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Cellulose nanostructures obtained using enzymatic cocktails with different compositions

Thalita J. Bondancia [a,b], Camila Florencio [a], Graziela S. Baccarin [a,c], Cristiane S. Farinas [a,b,*]

[a] National Nanotechnology Laboratory for Agribusiness (LNNA), Embrapa Instrumentation, São Carlos, SP, Brazil
[b] Graduate Program of Chemical Engineering, Federal University of São Carlos, SP, Brazil
[c] Chemistry Department, Federal University of São Carlos, SP, Brazil

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ABSTRACT

Cellulose nanostructures obtained from lignocellulosic biomass by the enzymatic route can offer advantages in terms of material properties and processing sustainability. However, most of the enzymatic cocktails commonly used in the saccharification of biomass are designed to promote the complete depolymerization of the cellulose structure into soluble sugars. Here, investigation was made of the way that the action of different commercially available cellulase enzyme cocktails can affect the production of nanocellulose. For this, enzymatic cocktails designed for complete or partial saccharification were compared, using eucalyptus cellulose pulp as a model feedstock. The results showed that all the enzymatic cocktails were effective in the formation of nanocellulose structures, with the complete saccharification enzymes being more efficient in promoting the coproduction of glucose (36.5 g/L, 87% cellulose conversion). The presence of auxiliary enzymes, especially xylanases, acted cooperatively to favor the production of nanostructures with higher crystallinity (up to 79%), higher surface charge (zeta potential up to −30.9 mV), and more uniform dimensions within the size range of cellulose nanocrystals (80 to 350 nm). Interestingly, for the enzymatic cocktails designed for partial saccharification, the xylanase activity was more important than the endoglucanase activity in the production of nanocellulose with improved properties. The findings showed that the composition of the enzymatic cocktails already used for complete biomass saccharification can be suitable for obtaining nanocellulose, together with the release of a glucose stream, in a format compatible with the biorefinery concept.

1. Introduction

There is an increasing demand for novel materials obtained from green and sustainable sources that can replace petroleum-based polymers in technological applications. Given its renewable nature and abundance, lignocellulosic biomass stands out as a promising feedstock to replace fossil resources in future biorefineries [1]. However, for the economic viability of biorefineries, it is essential to integrate large-scale biofuel production with the generation of other higher added-value bio-based products, such as nanocellulosic materials [2–4]. Nanocellulose can be produced in the form of cellulose nanofibrils (CNF) or cellulose nanocrystals (CNC), using different treatments (chemical, mechanical, enzymatic, or a combination of them). The enzymatic route has been mainly applied for the pretreatment of cellulose, in order to reduce the energy spent in the mechanical processes of refining and fibrillation for nanocellulose production [5], but the use of enzymes is very promising as a green and sustainable method for the extraction of nanocellulose. The enzymatic route has the advantages of producing nanostructures with high thermal stability, without the use of toxic reagents and with low consumption of water, while the soluble sugars released during the reaction process can be used to obtain biofuels and other bio-based products [6–9]. However, the enzymatic route often results in nanomaterials with considerable size heterogeneity [10,11], which makes it important to investigate more efficient combinations of enzymes for the release of cellulose nanostructures that have greater crystallinity and uniformity.

Cellulose hydrolysis by the action of enzymes requires the synergistic action of three main groups of cellulase enzymes: exoglucanases, composed of exo-1,4-β-D-glucanases, also known as cellobiohydrolases (CBH) and 1,4-β-D-glucanohydrolases or cellodextrins; endo-1,4-β-D-glucanases or endoglucanases (EGase); and 1,4-β-D-glucosidases, also called cellobiases [12,13]. Endoglucanases are considered key enzymes

* Corresponding author at: Embrapa Instrumentação, Rua XV de Novembro, 1452, São Carlos, SP 13561-206, Brazil.
E-mail address: cristiane.farinas@embrapa.br (C.S. Farinas).

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to obtain nanocellulose, given their action in promoting the defibrillation process, without compromising the crystalline regions [14,15]. Therefore, the use of monocomponent endoglucanases is more frequent in studies whose objective is to obtain nanocellulose, due to their action leading to the reduction of fiber length and increase of crystallinity, while largely preserving the mechanical properties [16]. Besides endoglucanases, combinations of enzymes such as xylanases and lytic polysaccharide monooxygenases (LPMO) have also been investigated for improving the release of nanostructures [10,17–22]. The use of these accessory enzymes can complement the nanocellulose production process, providing a more purified cellulosic material and/or assisting cellulases in the depolymerization process. However, the majority of the commercial cellulase enzyme cocktails commonly used in the saccharification of biomass also contain high amounts of β-glucosidase enzymes, since they are designed to promote the complete depolymerization of the cellulose structure into soluble sugars. Therefore, a comparison of commercially available cellulase enzyme cocktails that would be more suitable for removal of the amorphous regions of cellulose could contribute to further promoting the use of more sustainable routes to produce nanocellulose in future biorefineries.

This work investigates how the action of different commercially available cellulase enzyme cocktails can affect the production of nanocellulose from eucalyptus cellulose pulp. For this, comparison was made of enzymatic cocktails designed for complete or partial saccharification. The glucose released was quantified and the physical-chemical properties of the nanocellulose materials were evaluated in terms of crystallinity index (using X-ray diffraction), morphological alteration (using atomic force microscopy), and surface charge (apparent ζ-potential).

