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ORIGINAL RESEARCH

Enzyme-assisted production of cellulose nanofibers from bleached and bleached/sulfonated sugarcane bagasse: impact of sulfonation on nanocellulose properties and yields

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Abstract Partial enzymatic hydrolysis is a green alternative to chemical processes to facilitate the isolation of cellulose nanofibers (CNFs). In this work, we compared the production of CNFs from two sugarcane bagasse substrates (bleached and bleached-sulfonated) using partial enzymatic hydrolysis with the commercial cocktail Cellic CTec3®, followed by ultrasonication. The effect of pretreatments and enzyme dosage on CNF properties and yields were evaluated. Mild enzymatic hydrolysis applied to sulfonated samples using only 0.312 mg enzyme/g substrate for 6 h increased CNF yield up to 2.5-fold and resulted in micrometer length fibers with an average diameter between 5 and 6 nm, as demonstrated by detailed morphological characterization of the substrates. These results were achieved due to the combination of the delignification steps and sulfonation, which enhanced enzymatic hydrolysis and fibrillation efficiency. Furthermore, combining enzymatic hydrolysis and sulfonation increased the CNF thermal stability (56–111 °C for bleached and 87–97 °C for bleached and sulfonated samples). These results demonstrated a pivotal role of enzymes in green CNF production and revealed the optimized hydrolysis/pretreatment conditions for manufacturing CNFs with advanced properties using enzymatic mixtures.

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Introduction

Cellulose nanofibers (CNFs), also named nanofibrillated cellulose, are colloidal structures with an average diameter of 5–50 nm and a length of a few micrometers, forming long and flexible fiber-like nanostructures (Blanco et al. 2018). They find diverse applications as hydrogels (De France et al. 2017), aerogels (Ferreira et al. 2021), films (Bangar and Whiteside 2021), and emulsion stabilizers (Kedzior et al. 2021), to cite a few.

Various methodologies have been recently employed to produce CNFs from raw lignocellulosic substrates, resulting in particles with different morphologies and properties (Klemm et al. 2018). The canonical process of CNF production involves delignification procedures, followed by chemical or biological processes to facilitate the final step of mechanical fibrillation (Klemm et al. 2018). As mechanical fibrillation is an energy-demanding step in CNF production that commonly employs homogenizers, micro-fluidizers, grinders, or sonicators (Nechyporchuk et al. 2016), the use of chemical or enzymatic treatments applied to the cellulose-enriched lignocellulosic biomass aiming to make it more susceptible to fibrillation is pivotal for reducing the energy required for this process (Rambabu et al. 2016).

Enzyme-assisted hydrolysis is an approach that has been reported as a potential green alternative to chemical treatments to enable CNF production (Arantes et al. 2020). This process was applied to various substrates, including wood pulp (Zhou et al. 2019) and alternative sources, such as sisal fibers (Siqueira et al. 2010) and sugarcane bagasse (SCB) (de Campos et al. 2013; de Aguiar et al. 2020; Berto et al. 2021; Rossi et al. 2021). Chemical treatments, such as carboxymethylation, TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl)-mediated oxidation (Isogai and Zhou 2019), periodate–chlorite oxidation (Errokh et al. 2018), cationization (Silva et al. 2020), and acidification (Nascimento and Rezende 2018) can be applied to produce CNFs. These treatments act by adding charged groups (e.g., carboxyl or carboxymethyl) on the cellulose fiber surface, favoring delamination of the nanofibrils by charge repulsion during mechanical disintegration. However, various environmental problems are related to using chemicals for CNF production, particularly for TEMPO-mediated oxidation (Arantes et al. 2020). Furthermore, CNFs obtained via TEMPO oxidation are also known for their lower thermal stability due to incorporating sodium anhydroglucoronate units into the cellulose structure (Fukuzumi et al. 2010).

Enzyme-assisted treatments utilize enzymes with high specificity for particular biopolymers (e.g., amorphous cellulose, xylan), thus potentially reducing energy consumption in the following mechanical disintegration steps. Commercial cellulase cocktails, composed of several cellulose-hydrolyzing enzymes, including cellobiohydrolases, endoglucanases, and β- glycosidases, synergistically convert cellulose into cellulose glucose. However, commercial cellulase cocktails were chiefly developed for the total conversion of cellulose into glucose, which can significantly reduce the CNF yield due to excessive cellulose depolymerization. Yet, no commercially available enzymatic cocktails are developed explicitly for CNF production (Arantes et al. 2020). Therefore, there is still a lack of information on the most appropriate conditions for CNF production from different substrates using commercial cocktails.

The lignin removal from the raw substrates significantly increases the efficiency of enzymatic hydrolysis on lignocellulosic substrates. Nevertheless, other alternative pretreatments can be carried out to increase the enzymatic activity and to incorporate functional groups into CNF surface. A promising alternative to improve enzymatic efficiency and produce modified CNFs is to sulfonate cellulose before enzymatic hydrolysis (Han et al. 2020). Sulfonation is a chemical treatment that introduces sulfonic groups onto cellulose fibers, leading to CNFs with unique properties such as strong electronegativity, high solution stability, and good film formation. In addition, sulfonation hinders the unproductive adsorption of enzymes, which enhances enzymatic hydrolysis efficiency (Chandra et al. 2016; Han et al. 2020). Sulfonation can be carried out in aqueous neutral media using sodium sulfite (Na_2SO_3), an inexpensive, safe, and environmentally less hazardous reagent.

