytic and hemicellulolytic activities and non-catalytic protein (swollenin), followed by ultrasonication. This work represents an initial study of the implementation of non-catalytic proteins along with enzymes to extract NFC from biomass. Enzymatic pretreatment was performed to partially remove hemicellulose while enhancing cellulose accessibility for NFC extraction. Cellulase pretreatment with xylanase and swollenin supplementation increased cellulose accessibility and fiber swelling due to extensive hemicellulose removal (>80%) and fiber morphology changes. Subsequent ultrasonication was performed for cellulose nanofibrillation resulting in high NFC yields (61-97%), while keeping NFC properties almost unchanged. Through this process, cellulose nanofibers with diameters ranging from 3 nm to 10 nm were effectively isolated from BEKP, which allows to produce high quality NFC for further applications.

Keywords: nanofibrillated cellulose, enzymatic pretreatment, xylanase, swollenin, ultrasonication

1. Introduction

Cellulose-based materials have great potential to replace petroleum-based material in technological applications due to its abundance, biodegradability, and outstanding mechanical properties. The production of cellulose nanomaterials has gained increasing attention over the past few decades due to their potential applications such as aerogel and hydrogel, reinforcement in nanocomposites, packaging materials, and biomedical materials (Abitbol et al. 2016). Two different kinds of cellulose nanomaterials can be obtained from lignocellulosic biomass based on the size, morphology and extraction method: cellulose nanocrystals or nanocrystalline cellulose (NCC) and cellulose nanofibrils or nanofibrillated cellulose (NFC). Typically, NFCs have long, entangled and flexible fibrils with diameters and lengths ranging from 1-100 nm to 500-2000 nm, respectively, containing both crystalline and amorphous cellulose regions (Debiagi et al. 2020; Phanthong et al. 2018).

NFCs are typically produced by mechanical pretreatment such as mechanical refining and homogenization, ultrasonication, grinding, microfluidization, ball milling (Abdul Khalil et al. 2014; Pires et al. 2019). All these pretreatments operate under high shear force, resulting in the cleavage of cellulose fibers and fibrillation. However, high energy consumptions associated to the process (20,000-30,000 kWh/ton) and product heterogeneity after mechanical treatments are still limiting its implementation (Rajinipriya et al. 2018). To overcome this, combinations of chemicals and/or enzymatic pretreatments are being proposed to facilitate mechanical pretreatments by opening up the starting cellulosic material and further enhancing cellulose accessibility. Thus, the introduction of a chemical or enzymatic pretreatment could facilitate further mechanical pretreatment of microfibers and bring down the energy consumption up to 20 times (e.g. 1,000 kWh/ton) (Arantes et al. 2020; Rajinipriya et al. 2018; Ramakrishnan et al. 2019). Several chemical pretreatments (e.g. TEMPO-oxidation, carboxymethylation, sulfonation) have been successful in achieving this goal, but there are still many drawbacks associated to process efficiency, use of hazardous reagents and chemical recovery that limits their application (Pires et al. 2019; Ramakrishnan et al. 2019).

Enzymatic pretreatment represents an environmentally friendly alternative to chemical pretreatment for NFC production due to high enzyme specificity and less harmful reaction conditions (Michelin et al. 2020; Ribeiro et al. 2019). The use of enzymes during enzymatic pretreatment catalyze the hydrolysis of cellulose fibers and makes fiber fibrillation by following mechanical homogenization much easier. Most of the reported work on nanocellulose production through enzymatic pretreatment were focused mainly on the use of cellulase

enzymes, such as endoglucanases (Arantes et al. 2020; Di Giorgio et al. 2020; Long et al. 2017; Ribeiro et al. 2019). Endoglucanases are the type of enzymes with the highest interest for the production of nanocellulose, because they break the cellulose polymer into smaller length polymers by acting on the amorphous, or less organized, part of the cellulose. Although enzymatic pretreatment using endoglucanases has shown to be promising for cellulose nanofibrillation enhancement, its efficacy is still limited (Long et al. 2017). However, it has been shown a high degree of synergism between exoglucanases and endoglucanases, which could enhance cellulose hydrolysis and further nanofibrillation (Yarbrough et al. 2017). Additionally, it was recently demonstrated that exoglucanases can hydrolyze microcrystalline cellulose by peeling the cellulose chains from the microcrystalline structure (Merklein et al. 2016). Recently, it was found that the combination of cellulolytic enzymes (cellulases) with cellulase accessory enzymes such as hemicellulases (e.g. xylanases), laccases, and lytic polysaccharide monooxygenases (LPMO) could improve cellulose accessibility during biomass deconstruction, without directly hydrolyzing cellulose (Hu et al. 2018; Zhou et al. 2019). For instance, it was shown that the synergism between cellulases and xylanases increases cellulose hydrolysis efficiency by improving cellulose accessibility, not only due to xylan removal but also to changes in fiber morphology such as increasing fiber porosity and fiber swelling (Long et al. 2017). Even though several studies have been conducted in the past years to produce nanocellulose from different cellulosic substrates through an enzyme-mediated pretreatment, the selection of an effective and low-cost enzyme cocktail still remains a challenge.

Moreover, it has been reported that some non-catalytic proteins (CBM, swollenin) enhances the cellulolytic/hemicellulolytic activity by their effects on the dispersion of the cellulose microfibers and loosening of the cellulose macrofibers, causing a decrease in cellulose crystallinity and increase in cellulose accessibility (Adsul et al. 2020). This type of non-catalytic proteins is generally required at a very low amount and not present in base enzyme preparations. Swollenin represents one of these types of proteins, which was first isolated and characterized from the cellulolytic fungus *Trichoderma reesei*. Swollenin has been shown to facilitate delamination or fibrillation of substrates, or splitting of microfibrils, resulting in a greater exposure of new crystalline regions of cellulose without producing detectable amounts of reducing sugars (Gourlay et al. 2012; Saloheimo et al. 2002). Recently, studies on the supplementation of fungal swollenin into a cellulolytic enzyme preparation for improving enzymatic hydrolysis showed an increase in both cellulose and xylan hydrolysis of lignocellulosic pretreated substrates such as corn stover (Gourlay et al. 2013; Morrison et al.

2016). It was suggested that the enhancement on the enzymatic hydrolysis could be due to the opening up of the cellulosic substrate by weakening and/or disrupting of hydrogen bonds within the biomass, which enhanced the enzyme access to the carbohydrates. Moreover, it has been shown that swollenin could promote fiber fragmentation without the presence of non-cellulosic polymers (lignin and hemicellulose) such as dissolving pulps (Gourlay et al. 2013). However, to the best of the authors' knowledge, there are no previous studies on swollenin supplementation into enzyme cocktails for cellulose enzymatic pretreatment to enhance cellulose nanofibrillation.

Ultrasonication, which consists on the exposure of the suspension to ultrasonic waves, is one of the mechanical pretreatments used to produce NFC and it was shown to be very effective for cellulose depolymerization (Mahardika et al. 2018). During the ultrasonication process, highly intensive waves are produced by the formation, expansion, and implosion of microscopic gas bubbles when ultrasonic energy is absorbed by molecules, creating hydrodynamic forces that are used for cellulose nanofibers liberation from the material (Abdul et al. 2014). Process optimization using ultrasonication for cellulose fibrillation has previously been reported (Wang and Cheng 2009). Mainly, the effect of temperature, suspension concentration, sonication power and time on fibrillation efficiency was evaluated. However, these conditions greatly depend on the type of cellulosic material used, for example, suspension concentration depends on the size of the fibers, since longer size fiber needs to be treated at lower concentrations. On the other hand, few studies have been reported on combined enzymatic and ultrasonication pretreatment for NFC production. Filson et al. (2009) obtained cellulose nanofibers from recycled pulp using enzymatic pretreatment followed by sonication. Tsukamoto et al. (2013) proposed a method to isolate NFC from enzymatic hydrolysis residues of citrus processing waste using an ultrasonic processor. De Campos et al. (2013) produced NFC from dewaxed and bleached curauá and sugarcane bagasse using enzymes under different enzymatic pretreatment conditions, followed by sonication.

