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Cellulose nanofibers from eucalyptus pulp and their coproduction with biobutanol through enzyme- mediated treatment

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Resumen

La nanocelulosa tiene aplicaciones potenciales en varios sectores que van desde el campo biomédico hasta el medioambiental. La posibilidad de desarrollo de una amplia gama de aplicaciones potenciales se ve favorecida por la abundancia y sostenibilidad de los recursos necesarios. Aunque este biomaterial emergente presenta propiedades excepcionales y alta disponibilidad en la naturaleza, su extracción a partir de materias primas renovables como la biomasa lignocelulósica o los materiales celulósicos sigue siendo un desafío importante. La celulosa es un material estructural y, debido a su interacción con la lignina y la hemicelulosa, resulta ser bastante resistente a la descomposición. Para lograr la extracción de nanocelulosa, se requieren métodos energéticamente intensivos o el consumo de productos químicos o enzimas costosas, lo que limita la implementación de su procesamiento a gran escala.

En este trabajo, se propuso una combinación de pretratamiento enzimático seguido de fibrilación mecánica como estrategia alternativa para la extracción de nanofibras de celulosa (CNF del inglés *cellulose nanofibers*) a partir de pulpa de celulosa Kraft de eucalipto de una planta de celulosa local. El pretratamiento mediado por enzimas permitió disminuir la recalcitrancia intrínseca de las fibras mediante reducción del tamaño y degradación parcial, promoviendo la accesibilidad de la celulosa para la posterior extracción de CNF. Según el tipo de enzima utilizada y las condiciones de trabajo, la liberación de azúcares solubles de las fracciones de carbohidratos (celulosa y hemicelulosa) resultó inevitable. Cuando se utilizaron complejos de celulasa y/o xilanasas, se alcanzó una extensiva hidrólisis de celulosa (hasta 46%) y xilano (hasta 85%), incluso para tiempos de reacción cortos (4 h). Por lo tanto, azúcares fermentables como glucosa (20-40 g/L) y xilosa (6-13 g/L) resultaron disponibles como coproductos. Estos azúcares fermentables fueron recuperados y convertidos completamente a biobutanol y otros solventes (hasta 15 g/L) por fermentación microbiana empleando cepas del género *Clostridium* para un completo aprovechamiento de la materia prima. El biobutanol es considerado un compuesto atractivo ya que tiene propiedades superiores como biocombustible en comparación con el bioetanol, además de ser un químico importante en diferentes industrias. Cuando se utilizó endoglucanasa pura, la degradación de carbohidratos resultó baja (<5%), lo que limitó la fragmentación y la depolimerización de las fibras de celulosa para su posterior procesamiento mecánico.

Dos métodos mecánicos fueron evaluados para la extracción de CNF a partir de pulpa de eucalipto pretratada enzimáticamente. El método de ultrasonificación demostró ser eficaz para el aislamiento de CNF de la pulpa de eucalipto local, permitiendo alcanzar rendimientos de extracción de CNF de hasta 98% a partir de pulpa de celulosa pretratada con los complejos de celulasa y xilanasas. La caracterización fisicoquímica y microscópica de las CNF extraídas reveló una significativa

uniformidad de tamaños en la escala nanométrica (531-992 nm) con diámetros en el rango de 3-10 nm. Se obtuvieron films de nanocelulosa altamente transparentes y flexibles, lo que resulta atractivo para aplicaciones potenciales. Sin embargo, considerando que la aplicación del tratamiento de ultrasonificación a escala industrial es aún limitada, se evaluó también el tratamiento de molienda de bolas como un método mecánico alternativo para la extracción de CNF. El método de molienda de bolas permitió obtener suspensiones de CNF de mayor consistencia, pero las altas concentraciones de sólidos limitaron la efectividad del proceso de extracción de CNF resultando en rendimientos de extracción más bajos (hasta 35%) en comparación con el método de ultrasonificación. Se evaluó el tratamiento de molienda bajo una configuración secuencial (hasta 5 etapas), el cual permitió alcanzar un rendimiento de extracción de CNF de 48% a partir de pulpa pretratada con los complejos de celulasa y xilanasas. Se obtuvieron CNF con diámetros en el rango de 4-14 nm y relaciones de aspecto de 220-230 mediante tratamiento de molienda de bolas, con mejores propiedades térmicas y cristalinidad. Se obtuvieron films de nanocelulosa flexibles, cuya transparencia resultó menor en comparación con las CNF extraídas por ultrasonificación.

En resumen, este trabajo destaca la viabilidad de coproducir CNF y biobutanol como productos de alto valor agregado utilizando pulpa de celulosa Kraft de eucalipto de una planta de celulosa local en un concepto de biorrefinería forestal, lo que podría aumentar la rentabilidad del proceso. Además, pretende contribuir al conocimiento de nuevas técnicas de obtención de nanocelulosa derivada de fuentes renovables, y su potencial aplicación.

Abstract

Nanocellulose has potential applications in various sectors ranging from biomedical to environmental fields. The possibility of developing a wide range of potential applications could be attributed to the abundance and sustainability of its resources. Even though this emerging biomaterial has many outstanding properties and high availability in nature, the extraction of them from renewable feedstocks such as lignocellulosic biomass or cellulosic materials is still a main challenge. Cellulose is a structural material and, due to its association with lignin and hemicellulose, it is rather resistant to decomposition. To achieve the extraction of nanocellulose, energy-intensive methods or the consumption of chemicals or costly enzymes are required for its processing, which limits the implementation of its large-scale processing.

