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Published in:
ACS Sustainable Chemistry and Engineering

DOI:
10.1021/acssuschemeng.0c08162

Published: 08/02/2021

Document Version
Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

Please cite the original version:
Berto, G. L., Mattos, B. D., Rojas, O. J., & Arantes, V. (2021). Single-step fiber pretreatment with monocomponent endoglucanase : Defibrillation energy and cellulose nanofibril quality. *ACS Sustainable Chemistry and Engineering*, *9*(5), 2260-2270. https://doi.org/10.1021/acssuschemeng.0c08162

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Single-step fiber pre-treatment with monocomponent endoglucanase: defibrillation energy and cellulose nanofibril quality

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ABSTRACT

The combination of a multistep enzymatic pre-treatment of cellulose fibers and mechanical defibrillation has become a green and low-energy route to obtain cellulose nanofibrils (CNF). However, the variability in the properties of the as-produced CNF remains a major challenge that

needs to be addressed for any application to be realized. Herein, we study the effect of monocomponent endoglucanase (EG) on the energy consumed in defibrillation as well as the physical properties of the obtained CNF. This single-step enzymatic pre-treatment (0.5-25 EGU/g cellulose fibers for 1-3h) reduces the defibrillation energy (by up to 50%) at nearly 100% yield to obtain nanofibrils of similar morphology, crystallinity and crystal size compared to CNF obtained in the absence of pre-treatment. At a mild condition (5.6 EGU/g for 1h), aiming to minimize energy consumption while preserving rheological properties, EG pre-treatment increased the water retention value, reduced the molecular weight and promoted structural surface modification (amorphogenesis), without significant cellulose hydrolysis. In addition, the carbohydrate binding module of the EG was found to improve the interaction of the catalytic core with the substrate. The combination of the factors considered here boost the effect of the enzyme, even if used at low loadings, facilitating high-yield, more sustainable production of CNF.

Keywords: single-step pretreatment; energy reduction; enzyme-substrate interactions; endoglucanases

INTRODUCTION

Mechanical defibrillation is a widespread, effective method to isolate cellulose nanofibrils (CNF) from cellulosic biomass. High-pressure homogenization and microfluidization as well as disc ultra-refining are suitable approaches for industrial-scale production of CNF. They also fulfill the increased demand for new advanced materials based on such renewable nanoparticles.[1–3] Typically, a pre-treatment is required to allow better flow of the fiber suspension through the microchannels, chambers or confined spaces involved in defibrillation

with high-pressure systems. This is key to avoid clogging of the machinery, which otherwise results in an inefficient process, requiring high energy.[4–6]

One of the most used pre-treatments to produce nanocellulose is the oxidation of fibers by using 2,2,6,6-TetraMethylPiperidine-1-Oxyl (TEMPO).[7] TEMPO-oxidized CNF displays reduced fibril size and good colloidal stability but relatively low degree of polymerization (DP) and crystallinity.[8–10] Most importantly, TEMPO-oxidized CNF is not as easily digested by typical cellulases, hindering one the most appealing characteristics of cellulose, such as its biodegradation.[4] Chemical pre-treatments, however, are not required when defibrillating wood pulp into CNF with a disc ultra-refiner. This has been beneficial for the industrial production of CNF given the associated practical, economic and environmental benefits. Importantly, pre-treatment of the starting material remains as an option to improve the production efficiency, further reducing the energy cost.[2] Among the options, enzymatic pre-treatment has been proposed as sustainable and green alternative to the chemical counterparts. Several enzymes, such as endoglucanases, xylanases, lytic polysaccharide monooxygenase or enzymatic cocktails, have been considered to facilitate defibrillation of cellulose fibers into CNF.[6,11–16] Cellulose-hydrolyzing enzymes (e.g., monocomponent endoglucanases, EG) have been explored more widely due to their effectiveness in defibrillating and because their relative low cost, commercial availability and biosafety, both considering the nature of the enzyme and the released products.[5,17–21] The effectiveness of EG stems from their mechanism of action since it preferentially attacks the less ordered regions of the cellulose fibers, randomly breaking accessible glycosidic bonds and introducing new reducing and non-reducing ends. Because of its features, EG has the potential to promote specific modifications, which in turn can aid in the defibrillation process, without compromising the crystalline regions; consequently, the

mechanical properties can be largely preserved. The high mechanical performance of nanocellulose building blocks is a major factor driving their utilization in material development, as the cohesion of the cellulosic constructs tends to mirror those at the molecular level.[22]

Despite the fact that pre-treatment of cellulosic biomasses with monocomponent EG is beneficial for the production of CNF, the reported processing conditions differ greatly and no agreement exists with regards to the effect of reaction time, which spans short (few minutes) and long treatments (up to 72 h). The same can be said about the enzyme loading, ranging three orders of magnitude (from 0.85 to 351 EGU/g fiber).[5,20,21] Besides such wide range of conditions, there is no consensus about their effect on the final properties of the resulting nanofibers. For instance, a considerably mild enzymatic treatment (0.85 or 1 EGU/g, – 2 h) was reported to lead to 24% DP reduction, while harsher treatment (150 or 185 EGU/g – 2h) led to 55% DP reduction when combined with mechanical refining.[5,17] At intermediate severity (25 EGU/g – 2h), the DP was reduced by 50%, however this condition resulted in a smoother mechanical defibrillation.[5,11,20] Pre-treatments have been used combined with mechanical processes prior to the final defibrillation, however they have potential to be a standalone process. Overall, the conflicting results when obtaining CNF under a wide range of conditions and seemingly confusing effects on properties are major challenges that limit industrialization efforts. Therefore, we systematically investigated the effects of processing parameters on the properties of CNF produced after a single-step enzymatic pre-treatment followed by disc ultra-refining. We thoroughly discuss the effects of severity of enzyme treatment (load and time), as well as their synergistic combinations, on key variables such as energy requirements and resulting CNF properties (crystallinity, molecular weight, particle size and viscosity).

EXPERIMENTAL SECTION

Cellulosic Pulp and Enzymes. Never-dried bleached eucalyptus Kraft pulp (BEKP) was kindly supplied by Suzano S/A (Jacareí, State of São Paulo, Brazil). The chemical composition included 78.6% cellulose and 14.6% xylan as obtained by following the procedures described in Sluiter.[23] The monocomponent endoglucanase enzyme (FiberCare®, Lot CGK20074) was kindly provided by Novozymes (Araucária, State of Paraná, Brazil) and applied without further purification.

Enzymatic Activity. The EG enzymatic activity was determined using carboxymethyl cellulose (CMC) as substrate at 0.44% wt (medium viscosity) dissolved in buffer phosphate-citrate (50 mM pH 6.0). The activity of the enzyme was measured using dinitrosalicylic acid by a photometric procedure.[24] The EG activity unit (EGU) was determined as one μmol of glucose released per minute per mL of the enzyme.