Agro-industrial residues stand out as promising candidates for enzyme-assisted CNF production because of their wide availability and low prices. Amongst them, SCB can be highlighted since it has

relatively low recalcitrance compared to other biomasses (Pinto et al. 2019), thus becoming one of the preferable low-cost industrial residues for the production of nanocelluloses (Lima et al. 2014). However, studies of the efficient CNF production from SCB using enzyme-assisted hydrolysis are still limited (de Campos et al. 2013; de Aguiar et al. 2020; Berto et al. 2021; Rossi et al. 2021). In one of the previous studies, a mixture of xylanase, cellulase, and lytic polysaccharide monooxygenase (LPMO) and mild mechanical treatment were applied to produce CNFs from SCB (Rossi et al. 2021). In addition to the compelling advantages of using this eco-friendly approach, CNFs produced from enzyme-assisted treatments were longer and more thermostable than TEMPO-oxidized CNFs, indicating a room for a more comprehensive evaluation of different alternatives for CNF production based on partial enzymatic hydrolysis. Therefore, the use of pretreatment strategies, such as sulfonation, and the use of commercial cellulase cocktails can increase the methods to valorize SCB as a potential substrate to produce high value-added CNFs, mainly considering that the limits of the enzyme-assisted CNF method are frequently related to the availability and relatively high cost of enzymes. Considering the well-established production of commercial enzymatic cocktails, we investigated its potential use for CNF production.

In this work, we assessed the enzymatic CNF production from sugarcane bagasse using two different pretreatment strategies (bleaching only and sequential bleaching and sulfonation) to compare both approaches regarding CNF properties and yields. In addition, assays using different dosages of the commercial cocktail Cellic® CTec3 were also tested, including experiments in the absence of enzymes, to evaluate the relations between cellulose enzymatic degradation and CNF yields and to define the best conditions of enzymatic hydrolysis for CNF production. Our results revealed that including the sulfonating step and using different enzymatic dosages led to very different CNF yields and enzymatic hydrolysis efficiencies, rendering CNFs with similar physical properties. To the best of our knowledge, studies focused on the production of CNFs from sulfonated SCB using enzyme-assisted processes have not been reported so far.

Materials and methods

The CNF isolation process from sugarcane bagasse was divided into three steps (Fig. 1): pretreatment, partial enzymatic hydrolysis, and mechanical disintegration using ultrasonication. In terms of pretreatments, SCB underwent one of two different routes: Route 1—involving a delignification (or bleaching) step to obtain bleached bagasse (BB), and Route 2—where BB samples from Route 1 were sequentially sulfonated to obtain bleached-sulfonated bagasse (BSB). BB and BSB samples were hydrolyzed with three different enzymatic loadings (0; 0.312; and 0.625 mg/g), releasing glucose to the liquid fraction from the biomass samples. CNFs were then obtained by sonication and filtration of the partially hydrolyzed solids.

Pretreatments to obtain solids enriched in cellulose

Delignification

Dried and milled raw SCB was treated using a 1:1 solution of 4% (w/v) NaOH + 7% (v/v) H_2O_2 using a 1:20 (g/mL) solid:liquid ratio at 70 °C for 2 h and under mechanical stirring at 120 rpm, following a methodology adapted from Nascimento and Rezende (2018). Then, the solid fraction was recovered by filtration, washed with tap water until neutral pH, and oven-dried for 48 h at 40 °C.

Sulfonation

BB solid was sulfonated using 0.16 g of Na_2SO_3 per g of biomass in a 1:10 (g/mL) solid:liquid ratio for 2 h at 120 °C in an autoclave (Han et al. 2020). Next, the BSB solid was separated by filtration and oven-dried, as described previously.

Enzymatic hydrolysis

BB and BSB solids were hydrolyzed using the enzymatic cocktail Cellic® CTec3 at 50 °C for 6 h in citrate buffer (50 mmol/L, pH = 5) at a solid:liquid ratio of 1:10 (g/mL). The reactions were performed using different enzyme loadings: 0 (control), 0.312, and 0.625 mg enzyme/g of substrate. After the reaction period, hydrolyses were stopped by heating the reactions to 95 °C for 15 min and centrifuged at 3500 rpm

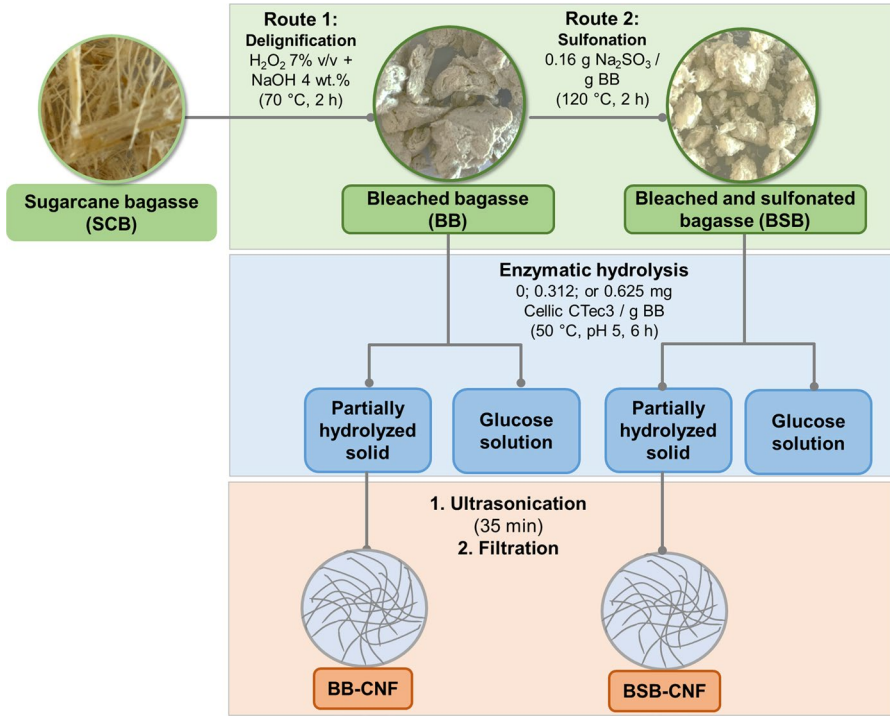


Fig. 1 Schematic representation of the CNF isolation process from sugarcane bagasse encompassing delignification and sulfonation, enzymatic hydrolysis, and ultrasonication/filtration

for 20 min. An aliquot of the supernatant was collected for glucose quantification, and the solids were stored at 4 °C without drying until ultrasonication (Fig. 1).