In this work, the potential of using commercial enzyme complex and non-catalytic protein during the enzymatic pretreatment process for NFC production to facilitate cellulose nanofibrillation was assessed. Bleached eucalyptus Kraft pulp (BEKP) was used as raw material due to its low cost and abundance. The BEKP was industrially obtained by treating eucalyptus wood chips through the Kraft process, followed by a bleaching process performed in a series of stages involving bleaching agents (chlorine dioxide, hydrogen peroxide and oxygen) and alkaline extractions. One of the main advantages of eucalyptus pulp compared to other cellulosic

materials is the shorter fibers, which could facilitate disintegration and reduce energy input. However, it contains considerable amount of hemicellulose (15-20%) which could have a negative effect on cellulose accessibility towards the enzymes, given that it is located on the outer surface and interfibrillar space of cellulose fibers. Thus, removal of hemicellulose from cellulosic biomass could be crucial to produce NFC, since it might be able to enhance opening up the biomass structure and facilitate cellulose nanofibrillation. The aim of this work was to investigate the enzymatic pretreatment consisting of xylanases as hemicellulolytic enzymes and swollenin as non-catalytic protein with commercial cellulase complex to produce NFC from BEKP, followed by an ultrasonication step. This work represents an initial study of the implementation of non-catalytic proteins along with enzymes for NFC production from biomass. The obtained materials were characterized according to their chemical composition, size distribution, fiber swelling, cellulose accessibility, degree of polymerization, crystallinity and thermal stability. NFC yields were calculated as an evaluation of the effects of the different enzymatic pretreatment conditions on the cellulose nanofibrillation.

2. Materials and Methods

2.1. Materials

Bleached eucalyptus Kraft pulp (BEKP) was used as raw material for the production of NFC, which was provided by a commercial local pulp mill (UPM Fray Bentos, Uruguay). The enzymes used in this study were Cellic CTec3 cellulase (279 mg/mL protein content) and HTec xylanase (53 mg/mL protein content) supplied by Novozymes (Davis California). Swollenin, expressed in *Trichoderma reesei*, was used as non-catalytic protein, which was provided by VTT Technical Research Center of Finland and produced as previously described (Gourlay et al. 2013).

2.2. BEKP chemical composition

BEKP was analyzed according to the klason protocol from the TAPPI standard method T222. Sugars were determined by a Dionex ICS-3000 HPLC (Sunnyvale, CA) equipped with an anion exchange column (Dionex CarboPac PA1). Acid soluble lignin was determined by measuring absorbance at 205 nm using Cary 60 UV-Vis spectrophotometer. Glucan, xylan, and lignin contents of BEKP were $77.3 \pm 0.6\%$, $17.6 \pm 0.1\%$ and 0.7 ± 0.1, respectively.

2.3. Enzymatic hydrolysis of BEKP

The enzymatic hydrolysis of BEKP was performed in duplicate at 50℃ and 200 rpm in acetate buffer (50 mM, pH 4.8) using CTec3 and HTec accordingly. Before enzyme addition, the slurries were equilibrated at 50℃ and 200 rpm for 1 h in an orbital shaker (MaxQ 4000, Barnstead/Lab-Line) for pulp disintegration. Enzymatic hydrolysis was carried out at a solid concentration of 2-8% (w/w) and an enzyme loading of 1-7 $mg_{protein}/g_{dry\ pulp}$. Samples were taken during hydrolysis and heated at 100℃ for 10 min to deactivate the enzyme. Samples were then centrifuged (5,000 rpm for 15 min) to separate the liquid fraction for sugar release analysis, and the residual solid fibers were washed three times with distilled water by centrifugation (5,000 rpm for 15 min). The effects of swollenin on enzymatic pretreatment were evaluated by adding the same amount of swollenin as enzyme loading on protein basis when corresponded. Liquid fractions from the enzymatic hydrolysis experiments were analyzed following NREL protocol (Sluiter et al. 2006), and monomeric sugars were measured by a Dionex ICS-3000 HPLC (Sunnyvale, CA) equipped with an anion exchange column (Dionex CarboPac PA1).

2.4. Ultrasonication

The enzyme-treated BEKP samples were redispersed in deionized water to reach solid consistencies of 0.07 to 0.5%. The resulting suspensions were subjected to ultrasonication (50-90% amplitude) with a Q700 sonicator (QSonica, Newtown, CT, USA) operating at 20-25 kHz frequency and equipped with a 0.5 inch flat tipped titanium probe. The suspensions were cooled in an ice bath during ultrasonication to avoid overheating, and temperature was kept below 30℃. The ultrasonication was performed with 5 s pulse-on time followed by 5 s of pulse-off time for 60 and 120 min when corresponded. The resulting preparations were centrifuged (5,000 rpm for 30 min) in order to separate the micro/nanofibrils.

2.5. NFC yield

Ultrasonicated suspensions were centrifuged at 5,000 rpm for 30 min to separate the nanofibrillated material (in supernatant fraction) from the unfibrillated or partially fibrillated ones, which settle down. The supernatant fraction was then dried to a constant weight at 105°C. The NFC yield was calculated from the following equation:

$$NFC\ yield\ (\%) = \frac{M_{NFC}\ .\ M_T}{M_{sample}\ .\ M_F}\ x\ 100 \quad (1)$$

where M_{NFC} denotes the mass of the dried NFC in the supernatant sample; M_T denotes the total mass of the ultrasonicated suspension; M_{sample} denotes the mass of the supernatant sample; M_F denotes the mass (oven-dry basis) of the pulp before ultrasonication.

2.6. Analytical methods

2.6.1. Water retention value

The water retention value (WRV) was measured in duplicate using the TAPPI Useful Method-256. Briefly, 0.5 g (oven-dry basis) of the pulp was soaked in 50 mL of water overnight prior to filtration through a 200-mesh screen. The resulting pulp pad was centrifuged at 900 g for 30 min, and finally oven-dried at 105°C overnight. WRV represents the percentage of retained water of the dried substrate, according to the following equation:

$$WRV = \frac{W_w - W_d}{W_d} \quad (2)$$

where W_w and W_d denote the weight of the wet sample after centrifugation and the dried pulp, respectively.

2.6.2. Simons' staining

The cellulose accessibility was assessed using Direct Blue (DB) as dye following a modified version of the Simons' staining procedure (Chandra et al. 2008). Briefly, 10 mg (oven-dry basis) of substrates were mixed with 1 M phosphate buffered saline solution (PBS, pH 6) and increasing DB dye concentrations in 1.5-mL screw cap centrifuge tubes. The tubes were incubated overnight at 70°C in an orbital shaker at 180 rpm. After incubation, the tubes were centrifuged at 5,000 rpm for 10 min and the absorbance of the supernatant was read at 455 nm on a Cary 60 UV-Vis spectrophotometer.

2.6.3. Degree of polymerization

The degree of polymerization (DP) of BEKP and enzyme-treated BEKP was determined by viscosity (25°C) of fiber solution in 0.5 M cupriethylenediamine (CED) solution using Ubbelohde viscometer according to TAPPI standard method T230. The DP was determined using the following equation (Immergut et al. 1953; Hamad and Hu 2010):

$$DP^{0.905} = 0.75[\eta] \quad (3)$$

where η denotes the intrinsic viscosity expressed in cm^3/g and calculated according to ASTM D1795-13.

193 *2.6.4. X-ray diffraction (XRD)*

194 BEKP and enzyme-treated BEKP were analyzed using X-ray diffractometer (MiniFlex600, Rigaku).
195 The samples were dried and ground into powders for XRD test. The diffracted intensity of CuK_{α} radiation
196 (40 kV and 15 mA) was measured in a 2θ range between 5° to 60° with increments of 0.05°, at a scanning rate
197 of 4°/min. The relative crystallinity index (CrI) was calculated using the Segal et al. (1959) method, as the
198 difference between the maximum intensity of the peak located at 2θ = 22°-23°, and the minimum intensity
199 located between the major peaks at 2θ = 18°-19°C divided by the intensity of the highest peak.