Design of Experiments. In order to fully understand the individual and combined effect of reaction time and enzyme loading on the pretreatment of BEKP, a 2^2 full factorial design with triplicates at the central point was employed (Table 1). The highest enzyme loading was 25 EGU/g with a minimum of 0.5 EGU/g and central point at 12.5 EGU/g. The longest reaction time used was 3 h with the shortest and central point at 1 h and 2 h, respectively. These values were chosen based on common values reported in recent literature. Statistical analyses (ANOVA and regression analyses) were carried out using the statistical software Minitab® 18.1 (Minitab Inc. State College, PA, USA) and the index of significance was 95% (p-value < 0.05). Two different scenarios were determined with the Response Optimizer tool in Minitab® 18.1 (Minitab Inc. State College, PA, USA). In Scenario #1, the target was to minimize the energy consumption and in Scenario #2, the target was set to minimize energy reduction and maximize the properties of CNF.

Enzymatic Pre-treatment. All single-step enzymatic pre-treatment experiments were conducted at 50 °C and 5% (w/w) solids (on a dry weight basis) in phosphate-citrate pH 6.0 buffer at a final concentration of 50 mM. The reaction volume (250 mL) was conditioned in 2 L Erlenmeyer flasks and kept in an orbital shaker (Thermoscientific) at a constant shaking speed (250 rpm). At the end of the pretreatment, the reaction was boiled in water for 30 min to inactive the enzyme. Then, the fiber suspension was vacuum-filtered, using miracloth membrane, with excess of distilled water to remove the enzymes. The liquid fraction was collected for quantification of soluble sugars, hydrolysis and solid yields (**Figure 1**). The processing parameters (enzyme loading and reaction time) were selected from reported values of energy savings and following a comprehensive analysis of CNF properties measured after application of a broad range of conditions. The conditions were limited to the enzyme loading of 25 EGU/g pulp and reaction time to 3 h, mainly because conditions over such thresholds have shown to be detrimental to CNF properties. [5,17–21]

Solid Yield of Enzymatic Pre-treatment. The liquid fraction collected after the enzymatic pretreatment was utilized for determination of the solubilized sugars and, therefore, the hydrolysis and solid yield. First, the liquid fraction was centrifuged (13,000 *g* for 5 min), filtrated through a 0.45 μm mesh and subjected to a mild acid hydrolysis (4% H_2SO_4) to hydrolyze any released oligomer to monomeric sugars.[23] The monomeric sugars were then quantified by high performance liquid chromatography (HPLC, Waters), equipped with a HPX87H column (Bio-Rad Laboratories) at 45 °C, eluted at a rate of 0.6 mL/min with 5 mM H_2SO_4, and using a temperature controlled refractive index detector at 35 °C. The method detects glucose concentration down to 0.1 g/L. Considering our processing conditions, the minimum reading accounts to an equivalent of 0.01% of cellulose conversion.

Mechanical Defibrillation. For the isolation of CNF, the enzymatically-pretreated fibers were diluted to 1% (w/w) using distilled water and further defibrillated with a disc ultra-refiner SuperMassColloider (Masuko, model MKCA6-5J, discs model MKGA10-80) (**Figure 1**). The distance between the grinding discs was fixed at amplitude of -100 μm and using 1,600 rpm.[25] The SuperMassColloider was connected to a digital energy monitor (ForLong DRT-341D), reporting the energy consumption in kWh. Simultaneously, after each grinding cycle, 1 mL aliquot was collected and rapidly analyzed in a particle size analyzer to determine the apparent wet surface area of the fibrillated fibers. A suspension of untreated BEKP was also defibrillated under the same mechanical conditions as a control.

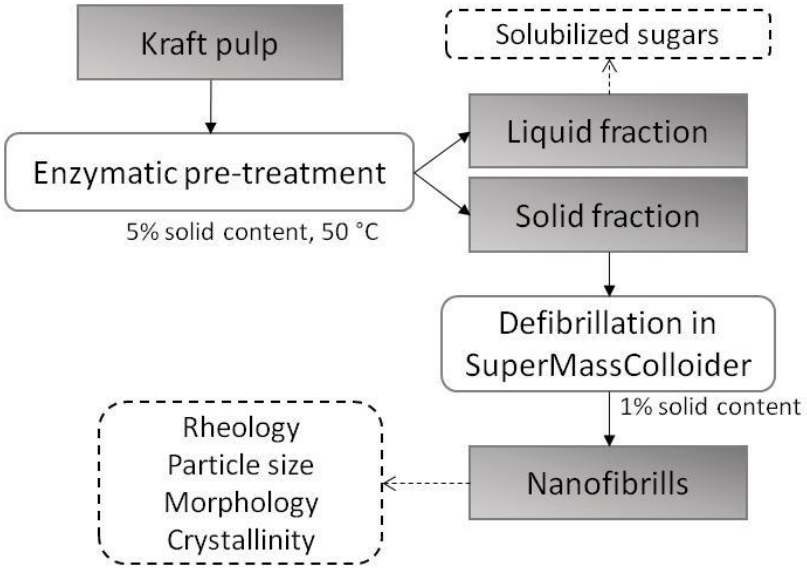

Figure 1. Schematic flow chart of fiber enzyme pre-treatment followed by mechanical isolation of cellulose nanofibrils.

CNF Particle Size and Specific Surface Area (SSA). The apparent particle size and the wet specific surface area of the nanofibrils were determined by low angle laser light scattering (LALLS), using the laser diffraction particle size analyzer Mastersizer 3000 (Malvern Instruments) as described in Berto and Arantes.[25] The particle size number refers to the

hydrodynamic diameter value of an equivalent spherical particle, which is calculated automatically during analysis. The reported values should be taken as relative to infer the degree of defibrillation.

CNF Colloidal behavior. The viscosity and shear stress of the CNF suspensions were determined in an automatic viscometer (Brookfield LVDV2T Pro digital). The spindle SCA-18 was coupled to the viscometer, the rotation set at 100 rpm and the temperature fixed at 25 °C using a water bath. The suspensions were prepared with a solids content of 0.25% (w/w) in distilled water. Each analysis was conducted for 5 minutes, with measurements every 30 seconds and the results are reported as the average of 10 measurements.

The rheology of the CNF suspensions – isolated at the optimized condition – was evaluated using an MCR 302 rheometer (Anton Paar, Austria), equipped with a parallel flat plate geometry and smooth bottom plate at controlled temperature of 23 °C. For the shear sweep it was applied a shear rate of 0.01 – 1000 s^{-1} at angular frequencies ranging from 100 to 0.01 rad/s with a strain amplitude of 0.1% (within the linear viscoelastic region). For each assay, triplicate measurements were conducted for each sample (validation 1 and 2), repeated separately, and the samples were changed for each collecting data.

