

## REVIEW

# Production of nanocellulose by enzymatic hydrolysis: Trends and challenges

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There is a great interest in increasing the levels of production of nanocellulose, either by adjusting production systems or by improving the raw material. Despite all the advantages and applications, nanocellulose still has a high cost compared to common fibers and to reverse this scenario the development of new, cheaper, and more efficient means of production is required. The market trend is to have an increase in the mass production of nanocellulose; there is a great expectation of world trade. In this sense, research in this sector is on the rise, because once the cost is not an obstacle to production, this material will have more and more market. Production of the cellulose fibers is determinant for the production of nanocellulose by a hydrolyzing agent with a reasonable yield. This work presents several aspects of this new material, mainly addressing the enzymatic pathway, presenting the hydrolysis conditions such as pH, biomass concentration, enzymatic loading, temperature, and time. Also, the commonly used characterization methods are presented, as well as aspects of the nanocellulose production market.

**KEYWORDS**

characterization methods, endoglucanase, enzymatic hydrolysis, market trend, nanocellulose

## 1 | INTRODUCTION

There is an increasing demand for more sustainable materials that can replace petroleum-based polymers in technological applications. More economically and environmentally friendly processes and raw materials are being developed to fulfill these demands [1]. Therefore, cellulose-based materials, such as nanocellulose composites, have great potential to assume this role in a near future because of its natural availability, biodegradability, and outstanding mechanical properties [2].

Cellulose is the most abundant renewable polymer on the earth and it is present mainly in plants and some species

of bacteria. It is commonly present in nature in lignocellulosic biomass, as a microfibril cellulose form, wrapped by other biopolymers such as lignin and hemicellulose. These two components act as a mechanical reinforcement and microbiological protection in plant cells [3,4].

As the demand for renewable fuels, such as bioethanol, increases, more and more biorefined residues are generated. The exploitation of these residues to produce second-generation ethanol with coproduction of nanocelluloses may represent a valuable increase in the profitability of the biorefinery industry and also provides a great solution to the environmental impact of the waste that would be generated-brk [5,6].

Nanocellulose has a broad variety of applications, depending on the range of the generated product physical properties. These properties are affected mainly by the production process utilized and by the composition of the lignocellulosic biomass [7].

Abbreviations: AFM, atomic force microscopy; CBH, cellobiohydrolases; CNF, cellulose nanofibre; CNC, cellulose nanocrystal; CD, catalytic domain; CrI, crystallinity index; EG, endoglucanases; XRD, X-ray diffraction; ZP, zeta potential

Nanocellulose is defined as cellulosic materials with at least one of its dimensions in the nanometric scale ( $1 \text{ nm} = 10^{-9} \text{ m}$ ) [8]. Its important features, such as high surface area, hydrophilicity, and high crystallinity, are due to intrinsic characteristics of cellulose and nanoparticles. Also, it presents high axial stiffness (up to 130 GPa), high aspect ratio (varying from 10 to 100), low density (approximately  $1.5 \text{ g/cm}^3$ ), and low coefficient of thermal expansion (approximately  $1 \text{ ppm/K}$ ). These features make it a promising material for mechanical reinforcements in polymer composites [9].

Cellulose fibers are divided into two domains: amorphous and crystalline. The first domain is the most susceptible for an enzyme or acid hydrolysis. The second one presents lower accessibility to those attacks, owing to the shield effect of lignin and hemicellulose. Amorphous regions are imperfections on the crystalline matrix and the proportion between them and crystalline domains depends strictly on the lignocellulosic biomass origin [10].

There are three types of nanocellulose morphologies that can be obtained: (a) Cellulose Nanofibre (CNF), which is normally obtained by an enzymatic and/or mechanical disintegration process; (b) Cellulose Nanocrystal (CNC), which is obtained by a treatment with concentrated mineral acids, mainly HCl or  $\text{H}_2\text{SO}_4$  [11]; and (c) Bacterial Nanocellulose, synthesized exclusively by a family of bacteria, known as *Gluconoacetobacter xylinus* [12]. However, the focus of this work is to present plant-derived nanocellulose.

The nanocellulose physical properties and biocompatibility are objects of interest for applications of these particles on drug delivery systems [13,14] and also food additives [15]. These features, along with the high surface area, make nanocellulose a strong candidate for biocompatible scaffolds for cell culture and tissue engineering applications [14,16].

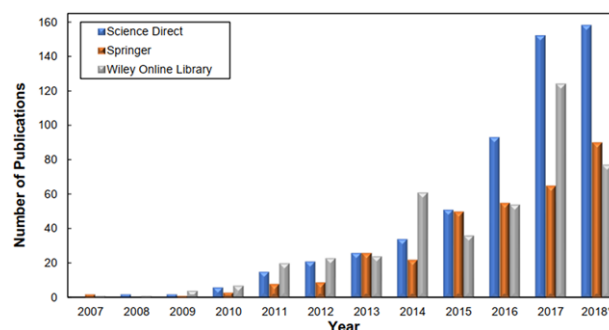
Most nanocellulose application studies aim the design and development of biopolymer composites. This can be explained by the outstanding mechanical properties and high surface area of these nanoparticles. Both features can provide composites with higher mechanical resistance and better particle aggregation [17].