2. Materials and methods

2.1. Materials

Kraft eucalyptus pulp kindly donated by the Suzano Pulp and Paper Company (São Paulo, Brazil) was used as a model feedstock. The chemical composition of the eucalyptus pulp was 75.6% ± 2.3% cellulose, 14.6% ± 0.6% hemicellulose, 6.7% ± 1.2% lignin, and 1.1% ± 0.2% ash, as determined previously by [6]. The commercial cellulase enzyme cocktails designed for complete saccharification (CS) used here were Cellic® Ctec3 (CS1) and Cellic® Ctec2 (CS2), both from Novozymes, Denmark. The enzyme blends designed for partial saccharification (PS) were Serzyme 50 (Sertec 20®, Spain) and Carezyme 1000L-C2605 (Sigma-Aldrich, USA), named here as PS1 and PS2, respectively.

2.2. Enzymatic hydrolysis for nanocellulose production

The enzymatic hydrolysis reactions were performed in 250 mL Erlenmeyer flasks, for 96 h, using an orbital shaker at 200 rpm and 50 °C. To each flask was added 5% (w/v) cellulose pulp with particle size smaller than 2 mm, together with 20 mg protein/g cellulose of each enzymatic cocktail in sodium citrate buffer (50 mM and pH 4.8). The hydrolysis conditions were based on previous study [8]. A control treatment using only cellulose pulp and sodium citrate buffer, without the presence of enzymes, was carried out under the same reaction conditions. Every 24 h, an aliquot was removed and centrifuged for 10 min, at 10,000 rpm, in order to separate the solid and liquid fractions. During the hydrolysis, the glucose and xylose concentrations were quantified using a high-performance liquid chromatography (HPLC). To quantify the sugars, the samples were filtered on 0.22 μm membranes and analyzed on a Shimadzu LC-10AD chromatograph, equipped with a RID-10A refractive index detector, Aminex HPX-87H column using mobile phase H_2SO_4 5 mM, flow rate of 0.5 mL/min and oven temperature of 65 °C. At the end of 96 h, the enzymes were denatured by boiling the entire suspension for 10 min at 100 °C. The residual pulp was washed with deionized water at least 4 times, followed by centrifugation under the same conditions described above, in order to separate the solid and remove residual glucose. Part of the material was separated from the supernatant containing nanocellulose. The separation was achieved after successive centrifugation steps at speeds at 1410*g*. The supernatant suspension was retained, while the portion containing the total residual material was frozen and dried by lyophilization. The lignin content of the samples was measured after enzymatic hydrolysis according to the TAPPI standard (TAPPI T222 om-02) [23]. All the assays were carried out in triplicate and the data were calculated as means ± standard deviations.

2.3. Enzymatic cellulose conversion

The enzymatic conversion of cellulose (ECC, %) to glucose was calculated according to Eq. (1):

$$ECC\ (\%) = \left(\frac{m^{t}_{\text{glucose}} - m^{0h}_{\text{glucose}}}{m^{0h}_{\text{cellulose}} \times 1.11} \right) \times 100 \quad (1)$$

where, m^t is the glucose mass at time t and the value 1.11 is the theoretical yield factor for the enzymatic conversion of cellulose to glucose.

2.4. Enzyme activity assays

Endoglucanase (EGase), filter paper (FPase), and β-glucosidase activities were determined according to the protocols of the International Union of Pure and Applied Chemistry (IUPAC) [24]. The substrates used for each enzyme were carboxymethylcellulose (CMC), Whatman no. 1 filter paper, and cellobiose (Sigma-Aldrich, USA), respectively. Xylanase activity was determined following standard methods [25], in the presence of beechwood xylan (Sigma-Aldrich, USA). Cellobiohydrolase (exoglucanase) activity was measured using Avicel (microcrystalline cellulose) as substrate, according to the method described by [26]. The release of reducing sugars was measured using the DNS method [27]. For β-glucosidase, glucose release was measured with a GOD-POD enzymatic kit (Labtest®, Brazil). One unit of EGase, FPase, β-glucosidase, xylanase, or cellobiohydrolase activity corresponded to 1 μmol of reducing sugars released per minute of reaction. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, USA). All the protein and enzymatic analyses were carried out in triplicate and the data were calculated as means ± standard deviations.

2.5. Atomic force microscopy (AFM)

The supernatant separated by centrifugation was dispersed in ultrapure water, under ultrasonication (Branson Ultrasonics 250), and an aliquot was deposited on a mica substrate. AFM images were obtained using a Multimode VIII instrument (Bruker AXS GmbH, Germany) equipped with a NanoScope V controller and a Si/Au probe (ScanAsyst-Fluid) operated in PeakForce tapping mode, with resonance frequency of 70 kHz, constant force of 0.4 N/m and scan rate of 1 Hz. Gwyddion v. 2.53 software was used for treatment and measurement of the images. Statistical evaluation of the quantitative data employed analysis of variance (ANOVA) and Tukey's test, adopting a 5% significance level.

2.6. Scanning electron microscopy (SEM)

The raw kraft eucalyptus pulp was evaluated by SEM micrographs at 10 kV using a JEOL JSM-6510 microscope with a tungsten filament. Samples were deposited to carbon tapes and gold-coated.

2.7. Nanocellulose yield

The yield of nanocellulose obtained after 96 h of enzymatic hydrolysis was determined by gravimetric analysis. The material was resuspended in ultrapure water and separated by centrifugation at 1410*g*.