Glucose yield (*GY*) was calculated according to Eq. 1

$$GY = \frac{GC \times V \times 0.9}{m} \quad (1)$$

where *GC* is the concentration of released glucose (mg/mL); *V* is the total volume of liquid in enzymatic hydrolysis (mL); 0.9 is the conversion factor of glucose, and *m* is the mass of biomass used for enzymatic hydrolysis (mg).

Mechanical disintegration

The enzymatically-treated substrates were mechanically treated in a Branson Ultrasonics SonifierTM (Digital Sonifier 250 W) equipped with the 3.2-mm-diameter tip at 50% of amplitude for 35 min. Prior to sonication, the concentration of substrate dispersions was adjusted to 5 g/L using deionized water. After ultrasonication, the dispersion was passed through a 150-thread mesh filter (pore size ca. 100 µm) to isolate CNFs from the non-converted fibers. The yield of CNFs was determined as follows:

$$Yield(CNF) = \frac{m_{(driedCNF)}}{m_{initialsubstrate}} \times 100 \quad (2)$$

where: $m_{(\text{dried CNFs})}$ is the mass of the filtrated after drying and $m_{(\text{initial substrate})}$ is the mass of the pulp that was submitted to the enzymatic hydrolysis.

Characterization

Chemical compositional analysis

The chemical compositions for raw SCB and pretreated (BB and BSB) solids were obtained according to the National Renewable Energy Laboratory (NREL) protocol (Sluiter et al. 2008). Briefly, substrates were hydrolyzed with an H_2SO_4 solution, and monosaccharides were quantified by high-performance liquid chromatography (HPLC) using an Aminex column (HPX-87 H, 300×7.8 mm, Bio-Rad, USA) in a Shimadzu LC-10AD chromatographer equipped with refractive index and UV-VIS detector. The mobile phase was H_2SO_4 (5.10^{-3} mol L^{-1}) with a flow rate of 0.6 mL min^{-1}. This isocratic condition

was maintained for 1 h at 65 °C. Acid-soluble lignin was analyzed by UV-Vis spectroscopy, while acid-insoluble lignin and ashes were quantified by gravimetry following previously published protocols (Santo et al. 2018). Extractives were quantified by Soxhlet extraction (Santo et al. 2018; Kane et al. 2022).

Energy-dispersive X-ray spectroscopy (EDS) analysis

Energy-dispersive X-ray spectroscopy (EDS) elemental analysis was carried out using the samples before and after sulfonation on a scanning electron microscope (Zeiss LEO 440). The samples were freeze-dried and fixed on a sample holder using a carbon tape. The analyses were carried out in two different regions of each sample.

Field-emission electron microscopy (FESEM)

Samples before and after chemical and enzymatic treatments were analyzed in an FEI Quanta FEG 250 operating at 2 kV. Samples after enzymatic hydrolysis were freeze-dried, fixed on the sample holder with carbon tape, and then coated with an iridium film (ca. 5 nm) using a BALTEC MED 020 sputter coater (13.4 mA for 70 s). At least 20 images were obtained from each sample to ensure the statistical validity of the results.

Atomic force microscopy (AFM)

CNF samples were analyzed by AFM under environmental conditions in the non-contact mode in a Shimadzu SPM-9600 microscope using silicon tips (NCHR Pointprobe, Nanoworld). Topography maps were obtained using a cantilever with a spring constant of 42 N/m and a nominal resonance of 318 kHz. The software Gwyddion 2.56 (gwyddion.net) was used for data treatment and particle measurements using the height image profiles (150 particles of each sample were measured to ensure the statistics). Samples were prepared by adding a drop (5 µL) of a nanoparticle dispersion (0.0025 wt%) on a cleaved mica substrate, which was dried inside a desiccator for 4 h.

Transmission electron microscopy (TEM)

TEM analysis of CNFs was performed in a Carl Zeiss LIBRA 120 equipment using a tungsten filament operating at 120 kV. Samples were prepared by adding a drop (5 µL) of nanoparticle dispersion (0.0025 wt%) on a copper grid, followed by drying in a desiccator.

Zeta potential (ζ-potential)

CNF ζ-potential was determined using a Zetasizer® 300 HS (Malvern, UK). All measurements were performed in triplicates with at least 10 scans each in backscattering (173°) mode. Samples were diluted to 0.5 wt% and the pH was adjusted to 7 before the analysis.

Conductometric titration

The acid groups in CNFs were quantified by the conductometric titration method, as described by (Katz et al. 1984).

Thermogravimetric analysis (TGA)

TGA of the CNFs was carried out in a PerkinElmer 4000 equipment under a nitrogen atmosphere at 50 mL min^{-1}. Samples with initial weights varying from 4 to 10 mg were placed in ceramic sample holders and heated from 30 to 600 °C under a 20 °C min^{-1} heating rate.

Results and discussion

Chemical composition

The enzyme-assisted production of CNFs is a sequential process that includes substrate delignification, partial enzymatic hydrolysis, and mechanical defibrillation, as represented in Fig. 1. The initial composition of untreated SCB in terms of cellulose, hemicellulose, and lignin was respectively 42, 25, and 20 wt% (Fig. 2). After the pretreatment steps, cellulose and hemicellulose retention and an efficient delignification can be noticed, resulting in 75 and 80% of lignin removal for BB and BSB, respectively. In addition, the cellulose fraction was higher than untreated SCB, and only a slight decrease in the hemicellulose fraction was observed for BB (25 to 23.8%). BSB exhibited similar chemical composition after pretreatments,