Environmentally friendly packaging polymers and even antimicrobial packaging materials containing nanocellulose in its matrix have been reported [18]. Many different polymers functionalization with nanocellulose can be carried out, depending on the aimed purpose [19]. The broad range of nanocellulose technical applications, that may be positively impacted by its development, reinforces the need for extensive studies to achieve plenitude of its potential.

The growing number of publications related to enzymatic production of nanocellulose between 2007 and 2018 highlights the academic and commercial potential of this study. Technological discoveries and developments about nanocellulose are in their maturation period, which means that is the

## PRACTICAL APPLICATION

The research shows various contributions and advantages that the production of nanocellulose provides from a scientific and economic point of view, focusing on its production through enzymatic hydrolysis. The study compiles several examples of production and techniques of characterization of nanocellulose found in the literature.



**FIGURE 1** Number of publications related to enzymatic production of nanocellulose from 2007 to 2018\* (specifically on December 8, 2018)

right moment to invest resources and intellectual activity on nanocellulose research. Medical and dental patents started to have a light decrease in registered documents. On the other hand, publications focusing on the improvement of the production process and on material applications is presenting a consistent increase during the last few years, especially the ones aiming at the development of nanocellulose composites [20].

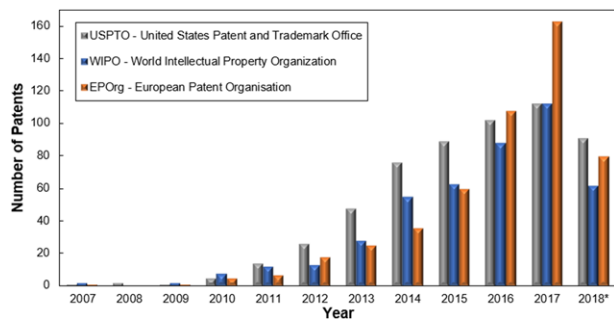
As can be seen in Figure 1, publications related to enzymatic production of nanocellulose have dramatically increased over the last 10 years. This tendency illustrates that this subject is at the early stages of development, with potential of developing novel and more efficient production methods, characterization, and innovative applications during the next few years.

The increasing number of patents related to the production of nanocellulose is a response to its high market value, as shown in Figure 2.

## 2 | NANOCELLULOSE PRODUCTION

### 2.1 | Current production methods

Concentrated mineral acid hydrolysis (mainly HCl or  $\text{H}_2\text{SO}_4$ ) is the most common method used for CNCs production. Although it provides higher yields when compared with enzyme hydrolysis, it requires higher capital investment,



**FIGURE 2** Patent publications over the years from different sources (WIPO, EPOrg, and USPTO), from 2007 to 2018\* (specifically on December 8, 2018)

because more expensive materials for reactors, tanks, and piping are required, on account of the corrosive nature of reactants. Besides, the effluent produced is harmful to the environment and operational costs may increase regarding the necessity of intensive effluent treatments [21].

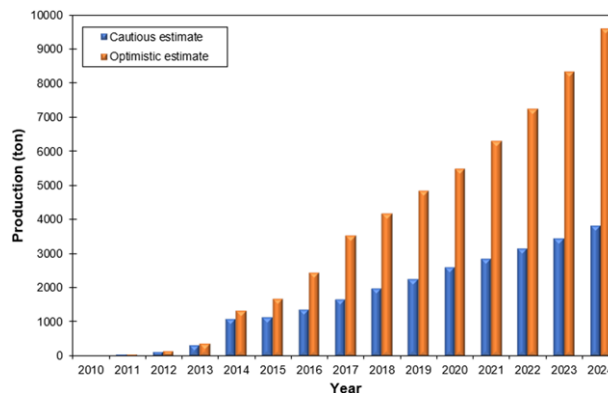
Cellulose fibers, transformed into smaller microfibrils by mechanical disintegration, are produced in many different ranges of width (in nanoscale) and length for more than three decades [22]. Even though mechanical disintegration with homogenization can be easily upscaled and operated continuously, this method requires high energy consumption, providing an expensive product [23].

As an emerging method, enzymatic hydrolysis may be an interesting pathway to nanocellulose fibrils production. In contrast, with both methods previously cited, enzyme hydrolysis does not generate toxic residues as mineral acid hydrolysis does. Moreover, enzymatic hydrolysis conditions are normally held in mild thermal and pressure conditions, resulting in a lower energy-intensive process. Also, nanocellulose produced by enzymes normally has more high-valued applications, because of its morphologies [24].