This procedure was repeated until the water was transparent. Afterwards, the total volume of suspension was measured, and a 20 mL aliquot was dried in a plate previously weighed to determine the mass. The yield was calculated according to Eq. (2):

$$Yield\ (\%) = \frac{\frac{m_2 - m_1 * Vt}{Va}}{m_i} \times 100 \quad (2)$$

where, m_2 is the plate mass containing an aliquot of the sample, m_1 is the plate mass, V_a is the aliquot volume of the sample, V_t is the total volume of sample and m_i is the initial mass of cellulose pulp used in enzymatic hydrolysis reaction.

2.8. X-ray diffraction (XRD)

The crystallinity index (CI) values of the samples were determined using a Shimadzu Model 6000 diffractometer operating with Cu Kα radiation (λ = 1.54 Å), at 30 kV and 30 mA, in the 2θ range from 5° to 40°, at a scan rate of 2°/min. The CI was calculated using the areas under the crystalline and amorphous peaks, obtained by deconvolution using Gaussian functions, after a baseline correction using Origin 9.0 software. This is the most appropriate method for this type of assessment [28]. The CI was obtained by dividing the summed areas of all the crystalline peaks by the total area.

2.9. Apparent ζ-potential

The surface charges of the samples were determined using a Zetasizer Nano ZS analyzer (Malvern Instruments Ltd., UK), at a wavelength of 633 nm. A suspension of 0.1 wt% nanocellulose in 5 mM NaCl was employed for the measurements.

3. Results and discussion

3.1. Effect of the enzyme cocktail on release of soluble sugars

It is important to recover the soluble glucose from the stream released during the nanocellulose production process, since it has considerable value as a precursor for a wide range of bio-based products. In this process, the choice of a suitable enzyme cocktail is crucial for retaining the integrity of the crystalline cellulose fractions during the saccharification reaction. Therefore, the first step of this study was to determine the enzymatic profiles of commercial cocktails, in order to identify the activities of the main enzymes present and investigate how they might influence the saccharification process. For this, the enzymatic cocktails were divided into two groups: cocktails designed for complete saccharification (CS) and for partial saccharification (PS). Table 1 shows the enzyme activity and specific activity values for the main cellulolytic enzymes and for xylanase. The cocktails designed for complete saccharification showed higher activities of all the quantified enzymes, notably with higher specific activities of β-glucosidase enzymes, which favor the release of glucose. On the other hand, the cocktails designed for partial saccharification were mainly rich in enzymes such as endoglucanase and xylanase that can hydrolyze the amorphous biomass components.

Table 1
Enzyme activity and specific activity of the main enzymes used in the saccharification process, for the evaluated enzymatic cocktails (CS1, CS2, PS1, and PS2).

Enzyme	Enzyme activity (IU/mL) Specific activity (IU/g protein)			
	CS1	CS2	PS1	PS2
Endoglucanase	1548 ± 12 5.3 ± 0.07	2479 ± 14 13.0 ± 0.3	219 ± 3 1.5 ± 0.006	335 ± 4 16.8 ± 1.4
Xylanase	7981 ± 223 27.3 ± 0.2	12,153 ± 145 64.0 ± 0.9	1971 ± 8 13.0 ± 0.1	49 ± 4 2.4 ± 0.2
Avicelase	61 ± 8 0.21 ± 0.03	45 ± 6 0.24 ± 0.04	9 ± 0.7 0.05 ± 0.003	14 ± 4 0.7 ± 0.1
β-Glucosidase	865 ± 6 2.9 ± 0.04	380 ± 3 2.0 ± 0.03	41 ± 3 0.27 ± 0.01	1.4 ± 0.2 0.07 ± 0.003
FPase	205 ± 3	167 ± 3	13 ± 0.1	n.d.
Protein (mg/mL)	292 ± 6	190 ± 4	151 ± 2	20 ± 2

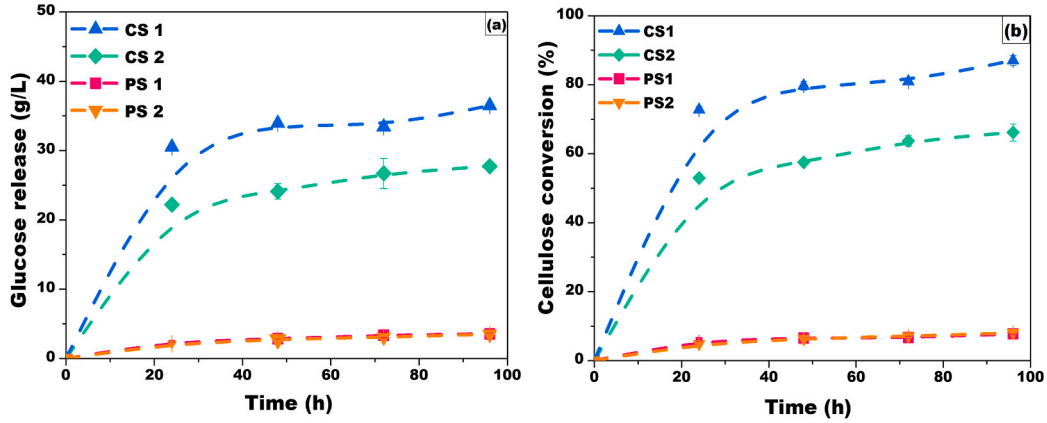
n.d.: not detected.