200 *2.6.5. Transmittance of NFC suspensions*

201 The light transmittance of NFC suspensions (0.1% w/v) from BEKP and enzyme-treated BEKP was
202 measured in the range of 400 nm to 800 nm using Cary 60 UV-Vis spectrophotometer.

203 *2.6.6. Thermogravimetric analysis (TGA)*

204 The thermal stability of BEKP, enzyme-treated BEKP and NFC was studied using thermogravimetric
205 analysis (TGA) and derivative thermogravimetric analysis (DTG) with a thermogravimetric analyzer
206 (TGA 4000, PerkinElmer, USA). The samples were analyzed under a nitrogen atmosphere with a gas flow of
207 20 mL/min by heating the material from 35°C to 600°C at a heating rate of 10°C/min.

208 *2.6.7. Morphological analysis - AFM*

209 The morphology of the NFCs was studied by atomic force microscopy (AFM) using Multimode AFM
210 Nanoscope-III from Veeco Instruments (Santa Barbara, CA, USA) with the PeakForce tapping mode. A freshly
211 cleaved mica was chemically modified with a cationic compound (3-aminopropyl)triethoxysilane (APTES)
212 according to previous studies (Aissa et al. 2019). Briefly, a drop of 0.1% w/v of APTES solution was placed on
213 a cleaved mica surface for 30 s and then thoroughly rinsed with nanopure water. The samples were prepared by
214 depositing a drop of diluted NFC suspension (0.01% wt) on the modified mica surface and leaving it to dry for
215 at least 1 h. AFM images were taken at a scan rate of 0.7 Hz using a RTESPA-150 cantilever proves with a
216 nominal spring constant of 7 N/m. AFM images were analyzed using NanoScope Software 8.10 (Veeco, Santa
217 Barbara, CA, USA).

218 *2.6.8. Fiber Quality Analysis (FQA)*

Fiber dimensions and fines content of BEKP and enzyme-treated BEKP were determined using a Hi-Resolution fiber quality analyzer (LDA02-series, OpTest Equipment Inc, Hawkesbury, Canada). About 10,000 fibers were collected to calculate the fiber length distribution in the range of 0.07 mm to 10.0 mm. Fines were defined as fiber lengths between 0.07 mm to 0.20 mm.

2.6.9. Dynamic Light Scattering (DLS)

The size distribution of enzyme-treated BEKP suspensions was also evaluated with a Malvern Mastersizer Hydro 2000G/S (Malvern Instruments Limited, Worcestershire, UK). The samples were suspended in water and analyzed with the particle size analyzer using dynamic light scattering (DSL).

3. Results and discussion

3.1. Enzymatic pretreatment of eucalyptus Kraft pulp: effect of cellulase, xylanase and swollenin preparation

In this study, a commercial BEKP was used as raw material to produce NFC using a combination of enzymatic and mechanical pretreatment. Enzymatic pretreatment was performed using enzyme cocktails (cellulase, xylanase) and non-catalytic protein (swollenin) to evaluate the partial removal of hemicellulose from BEKP and catalyze the hydrolysis of cellulose fibers to enhance cellulose accessibility and make fibrillation easier. Enzymatic hydrolysis experiments of BEKP were initially carried out for 24 h using CTec3 and varying the solids loading (2, 4 and 8% w/v) (Figure 1). Low and relatively high solids loading was selected in this study to evaluate possible effects due to mass transfer limitations and the recovery of released sugars at an increased concentration. As it was expected, higher glucose and xylose concentrations were obtained with increasing solids loading, reaching 45 g/L and 13 g/L, respectively, for 8% solids loading.

However, similar glucose and xylose concentrations were obtained during the first 4 h of hydrolysis when the solids loading was increased from 4% to 8%, indicating similar enzymatic hydrolysis rates. This suggests possible mass transfer limitations at the initial stages of hydrolysis due to the higher solids content. In order to determine if mass transfer or other limitations affected the hydrolysis performance, results were analyzed in terms of cellulose and xylan hydrolysis (Figure 1b). It can be observed that higher cellulose (33%) and xylan (52%) hydrolysis was reached at 4% solids loading, but further increasing solids loading up to 8%

w/v negatively affected the hydrolysis rates (15% and 30% for cellulose and xylan hydrolysis, respectively). Similar results in terms of hydrolysis rates were observed when the solids loading decreased to 2% (29% and 49% for cellulose and xylan hydrolysis, respectively) compared to 4% during the first 4 h of hydrolysis. However, lower glucose (5.0 g/L) and xylose (2.1 g/L) concentrations were reached in this case. Consequently, considering that after 24 h of hydrolysis significant cellulose degradation was achieved due to glucose release, further enzymatic hydrolysis experiments were performed at 4% solids loading for 4 h that minimizes cellulose loss and increases the released glucose (11.7 g/L) and xylose (4.1 g/L) concentrations. However, cellulase pretreatment of BEKP resulted in relatively low hemicellulose removal, considering that only 50% of the original xylan was effectively removed.

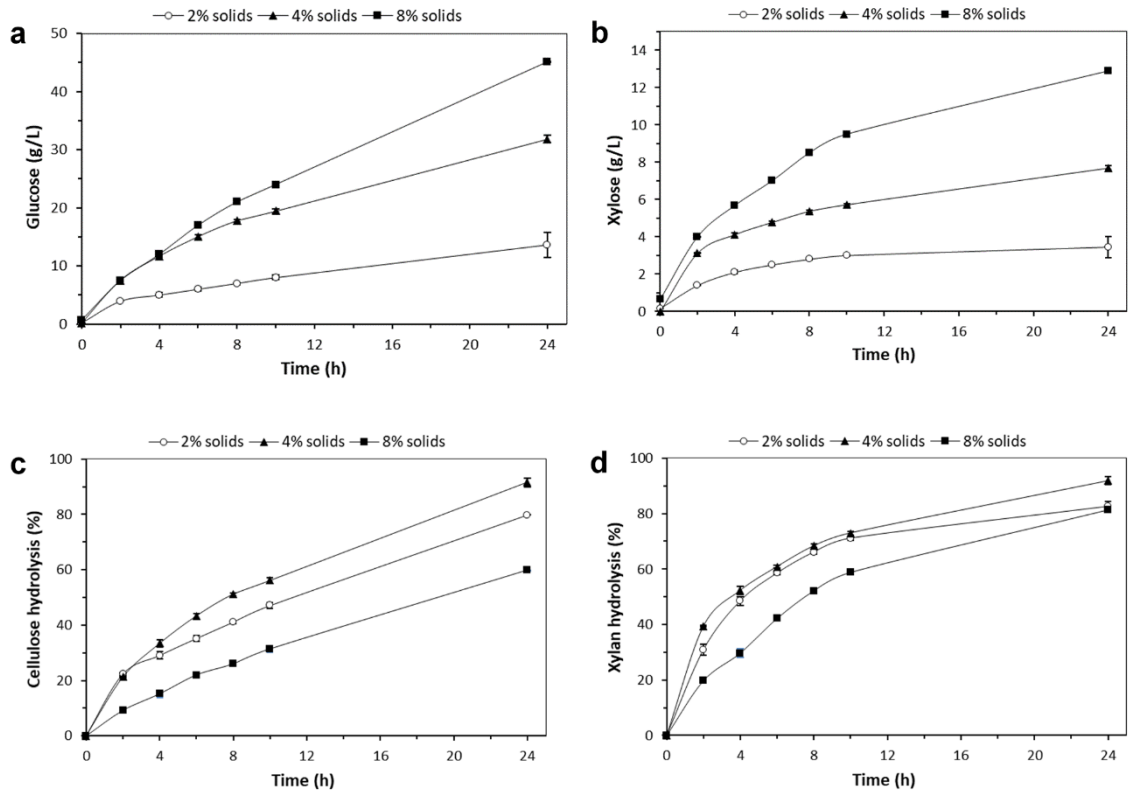

Fig 1. (a) Glucose, (b) xylose, (c) cellulose hydrolysis and (d) xylan hydrolysis profiles during enzymatic hydrolysis of BEKP under different solids loading conditions (2%, 4% and 8%).