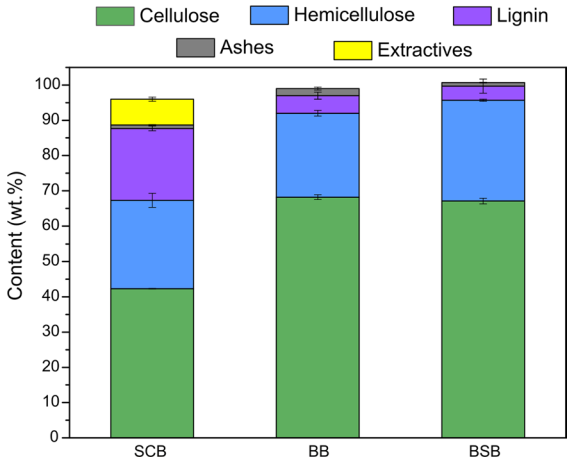


Fig. 2 Chemical composition of the raw SCB, bleached (BB), and bleached-sulfonated bagasse (BSB). Analyses were carried out in triplicate and error bars represent standard deviation values

indicating that the sulfonation step did not lead to significant degradation of carbohydrates.

Bleaching using H_2O_2 was chosen as a delignification step due to the efficient lignin removal by the combined action of sodium hydroxide, which removes lignin through irreversible hydrolysis of the ester bond of lignin-carbohydrate complexes, and H_2O_2, which oxidates the carbonyl and quinoid structures of the lignin side chain (Banerjee et al. 2011). In addition, the reaction between H_2O_2 and the benzoquinone structure of lignin increases the solubility of lignin. Moreover, H_2O_2 can react with the side chain carbonyl and carbon-carbon double bond of lignin leading to further oxidative degradation and enhancing lignin removal (Wu et al. 1993). The combined pretreatment using alkaline peroxide (H_2O_2/NaOH) was proven in previous studies to be very effective for removing the residual lignin while preserving the polysaccharide fractions (Nascimento and Rezende 2018; Hafemann et al. 2020).

Sulfonation of the BB substrate did not considerably modify the chemical composition of the substrate (Fig. 2). However, this process is expected to introduce sulfonic groups in lignin and cellulose. Indeed, conductometric titration (Table 1) indicated the addition of 0.4 mmol of sulfonic groups/g of substrate, while EDS measurements showed a sulfur content of $0.4 \pm 0.1\%$ in the BSB sample and no sulfur in the BB sample.

Sulfonation softens the lignin contained in the cell walls and increases its hydrophilicity as well as swelling capacity (Ämmälä et al. 2019). Furthermore, sulfonation effectively increases the enzymatic action, even in samples with a higher quantity of lignin, by reducing unproductive enzyme binding to the fiber surface (Han et al. 2020). Notably, the samples obtained in this work contained only a tiny residual lignin fraction, thus decreasing the impacts of residual lignin on CNF production. Sulfonation of cellulose enhanced the efficiency of fibrillation, allowing the production of CNFs with a reduced amount of lignin and additional sulfonic groups. Both lignin removal and sulfonation are advantageous for decreasing energy consumption in separating nanoscale fibrils (Khadraoui et al. 2023).

As shown in Fig. 2, there is a residual quantity of hemicellulose in substrates BB and BSB (23.8 and 28.6%, respectively). The presence of hemicelluloses is yet another factor that can contribute for production of CNFs because it allows the fibers to swell extensively, improving fibrillation (Hanhikoski et al. 2020). Indeed, residual hemicellulose in cellulose pulps can increase the CNF yields and avoid cellulose aggregation during fibrillation by inhibiting coalescence between fibrils (Norrrahim et al. 2021).

Glucose and CNF yields

BB and BSB samples were enzymatically hydrolyzed using 0.312 and 0.625 mg enzyme/g substrate (Fig. 3) in a relatively fast enzymatic hydrolysis process (6 h). As expected, higher enzyme dosages resulted in higher glucose yields for both substrates. Due to low enzymatic loads, a maximum of 3.6% of cellulose was converted to glucose. These low conversion yields are desirable for CNF production because higher quantities of partially hydrolyzed cellulose remain available in the solid substrate to be converted to CNFs, and only small amounts of the polysaccharide are converted to glucose. Likewise, enzymatic dosages higher than 0.625 mg/g are not suitable for CNF production because they lead to a higher rate of cellulose conversion into glucose, reducing the remaining cellulose fraction in solids to be converted into CNFs.

Next, all substrates (including control samples, not subjected to enzymatic hydrolysis) were sonicated to isolate CNFs. The positive impacts of enzymatic

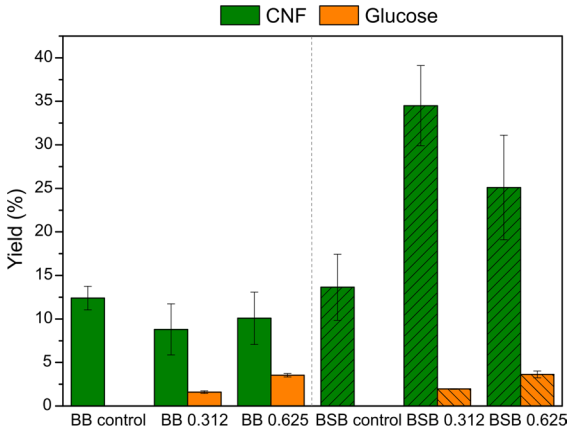


Fig. 3 CNF and glucose yields after enzymatic hydrolysis and fibrillation. Control samples did not release glucose. Analyses were carried out in triplicate and error bars are the standard deviation