CNF Crystallinity. For X-ray diffraction analyses, samples were dried in an air circulation oven at 33 °C for 24 hours. The measurements were performed in an X-ray diffractometer (XRD - 6000, Shimadzu) at room temperature, with CuKα radiation and a graphite monochromator (reflection mode analysis), and the conditions were $10 < 2\theta < 40$; 2θ step: 0.02°, 30 s per step, taken in duplicate. The XRD peaks were mathematically deconvoluted using a Gaussian function in the Origin software (version 2017, OriginLab). The CI of the CNFs was determined according to Segal.[26]

Fibril Morphology. Negative contrast scanning electronic microscopy (Neg-SEM) images were obtained following the methodology described by Mattos.[27] Briefly, for sample preparation, freshly cleaved mica discs were spin-coated with 4 nm of iridium, dipped in a polyethylenimine (PEI) solution (0.33% w/v), washed with MilliQ water, dipped in the CNF suspension (0.001% w/v) and dried at room temperature. The images were acquired in a field emission gun electron microscope (FEG-SEM) Zeiss Sigma VP (Germany) using the *in-lens* detector, acceleration voltage at 1.5 kV and working distance of 6 mm. The diameter of at least 20 fibers were measured using the software ImageJ.

CNF Thermal Stability. Thermogravimetric profiles of CNF obtained with and without enzymatic pretreatment were acquired in order to investigate possible structural modifications in the cellulosic matrix. Thermal stability analyses were performed in the thermal analyzer TGA-TA Instrument DST-Q500 in an inert atmosphere (N_2, flow rate 60 ml/min), with a heating rate of 10 °C/min and a temperature range of 30 - 600 °C. The samples were freeze-dried and approximately 5-8 mg was weighed in platinum pan.

Evaluation of the mechanism of EG action. Optimal enzymatic processing conditions, as far as rheological properties and energy input were used (Scenario #2): 50 °C, phosphate-citrate 50mM buffer pH 6.0, enzyme loading 5.6 EGU/g for 1 h) to investigate the effect of EG.

Degree of Polymerization. The typical behavior of depolymerization that EGs promote on cellulose chain was investigated by gel permeation chromatography (GPC), using pullulan as standard, as described in Potthast.[28] Briefly, a known mass of cellulosic fibers (500 mg) EG-pretreated and the control sample were dissolved in LiCl/DMAc (lithium chloride/N,N-dimethylacetamide) at a given final of sample concentration of 1.0 mg/mL. The samples were prepared in duplicate and each was run twice using LiCl/DMAc as eluent solution.

Water Retention Value. The water uptake caused by the fiber swelling by the EG was evaluated following a gravimetric methodology.[29] A known mass (approximately 0.2 g) of the enzymatically pretreated pulp and control was soaked in an adequate amount of distilled water to fully immerse the sample for at least 1 h (at room temperature). Afterward, the samples were drained using nylon membrane and the masses were measured. The difference of initial and the final mass was assumed to be the remaining water, which interacted with the cellulose fibers. The measurements were conducted in duplicate.

EG – Cellulose Interactions. The interaction of the EG monocomponent with the cellulosic substrate was investigated using a quartz crystal microbalance with dissipation (QCM-D, Q-Sense D-300). The frequency and dissipation data were collected using the 3rd, 5th, 7th and 11th overtones during the operation. The QCM-D gold crystals were treated with UV/ozone for 20 min and dipped in a PEI solution (0.4 mg/mL) before being covered by spin coating with a thin layer of mechanical fibrillated CNF (0.008 wt%). The crystals were placed in buffer to swell overnight and dried with nitrogen before being used. The crystals were placed in the cells and the buffer solution started being pumped at a continuous flow rate of 40 μL/min until the baseline reached the equilibrium. Afterwards, the enzyme solution was continuous pumped at the same flow rate for 60 min or until the baseline reached the equilibrium. Finally, the solution was switched back to buffer to wash the enzymes out of the system.

RESULTS AND DISCUSSION

Effect of the EG pretreatment on CNF properties. The wet specific surface area (SSA) of the fiber suspensions were monitored in real time (laser diffraction). The defibrillation process was stopped when the SSA value reached ~200 m^2/kg (**Table 1**), which corresponds to nanofibrils of ca. 20 nm width, as previously determined by atomic force microscopy.[25] Such value was used as

an internal measurement of the defibrillation process for BEKP, automatically calculated and reported in m^2/g as standard unit of this particular equipment. Despite the empirical nature of the wet method to determine SSA, it was found useful to monitor the defibrillation process in real-time (note: the SSA results are not to be taken as absolute surface areas since the measurement assumes a hydrodynamic diameter of an equivalent spherical particle, significantly underestimating the real surface area).

All enzymatic pre-treatments led to negligible release of glucose (**Table 1**) and xylan (data not shown), allowing to near perfect recovery for further processing into nanofibers. Therefore, it was assumed that the total solid yield after the enzymatic pretreatment was ~100% for all conditions tested; hence, the hydrolysis yield was not considered as a response variable in the statistical analyses. However, variations in the severity of enzymatic treatment led to notable changes in the properties of the obtained CNF and respective suspensions (**Table 1**).

Table 1. For the enzymatic pretreatment of BEKP, a 2^2 Full factorial experimental design matrix was used with repetitions at the central point. The outputs (response) were associated with the physicochemical properties of the obtained CNF suspensions, energy input and solubilized glucose.

Std. order	Variables		Outputs						
	Enzyme loading (EGU/g)	Reaction time (h)	SSA* (m^2/kg)	Glucose released (%)	CI** (%)	Particle size (µm)	Viscosity (cP)	Shear stress (Pa)	Input energy (kWh/kg)
1	0.5	1	208.1	0.00	60.8	10.9	23.8	3,14	16
2	25	1	209.5	0.00	55.5	12.5	16.7	2.21	12
3	0.5	3	207.2	0.00	56.1	10.8	18.2	2.40	21
4	25	3	204.1	0.03	62.5	13.6	15.1	1.99	10
5	12.5	2	224.6	0.01	52.3	11.9	19.7	2.61	13

6	12.5	2	214.0	0.01	62.3	12.2	17.8	2.35	13
7	12.5	2	226.7	0.01	55.4	12.0	19.3	2.55	11
Control	-	-	209.6	n.d.	54.7	11.3	23.1	3.05	20.5

SSA= specific surface area; CI** = crystallinity index; n.d. = not determined.*

The effect of enzymatic pretreatment on the CNF properties and the energy input was evaluated according to statistical differences and variances (ANOVA, **Table 2**). The significance of each main variable (factor) and their interaction are reported as *p*-value (values < 0.05 indicate a factor that is statistically significant). The curvature *p*-values are higher than the statistical significance of the factors to all responses; hence, indicating the suitability of the nominal model for each response.