Supplementation of hemicellulolytic enzymes (xylanase) and non-catalytic protein (swollenin) to the cellulase pretreatment of BEKP was performed to enhance xylan removal and thus improve cellulose

accessibility. Also, it has been previously demonstrated the synergistic effect between cellulases and xylanases to open up the cellulose fiber structure in order to enhance further cellulose nanofibrillation (Long et al. 2017). The enzymatic pretreatment was first evaluated using the commercial xylanase preparation commonly used for biomass deconstruction (HTec) alone and in combination with CTec3 using BEKP as substrate. The xylanase was added by replacing a portion of cellulase enzyme with xylanase enzyme (cellulase:xylanase ratios of 1:0, 0:1, 1:1, 2:1, 1:2 on mass basis) to avoid an increase in the total protein loading (Table 1). The highest degree of synergism between the enzymes was observed at a cellulase:xylanase ratio of 1:1, which resulted in a substantial increase in both cellulose and xylan hydrolysis (43% cellulose and 76% xylan hydrolysis, Table 1). In order to limit the amount of cellulose loss during hydrolysis, enzymatic pretreatment was carried out at lower total protein loading (Table 1). However, decreasing the total protein loading from 4 $mg_{protein}/g_{solid}$ to 3 $mg_{protein}/g_{solid}$ significantly reduced the xylan removal during enzymatic hydrolysis, which could have a negative effect on enhancing cellulose accessibility.

Table 1. Effect of xylanase and swollenin supplementation to cellulase pretreatment of BEKP on cellulose and xylan hydrolysis at 4% solids loading after 4 h.

Cellulase:xylanase ratio	Swollenin addition	Total protein loading ($mg_{protein}/g_{solid}$)	Cellulose hydrolysis (%)	Xylan hydrolysis (%)
1:0	No	7.0	33.4 ± 1.3	52.3 ± 1.5
0:1	No	1.4	6.3 ± 0.1	46.8 ± 1.0
2:1	No	5.2	34.2 ± 1.9	54.3 ± 2.1
1:2	No	3.2	30.9 ± 2.2	56.4 ± 2.8
1:1	No	4.2	43.1 ± 0.2	76.2 ± 0.4
1:1	No	3.0	28.5 ± 2.4	49.3 ± 2.2
1:1	No	1.5	19.9 ± 1.6	39.1 ± 1.3
1:1	No	1.0	9.8 ± 0.2	25.8 ± 0.1
1:1	Yes	8.4	46.3 ± 1.6	84.6 ± 2.0

On the other hand, swollenin was assessed in this study in combination with xylanase (Table 1), since it has been previously reported by other authors that swollenin has strong synergism with xylan degrading enzymes (Gourlay et al. 2012, 2013). According to the results obtained, swollenin was able to disrupt the relatively loosely ordered xylan structure during cellulase and xylanase enzymatic pretreatment, since an increased xylan hydrolysis (85%) was reached compared to enzymatic pretreatment without swollenin supplementation (76%). Moreover, no increased cellulose hydrolysis (46%) was observed with the use of swollenin, which suggests that swollenin does not cause cellulose degradation but has a significant effect on the hemicellulose component.

After enzymatic pretreatment, cellulosic fiber characteristics such as cellulose accessibility (Simon's staining techniques with Direct Blue, DB), fiber swelling (water retention value, WRV) and degree of polymerization (DP) were assessed (Figure 2). The cellulase pretreatment of BEKP has shown to significantly reduce the DP (from 1218 to 465). This was expected considering that cellulases could randomly cleave the glycosidic linkages in both the disorganized (also known as amorphous) and organized (also known as crystalline) regions of the cellulose fiber, which induces the cellulose DP reduction (Reese et al. 1957). Moreover, since xylan plays an important role in protecting cellulose chains against degradation (Gomes et al. 2014), xylan removal by cellulase pretreatment may have caused cellulose becomes more susceptible to depolymerization. DP reductions with enzyme pretreatment was also reported by other authors (Djafari Petroudy et al. 2015; Long et al. 2017; Wang et al 2014). On the other hand, even though xylan removal was increased during cellulase and xylanase enzymatic pretreatment, no further DP reduction was observed in this case. The greater exposure of cellulose due to extensive xylan removal could have caused a higher cellulose depolymerization and, thus, lower DP values. On the other hand, considering that xylans are categorized as short chain polysaccharides (Djafari Petroudy et al. 2015), removal of these low molecular weight carbohydrates may lead to an increase in the DP extent (Gomes et al. 2014). This effect may have offset the DP reduction by cellulose depolymerization occurring when xylan is removed and, thus, DP remained almost unchanged. Despite of this, the depolymerization reached could help the formation of NFC by subsequent ultrasonication. On the other hand, xylanase pretreatment of BEKP resulted in lower DP variations (from 1218 to 735) compared to cellulase pretreatment (Figure 2a). This was also shown by Zhou et al. (2019) and reflects the difference in the catalytic activity of the enzymes used.

306 The WRV, which can be an indicator of overall fiber accessibility, showed a contrary trend to the
307 observed for DP. Moreover, results showed that the enzymatic pretreatment may have increased the cellulose
308 accessibility, considering the significant increase in the DB adsorption and the WRV observed (Figure 2a and
309 Figure 2b). Even though the supplementation of xylanases to cellulase pretreatment had little effect on the fiber
310 swelling according to the WRV (Figure 2a), the synergism between cellulases and xylanases further improved
311 the cellulose accessibility of enzyme-treated BEKP according to DB adsorption (Figure 2b). The improvement
312 achieved on the cellulose accessibility indicated that the fiber structure was further opened up, which could be
313 beneficial for subsequent cellulose nanofibrillation. The partial replacement of cellulase by xylanase had very
314 little effect on the cellulose DP of the enzyme-treated BEKP obtained (Figure 2a). This indicates that the
315 combination of these enzymes was suitable for keeping cellulose properties while improving cellulose
316 accessibility, without causing further cellulose degradation. On the other hand, the addition of swollenin
317 considerably improved the cellulose accessibility and fiber swelling (Figure 2). It was apparent that the
318 increased xylan hydrolysis could have facilitated the enzyme access to the cellulosic component of BEKP thus
319 opening up the biomass structure and cellulose accessibility. Also, an increased WRV achieved with the use of
320 swollenin might suggest exposure of greater amount of cellulose due to xylan removal that enhanced water
321 retention, since it is likely that xylan acts as a physical barrier possibly coating cellulose microfibrils and/or
322 limiting cellulose fiber swelling. However, it was less able to alter the more highly-ordered cellulose structure,
323 since the degree of polymerization (DP) kept almost unchanged and no significant cellulose degradation
324 occurred (Figure 2a). Results obtained in this work showed how beneficial the addition of xylanases and
325 swollenin to cellulase pretreatment can be in enhancing major fiber characteristics such as fiber swelling and
326 overall cellulose accessibility, which could facilitate downstream cellulose nanofibrillation.
327 Regarding xylan release during enzymatic pretreatment, it should be noted that both cellulase (CTec3)
328 and xylanase (HTec) preparations showed good performance for xylan degradation (Figure 2b), since
329 considerable amounts of xylose (32-74%) and soluble xylooligomers (6-15%) were released from the original
330 xylan present in the BEKP. A higher proportion of xylooligomers than of xylose was released when xylanase
331 was employed alone (32%) and in combination with cellulase (20%) during enzymatic pretreatment, compared
332 to cellulase pretreatment (10%). This suggests that the commercial xylanase preparation used in this study may
333 lack of β-xylosidase activity to further convert xylooligomers to xylose by hydrolysis. However, the use of

swollenin in combination with cellulase and xylanase enhanced xylan hydrolysis, while decreasing the proportion of xylooligomers in the liquid fraction to 12%. This allows to get released sugars in a suitable way which represents a possible feedstock to produce other value-added chemicals (Bondancia et al. 2017; Pereira and Arantes, 2020).

