

## INNOVATIVE ENZYME TECHNOLOGIES FOR THE PRODUCTION OF CELLULOSE NANOMATERIALS

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### ABSTRACT

The unique properties of nanocelluloses make them attractive renewable building blocks for a broad spectrum of industrial and consumer applications. However, their commercialization has been slow due to the still high production costs and to the lack of commercial availability, at low cost, of nanocelluloses with different sets of properties, even within the same type of nanocellulose, a requisite to reach optimum performance in different applications. In this context, our efforts have been devoted to developing an innovative, viable, robust, and scalable enzyme-based platform technology that enables manufacturing nanocelluloses at various grades, suitable for achieving optimal performance in various applications. For this presentation, results will be presented and discussed to demonstrate that enzyme technology offers several advantages over some traditional mechanical and chemical process steps in producing nanocelluloses from hardwood Kraft pulp using commercial and in-house enzymes. Emphasis will be given to results that evidence the innovative enzyme technology's ability to produce CNCs and CNFs with high yield and customizable properties like morphology, rheological behavior, size, and surface chemistry, which are key to maximizing application performance.

**Keywords:** Lignin nanoparticles. Biorefinery. Nanocellulose. Prebiotic. Cellulosic biomass.

## 1 INTRODUCTION

The growing interest in cellulose-based products has led to the development of methods to systematically break down cellulose into cellulose nanoparticles (CNs), such as cellulose nanocrystals (CNCs) and cellulose nanofibrils (CNFs). These nanomaterials enhance the versatility of cellulose applications by leveraging their nanoscale features and superior properties for a range of high-volume, low-volume, and innovative applications. However, no single type of CNC or CNF is universally suitable. While all CNs share certain properties, factors like aspect ratio, crystallinity percentage, colloidal stability, surface chemistry, and biocompatibility vary based on the production technique and conditions, even if derived from identical cellulose sources. This variation presents an opportunity to use diverse production methods to achieve CNs tailored for specific applications. Yet, a singular production method that can be adjusted to produce CNs with varied properties from the same cellulose source remains elusive.

Among nanocellulose production techniques, enzyme-mediated methods are environmentally friendly and are likely more cost-effective than conventional methods. They also offer the distinct advantage of adjusting the process to customize nanocellulose properties for specific needs. While enzymatic hydrolysis holds significant promise for CNC isolation, it needs further refinement. Current enzymatic methods for CNC isolation typically yield a low hydrolysis rate (around 30%),<sup>1</sup> challenging the process's economic feasibility. The production yield must be optimized to enhance the commercial appeal of CNC production through enzymatic hydrolysis.<sup>2</sup> The prevalent strategy to boost CNC production yield via enzymatic hydrolysis involves pretreatment. Research indicates that pretreating cellulose fibers using techniques like refining, ultrasonics, or chemicals such as DMSO, NaOH, and HCl, or employing glycerin solution or high-pressure homogenization before enzymatic hydrolysis, can enhance enzyme access to cellulose, leading to better hydrolysis yields.<sup>3</sup> However, even with pretreatment in the enzyme-driven CNC production process, yields remain suboptimal. This shortfall may primarily stem from two reasons: pairing intense pretreatment with nonspecific enzymes or applying extreme hydrolysis conditions. The second reason might relate to using a multifaceted cellulosic material combined with a pretreatment that doesn't sufficiently reduce the starting material's resistance. In addition, enzyme-assisted modification of cellulose is seen as a promising approach to confer unique characteristics upon nanocelluloses, expanding their market potential. For instance, the hydroxyl groups on anhydrous glucose units are pivotal in CN aggregation. They are highly reactive, making CNs water-attractive and limiting their dispersion in water-repellent solvents or polymers. Their hydroxyl group reactivity can be covalently replaced with hydrophobic ends to improve CN compatibility with non-polar mediums. Lipases as catalysts have proven effective for this modification.