The sugars released during the hydrolysis reactions of the eucalyptus cellulose pulp showed similar profiles for the different cocktails, mainly varying in the concentrations (Fig. 1a). The control treatment without the addition of enzymes did not presented any soluble sugar. Higher glucose values were obtained with the CS1 and CS2 enzyme cocktails, reaching concentrations of 36.5 g/L (87% cellulose conversion) and 27.8 g/L (66% cellulose conversion), respectively, after 96 h of reaction (the conversion values are shown Fig. 1b). Since these two CS enzyme cocktails are produced by the same manufacturer, the updates mainly can relate to the addition of new accessory enzymes to strengthen their synergistic cooperation. Among these accessory enzymes, it is notable that the LPMO enzymes from the AA9 (Auxiliary Activity 9) family improve the performance of cellulases and contribute to glucose release [29].

For the biomass hydrolysis using the enzyme cocktails designed for partial saccharification (PS1 and PS2), the concentrations of glucose released were very similar, at around 3.5 g/L (8% conversion) after 96 h (Fig. 1a and b). As expected, these values were much lower than the ones achieved using the CS cocktails, in agreement with the enzymatic activity values (Table 1). In particular, the low activities of the exoglucanase (avicelase) and β-glycosidase enzymes in the PS cocktails could explain these low values of glucose released. The PS1 enzymatic cocktail is composed mainly of xylanase and endoglucanase, as well as exoglucanase and β-glycosidase with much lower activities (Table 1). The PS2 cocktail has an enzymatic composition similar to that of PS1, with high specific activity values for endoglucanase and xylanase, and lower values for exoglucanase and β-glycosidase. Comparison of the profile of the PS2 cocktail with the others shows that the specific activity values for xylanase and β-glycosidase were significantly lower. It should be noted that the main applications for these enzyme cocktails designed for partial saccharification are in the paper and cellulose industries, where their principal function is to assist in the refining and drainage stages of the processes [30].

In the enzymatic hydrolysis of biomass to obtain soluble sugars, it is desired to achieve high cellulose conversions. However, enzymatic hydrolysis reactions must be controlled, so that the crystalline segments are not completely converted and the material can be used for nanocellulose production [6]. In addition to releasing glucose (C6 sugar), hydrolysis in the presence of auxiliary enzymes releases other soluble sugars, such as xylose (C5 sugar) and oligosaccharides [31]. Fig. 1c shows the results for the release of total reducing sugars (TRS) and xylose release (g/L) using the different enzyme cocktails. The values for TRS and xylose release using the CS cocktails were higher than obtained using the PS cocktails. The use of the CS1 cocktail resulted in the highest release of TRS, reaching 47.9 ± 2.4 g/L in 96 h of hydrolysis. In terms of xylose release, it could be observed that the low values of xylanase activity in the PS2 cocktail resulted in no detected xylose in the supernatant. The measurement of TRS release provides useful information, since it shows the release of oligosaccharides and other soluble sugars, mainly due to the action of accessory enzymes. Xylanases have been shown to have important roles in both sugar release and nanocellulose production, due to their action on the structure of lignocellulosic fiber, with the removal of xylan from the surface leading to improvements in hydrolysis [17,32]. Therefore, it is important to evaluate how the compositions of these different enzymatic cocktails designed for complete and partial saccharification could influence the properties of the nanocellulose structures.

Besides the properties, the reaction yield related to the nanocellulose production by the enzymatic route is also an important aspect to consider during process development. After 96 h of hydrolysis, the yields



(c)

Time (h)	Total reducing sugar (g/L)			
	Xylose (g/L)			
	CS1	CS2	PS1	PS2
24	39.4 ± 1.4	29.3 ± 1.9	3.8 ± 0.4	2.5 ± 0.02
	6.56 ± 0.18	4.00 ± 0.20	0.86 ± 0.09	n.d
48	46.1 ± 1.2	35.7 ± 0.2	4.5 ± 0.5	2.8 ± 0.1
	7.20 ± 0.20	4.95 ± 0.15	0.95 ± 0.11	n.d
72	47.7 ± 2.6	38.9 ± 1.5	4.8 ± 0.5	3.6 ± 0.07
	7.42 ± 0.22	5.40 ±0.38	1.05 ± 0.13	n.d
96	47.9 ± 2.4	39.4 ± 1.6	7.1 ± 0.5	4.4 ± 0.05
	7.53 ±0.12	5.36 ± 0.14	1.02 ± 0.10	n.d

n.d.: not detected

Fig. 1. Temporal profiles for the enzymatic saccharification of cellulose pulp using different enzyme cocktails designed for complete (CS) and partial saccharification (PS). The reactions were carried out with a solids content of 5% (w/v) and enzyme load of 20 mg protein/g cellulose. (a) Glucose release; (b) cellulose conversion; (c) release of total reducing sugars and xylose.

of nanocellulose ranged from 2 to 6% when using the CS cocktails and from 10 to 13% for the PS cocktails. Despite being in agreement with the values reported in previous studies using the enzymatic route [8], these values can be explained by the low solids loading used here (5% w/v). Considering that further optimization of the reaction conditions could improve the yield, these results indicate the need for evaluating enzymatic cocktails better designed for nanocellulose production.

3.2. Effect of the enzyme cocktail on nanocellulose properties

3.2.1. Morphology and size

The supernatants separated from the solid material after 96 h of enzymatic hydrolysis reaction with the different commercial cocktails were evaluated using atomic force microscopy (AFM) to confirm the presence of nanocellulose (Fig. 2), as well as to estimate the sizes of the nanostructures (Fig. 3). For comparison purposes, scanning electron microscopy (SEM) of the starting cellulose pulp is shown in Fig. S1. The nanostructures obtained using the different enzyme cocktails were mostly thin and elongated, which is characteristic of nanocellulose. The average length of the nanostructures varied according to the enzyme cocktail used, whereas the diameters showed little variation (Fig. 3). Application of the Tukey test showed that the diameters of the nanostructures obtained using the PS1 enzyme cocktail were not significantly different from those obtained using the CS1 and CS2 cocktails. Only the diameters of the nanostructures obtained using the PS2 cocktail showed a statistically significant difference, compared to the other samples.