In this work, enzymatic pretreatment combined with mechanical fibrillation was proposed as an alternative strategy for the isolation of cellulose nanofibers (CNF) from eucalyptus cellulose pulp. The enzyme-mediated pretreatment allowed to relieve intrinsic recalcitrance of fibers by size reduction and partial degradation, promoting cellulose accessibility for subsequent CNF extraction. Depending on the type of enzyme used and the working conditions, the release of soluble sugars from the carbohydrate fractions (cellulose and xylan) results inevitable. When cellulase and/or xylanase complexes were used, extensive cellulose (up to 46%) and xylan (up to 85%) hydrolysis occurred, even for short reaction times (4 h). Thus, fermentable sugars such as glucose (20-40 g/L) and xylose (6-13 g/L) were available as coproducts. These fermentable sugars were recovered and completely converted to biobutanol and other solvents (up to 15 g/L) by microbial fermentation employing *Clostridium* strains for an integral exploitation of the raw material. Biobutanol is considered an attractive product since it has superior properties as a biofuel compared to bioethanol, as well as being an important chemical in different industries. When endoglucanase monocomponent was used, almost negligible carbohydrate degradation occurred (<5%), which limited fiber fragmentation and depolymerization for further mechanical processing.

Two mechanical methods were evaluated for the extraction of CNF from enzymatically pretreated eucalyptus pulp. Ultrasonication demonstrated to be an effective mechanical method for CNF isolation from local eucalyptus pulp, allowing to achieve CNF extraction yields of up to 98% from cellulase-treated pulp. Physicochemical and microscopic characterization of extracted CNF revealed significant uniformity of sizes in the nanometer scale (531-992 nm) with diameters in the range of 3 to 10 nm. Highly transparent and flexible nanocellulosic films were produced, which results attractive for potential applications. However, considering that the industrial application of ultrasonication is still limited, ball milling treatment was investigated as an

alternative mechanical method for CNF extraction. Ball milling allowed to obtain CNF suspensions at higher consistency, but the high solid concentrations limited the effectiveness of the CNF extraction process and lower extraction yields (up to 35%) were obtained compared to ultrasonication. A sequential ball milling treatment configuration (up to 5 stages) allowed to improve up to 48% the CNF extraction yield from cellulase-treated pulp. CNFs with diameters in the range of 4 to 14 nm and aspect ratios of 220-230 were obtained by ball milling treatment, with enhanced thermal properties and crystallinity. Flexible nanocellulosic films were produced, even though they were not highly transparent compared to ultrasonicated CNFs.

In summary, this work highlights the feasibility of coproducing CNF and biobutanol as high value-added products using eucalyptus cellulose Kraft pulp from a local pulp mill. This work provided insights into the coproduction of biomaterials and value-added chemicals in a forestry biorefinery concept, which could increase the overall revenue of the process. In addition, it intends to contribute to the knowledge of new techniques for obtaining nanocellulose derived from renewable sources, and its potential application.

Preface

All the work presented here was planned, conducted, and analyzed by Florencia Cebreiros. Florencia Cebreiros carried out most of the experimental work in the Department of Bioengineering laboratories at the Faculty of Engineering, Universidad de la República. The ultrasonication experiments were carried out by Florencia Cebreiros in the Forest Products Biotechnology/Bioenergy Laboratory at the University of British Columbia (Vancouver, Canada).

The publications listed below have been prepared from the research work presented here.

Peer-reviewed journal papers

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Conference presentations

- Florencia Cebreiros, Santiago Seiler, Claudia Lareo, Jack Saddler (2019). *Physicochemical characterization of enzyme-treated pulp and nanofibers from bleached eucalyptus Kraft pulp*. VIII Encuentro nacional de sólidos y primer encuentro bi-nacional Argentina-Uruguay, Montevideo, Uruguay.
- Florencia Cebreiros, Santiago Seiler, Sai Swaroop Dalli, Claudia Lareo, Jack Saddler (2020). *Nanofibrillated cellulose (NFC) production from eucalyptus pulp through an enzymatic approach*. 42nd Symposium on Biotechnology for Fuels and Chemicals, New Orleans, USA.
- Florencia Cebreiros, Santiago Seiler, Mario Daniel Ferrari, Claudia Lareo (2020). *Enzymatic pretreatment of eucalyptus pulp to produce cellulose nanofibers and biobutanol in an integrated biorefinery*. 9th Nordic Wood Biorefinery Conference, Stockholm, Sweden. Online.

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- Florencia Cebreiros, Mario Daniel Ferrari, Claudia Lareo (2021). *A green approach to produce cellulose nanofibers and biobutanol from eucalyptus cellulose pulp via the biochemical pathway*. 29th European Biomass Conference & Exhibition, Florence, Italy. Online.
 - Florencia Cebreiros, Mario Daniel Ferrari, Claudia Lareo (2022). *Evaluating the effects of enzymatic pretreatment on eucalyptus pulp for the extraction of cellulose nanofibers by ball milling*. XXIII SINAFERM XIV SHEB XIV ENZITEC, Buzios, Brazil.
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List of abbreviations

βG	β-glucosidase
ABE	Acetone-butanol-ethanol
AFM	Atomic force microscopy
ANOVA	Analysis of variance
APS	Ammonium persulfate
APTES	(3-aminopropyl)triethoxysilane
ASL	Acid soluble lignin
AIL	Acid insoluble lignin
BEKP	Bleached eucalyptus Kraft pulp
BET	Brunauer-Emmett-Teller
BJH	Barrett-Joyner-Halenda
BM	Ball milling
BNC	Bacterial nanocellulose
BSA	Bovine serum albumin
BSR	Ball to solid ratio
C:X	Cellulase to xylanase ratio
CBH	Cellobiohydrolase
CED	Cupriethylenediamine
CTec	Cellic® CTec3
CNF	Cellulose nanofiber
CNC	Cellulose nanocrystal
CNM	Cellulose nanomaterial
CrI	Crystallinity index
CSR	Cellulase to substrate ratio
DB	Direct Blue
DES	Deep eutectic solvent