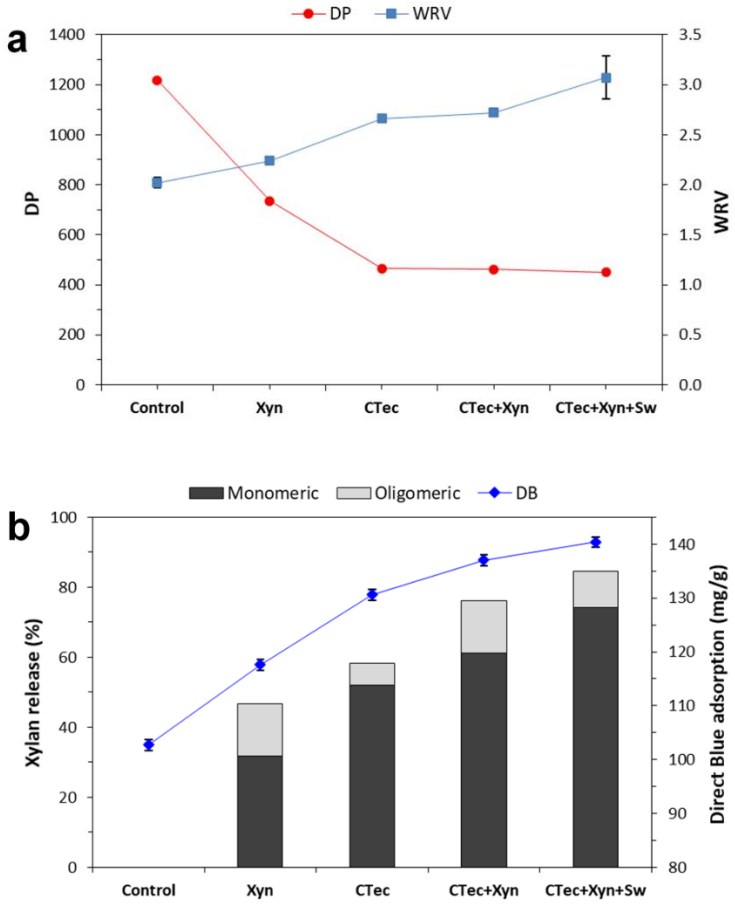

Fig 2. Characteristics of enzyme-treated BEKP: (a) fiber swelling by water retention value (WRV) and degree of depolymerization (DP), (b) xylan release in monomeric and oligomeric forms and cellulose accessibility by Simon's staining with Direct Blue (DB). CTec, Xyn and Sw are assigned to CTec3, HTec and swollenin, respectively.

3.2. Effect of enzymatic pretreatment on pulp fiber properties

Since it has been shown that hemicellulose contributes to fiber strength and its solubilization influences pulp fiber properties, fiber dimensions and fiber size distribution of BEKP and enzyme-treated BEKP samples

were determined by fiber quality analysis (FQA) (Figure 3a). FQA equipment has been extensively used in the past years for pulp and paper studies to evaluate changes at the fiber level. According to FQA results, BEKP was 15.1 µm in mean width and 1.02 mm in length, containing 25% of fines (fibers with length ranging from 0.07 mm to 0.2 mm). After enzymatic pretreatment, significant fiber fragmentation was observed using cellulase alone or in combination with xylanase and swollenin (Figure 3). When xylanase was used alone during enzymatic pretreatment of BEKP, the fiber length decreased up to 0.64 mm, with a fines content of 51%. However, cellulase pretreatment significantly increased the level of fibrillation, as the fines content increased up to 87% and 65% when used alone or in combination with xylanase, respectively. Due to the high fines content of the enzyme-treated BEKP, size distribution after enzymatic pretreatment using cellulase enzyme was determined by dynamic light scattering analysis (DLS) (Figure 3b).

Although microscopy analysis (e.g. TEM) could be used to determine size dimension and distribution of samples with high fiber fragmentation, the accuracy of microscopy measurements is limited to the small fraction of sample analysed relative to the whole sample. Because of this, DLS method was used in this study for a more complete evaluation of the size distribution of enzyme-treated BEKP samples. DLS is a well-known method which determined the hydrodynamic "apparent particle size" of cellulose micro/nanomaterials by measuring the scattered light intensity caused by particles undergoing Brownian motion (Yadav et al., 2017; Foster et al., 2018). By assuming that the particles have a single and constant rate of diffusion (e.g. spherical particles), the intensity is related to the particle size by the Stokes-Einstein equation (Foster et al., 2018). However, since rod-like fibers are treated as spherical particles, DLS measurements does not give absolute values for size distribution (Qua et al., 2011). Instead, DLS gives a hydrodynamic "apparent size" distribution that can be used to compare between samples that were prepared under different pretreatment conditions. Several authors used this technique to assess the size distribution of cellulose micro/nanomaterials (Qua et al., 2011; Yadav et al., 2017; Foster et al., 2018; Tibolla et al., 2018; Ramakrishnan et al., 2019; Ferreira et al., 2020).

According to DLS results, fibers fractionation was apparent during cellulase pretreatment of BEKP, reaching an average hydrodynamic size of 28.6 µm. However, there were still some large fibers in the range of 200-800 µm, which should be fractionated during subsequent ultrasonication. When cellulase was used in combination with xylanase during enzymatic pretreatment, the average hydrodynamic size remained relatively

constant (27.8 µm) and no significant changes was observed compared to cellulase pretreatment (Figure 3b). On the other hand, the addition of swollenin during enzymatic pretreatment allowed to achieve a more uniform hydrodynamic size distribution of the fibers, without significantly affecting the average hydrodynamic size (29.8 µm). This may be explained by the increased fiber swelling observed with the use of swollenin during enzymatic pretreatment, which enhanced fiber fragmentation and, thus, less larger fibers in the range of 60-800 µm were observed. It should be noted that in all cases between 80 and 90% of the fibers were less than 100 µm size on average, which is in the microfiber range. Also, considerable size reduction after enzymatic pretreatment of BEKP was reached, which implies that fibers became smaller due to increased degree of microfibrillation and thus, greater exposure of surface area on the fibrils occurred. This correlates quite well with the increased WRV of enzyme-treated BEKP samples related to both fibrils and microfibrils surface area.

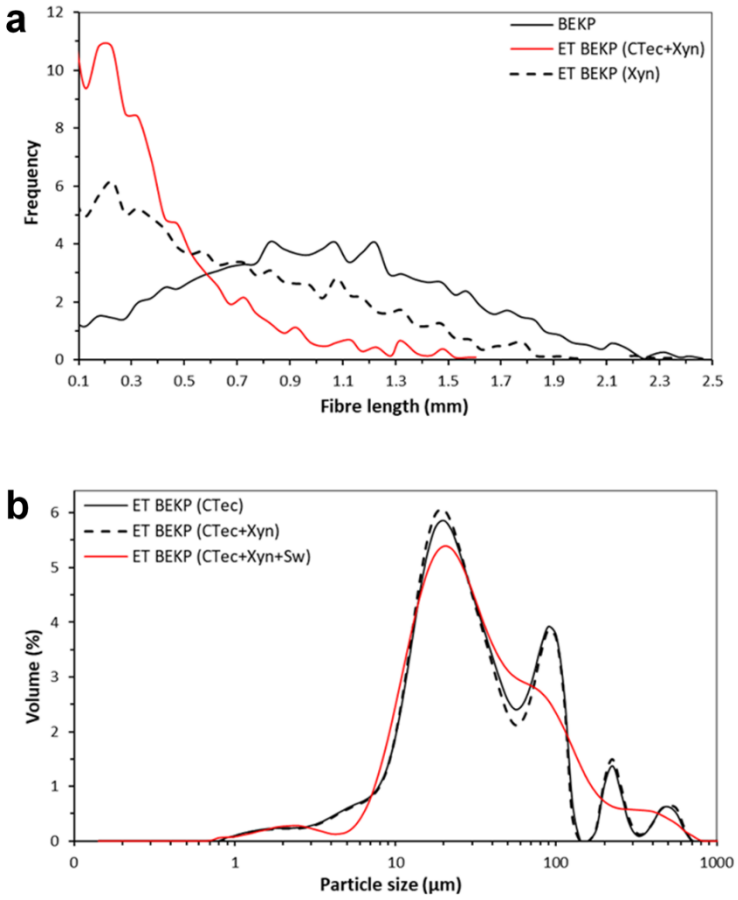

Fig 3. Size distribution of BEKP and enzyme-treated (ET) BEKP samples using (a) fiber quality analysis and (b) dynamic light scattering. CTec, Xyn and Sw are assigned to CTec3, HTec and swollenin, respectively.