Table 2. Response matrix with p-values of the ANOVA test

	Curvature	EL*	RT**	Interaction	R^2	R^2 adjusted	Adjusted model
CI***	0.656	0.908	0.811	0.282	37.39	-	-
Particle size	0.058	0.990	0.929	0.970	0.37	-	-
Viscosity	0.577	0.012	0.029	0.124	94.42	88.84	Viscosity = 24.91 – 0.2076 EL – 1.805 RT
Shear stress	0.576	0.012	0.029	0.125	94.35	88.71	Shear stress s=3.289 – 0.02733 EL –0.2388 RT
Input Energy	0.111	0.036	0.518	0.187	84.80	69.61	Energy = 17.62 – 0.3061 EL

EL= enzyme loading; RT**= reaction time; CI***= crystallinity index.*

CNF with the same degree of defibrillation, as determined by the given value of SSA (ca. 200 m^2/kg), displayed statistically equal CI%, regardless of the severity of the pre-treatment (**Table**

2). Therefore, CI% was not considered for further construction of predictive statistical models. The CI% of the CNF obtained from enzymatically-pretreated fibers ranged from 52 to 62%, regardless of the enzyme dosage or reaction time (**Table 1**). EG acts preferentially on the disordered regions of cellulose, which presumably should increase the crystallinity. However, while some authors have reported that EG treatment of cellulose increases CI%,[12,20,30] others have observed the opposite.[19,31] For example, Nechyporchuk[2] measured a CI increase from 82% for the starting cellulosic pulp to ca. 86% after enzymatic treatment (21, 210 or 315 EGU/g for 2h). Meanwhile, Siqueira[31] reported a CI reduction, from 90% for the starting material to 88% after EG treatment (400 EGU/g for 72h).

The relative average particle size of the CNF, as determined by laser diffraction, ranged from 11 to 13 μm (**Table 1**). There was no trend as far as the enzymatic loading and reaction time. Under harsh enzymatic pre-treatments, larger nanofibrils were produced. These points to the inherent variability of the mechanical fibrillation, which leads to highly polydisperse nanofibers, outweighing any effect of enzymatic pre-treatment on fibril size. In addition, particles of similar size were expected as the mechanical processing was halted after reaching the target SSA value, since both properties are closely related. The enzyme pretreatment promoted enhanced defibrillation and increased CNF networking ability. This assumption was confirmed by ANOVA analysis (**Table 2**). That is, for both main factors and their interaction, the *p*-value was > 0.05 (**Table 1**), indicating that neither the reaction time nor enzyme loading (or their interactions) promoted statistically significant changes in the CNF average particle size compared to the CNF control.

The effect of the EG pretreatment was more evident when observing the rheological properties of the CNF suspensions, which tracked with the collective colloidal behavior of the nanofibrils.

Both, enzymatic loading and reaction time, as well as their synergistic interactions, led to significant changes in the viscosity of the CNF suspensions (**Table 2**). There was a significant decrease in viscosity and shear stress after pre-treatment under the most severe conditions (25 EGU/g for 1 or 3 h) and, compared to the CNF control, a less pronounced change was noted for the central and milder conditions (12.5 EGU/g - 2h) (**Table 1**). Under the mildest condition (0.5 EGU/g), the prolonged reaction time outweighed the rheology changes. From the coefficients of the adjusted models, **Table 2**, one can see that the reaction time had a more significant effect (one order of magnitude higher) on both viscosity and shear stress, e.g., when compared to the enzyme loading. In fact, both the enzyme loading and the reaction time were significant (*p*-value < 0.05) as far as the rheology results of the CNF suspension; however, their interaction was not significant. Such observation arises from the most impactful effect of the reaction time that is independent of the enzyme loading. This effect is likely associated with the expected reduction in DP and surface accessibility. EGs can reach and break accessible glycosidic bonds, thus reducing the DP, as will be discussed later. This has been shown to have a strong influence on the viscosity and shear stress of CNF suspensions.[5,32] Considering that only a very limited fraction of cellulose is accessible to the enzymes, longer reaction times allowed higher EG-cellulose interactions, leading to an efficient activity of the enzyme over the cellulose chain.

*Effect of the EG pre-treatment on the defibrillation energy***.** The energy consumed during defibrillation was significantly affected by enzymatic loading (**Table 2**). With the higher enzymatic loading (25 EGU/g for 1 or 3h), the energy consumption was reduced from 20.5 (control sample) to ~10 kWh/kg, a 52% reduction. The energy consumed was reduced by 38%, from 20.5 to 13 kWh/kg, when lowering the enzyme loading to 12.5 EGU/g for a reaction time of 2 h. However, the energy consumption and the enzyme loading were not proportionally

related. For instance, a two-fold increase of enzyme loading (from 12.5 to 25 EGU/g) did not lead to equivalent energy reduction. This clearly marks a threshold of energy saving from the enzymatic pre-treatment, with no benefits from a high enzyme loading.[18–20] Considering that the extension of reaction time and its interaction with enzyme loading were not statically significant, both factors were removed from the models used to predict the optimal conditions to minimize the energy consumption while preserving the CNF properties.

The enzyme load, within the range tested, had a positive effect on energy reduction as evidenced by the negative term in the adjusted equation for energy consumption (**Table 2**). As previously discussed, the reaction time had no effect on energy reduction (p-value > 0.05, **Table 2**). This indicates that a short reaction time (at least 60 min) is sufficient for the enzyme to produce the intended modification that facilitates defibrillation; therefore, decreasing the required energy input. Similar to our findings, Tarrés[21] observed that reaction time (2-4 h) did not increase the nanofiber surface area, even at a higher enzyme loading . On the other hand, when the enzyme loading was increased, from 0.5 to 25 EGU/g, it intensified the action of the EG on the cellulose chains, consequently promoting defibrillation energy saving (52% reduction compared to the control values).

*Optimization of enzymatic pre-treatment for CNF production: trade-off between energy input and CNF properties***.** From the observed experimental responses for EG pre-treatment, it is possible to draw a more precise prediction of different scenarios or conditions for optimal conditions for EG pre-treatment, keeping the objectives of defibrillation energy saving and CNF suspension rheology. For the predictions, the same weight was considered for all the outputs (viscosity, shear stress and energy consumption), which were statistically significant according to the ANOVA analysis (**Table 2**). The optimization process was based on the creation of

scenarios leading to the largest energy reduction (Scenario #1) or to a compromise between the highest energy reduction and CNF properties (Scenario #2). As such, Scenario #1 targeted an energy reduction from 20 to 10 kWh/kg) and the predicted condition was the same achieved by applying the highest enzyme loading (25 EGU/g) for the longest reaction time (3 h). In this scenario, the energy saving was 50% compared to the control, but the penalty was sub-optimal rheological properties. Compared to the control sample, the EG pre-treatment (25 EGU/g for 3h) reduced the apparent viscosity, from 23 to 15 cP, along with a reduction in the shear stress, from 3.05 to 1.99 Pa. Controlling the viscosity of the CNF suspensions warrants applications where control of flow is necessary, as is the case of extrusion, paper making,[33] coating/printability,[34] 3D printing, filament spinning or spraying. Although viscosity can be readily adjusted with the CNF mass fraction, the need of a high solids content to achieve a given viscosity is detrimental to most applications. Therefore, although the noted enzymatic pretreatment leads to high energy saving, by 50%, it may not be suitable for large scale operations, given the detrimental effect on CNF properties.