DLS	Dynamic light scattering
DNS	Dinitrosalicylic acid
DP	Degree of polymerization
DTG	Derivative thermogravimetric analysis
DW	Dry weight
EBI	Electron beam irradiation
ECT	Elemental chlorine free
EDL	Electric double layer
EDS	Energy dispersive X-ray spectroscopy
EG	Endo-(1,4)- β -D-glucanase
EH	Enzymatic hydrolysate
ET	Enzyme-treated
FPU	Filter paper unit
FQA	Fiber quality analysis
GC	Gas chromatography
Ho	Initial suspension height
Hs	Sediment height
HPLC	High performance liquid chromatography
IBE	Isopropanol-butanol-ethanol
IUPAC	International Union of Pure and Applied Chemistry
LD	Laser diffraction
LOI	Loss on ignition
LPMO	Lytic polysaccharide monooxygenases
Lw	Length-weighted average
MW	Microwave
na	Not applicable
NCC	Nanocrystalline cellulose

nd	Not detected
NFC	Nanofibrillated cellulose
NREL	National Renewable Energy Laboratory
nr	Not reported
OD	Optical density
OM	Optical microscopy
PAA	Peracetic acid
PBS	Phosphate buffered saline solution
PDI	Polydispersity index
PG	Potato glucose medium
PNPG	<i>p</i> -nitrophenyl β -D-glucopyranoside
RCM	Reinforced clostridial medium
R _H	Hydrodynamic radius
rpm	Revolutions per minute
SEM	Scanning electron microscopy
SPP	Solution plasma processing
SF	Solid fraction
Sw	Swollenin
SY	Solid yield
TCF	Total chlorine free
TEM	Transmission electron microscopy
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TGA	Thermogravimetric analysis
US	Ultrasonication
USD	United States dollars
X	Dry cell weight
XOS	Xylo-oligosaccharides

XRD	X-ray diffraction
XRF	X-ray fluorescence
Xyn	Cellic® HTec
W	Width
WRV	Water retention value
ZP	Zeta potential

List of symbols

HAc	Acetic acid concentration [g/L]
ABE	Acetone-butanol-ethanol concentration [g/L]
AE	Acetone-ethanol concentration [g/L]
B	Butanol concentration [g/L]
HBu	Butyric acid concentration [g/L]
t_F	Fermentation time [h]
C_G	Glucose consumption [%]
G_i	Initial glucose concentration [g/L]
X_i	Initial xylose concentration [g/L]
IBE	Isopropanol-butanol-ethanol concentration [g/L]
IE	Isopropanol-ethanol concentration [g/L]
Q_{ABE}	Overall ABE productivity [g/Lh]
ΔX	Overall biomass growth [g/L]
Q_B	Overall butanol productivity [g/Lh]
Q_{IBE}	Overall IBE productivity [g/Lh]
$Y_{ABE/S}$	Overall sugars to ABE conversion yield [g/g]
$Y_{B/S}$	Overall sugars to butanol conversion yield [g/g]
$Y_{IBE/S}$	Overall sugars to IBE conversion yield [g/g]
v/v	Volume per volume [%]
w/w	Weight per weight [%]
C_X	Xylose consumption [%]

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Chapter 1. Introduction

Nowadays, there is an increasing interest in the efficient development of biofuels, platform biochemicals, and biomaterials derived from various biomass feedstocks because of the growing concern about the depletion of non-renewable petroleum resources as well as the global warming. In fact, achieving this goal is one of the most challenging issues since it requires the transition to a greener development based on a circular economy. In this context, cellulosic biofuels production has been the most important driving force for the development of lignocellulosic biorefineries over the last decades. Besides, the utilization of cellulosic biomass for biofuels or other value-added products has become more inexpensive due to their abundant availability. However, biofuels or other value-added chemicals (e.g. lactic acid, succinic acid, etc.) derived from lignocellulosic biomass are still not economically viable due to the elevated costs of investment and production, which are mainly related to the deconstruction of the recalcitrant structure of lignocellulosic biomass.

To overcome this barrier, efforts have been made on the production of value-added products from other components in the lignocellulosic biomass (e.g. hemicellulose, lignin) which can be coupled with the production of biofuels under the concept of a biorefinery. The concept of a modern biorefinery can be compared to the present-day petroleum refinery, in which a sustainably produced feedstock is fractionated and converted into a mixture of marketable products through different processes. Several lignocellulosic biorefinery schemes have been proposed in the literature in which cascading processes were developed for multiproduct facilities [1]. This could include transportation biofuels, chemicals, energy, and high value biomaterials.

In addition to its use for fermentable sugars production, cellulose is a well-known material used in the manufacturing of a wide range of products and materials [2]. Recently, the production of cellulosic material has increased significantly to fulfill the need for renewable and ecofriendly sustainable materials. Since ancient times, biomass fibers have been used mainly for building materials, paper, clothing, textiles, and energy production. Nowadays, the production of advanced and innovative materials from renewable and abundant bioresources, for example cellulose nanomaterials (CNMs), is becoming an important area of research. Chemical, biochemical and mechanical treatments provide ways to modify cellulose and yield CNMs which offers an unparalleled combination of high physical properties and environmental benefits producing a variety of high value-added products [3].

1.1. Cellulose

Cellulose is a renewable natural resource and the most abundant biopolymer on Earth that naturally exists in plants, animals, or bacteria, with a worldwide production of 7.5×10^{11} ton/year [1]. It is a complex carbohydrate or polysaccharide that consists of 3,000 or more units of β -D-glucose (Fig. 1.1). Chemically, cellulose is constituted by a linear homopolysaccharide of β -D-glucopyranose or β -D-glucose monomers linked by β -1,4-glycosidic bonds, with a degree of polymerization in the range of 10,000 to 15,000, which depends on the plant species. The repeating unit of the linear chains of cellulose is the dimer cellobiose, which consists of two β -D-glucose units.