3.3. NFC production using ultrasonication

NFCs were produced by enzymatic pretreatment followed by ultrasonication, being this last step important for fiber defibrillation in nanofibers by ultrasound hydrodynamic forces. Process conditions such as suspension consistency, sonication intensity (amplitude) and time were evaluated during ultrasonication of enzyme-treated BEKP, and results were analyzed in terms of NFC yields (Table 2). NFC yields of 61-93% were reached for enzyme-treated BEKP, which indicates that cavitation was effective for opening the microfiber structure, releasing the nanofibrils from the fiber cell wall. Also, relatively high NFC yield (33%) was obtained from BEKP at the highest ultrasonication intensity, which demonstrates the efficiency of the ultrasonication process for NFC production from this type of cellulosic material. However, it should be mentioned that eucalyptus pulp presents shorter fibers compared to other woody materials (e.g. Pinus pulp) which facilitates disintegration.

Table 2. NFC yields of different ultrasonication conditions obtained from untreated and enzyme-treated (ET) BEKP samples.

Substrate	Amplitude (%)	Solid consistency (%)	NFC yield (%)
BEKP	90	0.30	33.1 ± 0.1
ET BEKP (CTec)	50	0.30	61.3 ± 4.4
	70	0.30	74.5 ± 1.4
	90	0.07	78.9 ± 4.9
	90	0.15	92.7 ± 2.0
	90	0.20	93.0 ± 2.7
	90	0.30	92.7 ± 2.0
	90	0.50	77.0 ± 3.0
ET BEKP (CTec+Xyn)	90	0.30	97.3 ± 1.1
ET BEKP (Xyn)	90	0.30	73.0 ± 1.1
ET BEKP (CTec+Xyn+Sw)	90	0.30	95.9 ± 0.2

Ultrasonication pretreatment was performed for 60 min in all cases.

406 The yield of NFCs produced was increased from 79% to 93% by increasing solid consistency from
407 0.07% to 0.15%. While increasing the fibers concentration in the suspension, the hitting and crashing effects
408 among the fibers that are accelerated by the microbubbles aquatic force became significant, enhancing the
409 disintegration of the fibers and degree of nanofibrillation. No significant difference was observed in NFC yields
410 when increasing solid consistency up to 0.3%. However, NFC yield was significantly reduced to 77% when the
411 solid consistency was increased up to 0.5%, which suggests that further increasing solid consistencies
412 negatively affect cellulose nanofibrillation. This could be due to poor agitation and inadequate stirring at high
413 solid concentrations by the microbubbles force generated during ultrasonication, so the fibers have a lower
414 chance of passing the probe tip. A similar effect was also reported by Wang and Cheng (2009) with increasing
415 concentrations of cellulose suspensions. Consequently, a solid consistency of 0.3% was selected for further
416 experiments, which allowed to obtain higher concentrations of NFCs in supernatants.
417 Regarding ultrasonication intensity, NFC yield changed greatly depending on the ultrasonication
418 amplitude used (Table 2). Higher the amplitude (90%), higher the NFC yield achieved (93%). When an
419 amplitude of 50% was used during ultrasonication, the NFC yield was only 61%, which increased to 74% by
420 increasing amplitude to 70%. It was demonstrated that higher operating amplitude facilitated cellulose
421 nanofibrillation, which led to almost 100% conversion from microfibers to nanofibers (nanocellulose) in
422 enzyme-treated BEKP samples. Based on the NFC yields determination, the best conditions selected for the
423 ultrasonication process was: 90% ultrasonication amplitude, 60 min process time and 0.3% solid consistency.
424 However, ultrasonication was also performed for 120 min (data not shown), but no NFC yield improvement
425 was achieved for any of the different substrates.
426 Finally, the degree of nanofibrillation was compared among the different enzyme-treated BEKP
427 samples (Table 2). Results showed that the nanofibrillation degree was increased when cellulase pretreatment
428 was supplemented with xylanase enzyme, which allowed to achieve the highest NFC yield after ultrasonication
429 (97%). This correlates quite well with the increased cellulose accessibility and fiber swelling of the substrates
430 (Figure 2). No significant difference in cellulose nanofibrillation was observed with the addition of swollenin
431 during enzymatic pretreatment, which also allowed to achieve almost complete conversion of microfibers to
432 nanofibers (96%). This was expected due to the increased cellulose accessibility and fiber swelling observed
433 for this enzyme-treated sample, which was previously discussed. Moreover, these results demonstrated that the

xylan removal achieved using swollenin during enzymatic pretreatment facilitated the initial fibrillation to separate cellulose fibrils, but had no significant effect on nanofibrillation through subsequent ultrasonication. However, it should be noted that, despite the extensive xylan removal under this condition (85%), no negative effect was observed on cellulose nanofibrillation in this study.

On the other hand, lower cellulose nanofibrillation was obtained using enzyme-treated BEKP with xylanase alone (73%), probably due to the lower cellulose accessibility of the substrate after enzymatic pretreatment. Nevertheless, this cellulose nanofibrillation degree resulted higher than the BEKP nanofibrillation (33%), which demonstrates that enzymatic pretreatment, using cellulases and/or xylanases, effectively enhanced cellulose nanofibrillation during the ultrasonication process.

3.4. Morphology and size of NFCs

The morphology of the NFCs prepared through enzymatic and ultrasonication pretreatment was analyzed by AFM observations (Figure 4a and Figure 4b). For all samples, observations revealed networked and ribbonlike NFC samples, with a quite uniform size distribution. Though the fibers were entangled by the long lengths, they were well dispersed into almost elementary fibril levels. The diameter of the nanofibrils was estimated from the height profile and resulted in the range of 3-10 nm for individual non-aggregated nanofibrils (Figure 4c). This indicates efficient fibrillation and separation of NFC through the combination of enzymatic and mechanical pretreatment. Long et al. (2017) also produced NFCs from BEKP by endoglucanase pretreatment followed by ultrasonication. However, the diameters of NFCs obtained in this study (3-10 nm) were smaller than the nanocellulose products reported in their study (about 50-150 nm), which indicates improved cellulose nanofibrillation. Also, Baati et al. (2017) reported NFC production with a fiber size in the range of 2-5 nm after using a twin-screw extruder for fibers disintegration, which were chemically pretreated (TEMPO-Mediated oxidation) prior to mechanical pretreatment. In their study, smaller NFC sizes were mainly due to the stronger pretreatment which facilitated cellulose disintegration and further nanofibrillation, with a quite uniform width distribution.