In Scenario #2, the processing conditions were optimized to account for the minimum energy input (10 kWh/kg) and target rheological properties (shear stress and viscosity of 3.1 Pa and 23.8 cP, respectively). An enzyme loading of 5.6 EGU/g for 1 h reaction time should be used to fulfill such criteria. In these conditions and by combining the individual adjusted models of each response, the model predicted an energy input of 15.9 kWh/kg to produce CNF while preserving the rheological properties (viscosity 21.9 cP and shear stress 2.89 Pa) (**Table 3**). The desirability composite equal to 66 % indicates the combination of the variables is able to achieve a positive result for global combination of all responses. Considering that under this scenario the properties of the CNF were preserved, this condition was taken for the purpose to further investigate the

mechanism involved in energy reduction *via* enzymatic treatment. In order to validate Scenario #2, BEKP was enzymatically treated under the predicted optimal condition (5.6 U/g for 1h), defibrillated and characterized following the same methods previously described. The experimental responses for the CNF suspensions fell within the predicted values (**Table 3**). The experimental average energy consumption was 15 kWh/kg, 21.59±1.07 cP viscosity and 2.58±0.14 Pa shear stress. These values validate the predicted Scenario #2 and give optimized conditions to pretreat fibers, aiming at an efficient and more sustainable CNF defibrillation.

Table 3. Predicted and experimental responses for CNF generated under the optimal condition (5.6 U/g - 1 h) for scenario #2.

			Predicted		Experimental values		
	Scenario 2	Target	Solution	SI* 95%	#1	#2	Average
Input energy (kWh/kg)	Minimum	10	15.89	13.03-18.74	15	15	15
Viscosity (cP)	Maximum	23.76	21.93	19.52-24.34	23.72	19.45	21.59±1.07
Shear Stress (Pa)	Maximum	3.13	2.89	2.57-3.21	3.16	2.58	2.87±0.41
Desirability	-	-	0.662	-	-	-	-

**Significance index.*

The properties of the CNF produced from under optimized condition (5.6 EGU/g for 1h) were more deeply investigated as far as morphology, thermal stability and rheology. The CNF suspension obtained by the enzymatic pre-treatment (EG-CNF) was compared to the control sample (CNF).

The morphology of the nanofibrils was analyzed by negative contrast scanning electron microscopy (Neg-SEM). The overall morphology, as well as CNF lateral dimension and

distribution are shown in **Figure 2**. The general aspect of EG-CNF and CNF was similar, displaying a high aspect ratio and widths < 50 nm and producing an entangled nanofibril network. In both CNF suspensions, the nanofibrils with high level of defibrillation, with small widths, were found homogeneously distributed across the network. It was also possible to observe fibril bundles with large diameters (ca. 100 nm). This is a typical characteristic of CNF obtained by mechanical defibrillation.[35,36]

CNF is industrially competitive and its intrinsic morphological features allow to achieve improved material mechanical performance.[22] The median widths of the EG-CNF were slightly smaller (26 nm) compared to the control CNF (31 nm), with sizes spanning the range between 3 to 116 nm, compared to 8 to 150 nm, respectively (**Figure 2**). Considering a preserved fibril length (**Table 1**), a smaller width implies a slightly higher aspect ratio for the EG-CNF. The absence of bigger fiber bundles may warrant more homogenous materials assembled from such EG-CNF, as well as potentially stronger materials, considering that the interparticle interactions can be better controlled with narrower particle distribution. Indeed, a homogenous suspension displays a better networking capability, while heterogeneous ones include aggregates and are inefficient in networking.[37] The width distribution results (**Figure 2**) also show that EG-CNF has a considerable narrower diameter size range, clustering (85%) within 3 – 40 nm, and entangled fibrillar structures that leads to good networking formation. Compared to the reference CNF, the enzymatic treatment resulted in a slightly more uniform suspension, as CNF showed size clustering (78%) with widths distributed within the 8-to-40 nm range.

The high aspect ratio of the nanofibrils and their entangled network strongly influence the rheology of the suspension. The storage and loss modulus profiles of EG-CNF and CNF in aqueous suspension (1 % wt) indicate a gel-like behavior (**Figure 3A**). The storage modulus was

10-fold greater than the loss modulus, indicating the viscoelasticity of the suspensions, owing to the strong interfibril interactions within the entangled network, the same CNF profile obtained by mechanical and enzymatic pre-treatments described by Paakko.[17] The EG-CNF suspension has a slightly higher storage (G') and loss (G") moduli than the CNF suspension, which can be attributed not only to the entangled network but also to a greater surface interaction between the fibrils and water. A similar profile was observed in the profiles of shear stress as a function of strain (**Figure 3B**), showing that the EG-CNF suspension had a slightly higher elastic modulus compared to CNF.