One distinguishing and useful characteristic of cellulose is the high degree of hydroxylation along the polymer chain. Each β -D-glucopyranose unit contains three highly reactive hydroxyl (OH-) groups which are responsible for cellulose main characteristics such as hydrophilicity, chirality, and biodegradability [4]. The β -1,4-glycosidic bonds in cellulose build an ordered crystalline structure by van der Waals forces and inter- and intramolecular hydrogen bonding. The OH-groups present in the β -D-glucopyranose unit form strong intramolecular hydrogen bonding networks with the following glucose unit in the same chain, and intermolecular hydrogen bonding networks with a glucose unit in a different chain (Fig. 1.1). In the crystalline regions, these bonding networks are strong and packed, which confers interesting characteristics such as toughness, strength, fibrous, insoluble in water, and highly resistant to organic solvents. Moreover, primary OH- groups are readily chemically modified (e.g. through phosphorylation) which provides large scope for these materials [5]. The hydrogen bonds existing between cellulose chains make it highly stable but poorly soluble in water and other solvents.

Cellulose does not exist as an isolated individual molecule in nature, but it is found as assemblies of individual cellulose chain-forming fibers. Two of the most important properties of cellulose are the structure forming microfibrils and the hierarchical organization which includes crystalline and amorphous regions. In plant cell walls, around 36 individual cellulose molecular chains are typically connected with each other to form larger units known as elementary fibrils or nanocellulose. These elementary fibrils are further packed into larger microfibrils with a diameter of 5-50 nm and several micrometers in length, which are assembled into cellulose fibers. Fig. 1.1 illustrates the hierarchical structure of cellulose which is composed of microfibrils with a highly ordered region (crystalline structure) and a disordered region (amorphous structure).

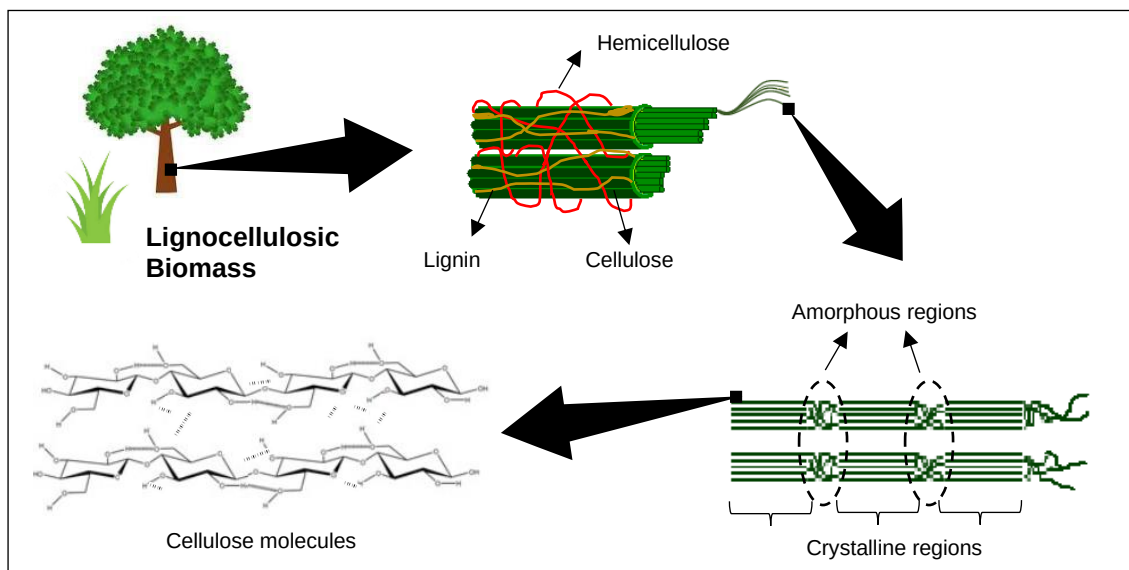


Figure 1.1. Hierarchical polymeric structure of cellulose.

The hydrogen bonding networks in cellulose and the molecular arrangement result in polymorphs or allomorphs. The native allomorph of cellulose is known as cellulose I, which can be chemically or thermally transformed into other polymorphs or allomorphs depending on the plant source. Cellulose type I arises in I_{α} and I_{β} allomorphs. There are six interconvertible identified polymorphs of cellulose defined as cellulose I, II, III_I , III_{II} , IV_I , and IV_{II} [1]. Cellulose II can be obtained from chemical regeneration or by mercerization. Cellulose III is formed when cellulose I or II is treated with ammonia or various amines, and the polymorphs IV_I and IV_{II} are produced by heating cellulose III_I or III_{II} , respectively, at 260°C in glycerol [4].

1.2. Processes for cellulose extraction from biomass resources

Cellulose can be extracted from several biomass sources such as grasses, wood, algae, among others. Lignocellulose is the major structural component of plants and by far the most abundant type of biomass [6]. Lignocellulosic biomass resources are composed of cellulose (40-60%), hemicellulose (10-40%) and lignin (15-30%), with minor amounts of extractives, proteins, and inorganic materials [6].

Nowadays, wood and cotton are the most important sources of cellulose for industrial processes, which are considered to be primary source. Wood can be classified as hardwood (such as eucalyptus, birch, aspen, poplar) and softwood (pine, spruce, fir, larch, hemlock) [7]. However, other sources are being investigated to find new techniques for natural cellulose extraction. For instance, there is a renewed interest in the use of non-woody raw materials such as agriculture and industrial residues (sugarcane bagasse, wheat and rice straws, corncob, peels) because of their abundance as low-cost fibers source and because they are sometimes the only exploitable fibers

source in certain geographical areas [6]. Cellulose can also be obtained from some other small class of sources like marine animals (tunicate), algae, fungi, and bacteria.