## 2 MATERIAL & METHODS

Bleached eucalyptus Kraft pulp (BEKP), the initial material, was either defibrillated through disc ultra-refining (BEKP-PTUR) as described by Berto & Arantes<sup>4</sup> or subjected to cavitation using a laboratory hydrodynamic cavitation (HC) system. This system consists of a 2.5L jacketed tank with a media volume of 2.3L, circulated through the cavitation zone by a 1.04 kW centrifugal pump (Grundfos, model MG80BA-O-D1-CMS1B), as outlined by Yupanqui-Mendoza et al.<sup>5</sup> Subsequently, BEKP-PTUR hydrolyzed in 50 mL Erlenmeyer flasks containing 0.5% (w/w) of pulp, incubated for 72 h at 50°C. Two distinct hydrolysis experiments were set up. The first experiment utilized endoglucanase and xylanase, individually and in various combined ratios, with enzyme loadings of 100 and 200 U/g pulp. The second experiment employed the cellulase preparation Cellubrix, with total endoglucanase activity ranging from 5 to 200 U/g pulp. For accuracy, all enzymatic hydrolysis procedures were conducted in pairs. After 72 h, each

hydrolysis process was ended by boiling the reaction mixture at 100°C for 10 minutes. The mixtures were then centrifuged at 13,000 ×g for another 10 min. The resultant solid fraction underwent sonication to extract cellulose nanoparticles.

Cellulose nanofibers (CNFs) were derived from bleached eucalyptus kraft pulp through endoglucanase-assisted ultra-refining, following the methodology presented by Las-Casas & Arantes.<sup>6</sup> The research explored how processing conditions, specifically variables like enzyme loading, reaction duration, and defibrillation degree, influenced CNF properties. These attributes, termed response variables, included the aspect ratio, crystallinity index, surface area, polymerization degree, and viscosity. A central composite design (CCD) was employed for this examination. Based on the findings, the CNF production method was optimized to produce CNFs with specific characteristics that enhance barrier efficiency in two film production contexts: i) In the casting approach, the goal was to maximize viscosity; ii) For the coating method, the aim was to reduce viscosity.

CNF films were produced using a vacuum-assisted filtration method. To begin, 0.3 wt% CNF suspensions (315 mL) were stirred magnetically for 5 minutes. Each suspension was then filtered through a 22 μm nylon membrane with a 20 cm diameter for 30 minutes. The formed CNF films were gently removed from the filter membrane and left to dry overnight at 35°C on a stove. All films were crafted with a set grammage of 30 g/m<sup>2</sup> to maintain a consistent thickness. Both untreated CNF film samples with varying DDs (CNF5, CNF12.5, and CNF20) and the optimized CNF film samples (EG-25-3-CNF12.5 and EG-0.5-1-CNF12.5) underwent characterization. The barrier properties of the CNF films were evaluated based on the oil resistance, water vapor transmission rate (WVTR), and oxygen transmission rate (OTR). Oil resistance tests were carried out using a commercial kit from 3 M. The kit consists of mixtures numbered 1 to 12, each with different chemical characteristics. As the mixture number increases, the resistance to oil penetration becomes more challenging, indicating higher oil resistance of the material. WVTR experiments were conducted according to TAPPI-464 om-18 at two different conditions: 25 °C and 50 % RH, as well as 38 °C at 90 % RH. These experiments were conducted using a climatic chamber (Mecalor, EE/0.5/AR-URC, São Paulo, Brazil). The OTR of the films was measured using an OX-Tran 2/22 (Minneapolis, USA) at 50 % RH. A commercial immobilized lipase was used to esterify CNFs, with butanoic acid as the acyl group (COO-R) donor candidate.