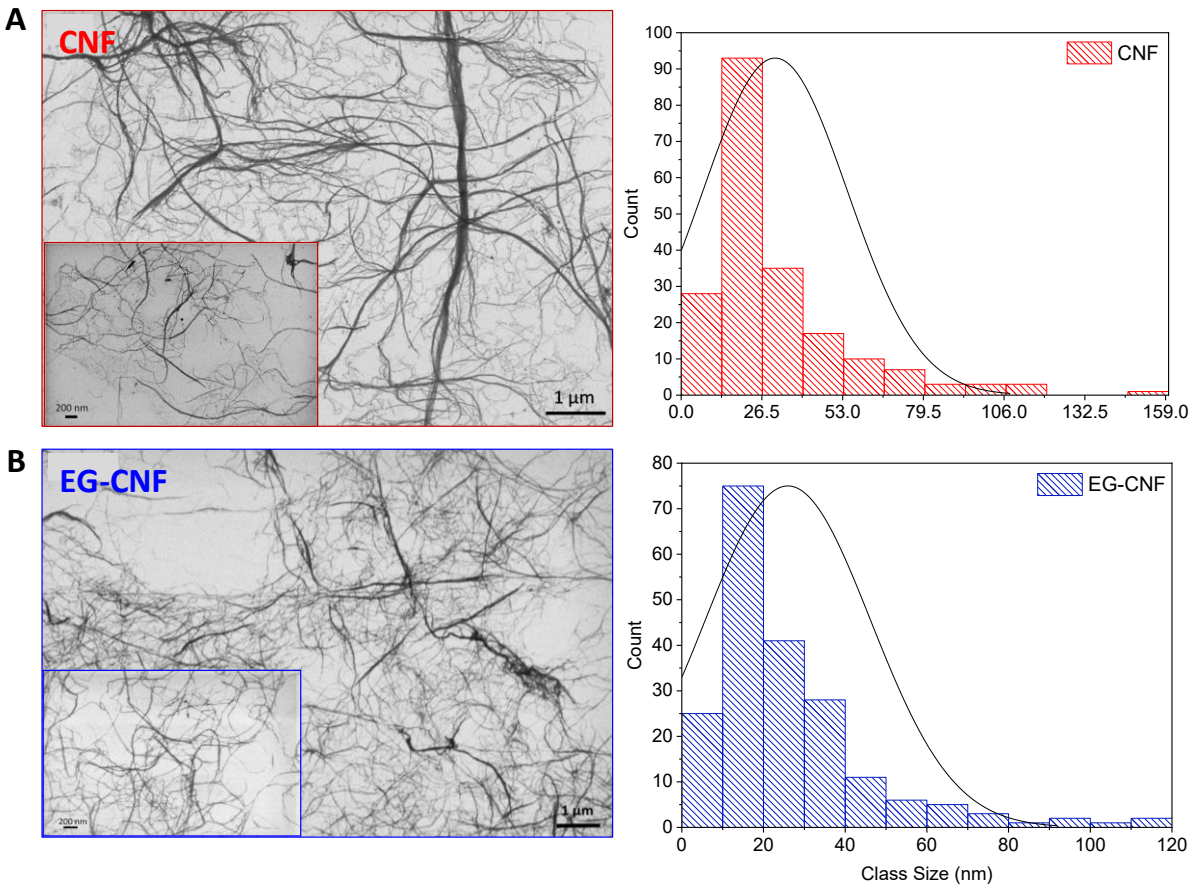

Figure 2. Morphology accessed by high contrast SEM images and the respective distribution of

lateral sizes of nanofibrils isolated from BEKP *via* defibrillation with a SuperMassColloider: (A) without and (B) with EG pre-treatment (scenario #2, 5.6 U/g - 1 h).

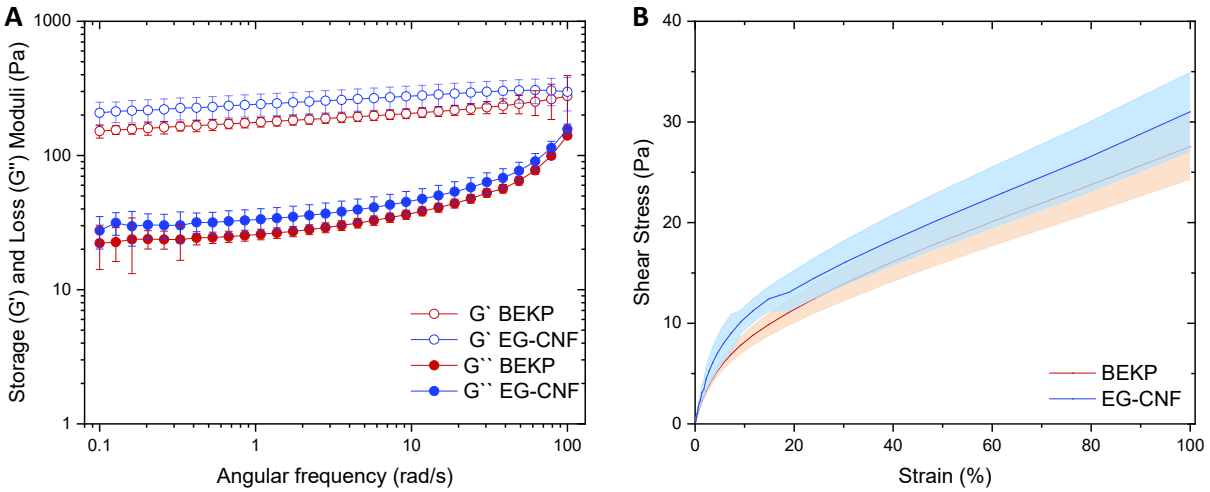

Figure 3. Rheological characterization of CNF obtained after optimal EG pre-treatment (blue, EG-CNF) and in the absence of enzymatic treatment (red, CNF). (A) Storage (empty symbols) and loss (filled symbols) moduli as function of angular frequency at 1% of solid content using parallel flat plate geometry. (B) Shear stress-strain profiles.

The pre-treatment with EG led to a significant reduction in the defibrillation energy. The action of the enzyme on cellulose under mild enzymatic pretreatment (5.6 EGU/g – 1 h) improved the cellulose surface reactivity, with no significant release of soluble products. This allowed better interactions with water, thus modifying the rheological behavior of the suspension. In a related study, Ibarra[32] demonstrated that this specific monocomponent EG was very efficient to increase the cellulose surface reactivity. The authors attributed this increase to i) the inverting catalytic mechanism, which it is more efficient on the disordered regions along the cellulose fibrillar structure and, ii) the presence of a secondary protein domains, the carbohydrate binding module (CBM), which gives EG a high binding efficiency.[32]

A key property of CNF is its thermal stability, especially considering applications such as reinforcement of polymeric composites, usually processed under heat. The thermogravimetric curves (derivatives) of both EG-CNF and CNF displayed similar profiles. The initial degradation temperature (T_{onset}) of EG-CNF and CNF was found to be ca. 230 °C, which agrees with the thermal degradation of pure cellulose.[38] CNF, however, was slightly more stable at T_{onset} than EG-CNF (**Figure 4 inset**). This is likely a result of EG action on the disordered segments of cellulose that were deconstructed into fragments with chains of smaller molecular weight (Mw), more susceptible to thermal degradation. This can be attributed to the action of EG that decreases the DP, resulting in an early-initial thermal degradation.

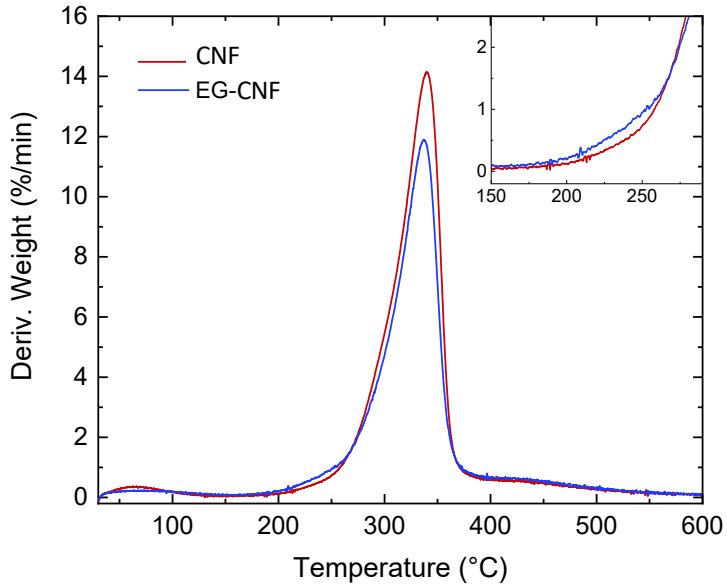

Figure 4. Derivative thermogravimetric (DTG) curves of EG-CNF and CNF lyophilized samples. Inset: detailed DTG view from 150 to 300 ℃.