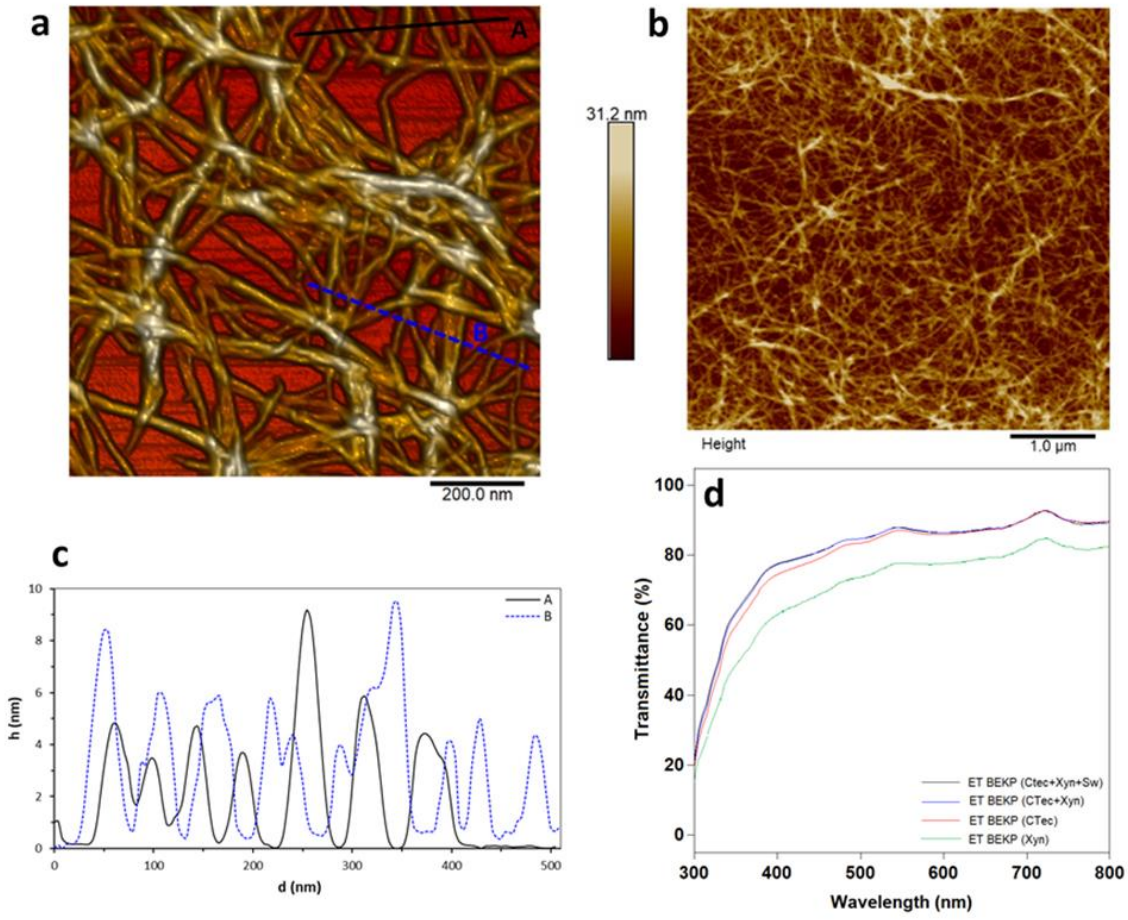

Fig 4. (a,b) AFM images of NFCs produced after ultrasonication of enzyme-treated BEKP, (c) cross-section profile analysis (the arrows (A, B) mark the points used for height measurements) and (d) UV–Vis transmittance spectra of NFC suspensions (0.1% w/v approx.). CTec, Xyn and Sw are assigned to CTec3, HTec and swollenin, respectively.

Recently, turbidity measured as the amount of light transmitted has been proposed as a quick method to assess the dispersion of cellulose nanomaterials (Foster et al. 2018). Thus, lower turbidity of suspensions means more fibrillated NFCs. Light transmittance was determined by spectrometry to evaluate the opacity and ensure uniform nanofibrils morphology of NFC suspensions (Figure 4d). Transmittance values of over 80% at 500 nm demonstrates NFC suspensions well dispersed in water and with nano-properties. As expected, the use of xylanase enzymes and swollenin during cellulase pretreatment improved the transparency of NFC suspensions after ultrasonication (82-84% at 500 nm), which demonstrates an improved cellulose

nanofibrillation. However, lower transparency values (73% at 500 nm) were obtained by using xylanase enzymes alone during enzymatic pretreatment, which suggests lower cellulose nanofibrillation degree. These results correlate quite well with the NFC yields previously discussed (Table 2).

3.5. X-ray diffraction

X-ray diffraction (XRD) was used in this study to evaluate changes in the relative crystallinity index (CrI) of BEKP until the production of NFC by enzymatic pretreatment since it is widely used to investigate the crystal structure of cellulosic materials (Foster et al., 2018). CrI is based on the Segal method (Segal et al., 1959) which, despite some objections and fault found by other authors (Park et al., 2010; French and Santiago-Cintrón, 2013), it is commonly used to analyse XRD spectra to determine CrI due to its simplicity. XRD curves and CrI of BEKP, enzyme-treated BEKP, and NFC samples are shown in Figure 5. According to Figure 5b, the CrI of BEKP, enzyme-treated BEKP samples, and NFC from BEKP were 68%, 76-79%, and 67-76%, respectively. The CrI of the enzyme-treated BEKP samples resulted higher (12-16% increase) than the CrI of BEKP, which was mainly due to the removal of cellulose disordered regions and amorphous hemicellulose during enzymatic pretreatment. Even though the CrI of the enzyme-treated BEKP samples resulted higher than the CrI of BEKP, differences in the CrI values were observed for the different enzyme-treated samples. These differences may be related to the hemicellulose removal during enzymatic pretreatment under the different conditions evaluated, since it was previously shown that decreasing hemicellulose content in pulps increases crystallinity, possibly by the apparent partial recrystallization of amorphous cellulose regions to crystalline, or partial co-crystallization of crystallites in adjacent fibers (Wan et al., 2010). However, it should be noted that further increasing hemicellulose removal (e.g. by swollenin addition) did not increase CrI of enzyme-treated BEKP. This may be explained by the possible presence of grooves in cellulose structure due to high hemicellulose extensive removal, which was previously shown to result in lower CrI values (Wan et al., 2010).

On the other hand, XRD measurements showed that NFC obtained after ultrasonication resulted in lower CrI values (67-76%) compared to that of the enzyme-treated BEKP samples. This indicates breakage of the intermolecular hydrogen bonds between cellulose chains during mechanical pretreatment, which caused damage or peeling of the cellulose crystalline structure. This was also observed by Tonoli et al. (2012), who

reported a certain degree of damage to the NFC crystalline region when ultrasonication pretreatment was performed.

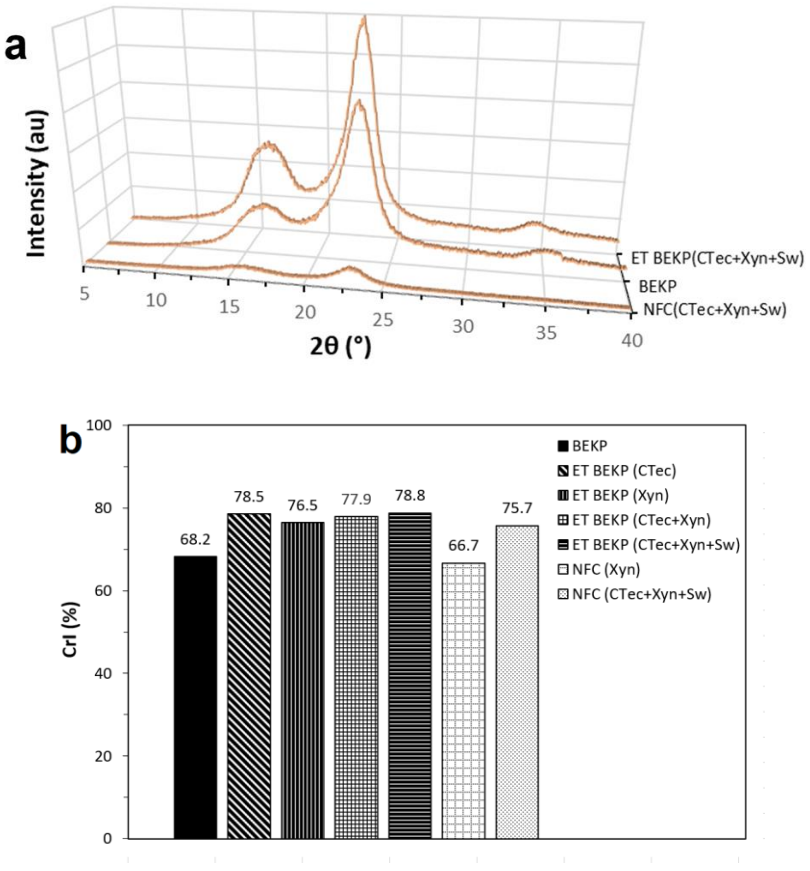

Fig 5. (a) XRD pattern and (b) CrI of BEKP, enzyme-treated (ET) BEKP and NFC obtained under different enzymatic pretreatment conditions. CTec, Xyn and Sw are assigned to CTec3, HTec and swollenin, respectively.