Recalcitrance, which is commonly refer to the resistance of plant cells to deconstruction, is a major obstruction in the separation of cellulose from the complex polymeric matrix of hemicellulose and lignin in which is embedded. Pretreatments are an earlier and necessary step in the process of getting cellulose extracted from the biomass resources. By causing both structural and compositional changes in the biomass, pretreatment aim to surpass the recalcitrance obstacle. Thus, the selection of an appropriate pretreatment is intended to decrease crystallinity and degree of polymerization, increase porosity, inner surface and reactivity, and break the hydrogen bonds between cellulose and non-cellulosic compounds to promote the accessibility towards the cellulosic fraction [3]. Therefore, an ideal and effective pretreatment should avoid the loss or rupture of cellulose, hemicellulose, and lignin while separating the cellulose from the remaining components, minimize the energy input for cost-effectiveness, and minimize the generation of hazardous and toxic wastes.

The most important pretreatments required for cellulose extraction (cellulose pulp) are the preparation of the raw material, delignification (or pulping) process and the bleaching, which are independent of the lignocellulosic biomass source. In general, non-woody raw materials have a lower density and more porous structure, and also less lignin content in most of the cases, which means less energy and chemicals are required for cellulose extraction during cellulose pulp production.

1.2.1. Delignification

The delignification process (also known as pulping) aims to remove the non-cellulosic fractions, particularly lignin and a significant portion of hemicellulose, with only modest damages to the cellulose molecules in the wood. This chemical process allows the separation of individual cellulosic fibers based on an alkaline treatment with either sodium hydroxide (NaOH) or potassium hydroxide (KOH) and mineral acids. During the pulping process, wood is reduced to wood chips, and lignin is solubilized to obtain cellulose pulp using several chemical methods. The two main chemical pulping processes are referred to as Kraft (alkaline) and sulfite (acid) pulping [8].

The Kraft pulping consists of a process under alkaline condition in which the wood chips are submitted to NaOH and sodium sulfide (Na_2S) treatment using high temperature and pressure to remove lignin from cellulose. During Kraft pulping, alkaline degradation of polysaccharides results in considerable carbohydrate loss. The main reaction, which is called the peeling reaction,

occurs at the reducing end groups of polysaccharides. The primary peeling reaction occurs during the early stage of cooking. As cooking temperature increases, the alkaline hydrolysis of glycosidic bonds results in new reducing ends and gives rise to additional degradation, which is called the secondary peeling. As a result, cellulose chains on the surface of cellulosic fibers, which are more exposed to the chemicals, degrade more than the cellulose chains inside the cellulosic fiber. The peeling reaction is ended by a different reaction converting the reducing end groups to stable carboxylic acid group.

The acid sulfite (and bisulfite) pulping is a process where the wood chips are treated with sulfurous acid (H_2SO_3) and bisulfite ions (HSO_2^-) to disrupt lignin from cellulose fibers, while hemicelluloses are also hydrolyzed into simple sugars. Sulfite pulp is relatively weak, but is easy to bleach and refine, which makes it excellent material for use in tissue and printing paper. During the acid sulfite (and bisulfite) pulping process, the degradation of both cellulose and hemicellulose carbohydrates is intended to be minimized, but damage is unavoidable, due to the sensitivity of glycosidic bonds towards acid hydrolysis at elevated temperatures. Compared with hemicellulose, the ordered structure protects cellulose from decomposition, and it is the least damaged of the major components. Only a moderate depolymerization of cellulose occurs during sulfite pulping and little cellulose loss takes place unless the delignification is aimed at very low lignin content, which is the case for dissolving pulp production [9].

1.2.2. Bleaching

Finally, the bleaching process consists of the complete removal of lignin using chemical, gases, and steam, to provide the required color of pulp for paper manufacturing. The bleaching process aims to completely remove lignin and remove hemicellulose that was not removed during delignification from the lignocellulosic biomass, while keeping the yield and integrity of the cellulose pulp fibers. Since lignin is a complex molecule with different types of linkages, the bleaching process is accomplished with various compounds containing chlorine or oxygen in several stages to break the various types of bonds within the lignin. Apart from lignin removal, bleaching of the cellulose fibers can clean the fiber surface, chemically modify the surface, stop the moisture absorption process, and increase the surface roughness. Also, some features including surface area, crystallinity, and aspect ratio can be improved.

The bleaching process involves single or multiple stages depending on the end use applications of the cellulose pulp. Bleaching chemicals can be divided into three groups according to their functions: (i) chlorine gas (Cl_2), ozone (O_3) and hydrogen peroxide (H_2O_2) which react with the aromatic lignin units; (ii) chlorine dioxide (ClO_2) and oxygen gas (O_2) which react with lignin structures that have free phenolic hydroxyl groups; (iii) hypochlorite (NaClO or $\text{Ca}(\text{ClO})_2$) and

hydrogen peroxide (H_2O_2) which react with only certain functional groups such as α -carbonyls [10]. Generally, the most frequently used bleaching agents are sodium chlorite (NaClO_2), hydrogen peroxide (H_2O_2), oxygen (O_2) or ozone (O_3).

Among the commercially used bleaching agents, ClO_2 is the most selective lignin oxidant and reacts very slowly with polysaccharides [9]. However, due to environmental concerns, chlorine-free agents have recently received more attention. Therefore, alternative processes for pulp bleaching have also been proposed by using, for instance, H_2O_2 or O_3 to avoid the use of chlorine in this process. For instance, O_3 is being used by different paper mills in their elemental chlorine free (ECF) and total chlorine free (TCF) pulp bleaching sequences for producing different kinds of end products like writing and printing papers, tissue papers and rayon grade pulp [11]. Besides, H_2O_2 and/or sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) or sodium hydrosulfite (NaS_2O_4) are cheap and environmentally safe bleaching agents with their effectiveness being dependent on the low content of lignin and extractives in wood species. Peracetic acid (PAA) is a promising lignin-selective reagent which reduces the molecular weight of lignin polymers by cleaving β -aryl ether bonds, and C-C and C-O bonds linked to the aromatic ring. Also, it increases the water solubility of lignin by breaking the aromatic rings. PAA does not react with most of the carbohydrates, though it could oxidize the reducing end of carbohydrates.