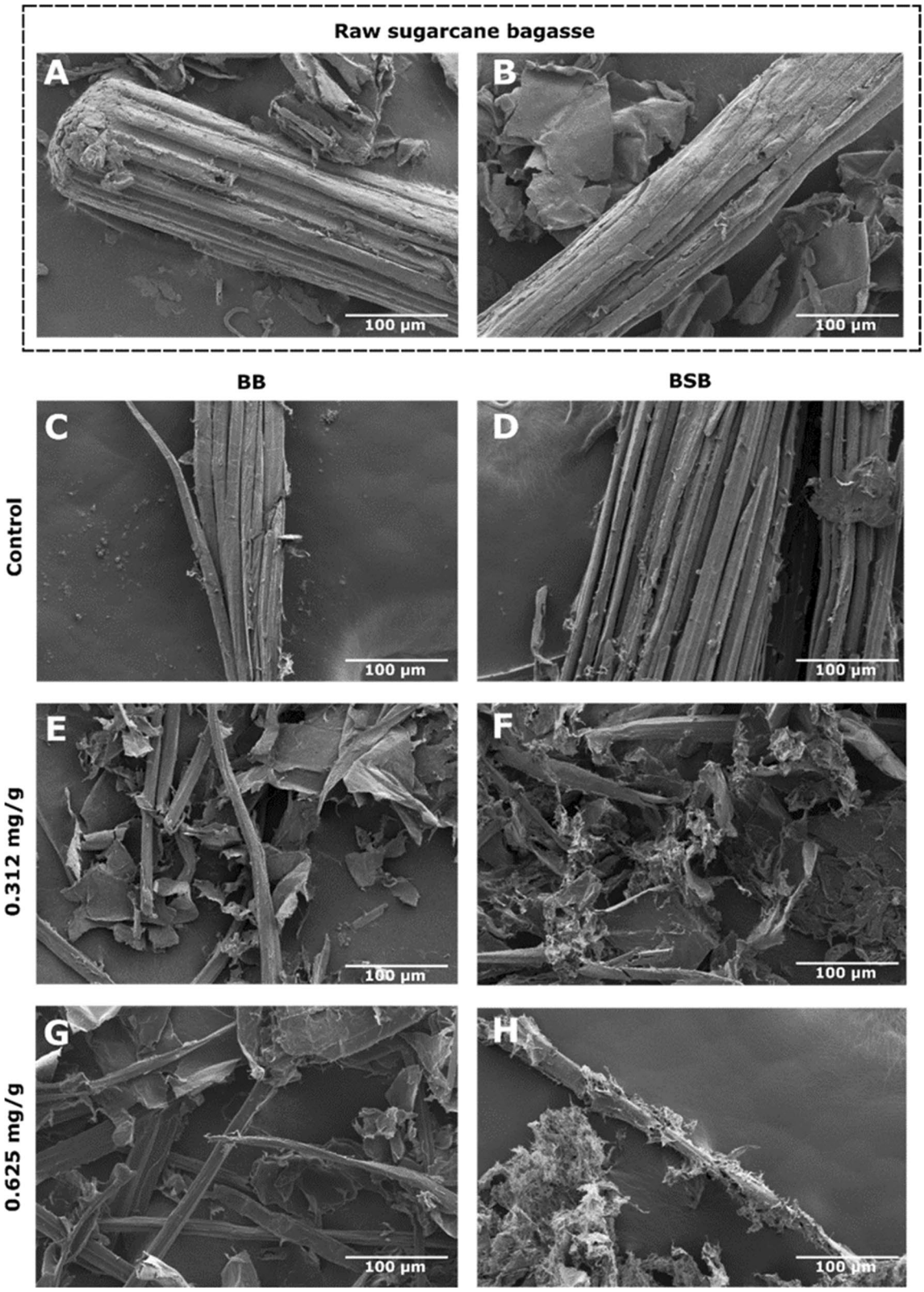
hydrolysis on the fibrillation process and the CNF yields became evident for BSB samples (Fig. 2). Comparing the CNF yields between control and enzyme-treated (0.312 mg/g) samples, cellulose conversion to CNFs increased from 13 to 34% for BSB. Therefore, the yield is up to 2.5-fold higher by carrying out an enzymatic hydrolysis step in the sulfonated substrate using the same fibrillation conditions. No statistical differences in cellulose conversion to CNFs were observed by increasing enzyme load in both pretreatment conditions (BB and BSB), indicating that 0.312 mg/g is the most suitable condition. A possible explanation for this observation can be related to the ability of the enzymes to convert the more available cellulose into glucose and cellooligosaccharides, which reduces the CNF yield (Han et al. 2020).

Considering the process mass balance (Fig. S1), introducing an additional sulfonation step increased about 2-fold the total mass of CNFs produced in the best conditions: starting from 100 g of SCB, it is possible to produce 12.3 g of BSB-CNFs using 0.312 mg of enzyme/g vs. 5.5 g of BB-CNFs using no enzymatic treatment. However, even considering the significant increase in CNF yields, sulfonation is an additional step for CNF production, adding other expenses. Therefore, the evaluation of the economic aspects of CNF production should be further considered.

This increase in the CNF yields is consistent with previous studies that reported improved enzymatic hydrolysis efficiency in bleached softwood sulfonated thermomechanical pulps (Han et al. 2020). The CNF yields achieved in the present study under the best conditions were higher than those obtained by Han et al. (2020) from the bleached and sulfonated chemi-thermomechanical pulp (15–29%). In a separate study, combining cellulase activity with those of LPMO or laccase allowed CNF production from cotton linters with 10–23% yields using high-pressure homogenization (Valls et al. 2019). Furthermore, combining pectinase and a mild-physical blender treatment resulted in CNF production from orange peels with a 15% yield (Hideno et al. 2014). Nonetheless, it is essential to consider that the biomass, lignin content, defibrillation, and enzymatic treatment procedures differed, which might affect the CNF yields. Under current study conditions, enzymatic low-loading conditions were sufficient to significatively improve the CNF conversion, indicating that sulfonation of the pretreated SCB can help advance establishing the optimized enzymatic dosages for the processes focused on CNF isolation using commercial enzymatic cocktails. Since the mechanical disintegration processes are energy-demanding, partial enzymatic hydrolysis can thus be a key alternative to significantly improve biomass conversion into CNFs.