The histograms shown in Fig. 3 present the distributions of length and diameter for the different structures. Additional AFM images used to size estimates are shown in Fig. S2. The samples obtained using the CS1 and CS2 cocktails presented average lengths in the ranges from 80 to 350 nm and from 67 to 430 nm, respectively (Fig. 3a and b). The nanocellulose obtained using the PS1 cocktail showed lengths varying from 95 to 350 nm (Fig. 3c), with greater uniformity of the structures and dimensions within the range attributed to cellulose nanocrystals (from 50 to 350 nm) [33]. On the other hand, the nanostructures obtained using the PS2 cocktail showed a broader size distribution between 228 and 750 nm (Fig. 3d).

The physical characteristics, in terms of size and diameter, were similar for the nanocelluloses obtained using the CS1, CS2, and PS1 cocktails. Interestingly, the highest xylanase activities were observed in these three cocktails (Table 1). It is possible that the formation of better defined nanostructures was favored by the presence of xylanases, since the synergistic action of these enzymes and cellulases could improve the

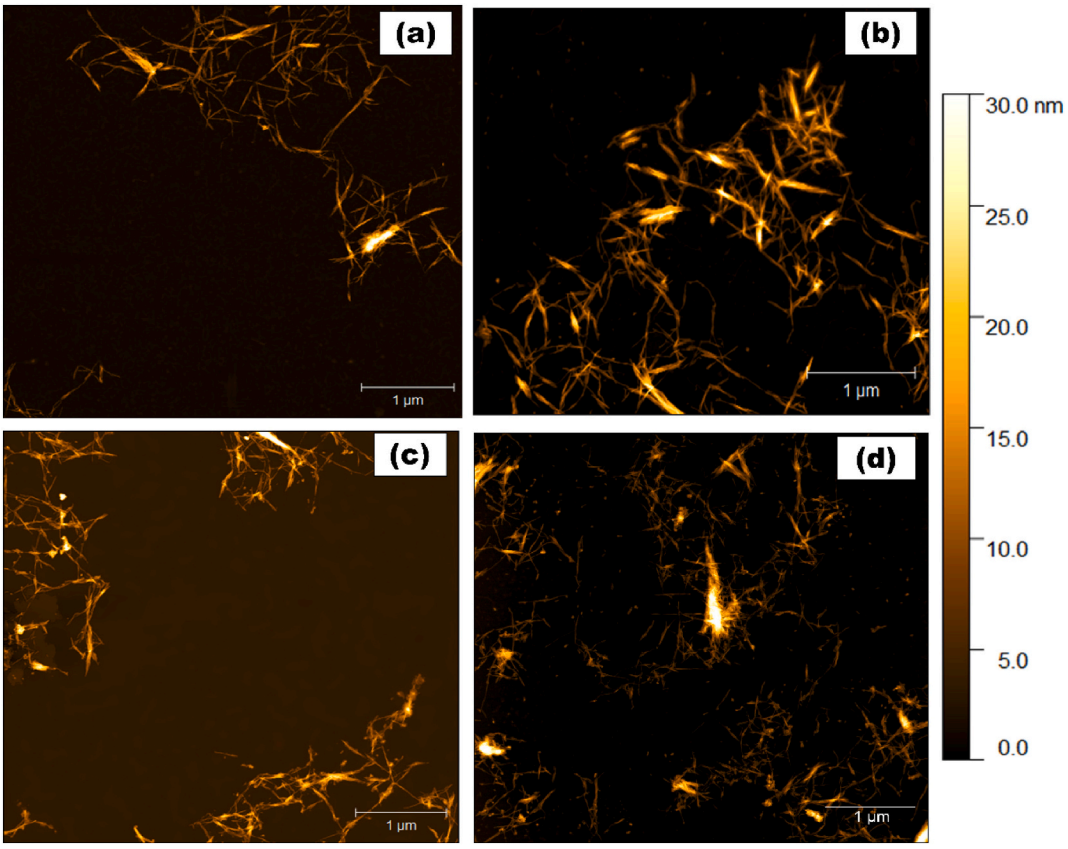


Fig. 2. Atomic force microscopy analyses of the nanocellulose samples obtained by the enzymatic saccharification of cellulose pulp using different enzyme cocktails designed for complete (CS) and partial saccharification (PS). (a) CS1; (b) CS2; (c) PS1; (d) PS2.

swelling of the fibers and porosity, consequently increasing the access of cellulases to the cellulose [34]. A recent study has also shown that the presence of accessory oxidative enzymes (AA9), in association with endoglucanase and xylanases, favored the release of more uniform and dispersed nanofibrils after stages of fibrillation and sonication [10]. These oxidative enzymes have been reported to be present in higher amounts in the CS1 cocktail, compared to CS2 [29], suggesting that they could have contributed to the uniformity of the nanostructures observed in this work.