Herein, Scenario #2 for enzymatic pretreatment of BEKP smoothly shifted the rheology, morphology and the thermal stability, in addition to saving defibrillation energy. Apparently, it is sufficient to apply EG at low dosages and short reaction times to promote cellulose modification.

Moreover, this slight modification facilitates the defibrillation process, saving energy and preserving key properties. Our work agrees with previous efforts,[17,18,21] confirming the positive effect of a single-step enzymatic pre-treatment. However, the mechanism of enzymatic action during defibrillation still require elucidation, which is a topic that we discuss next.

EG mechanism of action in defibrillation. Under the tested conditions, the monocomponent enzyme used herein (EG) did not release soluble products from cellulose, even when applied at the highest severity (**Table 1**). Nevertheless, it was possible to save up to 50% defibrillation energy in (25% when the pretreatment condition was optimized to preserve rheological properties). To better understand how the EG promotes changes in the substrate, leading to efficient defibrillation at low energy input, we investigated structural modifications of cellulose fibers promoted by the enzyme. Further, the substrate-enzyme interactions were investigated by using quartz crystal microgravimetric analyses (QCM). The structural changes in the cellulose chains were investigated in the treated fiber. The BEKP treated enzymatically by the monocomponent EG, at the optimum condition (Scenario #2, EG-BEKP) was compared with the non-pretreated BEKP.

EGs are well-known to reduce the DP by randomly cleaving the glycosidic bonds and inserting new non-reducing and reducing ends in less organized regions of cellulose. This tends to reduce the molecular size of cellulose chains, leading to smaller cellulose fragments at the molecular level (**Figure 5A**). The analyses of molecular weight distribution of the EG-BEKP and BEKP allowed identification of structural differences between the two fiber types. The EG-BEKP treated under Scenario #2 displayed an average molecular weight of approximately 284 kDa, a reduction of 17 % compared to the BEKP (341 kDa) (**Figure 5B**). Ibarra[32] reported a reduction of molecular weight of ~8% using the same enzyme and dissolved pulp from hardwood as

substrate. This different extent of reduction is likely related to the differences of the substrate and their recalcitrance to hydrolysis. By analyzing the molecular weight distribution, one can observe that the starting material (BEKP) was heterogeneous and clustered as fragments with two clear peaks. The same profile was observed for the treated fibers (EG-BEKP). However, EG-BEKP had its molecular weight distribution shifted towards the lower molecular mass region. This confirms the typical action of EG, e.g., reducing the DP, even under a very mild reaction condition, making the cellulose surface more reactive,[32] therefore facilitating defibrillation.

The surface modification promoted by EG can improve the interaction of cellulose with water. Specifically, our EG has been described to efficiently improve fiber swelling, having an evolutionary similarity with enzymes specialized in swelling of cellulosic fibers (i.e., swollenin).[39–41] In related inquiries, we determined the water retention value (WRV) (tea-bag method, **Figure 5C**): EG-BEKP had higher WRV when compared to the control fibers (BEKP), indicating a possible increase in the swelling capacity of the fibers accentuated by the EG treatment. WRV was also increased by the surface activation caused by the CBMs. The interaction/penetration of CBM in the cellulose hierarchical structure promoted a re-arrangement of the cellulose chains, without hydrolytic effects, and more water interacted with the cellulose surface, a phenomenon called amorphogenesis. [42,43]

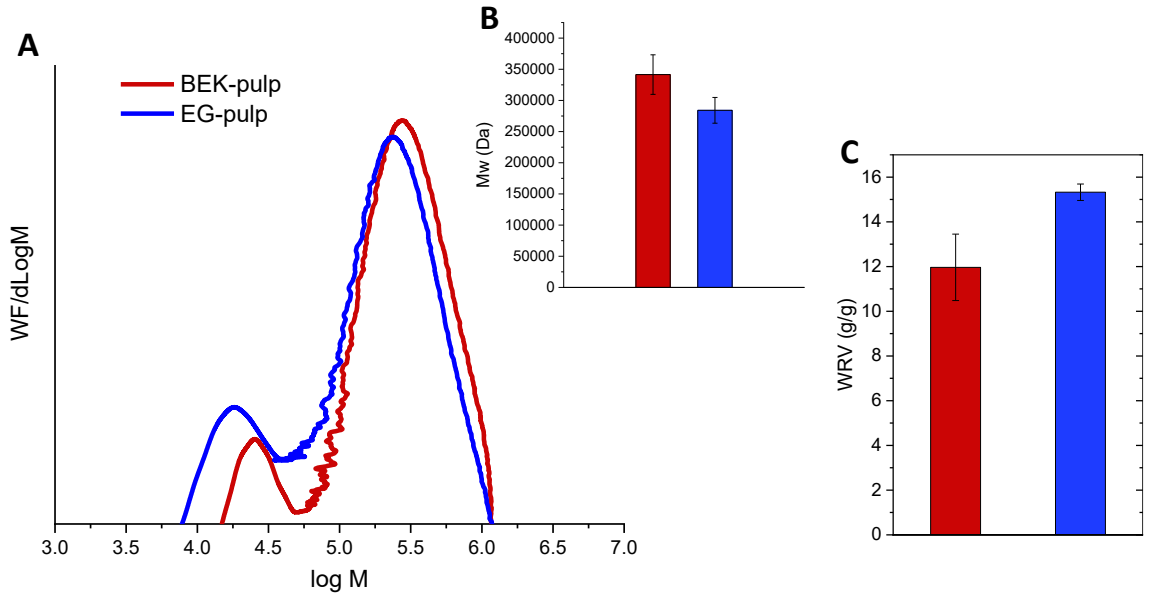

Figure 5. Molecular weight (gel-permeation chromatography) of non-treated fibers (red, BEKP) and those treated with EG (blue, EG-BEKP). (A) Distribution of retention volume versus RI of molecular weight; (B) average molecular weight and; (C) water retention value (WRV) by tea-bag methodology.

The cellulose-enzyme interfacial interactions were evaluated by QCM-D using CNF model surfaces (50 °C, phosphate-citrate 50 mM pH 6.0, (**Figure 6**) subjected to EG at 5.6 EGU/g (40 µL/min for at least 60 min). EG showed a rapid adsorption on the cellulosic surface, right after injection in the system (first 15 min) followed by a gradual adsorption over 1h that did not reach a plateau value (**Figure 6** – black profile). Such profile shows a high time-dependency for the enzyme to efficiently adsorb on the cellulose surfaces, explaining why the reaction time was a significant variable in modifying the viscosity and shear stress of the CNF suspension produced from EG-BEKP. The enzyme was more efficient to adsorb on the cellulose surface at the beginning of injection, which correlates closely with the available free-surface of the substrate.