### 3 RESULTS & DISCUSSION

Utilizing a combination of mechanical pretreatment and specific enzymatic hydrolysis significantly enhanced the production yield of cellulose nanomaterials (CNs). This approach achieved high yields of rod-like and spherical CNs, with up to 83% yield for cellulose nanocrystals (CNCs). The morphology, yield, and size of the CNCs depended on the type, ratio, and loading of enzymes used. For example, 100 U and 200 U of endoglucanase (EG) produced peak CN yields, while 200 U of endoxylanase (EX) resulted in the purest and most abundant CNs. A combination of 50 U of EG and 150 U of EX produced spherical CNs, and the cellulase preparation Cellubrix created more thermostable CNs. Higher-purity CNCs were generally produced at lower yields, though these yields were still higher than typical literature values for enzymatic hydrolysis. The pretreatment reduced the crystallinity index and thermal stability of the cellulose fibers, but enzymatic hydrolysis targeted less structured cellulose regions, resulting in CNs with enhanced crystallinity. The enzymatic hydrolysis conditions did not significantly affect the crystallinity and thermal stability of the resulting CNs. Beyond ultra-refining, cavitation stands as an alternative pretreatment method for CNC production. Specifically, hydrodynamic cavitation (HC) is emerging as a promising technology for pretreating cellulose fibers before CNC production through enzymatic hydrolysis.<sup>8</sup> HC involves channeling liquid through an orifice plate or a venturi-style constriction. This process leads to the creation of vapor microbubbles in the flow. These bubbles expand and subsequently collapse due to abrupt local pressure drops. Recent research indicates that HC can effectively modify lignocellulosic biomass, triggering physical and chemical changes that aid in breaking down the polymeric matrix. As corroborated by other studies, The shear forces generated by this method can induce cracks on the biomass surface, facilitating its fragmentation and size reduction.

**Table 1.** Energy consumption derived from each scenario and CNC morphology determined atomic

Pre-treatment	Pre-treatment energy (kWh/kg)	Total energy (kWh/kg)	Length (nm)	Width (nm)
S1 <sup>a</sup>	41.74	63.75	439 ± 134	5.1 ± 1.5
S2 <sup>b</sup>	0.52	25.48	>1000	6 ± 2.6
S3 <sup>c</sup>	24.87	46.52	503 ± 199	6.1 ± 1.6
S4 <sup>d</sup>	28.09	46.60	556 ± 196	10.1 ± 5.4
S5 <sup>e</sup>	18.35	37.79	321 ± 85	4.9 ± 1.6
S6 <sup>f</sup>	31.25	53.23	496 ± 254	4.7 ± 1.7

By merging HC pretreatment with enzymatic hydrolysis, we observed impressive outcomes. We achieved CNCs with high yields of around 60%, boasting a crystallinity index between 81% and 85%. Notably, this approach led to a substantial energy consumption reduction by roughly 56%, bolstering its economic appeal (Table 1). Additionally, the process yielded CNCs of varied morphologies, aspect ratios, and viscosity characteristics, allowing for customization of their attributes for specialized and innovative applications. A primary challenge in using CNF for food packaging is their strong water affinity, which results in high water permeability. This study found that the water vapor transmission rate (WVTR) of CNF films at 50% relative humidity (RH) ranged from 282.28 to 311.02 g.m<sup>-2</sup>.day<sup>-1</sup>, with EG25-3-CNF12.5 showing the lowest permeability. The oxygen transmission rate (OTR) tests yielded similar results, with EG25-3-CNF12.5 recording 1.00 cc.m<sup>-2</sup>.day<sup>-1</sup>. These improved barrier properties are attributed to the high crystallinity of CNF films from EG-pretreated nanofibrils, which create non-permeable barriers and increase the diffusion path for permeating molecules. However, when RH increased to 90%, the WVTR of CNF films significantly rose to

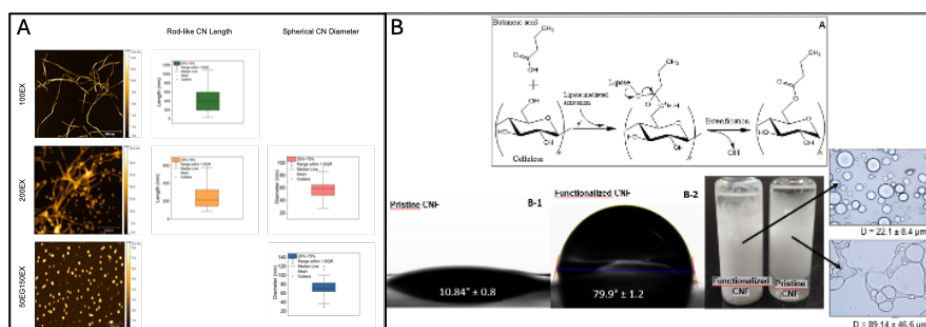
between 1640.80 and 1699.41 g.m<sup>-2</sup>.day<sup>-1</sup>. This increase in permeability at high RH is due to water's disruption of nanofibril interactions, indicating that enhancing CNF structure alone is insufficient to combat high humidity conditions.