## 2.2 | Commercialization of nanocellulose

The market trend points to an increase in mass production of nanocellulose; there is a great expectation of worldwide trade. The consulting company, Future Markets Inc, estimated the data presented in Figure 3 that better analyze this tendency. According to this source, it is believed that there will be a production of up to more than 9000 tons of nanocellulose per year.

The bacterial nanocellulose production is very limited compared to the large-scale production of CNCs and cellulose nanofibrils because of the high cost to support bacterial growth and low yield [14]. Tables 1 and 2 present some companies that elaborate and have been developing technology to produce nanocellulose (nanocrystals or nanofibrils) in the large scale.



**FIGURE 3** Estimates of nanocellulose production (adapted from Future Markets Inc, The Global Market for Nanocellulose to 2024) <http://www.vtt.fi/inf/pdf/technology/2014/t199.pdf>

## 3 | NANOCELLULOSE CHARACTERIZATION METHODS

There are several available techniques used for nanocellulose particle characterization. They provide highly relevant information about particle morphology and their behavior in other matrices such as suspensions and polymer composites. There are many different types of analysis that could provide valuable information from the experiments. Some of them are briefly described next.

### 3.1 | Atomic force microscopy (AFM)

This technique is largely used to study a 3D surface morphology. In terms of nanocellulose, it can be used to measure the particle dimensions [25] and also patterns of aggregation or dispersion on the surface [26].

### 3.2 | Transmission electron microscopy and scanning electron microscopy (SEM)

Both techniques provide high-resolution images in nanometric scales. It gives a clear view of nanocellulose, size, shape, and uniformity [27]. When used to compare pretreated particles with the nanocellulose obtained, it can give valuable information about changes occurred during the treatment [28].

### 3.3 | X-ray diffraction (XRD)

The ratio between the crystalline and amorphous regions determines the crystallinity index (CrI) of the cellulose, which together with the orientation of the crystalline and amorphous domains in the fibers affect the mechanical properties of the cellulose fibers. The standard technique used to measure the CrI is XRD [29], which consists of the incidence of an electron beam that, when being diffracted, contains information about the structure of the sample. The decrease in the degree

**TABLE 1** International Company involved with production of cellulose nanocrystals (Adapted from Tappinano, “Summary of International Activities on Cellulosic Nanomaterials”) <http://www.tappinano.org/media/1096/tc6-world-cnm-activities-summary-july-29-2015.pdf>

Cellulose nanocrystals (CNCs)					
Country	Company	Product/trade name	Production capacity	Source	Method
USA	American Process Inc.	BioPlus; CNCs	0.5 ton/day (estimated)	Wood chips; Agricultural residues (bamboo, grasses)	Sulfur dioxide and ethanol pretreatment
	USDA Forest Service Forest Products Laboratory (FPL)	CNCs	50 kg/week	Wood pulp	Sulfuric acid hydrolysis
Sweden	MoRe Research	Nanocrystalline cellulose	0.1 ton/day (pilot plant in place during first half of 2016)	Paper industry sludge	Controlled sulfuric acid hydrolysis, washing, and sonication
China	Tianjin Haojia Cellulose Co., Ltd.	NCC	–	Dissolving pulp; cotton; bleached kraft pulp	Mechanical shearing combined with enzymatic and acid hydrolysis
India	Indian Council of Agricultural Research–Central Institute for Research on Cotton Technology (ICAR-CIRCOT)	Nanowhiskers	10 kg/8 h (pilot plant)	Cotton and cotton linters	Novel microbial, enzymatic and chemomechanical processes

of crystallinity is evidenced by XRD by the decrease of the intensity and increase of the width at half-height of the diffraction peaks, the same can be observed for crystallites less than 1  $\mu\text{m}$  [30]. It also can help to the evaluate efficiency of the enzymatic reaction by monitoring the increase of crystallinity between feedstock and product; the reduction of the amorphous region by enzymatic action leads to an increase of crystallinity.

### 3.4 | Zeta potential (ZP)

This technique is a useful indicator of the effective electrical charge on its surface in the particles, it correlates with the attractiveness or repulsion electrostatic intensity of particles in colloidal suspensions. ZP can be measured by an instrument in which a small sample of the suspension is inserted, providing readability in mV, which can be a positive or negative number. The higher the ZP, the more likely the suspension to be stable as the charged particles repel each other and this force overcomes the natural tendency to aggregate. It can provide valuable information about behavior of particles in emulsions, foams, and also an aggregation of nanocellulose, which should be low for good results as a reinforcement agent [31].