Sulfonation action was also evidenced when comparing BB samples before and after enzymatic hydrolysis. Regardless of the quantity of enzyme used in the tested conditions, all BB substrates had similar CNF yields after enzymatic hydrolysis (Fig. 3). This indicates that the effect of partial enzymatic hydrolysis was less pronounced under the tested conditions when the samples were not sulfonated, and samples after enzymatic hydrolysis had similar properties to the controls. Since low enzymatic dosages were used in this work, they were insufficient to improve the mechanical disintegration of BB samples. Likewise, a possible alternative to enhance the effect of enzymatic hydrolysis may be using higher enzymatic loadings or increasing the enzymatic hydrolysis time. However, these might lead to additional expenses, higher cellulose degradation, and glucose production, which might not necessarily compensate for improved mechanical disintegration.

BB and BSB control samples had similar CNF yields, indicating that combining partial enzymatic hydrolysis with sulfonation enhanced the CNF yields but not the sulfonation itself. As shown in


Raw sugarcane bagasse
A
100 µm
B
100 µm
BB
BSB
Control
C
100 µm
D
100 µm
0.312 mg/g
E
100 µm
F
100 µm
0.625 mg/g
G
100 µm
H
100 µm

◂**Fig. 4** FESEM images of the raw substrate **A**–**B**; after BB **C** and BSB **D** pretreatment; and after partial enzymatic hydrolysis using: 0.312 mg enzyme/g enzyme in BB **E** and BSB **F**; and 0.625 mg enzyme/g in BB **G** and BSB (**H**)

Table 1, the sulfonation step added up to 0.4 mmol sulfonic groups/g biomass, which was a possible reason for the improved enzymatic action. Indeed, sulfonic groups should enhance substrate wettability and accessibility to enzymes. Besides that, improved wettability of sulfonated substrates facilitates the mechanical disintegration step.

Morphological analysis of the chemical and enzymatically treated substrates

Morphological analysis of the substrates before and after chemical and enzymatic treatments (Fig. 4) corroborates the aforementioned improved enzymatic action caused by sulfonation. Initially, the raw substrate shows the typical SCB morphology, with plant fibers and parenchymal tissue (Fig. 4A–B) (Rezende et al. 2011). After BB and BSB treatments (Fig. 4C–D, respectively), cellulose microfibers were significantly exposed and separated, which is related mainly to the delignification processes (Rezende et al. 2011).

After enzymatic hydrolysis, the fibers were more unstructured and detached as a consequence of enzymatic action both for BB and BSB samples (Fig. 4E–H). Figure 4F and H, in particular, show cellulose microfibers covered by partially attached nanofibers, revealing an advanced stage of enzymatic action. This partial fibrillation during hydrolysis is a probable consequence of substrate sulfonation, which justifies the best CNF yields for BSB samples.

The results are in agreement with the morphological modifications caused by enzymatic treatment to improve pulp refining to produce nanocellulose or to improve paper properties, for example (Lin et al. 2018). Morphological characterization showed that enzymatic treatment promoted the increase in the surface area, resulting in improved water absorption, swelling, and fibrillation. All these factors together make the fibrillation step easier, which is important for energy reduction, for example.

CNF characterization

Morphology of BB-CNFs and BSB-CNFs

In addition to the process yields, nanoparticle properties should be considered to decide on adequate conditions for CNF production for different applications. Morphological analysis carried out by AFM and TEM (Fig. 5) of the CNF isolated from control substrates and after enzymatic hydrolysis using 0.312 mg enzyme/g demonstrates thin and long fibers (in the micrometer scale), which is a typical characteristic of CNFs. Indeed, all samples (including samples treated with 0.625 mg enzyme/g, Supplementary Information S2) had similar average diameters (Table 2), varying between 5 and 6 nm, with most of the fiber diameters ranging from 1 to 13 nm. Comparing control experiments with those carried out using enzymes in the two dosages, the average diameter and the histograms were similar, indicating that enzymatic treatments did not affect significantly the CNF morphology.

The average diameters of CNFs were very similar to those obtained in previous studies of CNF production from sugarcane bagasse using recombinant enzymes (Liu et al. 2020; Rossi et al. 2021). The longer lengths are an essential characteristic of CNFs produced by partial enzymatic hydrolysis compared to CNFs produced from chemical methods (i.e., TEMPO-oxidation), as was previously discussed (Liu et al. 2020; Rossi et al. 2021). Similar entanglement profiles were also observed in CNF produced from sugarcane bagasse using a monocomponent endoglucanase enzyme, indicating that the set of enzymes in the commercial cocktails did not influence this CNF property (Berto et al. 2021). This is an advantage since longer fibers are easily entangled and suitable for preparing hydrogels and aerogels (De France et al. 2017), stabilizing emulsions (Kedzior et al. 2021), and manufacturing films (Camargos and Rezende 2021).

ζ-potential

ζ-potential is an important indicator of the colloidal stability of particles in dispersion and an important parameter for applying CNFs in composites or aqueous media. CNFs prepared from BB and BSB substrates showed negative ζ-potential due to the negatively charged groups at the particle surfaces