Dynamic contact angle tests highlighted a decrease in the hydrophilicity of the functionalized CNFs. Contact angles reached up to 79.9° within two minutes of water droplet application, whereas the untreated CNF absorbed the water immediately. Additionally, the functionalized CNF exhibited enhanced emulsifying capabilities in oil-in-water emulsions when various organic solvents, such as toluene, hexane, and ethyl acetate, were used as the apolar phase. In summary, the enzymatic process under investigation effectively diminishes the hydrophilicity of CNF. However, the process conditions require further optimization for industrial applications. Potential areas of exploration include the effects of functionalization on interfibril interactions during the drying phase.

**Table 3.** Barrier properties of CNF films

Films	OTR 50 % RH (cc.m <sup>-2</sup> .day <sup>-1</sup> )(m)	WVTR 50 % RH (g H <sub>2</sub> O.m <sup>-2</sup> .day <sup>-1</sup> )	WVTR 90 % RH (g H <sub>2</sub> O.m <sup>-2</sup> .day <sup>-1</sup> )	Kit Oil
CNF5	2.08	304.78 ± 2.94 <sup>ab</sup>	1640.80 ± 36.32 <sup>a</sup>	12
CNF12.5	2.22	311.02 ± 21.44 <sup>ab</sup>	1672.23 ± 91.35 <sup>a</sup>	12
CNF20	1.48	321.91 ± 22.32 <sup>a</sup>	1654.07 ± 58.84 <sup>a</sup>	12
EG0.5-1-CNF12.5	3.17	307.24 ± 21.8 <sup>ab</sup>	1688.31 ± 75.16 <sup>a</sup>	12
EG25-3-CNF-12.5	1.00	282.28 ± 12.38 <sup>b</sup>	1699.41 ± 56.28 <sup>a</sup>	12
CNF5	2.08	304.78 ± 2.94 <sup>ab</sup>	1640.80 ± 36.32 <sup>a</sup>	12
CNF12.5	2.22	311.02 ± 21.44 <sup>ab</sup>	1672.23 ± 91.35 <sup>a</sup>	12

Notes: The values are presented as mean ± standard deviation. Values in the same column with different letters differ significantly (p < 0.1). OTR – Oxygen transmission rate; WVTR – Water vapor transmission rate; RH – relative humidity.



**Figure 1:** A) Representative atomic force microscopy images and length/diameter distribution of the CNs produced from bleached eucalyptus Kraft pulp pretreated by disc ultra-refining (BEKP-PTUR) followed by enzymatic hydrolysis under different conditions. B) a) lipase-induced mechanism of CNF functionalization; b-1) pristine and b-2) functionalized CNF contact angles; C) Emulsifying capacity and dispersion of oil droplets in Pickering emulsions with CNF.

## 4 CONCLUSION

The potential of nanocelluloses as renewable building blocks for various applications is significant, yet commercialization has faced production challenges. This study highlights the promise of innovative enzyme technologies in overcoming these challenges, providing a scalable and eco-friendly method to produce nanocelluloses with tailored properties. The enzyme-based platform successfully produced CNCs and CNFs with high yields and customizable attributes, surpassing some traditional methods. Additionally, enzyme-assisted cellulose modification has improved the compatibility of cellulose nanomaterials with non-polar mediums, broadening their application potential. Although further optimization is needed for industrial feasibility, these advancements pave the way for wider adoption of cellulose nanomaterials in various industries, driven by the growing demand for sustainable and versatile materials.

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