### 3.5 | Thermal analysis

The thermal analysis of nanomaterials is becoming more common as the manipulation of its surface expands and new ideas for its use are diffused. The main techniques used widely to provide rich information on thermal decomposition and

impurities of nanocellulose are the thermogravimetric analysis, differential thermogravimetry, and differential scanning calorimetry [11]. It is known that thermogravimetric analysis thermograms show that the degradation temperatures for all nanocrystals, which are constituted essentially of three events. The first major decomposition event occurs at temperatures below 100°C due to the evaporation of adsorbed and bound water and/or low molecular weight compounds adsorbed on the surface of the nanocrystals and represents on average of approximately 10% of the total weight loss of the samples. For all CNCs, the initial weight loss is followed by a plateau that extends to the beginning of the second major decomposition event. The second prevailing decomposition event corresponds to the degradation of cellulose, which consists of several competing processes, such as depolymerization, dehydration, and decomposition of glycosidic units. The third major decomposition event is attributed to the oxidation and decomposition of carbonized residues to form gaseous products of low molecular weight. The thermal stability of nanocrystalline cellulose is mainly due to the sulphate groups on the surface of them. If these are modified or sodium is used as counter ion, the thermal stability is increased [58,59].

### 3.6 | Dynamic light scattering

This is a light scattering technique used to determine the hydrodynamic diameter of nanoparticles in suspension. The diffusion coefficient of the particles is measured by the light scattering characteristics applied by the device. The coefficient obtained is used to calculate the hydrodynamic

**TABLE 2** International Company involved with production of cellulose nanofibrils (adapted from Tappinano, “Summary of International Activities on Cellulosic Nanomaterials”) <http://www.tappinano.org/media/1096/tc6-world-cnm-activities-summary-july-29-2015.pdf>

Cellulose nanofibrils (CNFs)					
Country	Company	Product/trade name	Production capacity	Source	Method
Canada	Kruger Bioproducts Inc.	FILOCELL; Cellulose filaments	5 tons/day	Wood pulp fiber	Mechanical treatment
USA	American Process Inc. (AVAPCO)	BioPlus CNFs	0.5 ton/day (estimated)	low cost woody and non-woody biomass (forest and agricultural residues, bamboo, and grasses)	SO <sub>2</sub> and ethanol to fractione biomass; Mechanical treatment
	USDA Forest Service Forest Products Laboratory (FPL)	CNFs	5 kg/week (pilot plant in place during 2012)	Wood pulp	TEMPO oxidation and mechanical treatment
Sweden	Innventia AB	CMFs	100 kg/day (pilot plant in place during first half of 2014)	Wood fibers	Chemical and/or enzyme pretreatment, Mechanical treatment (homogenization)
Finland	UPM-Kymmene Ltd.	Biofibrils	–	Wood fibers	Mechanical treatment
UK	Zelfo Technology GmbH	MFC	–	Cellulose fibres, fibre-based waste (recycled)	Cellulose Optimization Resource Efficient (CORE). “Technology that allows a modification of cellulose fibres using minimum energy and water”
	Imerys	FibreLean; MFC	1000 to > 10,000 tons/year (pilot plant)	Range of (wood) pulp species	No fiber pretreatment; co-grinding mineral with fiber
China	Tianjin Haojia Cellulose Co., Ltd.	NFC	–	Dissolving cotton pulp; Bleached sulfite pulp (soft- and hardwood)	TEMPO oxidation and mechanical treatment
Japan	Daicel	Microfibrillated Cellulose; Celish™	–	Purified pulp	Mechanical treatment
	Nippon Paper Industries	CNFs; Cellenpia	30 ton/year	Wood pulp	TEMPO oxidation; carboxymethylation; mechanical treatment

diameter (Hd) of particles using the Stokes-Einstein equation. This technique presents a simple and fast operation; however, it has limitations in solutions that have polydispersed samples, due to the fact that clustered or very large particles can overlap and trap in the reading of smaller particles [32–34].

Table 3 shows examples of nanocellulose characterization that were obtained by enzymatic techniques mentioned.

### 3.7 | Crystallinity index

The cellulosic materials are characterized by having in their molecular structure ordered, considered crystalline, and disordered, considered amorphous, regions [42]. In addition, many nanocellulose production researches focus on the analysis of the CrIs of cellulosic materials [43]. The measurement of the

CrI is important, because it is related to the tenacity of the fibers and the absorption of the enzyme by the cellulose in enzymatic hydrolysis processes [30,44].

The techniques generally used to measure the CrI are: XRD, nuclear magnetic resonance, Fourier transform Infrared and Raman spectroscopy [45]. However, most research use XRD in the analyzes, through the semiempirical method of the Segal et al., due to its simpler method of analysis [46]. Equation (1) and Figure 4 illustrate and explain the method of measuring the CrI.