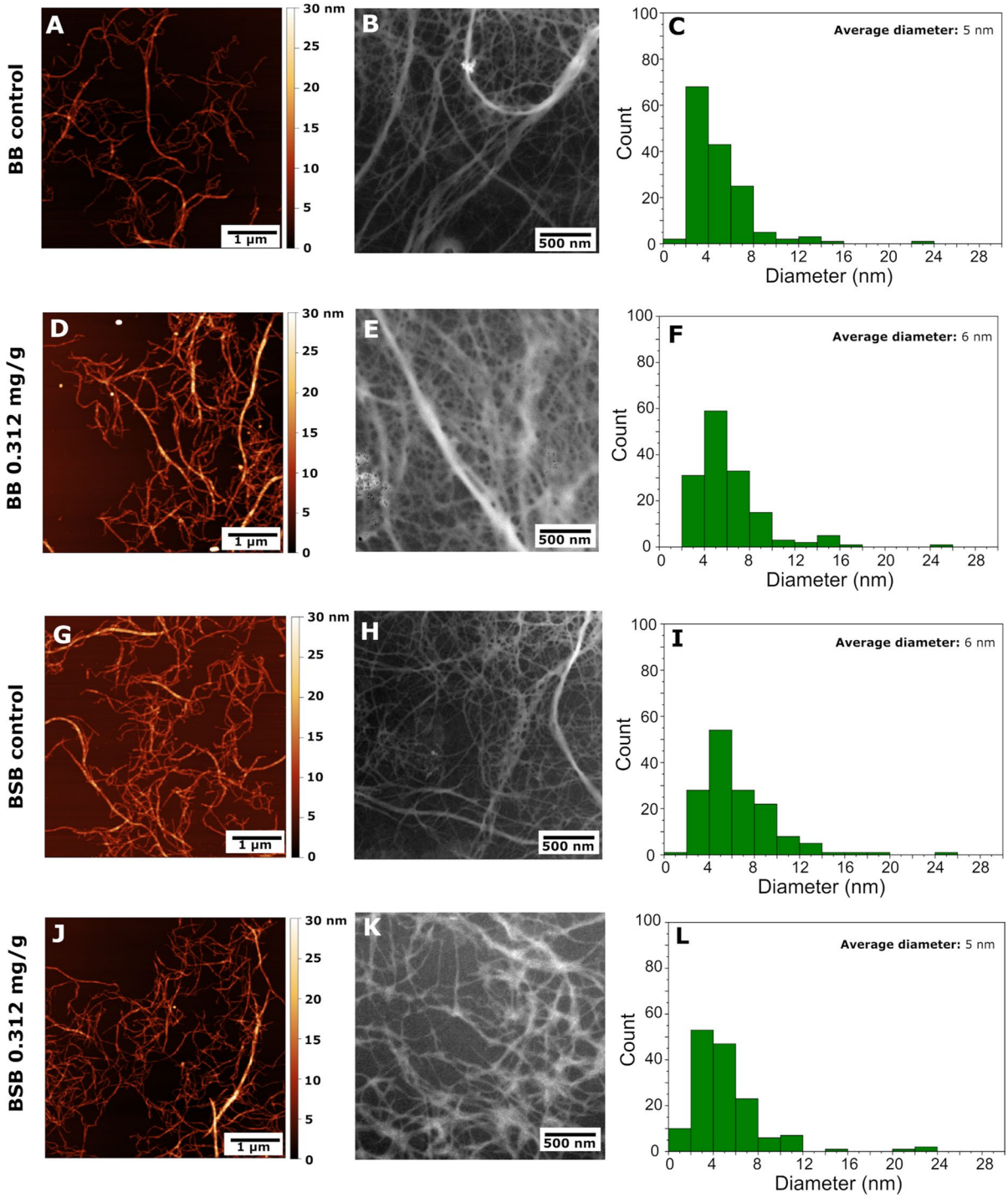


Fig. 5 Images of AFM (topography), TEM, and distribution of the CNF diameters (obtained from AFM images): BB-CNFs control (**A**–**C**); BB-CNFs 0.312 mg/g (**D**–**F**); BSB-CNFs control (**G**–**I**); and BSB-CNFs 0.312 mg/g (**J**–**L**). TEM images were acquired by using an energy loss of 25 eV. Diameters were measured by the height of 150 particles in AFM images. The average value refers to the arithmetic mean

Table 1 Summary of CNF properties: average diameter, zeta-potential, strong and weak acid groups, and quantity of added sulfonic groups calculated by conductometric titration

Sample	Average diameter (nm)	ζ-potential (mV)	Strong acid groups (mmol/g)	Weak acid groups (mmol/g)	Added sulfonic groups (mmol/g)
BB-CNFs control	5	− 28 ± 2	0.4 ± 0.1	1.1 ± 0.4	–
BB-CNFs 0.312	6	− 28 ± 2	0.4 ± 0.1	1.5 ± 0.1	–
BB-CNFs 0.625	5	− 31 ± 2	0.5 ± 0.1	1.6 ± 0.1	–
BSB-CNFs control	6	− 33 ± 3	0.8 ± 0.1	0.7 ± 0.1	0.4 ± 0.1
BSB-CNFs 0.312	5	− 35 ± 3	0.8 ± 0.1	1.2 ± 0.3	0.4 ± 0.1
BSB-CNFs 0.625	5	− 28 ± 2	0.8 ± 0.1	1.3 ± 0.1	0.3 ± 0.1

under the analyzed conditions (pH 7). Their experimental values varied from − 35 to − 28 mV (Table 1), with BSB-CNFs having a slightly more negative ζ-potential than BB-CNFs, which is indicative of better stability. Indeed, values of ζ-potential above 20 mV (in absolute value) can be used to consider a dispersion as "stable" (Bhattacharjee 2016). Therefore, CNFs produced from both substrates met this requirement. As sulfonation added 0.3–0.4 mmol of sulfonic groups per gram of CNFs, the surface sulfonic groups are contributing to the additional electrostatic repulsion, thus further improving the suspension stability in comparison to the non-sulfonated CNFs. Comparable values of ζ-potential were achieved when thermomechanical pulps were sulfonated to produce CNFs (Han et al. 2020). Still, CNFs produced from TEMPO-oxidation usually has more negative values (up to − 75 mV, for example) due to the higher quantity of negatively charged groups (Pinto et al. 2019). However, other factors should be considered in the colloidal stability of nanocellulose dispersions, such as the presence of residual hemicellulose, which can contribute to increase the dispersion stability (Siqueira et al. 2019). In addition to the slight improvement of the colloidal stability, the presence of sulfonic groups can be decisive for certain CNF applications. It can be used for instance, to enhance the conductivity of membranes for fuel cells without compromising the membrane mechanical properties (Bayer et al. 2021).