1.2.3. Alternative methods

As an alternative to the traditional delignification process previously described, the use of advanced cascading conversion technologies, which enables the application of a biorefinery approach, is also investigated. For instance, autohydrolysis for hemicellulose solubilization followed by an organosolv-acetosolv process was used as an alternative combined method to conventional NaOH pretreatment for cellulose separation to produce CNMs from apple tree pruning and pea stalks [12]. Also, hydrothermal pretreatment combined with an organosolv process was also evaluated for sugarcane bagasse delignification, followed by refining by Bauer disk, yielding a pulp with 92% of cellulose that was used to produce nanocellulosic films [13]. Other alternative solvents, such as the ionic liquid 1-butyl-3-methyl imidazolium chloride ([bmim]Cl), assisted by microwave heating were used for cellulose extraction from sugarcane bagasse [14].

On the other hand, it has been reported that the use of enzymes (mainly xylanases and laccases) has been increasingly employed as an environmentally friendly strategy to eliminate the remaining lignin in the pulp and to reduce the chemicals consumption in the process [15].

1.3. Nanocellulose

Nanocellulose, which is also known as cellulose nanomaterial (CNM), represents a term which refers to cellulose structures with at least one dimension in a nanometer scale. Nanocellulose can be produced by reducing the size of a common cellulose fiber, which is tens of microns in diameter. Despite being cellulose the most available natural biopolymer on Earth, it has been only recently that cellulose has gained attention and notoriety as a nanoscaled material.

Due to its nanometric size, CNM has different and more advantageous properties than the bulk material such as special morphology and geometrical dimensions, crystallinity, high specific surface area, rheological properties, liquid crystalline behavior, alignment and orientation, mechanical reinforcement, barrier properties, surface chemical reactivity, biocompatibility, biodegradability and absence of toxicity [3]. Besides, it presents higher structural characteristics compared to microcrystalline cellulose, like high mechanical strength, and can be easily surface modified through different strategies. Due to these outstanding properties, CNMs have potential for various types of applications and new value-added products [3, 5].

Based on the original resource and preparation method used for its extraction, CNMs can be classified into three categories: (i) cellulose nanocrystals or nanocrystalline cellulose (CNCs or NCC); (ii) cellulose nanofibers or nanofibrillated cellulose (CNFs or NFC); (iii) bacterial nanocellulose (BNC). These nanocellulose types possess similar chemical compositions but differ from each other in morphology and physical properties such as particle size, crystallinity, and appearance. Moreover, the extraction method primarily determines their structure, dimensions, and properties. For instance, the preparation of CNCs and CNFs is a top-down process in which nanocellulose is prepared by disintegrating the cellulose fibers from the lignocellulosic resources to their nanometer size. Acid hydrolysis results in nanometer-long and highly crystalline rod-like fragments which are referred to CNCs, while mechanical shearing techniques disintegrate cellulose fibers into their substructural nanoscale units which are typically longer and micrometric in length, referred to CNFs. BNC preparation is a bottom-up process in which nanocellulose is produced by building up nanofibers from low molecular weight sugars employing certain bacterial species. Fig. 1.2 shows a schematic representation of the preparation and synthesis of the different CNMs: CNCs, CNFs, and BNC.

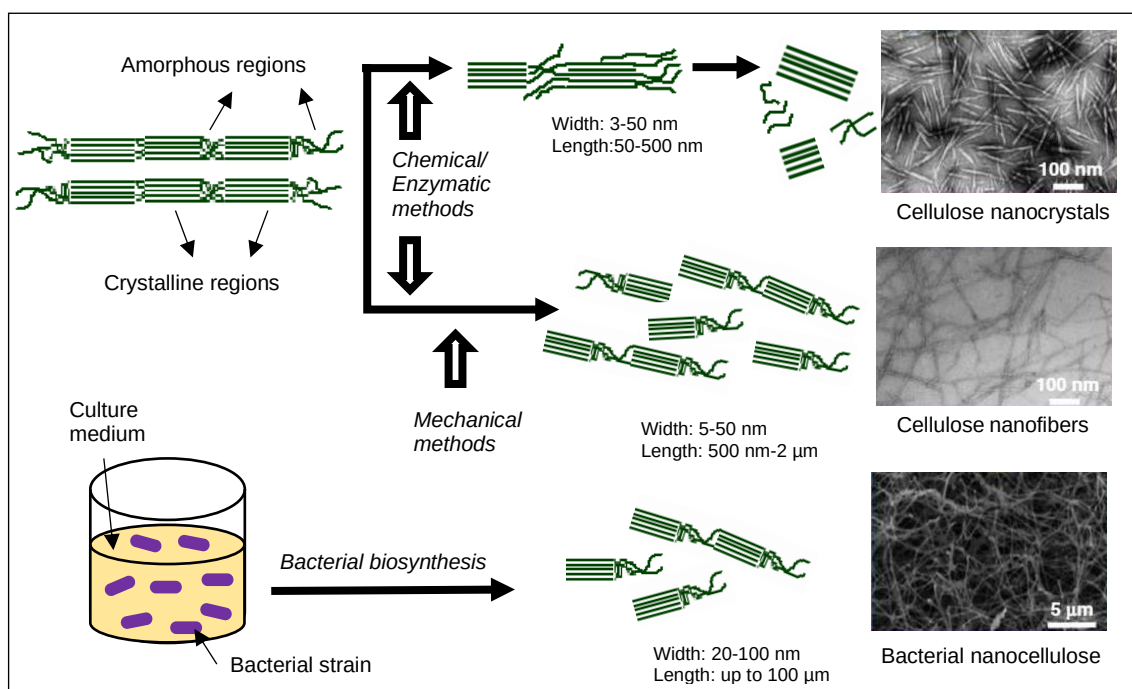


Figure 1.2. Schematic illustration of the preparation and synthesis process of cellulose nanomaterials (TEM images of CNC and CNF, and SEM image of BC adapted from [16], [17] and [18], respectively).