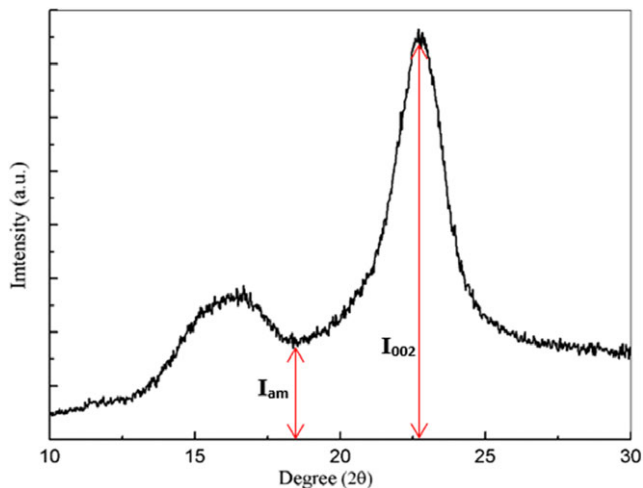
$$\text{CrI}(\%) = \left( \frac{I_{002} - I_{\text{am}}}{I_{200}} \right) \times 100 \quad (1)$$

where:

CrI (%) is the CrI;

TABLE 3 Examples of nanocellulose characterization found in the literature

Method	Source	Techniques of analysis	Responses	References
Enzymatic	Bleached Kraft eucalyptus pulp	SEM TEM	Cellulose nanofibril image	[27]
	Recycled pulp	SEM TEM DLS ZP	Cellulose nanofibril image  Hd: 100 to 3.5 nm −31.37 mV	[30]
	Commercial MFC; Eucalyptus holocellulose; Sugarcane bagasse; Wood Pulp from Pine	AFM SEM XRD	Cellulose nanofibril image  Commercial MFC Eucalyptus holocellulose Sugarcane bagasse Wood Pulp from Pine	[26]  CrI: 72.76 to 81.02% CrI: 58.42 to 65.94% CrI: 59.60 to 63.95% CrI: 59.33 to 68.48%
	Wood fiber pulp	AFM SEM	Cellulose nanofibril image	[25]
	Orange waste residue	SEM	Cellulose nanofibril image	[28]
	Sugarcane bagasse	AFM SEM TEM XRD	Cellulose nanocrystal image  CrI: 52 to 83.7%	[31]
	Soybean straw	TEM XRD ZP	Cellulose nanofibril image CrI: 45 to 68% −20.8 mV to −24.5 mV	[35]
	Bleached Kraft maple pulp	SEM TEM	Cellulose nanofibril image	[6]
Non-enzymatic	Bleached aspen kraft pulp	AFM XRD	Cellulose nanocrystal image CrI: 80.42 to 84.86%	[36]
	Banana rachis; Sisal; Kapok; Pineapple leaf; Coir	AFM SEM TEM XRD	Cellulose nanofibril image  Banana rachis Sisal plant Kapok tree Pineapple leaf Coir	[37]  CrI: 80.9% CrI: 91.3% CrI: 86.5% CrI: 92.3% CrI: 84.5%
	Bleached sulfite chemical; Pulp; Cotton; Microcrystalline cellulose	SEM  DLS	Cellulose nanocrystal image  Hd: 50 nm to 5 μm	[38]
	Rice straw	AFM SEM TEM XRD	Cellulose nanocrystal image  CrI: 61.8 to 91.2%	[39]
	Oil Palm Fibers Xianxing	SEM TEM XRD	Cellulose nanofibril image  CrI: 59.35%	[40]
	Sugarcane Bagass	AFM SEM TEM XRD	Cellulose nanocrystal image  CrI: 72.5%	[41]



**FIGURE 4** X-ray diffractogram of cellulosic material

$I_{002}$  is the maximum diffraction intensity (the 002 plane), located around  $2\theta = 22.5^\circ$  and corresponds to the crystalline material;

$I_{am}$  is the minimum diffraction intensity located around  $2\theta = 18^\circ$  and corresponds to the amorphous material.

## 4 | ENZYMATIC PRODUCTION OF NANOCELLULOSE

The transformation of cellulose into monosaccharides can be achieved in a chemical process carried out by a special group of enzymes known as cellulases. Cellulases are a group of enzymes that catalyze the breakdown of cellulose polymer into smaller polymer branches or even to cellobiose and glucose. Traditionally, these enzymes are divided into three groups: endoglucanases, exoglucanases, and cellobiohydrolases (CBH) [47–49].

It is widely accepted by the scientific literature that the complete hydrolysis of cellulose into glucose requires the synergistic action of at least two of the three enzymes: First, the amorphous regions of the cellulose chain are more accessible by endoglucanases (EG, endo-1,4- $\beta$ -D-glucanase, EC 3.2.1.4), and randomly separates the internal bonds of the glycan chains, thus resulting, nonreducing ends of cellooligosaccharides, for subsequent action of CBH or exoglucanase (1,4- $\beta$ -D-glucan-cellobiohydrolase, EC 3.2.1.91). The CBH then hydrolyzes these ends of the chain progressively, producing cellobiose as the main product. Finally,  $\beta$ -glucosidase (BG, cellobiase,  $\beta$ -D-glucoside glucanohydrolase, EC 3.2.1.21) hydrolyses cellobiose to glucose and also releases glucose from the nonreducing ends of the soluble cellooligosaccharides. There is a high degree of synergy between CBH (exoglucanases) and endoglucanases, which is necessary for better hydrolysis of cellulose. Although this division

is clear and well described, the role of proteins involved in the process is still poorly characterized [50–52].