3.6. Thermal analysis

Thermal stability of BEKP, enzyme-treated BEKP, and NFC samples was investigated through sample mass change using thermogravimetric analysis (TGA). Figure 6 shows the TGA and derivative thermogravimetric (DTG) curves of the different samples. According to the TGA curves, the material weight loss during sample heating can be divided into three regions of temperature degradation. The first region starts below 150°C and is attributed to water evaporation on the sample's surface. The second region starts above

200°C until 300°C and represents the thermal decomposition of hemicelluloses, which result more susceptible to thermal degradation than cellulose and lignin mainly due to the presence of acetyl groups. The third and major weight loss happens at high temperatures (310-390°C) due to cellulose thermal decomposition. Thus, a dominant peak is observed in the DTG curves at maximum weight loss. On the other hand, small portions of lignin in the biomass decompose over a broader degradation temperature range (250-700°C) than cellulose and hemicellulose components due to its aromatic ring structure. Table 3 summarizes the thermal properties of the different samples. The thermal decomposition onset temperature (T_{on}) represents the temperature of the beginning of the degradation, and the maximal weight loss temperature (T_{max}) represents the temperature of the maximum degradation rate. An increase on the T_{on} and T_{max} was observed for the enzyme-treated BEKP samples compared to BEKP, probably due to the removal of amorphous hemicellulose and non-crystalline cellulose regions during enzymatic pretreatment. Among the different enzyme-treated BEKP samples, it can be observed that higher T_{on} values (340-342°C) were obtained for samples with higher xylan release (76-85%) during enzymatic pretreatment, which demonstrates that the removal of non-cellulosic components (e.g. xylan) helps to increase the thermal stability.

On the other hand, the obtained NFC samples had a lower T_{on} and T_{max}, due to damages in cellulose crystalline region that occurred during ultrasonication, as it was already observed by XRD analysis. Moreover, several factors such as size reduction, degree of polymerization decrease, and specific surface area increase that occurred during ultrasonication may decrease the thermal stability of the samples. However, the thermal stability of NFC samples obtained in this study resulted better than that previously reported for NFCs produced by TEMPO-mediation oxidation or acid hydrolysis (Fukuzumi et al. 2010; Lv et al. 2019).

Table 3. Thermal properties of BEKP, enzyme-treated (ET) BEKP and NFC samples.

Sample	T_{on} (°C)	T_{max} (°C)	Residue (%)
BEKP	314	365	13.8
ET BEKP (CTec)	335	374	12.3
ET BEKP (Xyn)	330	372	12.2
ET BEKP (CTec+Xyn)	340	375	12.0
ET BEKP (CTec+Xyn+Sw)	342	375	11.2
NFC (CTec)	300	350	14.9
NFC (Xyn)	292	345	19.0
NFC (CTec+Xyn)	324	368	8.3
NFC (CTec+Xyn+Sw)	320	360	16.0

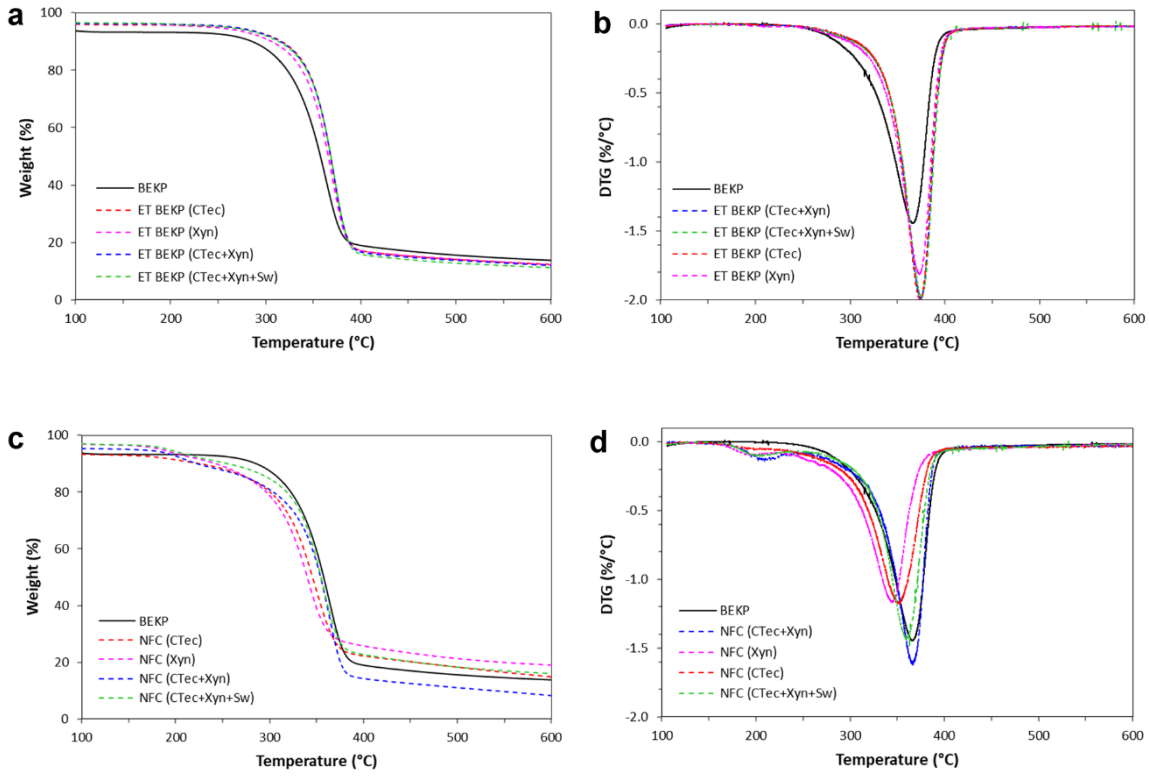

Fig 6. (a, c) Thermogravimetric analysis and (b, d) derivative thermogravimetric (DTG) curves of BEKP, enzyme-treated (ET) BEKP and NFC produced under different enzymatic pretreatment conditions. CTec, Xyn and Sw are assigned to CTec3, HTec and swollenin, respectively.

4. Conclusions

Enzyme-treated BEKP was effectively fibrillated by ultrasonication. The effects of enzymatic pretreatment on both enzyme-pretreated pulp and NFC properties were investigated. Physicochemical characteristics of enzyme-treated fibers demonstrated that the synergism effect between xylanase enzyme cocktail and swollenin during cellulase pretreatment could greatly open up the eucalyptus pulp fibers by selective removal of hemicellulose and fiber morphology changes, increasing cellulose accessibility without compromising cellulose properties. Thus, relatively high NFC yields were achieved with selective enzyme cocktail mediated pretreatment of cellulose, followed by ultrasonication. The combination of enzymatic and ultrasonication yielded cellulose nanofibers with diameters of 3-10 nm, constituting a potential strategy to isolate nanofibers from different BEKP. Production of NFC from BEKP by the proposed process also affords significant amount of sugars released as a coproduct, which can have further biotechnological applications through the production of value-added chemicals.

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