3.2.2. *Crystallinity index*

Possible changes in the crystalline structure of the cellulose were investigated by XRD analysis of the residual materials obtained after the hydrolysis reactions using the different enzyme cocktails (Fig. S3). Fig. 4a shows the evolution of the crystallinity index, as a function of time, for each of the enzyme cocktails. An important assumption for the results of this analysis is that conversion of amorphous constituents was the main contributor to the crystalline index variation. The crystallinity indexes increased by between 3 and 14%, due to the removal of amorphous components, with this effect being more pronounced for hydrolyses using the CS1 and CS2 cocktails. The greatest increases in crystallinity were for the samples obtained using CS1 (79%) and CS2 (76%), in agreement with the sugar release results (Fig. 1a), suggesting that saccharification of amorphous components of the material occurred. Hu and collaborators [10] reported that the presence of endoglucanase in the hydrolysis step of cellulose pulp led to a crystallinity index increase of around 7%, with the value increasing after addition of AA9 or xylanase enzymes to the cocktails.

The residual solids from the biomass hydrolysis using the PS1 cocktail presented a higher crystallinity index (74%), compared to the value for the residual material obtained using the PS2 cocktail (70%). It is possible that the higher xylanase specific activity in the PS1 cocktail (Table 1) could have influenced the enzymatic hydrolysis reaction, leading to alteration of the proportion of crystalline cellulose in the final solid. The findings suggested that due to the lower xylanase specific activity, the nanostructures obtained by hydrolysis using the PS2 cocktail showed less alteration of the crystallinity index, compared to those obtained using the other cocktails that contained auxiliary enzymes. Similar results were reported by Nie and collaborators [35], who attributed higher crystallinity levels to the removal of amorphous material, especially structures present in hemicellulose, when the hydrolysis was performed in the presence of higher xylanase activity. Increase of the crystallinity index has been observed in studies of the hydrolysis of xylan in kraft eucalyptus pulp by the action of endoglucanases and cellobiohydrolases [10,32]. It is evident that milder treatments for the production of nanocellulose, such as the enzymatic route, are effective in preserving the native structures of the cellulose, in addition to leading to gains in the crystallinity index, reflecting an increase in the crystalline fraction of the final material. Besides, it is important to mention that the nanocellulose structures presented total lignin content similar to the original kraft eucalyptus pulp for all the samples, being of 6.11 ± 0.41% for the CS1; 6.18 ± 0.92% for the CS2; 6.32 ± 0.39% for the PS1, and 6.52 ± 0.09% for the PS2 sample. These data indicate that the enzymes cocktails were acting mainly on the non-lignin fractions of the pulp.

3.2.3. *Apparent ζ-potential*

Suspensions with apparent ζ-potential above 30 (positive or negative) establish structures with good colloidal stability, reducing the possibility of agglomeration [36]. Therefore, determination of the ζ-potential is important since it provides a tool for predicting and describing the colloidal behavior of nanocellulose suspensions. Electrophoretic mobility measurements were used to assess the surface