### 4.1 | Catalytic mechanism of endoglucanase

In terms of enzymatic production of nanocellulose, endoglucanases are the ones with the highest interest, because their action is focused in the amorphous region of the cellulose, breaking the polymer into smaller length polymers [48].

Current studies on enzymatic hydrolysis of cellulose focus their attention to the synergistic effect of the different enzymes, allied with mechanical treatments or acid reactants. A broader comprehension of the synergistic effects of those mechanisms can lead to higher product properties predictability, which is essential to the development of reliable industrial products ready for commercialization. Also, the effect of enzyme hydrolysis can help to reduce energy costs during the mechanical treatment (the next step) [53].

Once cellulose crystals are produced from the crystalline region, the selectivity of endoglucanase enzyme is used to explore the hydrolysis of the amorphous regions, which yield CNCs, while the crystalline region is fragmented into cellulose nanofibrils by mechanical treatments such as ultrasonication.

Orłowski et al. used atomistic molecular dynamics simulations to study one of the endoglucanases of the *T-fungus reesei*, known as endoglucanase II (EG II or Cel5A), which is one of the proteins of this group. Based on the results of the atomistic simulation, the authors discuss how the enzyme Cel5A interacts with cellulose fibrils composed of crystalline and amorphous regions. Cel5A and many related proteins contain a carbohydrate binding modulus, a catalytic domain (CD), and a linker that unifies them. Cel5A is one of the most abundant endoglucanases with its highest activity at pH 4.6–4.8. In essence, this work shows that the carbohydrate binding domain of the enzyme prefers to interact with crystalline regions of cellulose, while the CD has a high affinity for the amorphous regions of the fibrils. Particularly, by electrostatic interactions, the CD attracts loose glucose chains to its catalytic slit [54].

## 5 | PRODUCTION TECHNOLOGY

Cellulose fibers exposure to a hydrolyzing agent is determinant to the nanocellulose crystals production, with a reasonable yield. Thus, lignin and hemicellulose, which induce a shield effect to the cellulose, must be removed from the lignocellulosic biomass. There are several methods of lignin and hemicellulose removal, by chemical pretreatments such as sodium carbonate treatment, periodate-chlorite oxidation, sulfonation, and many others [8]. Each lignocellulosic matrix

TABLE 4 Summary of some reports of enzymatic production of nanocellulose

Material	Description of the treatment	Enzymes	Conditions of hydrolysis			Dimensions		References	
			pH	Concentration of biomass	Enzyme concentration	Temperature—Time	Diameter		Length
Bleached Kraft eucalyptus pulp	Enzymatic hydrolysis followed by microfluidization	EG and CBH	4.8	10% pulp	1.5 FPU/gram of substrate	50°C—48 h	20 nm	Above 500 nm	[27]
Recycled pulp	Enzymatic hydrolysis with microwave and conventional heating	EG	6.8	0.8% (0.2 g/25 ml)	420 EGU/gram of substrate	50°C—1 h	30–80 nm	100 nm–1.8 μm	[30]
Commercial MFC; eucalyptus holocellulose; sugarcane bagasse; Wood Pulp from Pine	Wet disk milling and Enzymatic hydrolysis	EG and BG	5	1% biomass	345 EGU/g of substrate	45°C—72 h	15–30 nm	Several micrometers	[26]
Wood fiber pulp	Enzymatic hydrolysis followed by mechanical shearing	EG	7	3% pulp	0.02%–3% enzyme concentration	50°C—2 h	5–30 nm	Several micrometers	[25]
Orange waste residue	Enzymatic hydrolysis followed by bleaching	BG and Commercial Enzymes	4	17% orange waste residue	3.4 mg of pulpzyme HA, 3.4 mg of cellulast, and 0.6 mg of β-galactosidase per gram of substrate	50°C—48 h	180 nm	1.3 mm	[28]
Sugarcane bagasse	Pretreatment with liquid hot water (90°C) and ten minutes followed by enzymatic hydrolysis	EG and BG	5	10% bagasse	7, 12, and 22 mg of protein per gram of cellulose	50°C—24 h	14–18 nm	195–250 nm	[31]
Soybean straw	Enzymatic hydrolysis followed by pretreatment with NaOH and H <sub>2</sub> O <sub>2</sub>	BG and CBH	4	0.02% (3 g/150 mL)	93 μL of Optimash VR per gram of substrate	50°C—42 h	15 nm	~300 nm	[35]
Bleached Kraft maple pulp	Enzymatic hydrolysis followed by ultrasound	Cellic CTec 2 and Cellic HTec 2 (commercial enzymes)	4.8	5% (5 g/100 mL)	0.04 mL CTec 2 + 0.02 mL HTec 2 per gram of substrate	50°C—72 h	5–10 nm	1 μm	[6]



composition would require different pretreatments steps [19]. Table 4 presents some relevant works of enzymatic production of nanocellulose.