Thermal Stability

The thermal properties of the CNFs measured by TGA (Fig. 6) indicate that sulfonation and partial enzymatic hydrolysis enhanced the thermal stability of samples compared to control samples. There is only a slight reduction in the mass loss in the first stage of thermal analysis (until around 200 °C), which can be attributed to the loss of moisture (Zhang et al. 2018b). The initial decomposition temperature (T_{onset}) and temperature of maximum weight loss (T_{max}) (Table 2) are lower for BB-CNFs control (185 and 295 °C, respectively). The enzymatic treatment increased the T_{onset} of the BB samples to 240–296 °C, representing an increase in thermal stability. Regarding sulfonated samples, BSB-CNFs control is more thermally stable than BB-CNFs control, showing T_{onset} of 202 °C. Accordingly, the enzymatic treatment also increased the T_{onset} of the sulfonated samples.

Overall, hemicellulose and cellulose thermal decomposition occur respectively at 220–315 and 315–400 °C (Zhang et al. 2018b). Thermal degradation of control samples (both BB and BSB) starts at lower temperatures than enzymatic-treated samples, which can be attributed to the presence of cellulose fragments with lower molecular weights, which have lower temperatures of degradation (Berto et al. 2021). These experimentally obtained values are similar to those previously reported for CNFs produced by enzyme-assisted processes from bagasse and softwood pulps (Han et al. 2020; Berto et al. 2021; Rossi et al. 2021).

The results show that the isolated CNFs, particularly those obtained from sulfonated samples, have good thermal stability, which is essential for CNF final applications. For instance, thermal stability is pivotal for producing electronic components and incorporating CNFs into nanocomposites that will be extruded or molded. Here thermal stability is important due to the strong variations in temperature. TEMPO-oxidized and cationic CNFs are

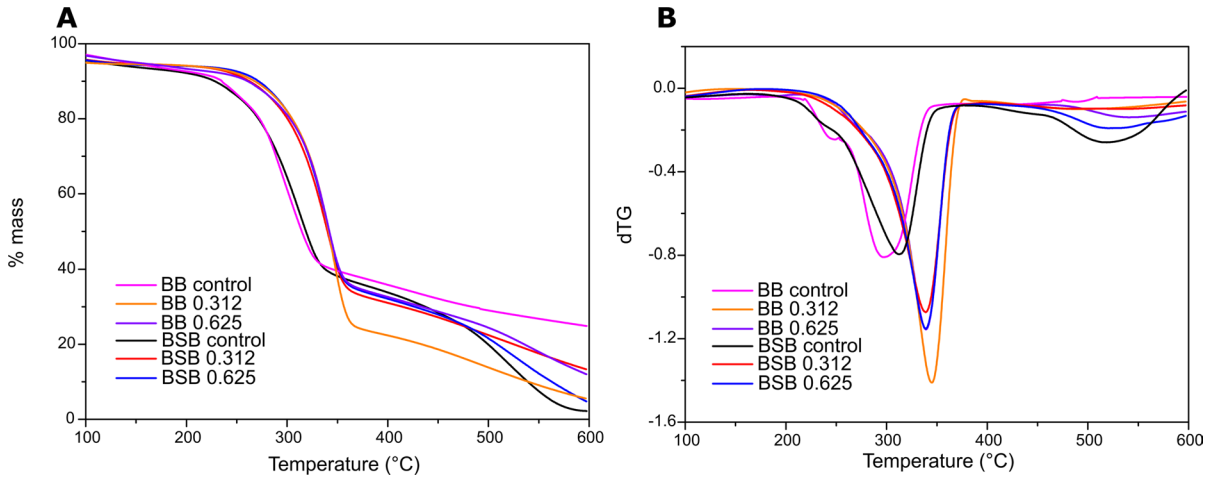


Fig. 6 **A** TGA; and **B** Derivative thermogravimetric curves of BB-CNFs and BSB-CNFs samples before and after enzymatic hydrolysis

Table 2 Initial decomposition temperature (T_{onset}) and temperature of maximum mass loss (T_{max}) of CNFs obtained from TGA

Sample	T_{onset} (°C)	T_{max} (°C)
BB-CNFs control	185	295
BB-CNFs 0.312	296	345
BB-CNFs 0.625	241	339
BSB-CNFs control	202	311
BSB-CNFs 0.312	279	338
BSB-CNFs 0.625	289	339

usually less thermally stable than enzymatic CNFs (Rol et al. 2019; Rossi et al. 2021), demonstrating the positive aspect of using enzymatic CNFs: For example, decomposition of the TEMPO-oxidized CNFs starts at ca. 190 °C (Rossi et al. 2021) whereas the cationic CNFs have degradation temperature which is about 35 °C lower than that of enzymatically produced CNFs (Rol et al. 2019).

The characterization of CNF samples prepared from bleached or bleached and sulfonated bagasse samples showed that including the enzymatic hydrolysis step in the process does not significantly change the morphology, dimensions, or zeta potential of the nanofibrils obtained. On the other hand, the enzymatic action positively affects the yield of fibrils obtained and their thermal stability.

Conclusions

Here, we demonstrated that the sulfonation step significantly enhances the yields of CNFs isolated from sugarcane bagasse. The overall process encompassed bleaching and a sulfonation step as pretreatments, mild enzymatic hydrolysis, and ultrasonication for mechanical disintegration. The isolated thermally and colloidally stable fibers, longer than those typically obtained by chemical methods, have an average diameter between 5 and 6 nm. Comparison with non-sulfonated samples revealed that adding sulfonic groups enhanced the enzymatic hydrolysis efficiency allowing CNFs to be obtained at improved yields by mechanical disintegration of the substrates. CNFs produced using enzyme-assisted technology have chemical and mechanical properties that enable their potential applications in packaging, nanocomposite reinforcement, and rheology modifier agents.

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Data Availability Not applicable.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval and consent to participate Not applicable.

Consent for publication Not applicable.

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