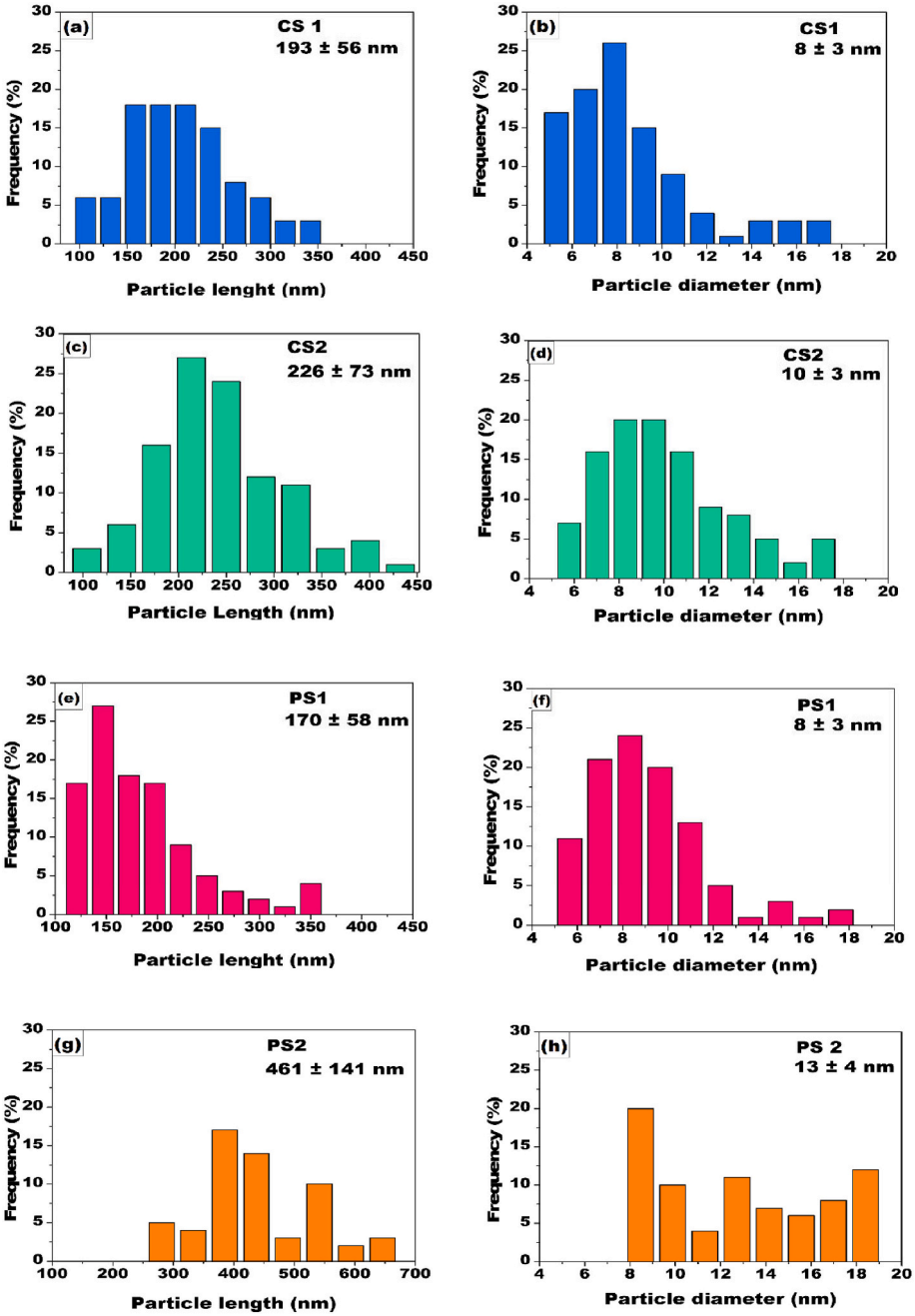


Fig. 3. Histograms of the length and diameter distributions (nm) for measurements of the nanocellulose samples obtained after the enzymatic saccharification of cellulose pulp using different enzyme cocktails designed for complete (CS) and partial saccharification (PS). (a, b) CS1; (c, d) CS2; (e, f) PS1; (g, h) PS2.

charges of the nanocellulose particles in the samples obtained after 96 h of hydrolysis using the different commercial cocktails (Fig. 4b). The ζ-potentials for the different samples varied from −30.9 to −15.8 mV. These values were expected, since enzymatic treatments do not install negatively charged groups on the surface, in contrast to sulfuric acid hydrolysis, where the ζ-potentials can vary from −50 to −20 mV [37]. Similar values to those obtained here were reported previously for nanocellulose production employing cellulose pulp and tailor-made enzymes [8].

The ζ-potentials obtained using the cocktails for complete saccharification were higher than for partial saccharification (Fig. 4b), with values of −30.9 ± 0.2 and −26.2 ± 1.9 for CS1 and CS2, respectively. Hu et al. (2018) [10] reported that the ζ-potentials for structures obtained using enzymatic cocktails containing AA9 enzymes were higher than the values obtained using cocktails without these enzymes. The same was observed in the present work, since the presence of this enzyme, especially in the CS1 cocktail, facilitated generation of the net charge associated with the exposure of charged groups. These groups can be introduced by oxidative cleavage by the AA9 enzyme at the C1 or C4 carbon of cellulose, improving the hydrogen bonding network and exposing hydroxyl groups [10,38].

The nanocellulose samples obtained using the PS1 cocktail, whose composition included higher xylanase specific activity, showed higher apparent ζ-potential, compared to the samples obtained using the PS2 cocktail. The composition of the PS2 cocktail included higher specific activity of endoglucanase, compared to xylanases. It was reported previously that the presence of xylanase in the hydrolysis step can facilitate exposure of the charged carboxylic groups on the surfaces of the fibers. This exposure leads to more electrostatically repulsive nanostructures, consequently improving the colloidal characteristics of the suspensions

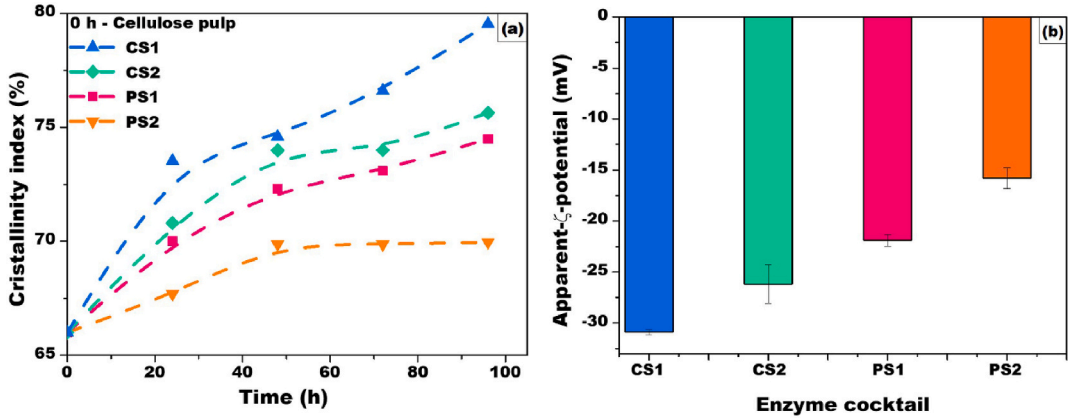


Fig. 4. (a) Evolution of crystallinity index, as a function of time, for samples obtained during the enzymatic saccharification of cellulose pulp using different enzyme cocktails designed for complete (CS) and partial saccharification (PS); (b) apparent ζ-potential of the nanocellulose samples obtained after 96 h of reaction.

[35]. Tibolla and collaborators [39] reported apparent ζ-potential values between −21.2 and −29.5 mV for nanocellulose suspensions obtained by the enzymatic route, employing xylanases from banana peel. Therefore, the presence of higher xylanase activity in the PS1 cocktail may have contributed to the higher apparent ζ-potential for the nanostructures obtained using this cocktail.