Combined treatment with endoglucanases and mechanical shearing of wood fiber pulps provided nanofibers with higher aspect ratio and molecular mass than nanofibers resulting from acidic treatment [25]. Besides producing microfibrils with interesting properties for reinforcement polymers, this method gives an environmentally friendly method, free of toxic and corrosive chemicals that are commonly used in acidic treatments.

The intensification of enzymatic hydrolysis time of bleached Kraft eucalyptus pulp has shown to significantly reduce solid cellulose degree of polymerization. Such depolymerization helped the formation of cellulose nanofibers by mechanical treatments [27].

It was shown that nanocellulose is a potential high-valued coproduct of 2G ethanol production chain. The fibers could be obtained by enzyme hydrolysis of sugarcane bagasse pretreated with liquid hot water. For this source of biomass, more efficient disruption of the lignocellulosic materials can lead to better results in overcoming cellulose recalcitrance [31].

The combination of wet disk milling with enzymatic hydrolysis has been shown as an alternative to coproduce CNCs during the saccharification process in biorefineries. This method has been proved efficient not only for sugarcane bagasse, but also for other lignocellulosic biomasses [26]. Soybean straws pretreated with NaOH or H<sub>2</sub>O<sub>2</sub> have been reported also as a good source of cellulose nanofibers by enzymatic treatment [35]. Depending on the pretreatment employed, different properties may be yield, leading to different possibilities of applications. The use of this kind of processes also produces sugars, which can be used for fermentation processes such as ethanol production.

Another interesting case report was the production of nanocellulose from orange waste residue. The use of this residue to produce nanocellulose could be economically feasible by integration with the orange juice industry, D-limonene, and ethanol, once throughout the process, all these components can be extracted from the orange fruits. These integrations would not only help the economic balance of the process, but also would help to give an end to solid residues that could generate a negative environmental impact [28].

Microwave heating has been shown to be an interesting alternative to conventional heating to provide higher yields of nanocellulose crystals using endoglucanase as the hydrolysis agent. Besides, high-negative ZPs were also obtained, suggesting the suitability for those particles as nanofillers in reinforced polymer composites [30].

A high aspect ratio is highly desirable when nanocellulose production is directed toward the development of reinforced, because it enables critical length for stress transfer to the reinforcing phase [55].

## 6 | PRODUCTION VIA ENZYMATIC HYDROLYSIS

### 6.1 | Characteristic of production via enzymatic hydrolysis

The method of production of nanocellulose through enzymatic hydrolysis is less explored in the literature than nonenzymatic, which is often alkaline or acidic processes [12]. This methodology presents some peculiarities and characteristics that may be advantageous than the other. Because of this, there is a necessity to develop and invest production through enzymatic methods.

The preparation applied via enzymatic hydrolysis in their procedures, usually, a temperature close to 50°C and pH 5, different from the nonenzymatic procedures that present less harmful conditions due to the fact of applying temperatures commonly around 100°C with very high or very low pH. These differences make the first procedure produces less toxic waste and less corrosive. This feature facilitates the application of nanocellulose via enzymatic hydrolysis in possible cosmetic and pharmaceutical products [40,56].

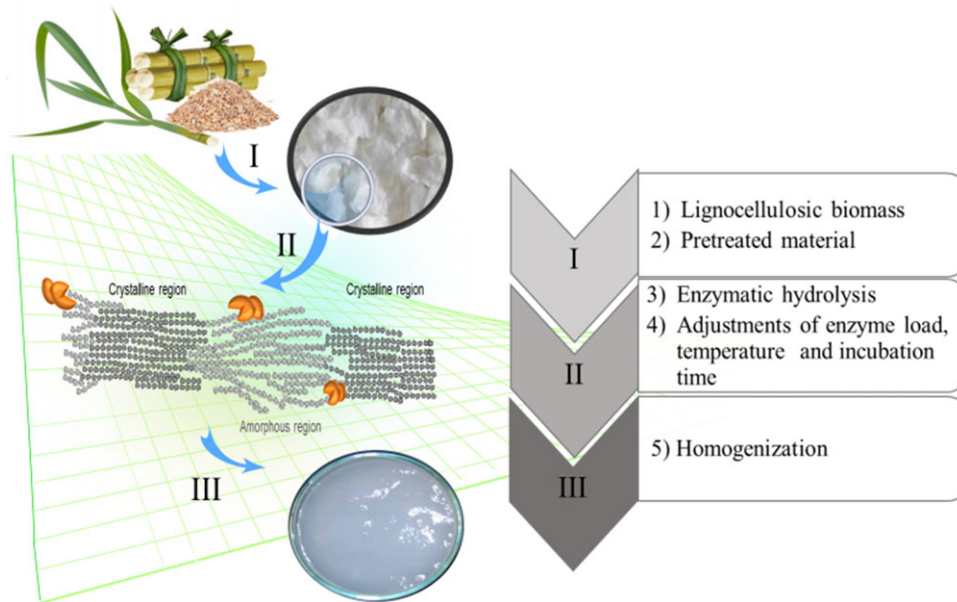
However, the enzymatic process requires more time to prepare the particles than the nonenzymatic. The first preparation usually takes on the scale of days, while the other takes on the scale of hours [4,40].