The dynamics of adsorption, slowing down rapidly, is possibly driven by the more intense traffic of enzyme on the cellulose surface. However, the frequency continued to decrease at a lower rate compared to the first 15 minutes, which can be rationalized if one assumes that the enzyme was still adsorbing and water molecules interacted strongly with the surface. However, possible adsorption in multilayers cannot be ruled out. The increase in WRV (**Figure 5C**) and the decrease in the molecular weight (**Figure 5A and 5B**) agree with this observation. The frequency shift, which correlates with the mass adsorbed on the substrate, was remarkably lower when compared to the adsorption of other proteins on cellulosic surfaces.[44] The difference in frequency intensity is closely related to the total protein mass applied in each study. While Josefsson[44] applied 10 μg of the EG Novozyme 476, herein enzyme solution was used at 5.6 EGU/g. Although much higher enzyme dosages achieve faster adsorption, they are shown to be unnecessary or excessive for defibrillation.

The dissipation reached a plateau value after 30 min of injection (**Figure 6)**. Thus, the modification of the viscoelasticity property of the cellulose substrate occurred within the first 30 min of enzyme action. Such modification is a result of two distinct phenomena, i) the adsorption of enzyme on the cellulose surface, adding a new layer of protein onto the surface and, ii) structural modification on the cellulose surface that allows better water interaction with the cellulose. As the energy consumption response was not affected by the reaction time, it is likely that the effects are closely associated with the modification of viscoelasticity rather than solely the enzyme adsorption. The presence of CBM is a key feature for efficient energy reduction, as such domains promote structural modification[42,43] and bind to the cellulose surface in a short period of time. In our study, the CBM is expected to assist the binding of EG on the surface of the cellulose fibers, considering the low enzyme dosage and short reaction times used. The

catalytic domain could efficiently act and promote superficial modification, without having a more drastic catalytic activity, for example, realizing products and preserving the morphology and rheology, in addition to saving 25% of energy during the defibrillation process.

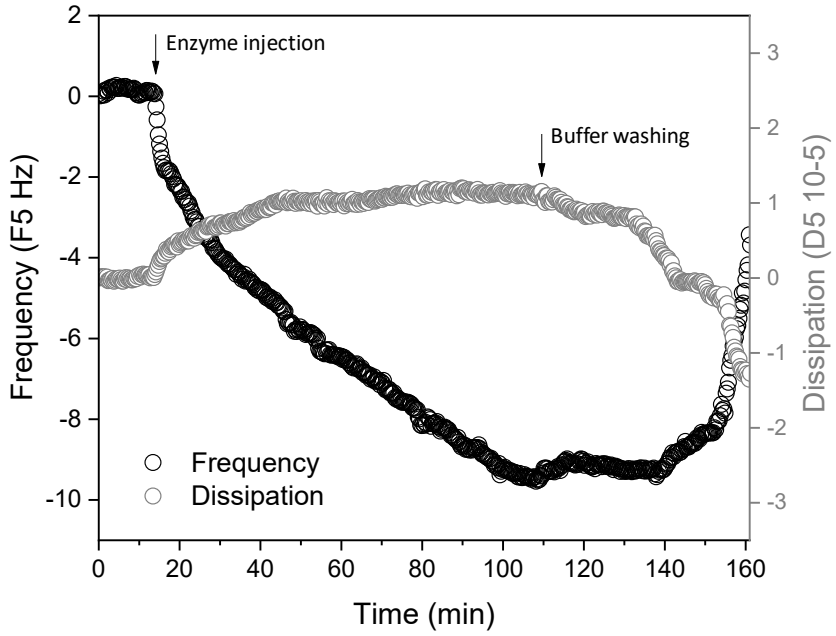

Figure 6. Interaction of nanocellulose surface with monocomponent EG as function of time by the QMC-D technique, where the black line is the frequency curve and grey line is the dissipation curve.

The analyses of molecular weight distribution, water retention value (WRV) and enzyme-cellulose interaction, all suggest that the monocomponent EG, when used at the optimum conditions (5.6 EGU/g for 1h), slightly reduced the DP, swelled the fiber to a great extent and adsorbed to the cellulose surface. Altogether, EG changed the molecular structure of the cellulose and its viscoelasticity behavior. Such mild modification facilitated the defibrillation process of BEKP, reducing the fibrillation energy, while preserving the morphology of the nanofibrils and improving the rheology of the obtained EG-CNF suspension.

The enzyme action did not lead to sugar release, especially at the optimized pretreatment condition (5.6 EGU/g – 1 h). Therefore, there was no contribution of the product solubilization on the reduction of the energy demand in the defibrillation process. However, it has been shown that EG typically can also promote other modifications such as reduce the viscosity of cellulose suspensions.[32] Thus, it appears that the structural modifications in cellulose promoted by the EG and its CBM were most relevant to improve the CNF production by disc ultra-refining than the hydrolytic effect to solubilize products. This insight allows new opportunities to explore hydrolytic enzymes, screening new enzymes and engineering proteins to promote structural modifications with very low or no hydrolytic effects and, potentially, to improve the enzymatic pretreatment for CNF production.

CONCLUSION

A comprehensive investigation was carried out by a single-step enzymatic pretreatment using a monocomponent EG for CNF production via disc ultra-refiner. By varying the enzyme loading and reaction time, it was possible to significantly reduce (25-50%) the energy input for defibrillation. Severe EG pre-treatment conditions led to a reduced energy consumption (50%) at the cost of reduced quality. Meanwhile, a milder condition led to 25% energy reduction while achieving a good CNFs rheology profile. Thus, EG pre-treatment allows to select the conditions to achieve the desired final property and energy saving. The process conditions were optimized to find a compromise between energy input and quality of the defibrillation. It was demonstrated that the EG applied at 5.6 EGU/g for 1 h was optimal as far as a balance between energy input and properties. The EG pretreatment, with negligible hydrolytic effect i) improved the cellulose-water interactions and led to greater fiber swelling, ii) reduced slightly the molecular weight,

introducing new reducing and non-reducing ends and, iii) modified the cellulose due to the strong binding of the CBM. The combination of these effects and the tailored condition for enzymatic pretreatment step, enabled a reduced defibrillation energy and allowed isolation of CNF. Moreover, the enzymatic pretreatment reported can be easily conducted in only a single-step, in addition to being a green and sustainable route to facilitate the CNF production from bleached fibers.

ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation (FAPESP, grant number 2015/02862-5) for financial support. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Finance Code 001. The authors also acknowledge support by the European Research Council under the European Union's Horizon 2020 research and innovation program (ERC Advanced Grant Agreement No. 788489, "BioElCell"), and the Canada Excellence Research Chair initiative. We gratefully acknowledge Suzano Cellulose (Jacareí, Brazil) and Novozymes (Araucaria, Brazil) for supplying the cellulose pulp and the enzymes, respectively. Finally, we thank Dr. Rafael Grande for support with microscopy imaging.

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Table of Content Graphics

Energy consumption and properties of cellulose nanofibers produced after single-step, low-loading enzyme pretreatment of wood pulp.