3.2.4. Suggested mechanism

Based on the results presented here and making an analogy with previous studies using mechanical defibrillation, a mechanism could be suggested for the formation of cellulose nanostructures by enzymatic hydrolysis (Fig. 5). The mechanism is based on enzyme-mediated deconstruction and occurs due to the synergism of enzymes that increase the overall accessibility to the substrate, in which the surface layers of cellulose are gradually hydrolyzed [10]. Some authors suggest that depending on the group of enzymes used for isolation of cellulose nanostructures, hydrolysis can be localized rather than processive [40]. Since kraft eucalyptus pulp has a significant amount of xylan associated with the cellulose fibers, it is of interest that this polymer should be removed. Hence, the cellulose chain is cleaved by endoglucanases and AA9 (present only in the CS cocktails), after the action of xylanases. As the saccharification proceeds, the outermost layers of cellulose are removed and the internal layers are exposed [41].

Similarly, in this work, as can be seen in the microscopy images (Fig. 2), it is likely that the depolymerization of cellulose to form nanostructures occurred by removal of the upper layers of biomass, with subsequent defibrillation resulting in disaggregation of the fibers and their reduction into smaller segments. These results suggested an important role of xylan in fibrillation, so its removal is essential for saccharification [10]. Therefore, the enzyme cocktails evaluated in the present work, which presented higher specific xylanase activities (Table 1), were likely to be more effective in producing homogeneous

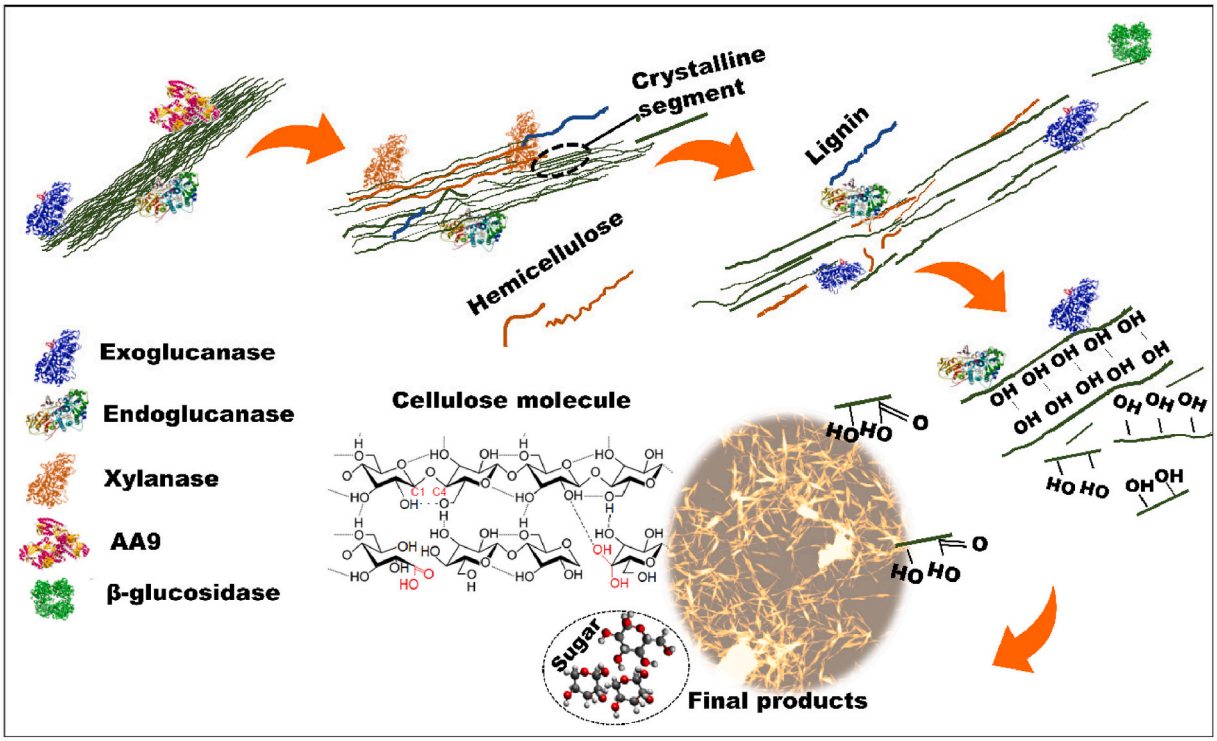


Fig. 5. Representative diagram of nanocellulose extraction by the enzymatic route, and mechanism of action of the main enzymes present in the commercial enzymatic cocktails evaluated, highlighting the feature of the xylanase enzymes.

cellulose nanostructures (Fig. 3). This was the case for both cocktails designed for complete saccharification (CS1 and CS2), while the xylanase activity of the PS1 cocktail was higher than that of PS2. Therefore, the higher endoglucanase activity of PS2, compared to PS1, did not prevail over the importance of the xylanase activity for obtaining nanostructures with properties characteristic of cellulose nanocrystals.

4. Conclusions

The commercial enzymatic cocktails designed for complete and partial saccharification, evaluated here, were effective in the formation of nanocellulose structures. The presence of auxiliary enzymes, especially xylanases, in the cocktails favored the production of nanostructures with higher crystallinity, liquid surface charge, and uniformity. For the enzymatic cocktails designed for partial saccharification, the xylanase activity was more important than the endoglucanase activity in the production of nanocellulose. The findings demonstrated that the compositions of the enzymatic cocktails already used for complete saccharification in biorefineries can also be suitable for obtaining nanocellulose, together with the release of a glucose stream.

CRediT authorship contribution statement

T.J.B.: Conceptualization, Methodology, Investigation, Writing - Original draft preparation C.F.: Writing - Reviewing and editing G.S.B.: Investigation, Writing - Reviewing and editing C.S.F.: Funding acquisition, Supervision, Validation, Writing - Reviewing and editing.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.03.007.

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