Generally, the processes of nanocellulose production through enzymatic hydrolysis generate cellulose nanofibrils while nonenzymatic processes generate CNCs [12]. One of the characteristics of CNCs is that it generally has a higher CrI than the other, due to the fact that it has more hydrolyzed amorphous regions, and has a shorter length. While the cellulose nanofibrils present greater flexibility due to the presence of a longer structure with some amorphous regions, this characteristic allows this type of nanocellulose to have a greater mechanical resistance [9,12].

### 6.2 | Nanocellulose production route via enzymatic hydrolysis

The disadvantages of the enzymatic hydrolysis technology are still the high costs of the enzymes and the process is slower. However, the reduction of these costs has attracted the interest of research centers and companies in recent years, due to the high potential of high saccharification efficiency and high penetration, as well as the low level of pollution. With the use of enzymatic hydrolysis, high yields and almost pure cellulose material are favored, in addition to, reducing environmental impact and obtaining products suitable for biomedical applications [61].

The processes of nanocellulose production via enzymatic hydrolysis have several steps. Figure 5 shows, in a simplified way, the main procedures that allow the production of



**FIGURE 5** Nanocellulose production diagram via enzymatic hydrolysis

nanocellulose. This whole route of production was based on the literature and summarizes some production procedures discussed to follow.

The process between the steps, according to the enumerations: The first stage (I), consists of the pretreatment of lignocellulosic biomass. The objective of this pretreatment applied in lignocellulose is to break the interconnecting structure of its composition. This exposure raises the surface area of the polymers and facilitates the effectiveness of the enzymatic treatment [56]. The main techniques applied as pretreatment are the mechanical processes combined with a washing process [25,26,31]. The second step (II) consists of the controlled enzymatic hydrolysis of fiber samples and it is the main step for the production of nanocellulose. In this phase, the pretreated material is solubilized in the buffer solution with an enzymatic cocktail. The enzymatic cocktail has the function of breaking the polymer of the cellulose into smaller polymers. The endoglucanases have a relatively moderate activity, which is suitable for the hydrolysis of amorphous cellulose regions without complete hydrolysis for glucose, but preserving the crystalline cellulose domain accordingly [47–49]. The added buffer ensures a more constant enzymatic activity. The pH range that is used varies from 4 to 7, according to the literature [6,25–28,30,31,35]. The reagents are mixed constantly, usually at a constant temperature range from 45 to 50°C. The mixing time, can range from 1 to 72 h. This time discrepancy is probably due to the different types and conditions of the lignocellulosic materials, the mixing speed, the distinct enzymatic cocktails and pH, which are presented in the literature. All these variations result in different times allowing the material to be hydrolyzed to obtain

nanocellulose [4,6–28,30,31,35]; Finally, the third step (III) is the homogenization process of the enzymatically treated material by a simple washing, suspension, or mechanical homogenization. These final procedures allow the production of nanocellulose with homogeneous consistency [6,25–28,30,35].

## 7 | FINAL REMARKS

### 7.1 | Challenges and future perspectives

One major issue involving the use of nanocellulose in composites is the fact that most polymers used are hydrophobic and nanocellulose is hydrophilic. This can be a problem during the mixture of the components, increasing the tendency of nanocellulose to form aggregates that would not only give nondispersed matrices, but would attenuate the nanoscale properties to a reinforcement agent. The hydrophobicity present in the nanocellulose can be resolved with the application of some organic solvents, for example, N,N-dimethyl formamide, dimethyl sulfoxide, and N-methyl pyrrolidone, as reported in the literature [2,57].

Current methods provide typically a low yield and products with a quite broad range of sizes. In order to turn the product more viable to commercialization or industrial applications, these results need to be improved. The most common pathways to achieve better yields are concentrated on pretreatment improvements. One possible way to financially overcome the lower yield provided by the enzymes is the integration of nanocellulose production with other

industries such as biorefineries, food and juice manufacturing processes. The residual biomass of these industries can be used in the production of nanocellulose, as a way to improve profitability. Other pathways to achieve better yields are concentrated on pretreatment improvements.

The growth tendency of studies related to the production of nanocellulose using enzyme treatments highlights the potential of this method. Despite some pointed difficulties that have to be overcome, enzymatic production of nanocellulose shows itself as an environmental friendly pathway to give a product with properties that meet the physical requirements for many valuable applications, specially reinforced polymer composites.

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## CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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