1.3.1. Cellulose nanocrystals (CNCs)

CNC, also known as nanowhisker, has a short crystalline rod-like or whisker shape with a width in the range of 3 to 50 nm and a length in the range of 50 to 500 nm. It is chemically composed of 100% cellulose, primarily in crystalline regions, which confers a high rigidity compared to CNF. The degree of crystallinity in CNCs ranges from 54 to 88% [19].

The CNCs are extracted by removing the amorphous regions between crystalline sections in cellulosic microfibrils via acid hydrolysis. Although processes involving enzymatic hydrolysis exist to produce CNC, strong acid hydrolysis is the most common preparation method. Several types of acids have been used to produce CNC such as sulfuric, formic, acetic, hydrochloric, maleic, oxalic, citric, and phosphoric [20, 21]. During acid hydrolysis, the cellulose amorphous regions are preferentially hydrolyzed because they are more sensitive to acidic attacks, while crystalline regions present a higher resistance to acids. This explains the high crystallinity of CNCs. After acid pretreatment, post-treatment steps are required for CNCs separation, such as centrifugation, neutralization with alkaline reagents, rinsing with distilled water to remove chemicals. Then, dialysis is usually employed to remove free acid molecules, and ultrasonication is used to disperse the CNCs and obtain a stable suspension. Finally, concentration and drying (e.g. freeze-drying or spray-drying) of the suspension may be carried out as required to reduce the cost of material packaging and transportation and to ease its use in industrial applications [2].

Both the reaction conditions (type of acid, acid concentration, reaction time and temperature) and the cellulose source have an impact on the obtained properties of CNCs such as morphology, degree of crystallinity, aspect ratio and dimensional dispersity [5]. CNCs can be obtained from several cellulose sources and employing different methods. However, the use of wood or cotton as the main source of cellulose and the acid hydrolysis with sulfuric acid (H_2SO_4) as preparation method are the most common approaches for CNCs production. However, one of the main drawbacks of this approach is the use of strong concentrated acid that is corrosive and its incompatibility with an environmentally friendly process [1]. Besides, it produces effluents that require adequate treatment for disposal, which will increase the processing costs.

1.3.2. Cellulose nanofibers (CNFs)

CNF, which is also known as NFC, has generally a width in the range of 5 to 50 nm and a length in the range of 500 to 2000 nm or several micrometers. Unlike CNCs, CNFs are entangled and flexible fibrils with a large surface area which contain both amorphous and crystalline cellulose domains within the single fibers.

Mechanical, chemical, and biological approaches have been explored to isolate CNFs from various sources. Ideally, CNFs are produced using intensive mechanical delamination of cellulosic fibers. Refining, homogenization, high pressure homogenization, microfluidization, cryo-crushing, and grinding are some of the mechanical treatments commonly employed to extract CNFs from cellulosic materials. The main disadvantage of the mechanical disintegration of cellulose fibers in nanofibers is the high energy consumption associated to the process. This condition continues to be the main barrier to the successful introduction of CNFs in the industry. Chemical methods, which include alkali treatments, and biological methods, which include mostly the use of enzymes, can be combined with mechanical methods to get the desired product. The introduction of different pretreatment before the mechanical process aim to reduce the energy consumption and to make the surface hydrophobic, so that CNF can be more attractive as a material for commercial applications [20]. Moreover, the introduction of repulsive charges by chemical pretreatment increases the efficiency of fibrillation. The extracted CNFs are more regular and have a lower fiber width compared to CNFs produced only by mechanical method [22]. For instance, the literature has highlighted the potentialities of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated oxidation as a pretreatment that transforms the C6-hydroxyls groups present on the native cellulose surface into carboxylate groups, preserving the crystallinity of the original sample. This pretreatment promotes the nanofibrillation process and is considered a chemical route to increase crystallinity and water dispersion of nanocellulose [20]. Other pretreatments like periodate-chlorite oxidation, periodate-bisulfite sulfonation,

carboxymethylation, and phosphorylation were used to insert negative charges onto the cellulose surface to fade the inter-fibril bonding.

Like CNCs, the properties of CNFs vary with the raw material and the extraction method employed. Depending on the treatment method, the resulting CNFs can dramatically differ in morphology, degree of fibrillation, shape, surface chemistry, and properties. CNF exhibits some attractive properties such as non-toxic and non-abrasive nature, very high elastic modulus, large specific surface area, and low thermal expansion [23]. Besides, it possesses a high aspect ratio and forms gels in water with shear-thinning and thixotropic behavior [22].

1.3.3. Bacterial nanocellulose (BNC)

BNC is extremely pure since it is produced biotechnologically by bacteria, unlike CNM extracted from biomass which is typically mixed with lignin, hemicelluloses, and/or pectin. It is commonly synthesized and secreted by *Gluconoacetobacter xylinus* (an aerobic acetic acid bacteria), even though other bacterial species such as *Agrobacterium*, *Pseudomonas*, *Rhizobium*, and *Sarcina* also produce BNC [7].

These bacteria can consume a variety of substrates as a carbon source, including glucose, fructose, sucrose, xylose, arabinose, mannitol, arabitol, glycerol, and oligosaccharides [24]. However, high yields of BNC require the addition of various nutrient supplements which increases the cost of BNC production process and, thus, limits its industrial implementation. To overcome this, promising alternatives as low-cost carbon and nitrogen sources have been investigated for BNC production. For instance, molasses, which is a byproduct of the sugar industry, has been investigated as an effective fermentation medium in the production of BNC. Also, a combination of molasses and corn steep liquor which provides both a carbon source and a nitrogen source represents an ideal medium for the development of *Acetobacter sp.* without expensive supplementation, reducing the cost of BNC production. Furthermore, residues from the food industry have been exploited for the production of BNC, such as grape pomace (residue of juice extraction) and dry olive mill residue (by-product of the olive oil industry) [25, 26]. For instance, grape pomace contains the fermentable sugars D-glucose and D-fructose which served as renewable carbon sources for the cost-effective production of BNC by *Gluconacetobacter xylinus* in a medium containing both grape pomace extract and corn steep liquor.

BNC is generally produced by cultivating the bacteria for a few days in an aqueous culture media containing glucose, phosphate, carbon, and nitrogen sources. During cultivation, cellulose microfibrils are formed by the combination of glucose chains and aggregated as ribbons. It has been reported recently that the structures and properties of BNC tubes can be regulated by

cultivation conditions including nutrient source, oxygen ratio, bacterial strain type, incubation time, and cultivation in a bioreactor [27, 5].

BNCs have potential to appear in functional materials with attractive magnetic, optical, and mechanical properties due to their low apparent density and high surface area [5]. BNC combines high physical strength, an extremely hydrophilic surface, and an interpenetrating structure, which recommends this material for biomedical applications such as implants and scaffolds for tissue engineering, wound dressings, and artificial skin, and as drug delivery carriers [5]. Besides, BNC membranes could be used in regenerative medicine, including guiding tissue regeneration, in periodontal treatments, and as membrane replacements.

Table 1.1 summarizes the main differences between CNC, CNF, and BNC. The focus of this study will be on CNCs and CNFs, since they are the two types of CNMs that can be extracted from lignocellulosic biomass.

1.4. Processes for nanocellulose extraction from cellulose sources

The extraction process of CNMs from cellulose sources is performed by breaking down the isolated cellulose fiber to the nanometer scale and, thus, convert it into one of the two specimens of nanosized cellulosic materials (CNCs or CNFs). Several types of methods have been reported in the literature for the preparation of CNMs from cellulosic materials [4]. The way CNMs are isolated from cellulose sources has a large effect on the morphology and properties of the product.

Traditionally, the pulp and paper industry is the most important supplier of cellulose for CNMs production, using delignified and bleached pulps [21]. Most of the reported works include the use of purified cellulose such as microcrystalline cellulose of bleached Kraft pulp [1]. Nevertheless, other processed residues from this industry, such as bleached pulp wastes or wastepaper, which is composed mainly of cellulose (60–70%), has also been used for CNMs production [1, 28]. These residues are considered a promising source of cellulose since a previous treatment for cellulose extraction is not required. In fact, wastepaper is shown as a cellulose source with high potential since harsh conditions to produce CNMs are not necessary, which can yield up to 64% depending on the wastepaper used, reducing the cost of the process and consumption of chemicals and energy. Moreover, recycled paper mill sludge was also evaluated for CNMs production [28]. Other byproducts from the pulp and paper industry such as wood bark or wood chips which contains around 45% of cellulose [29, 30], could also be an interesting raw material. In this case, a first pretreatment for cellulose fiber isolation (e.g. pulping/delignification and bleaching) is required to obtain more pure cellulosic fibers.

Table 1.1. Main differences between cellulose nanocrystals (CNCs), cellulose nanofibers (CNFs), and bacterial nanocellulose (BNC) (adapted from [5]).

	CNC	CNF	BNC
Nature	Nanocrystals of cellulose	Nanofibers of cellulose	Microbial cellulose fibers
Preparation method	Size reduction by chemically induced hydrolysis or oxidation of large lignocellulosic fibers (mm) to smaller units (nm)	Size reduction by mechanically induced deconstruction/delamination of large lignocellulosic fibers (mm) to smaller units (nm)	Synthesis using bacteria; construction in microbial culture from tiny units (Å) to small units (nm)
Morphology, dimensions, and aspect ratio ^a	Elongated rod-like crystals, whiskers or spherical shape; width: 5-30 nm; length: 100-500 nm; low aspect ratio	Fibrillar and network shape; width: 10-100 nm; length: 500-10,000 nm; high aspect ratio	Nanofiber network; width: 20-100 nm; length: up to 100 µm; high aspect ratio
Crystallinity	Highly crystalline; crystalline regions due to the removal or amorphous regions	Individual and aggregated nanofibrils with crystalline and amorphous regions	Microfibers formed and aggregated as ribbons with crystalline and amorphous regions
Requirements	Corrosive chemical reagents; energy consumption	High energy consumption; corrosive chemical reagents	Non-corrosive chemicals; suitable culture medium
Cost	Relatively low ^b	Relatively low ^b	High cost of suitable culture medium
Yield	Relatively low	Relatively low	Very low
Main advantages	Crystallinity; tensile strength; biodegradable; renewable; high axial stiffness; low coefficient of thermal expansion	Biodegradable; less abrasive to processing equipment; high surface area; low density; mechanical strength; ease to chemical modification	High biological affinity; biocompatibility; biodegradability; high DP; high porosity; high purity; renewable
Potential applications	Transparent barrier films; drug carriers; composite materials; optical and electronic devices; actuation and sensing; selective filtration; packaging; rheology modifiers; paints; coatings; adhesives; lacquers; cosmetics; construction; food industry, among others.	Barrier films; cosmetics; food industry; composite reinforcing; drug delivery; membranes for water treatment, biomedical devices; traditional high-volume applications (paper, packaging, paints); energy storage; flexible electronics; construction, among others.	Biomedical (antimicrobial products, skin therapy, artificial blood vessels, protein delivery, contact lenses); food industry; paper industry; biosensors; among others.

DP: degree of polymerization

^aAspect ratio is defined as the length divided by the width.^bUse of renewable biomass and inexpensive chemicals.

The choice of the method for CNMs extraction depends on the type of material being prepared, for instance, CNCs or CNFs. Despite the common idea that CNCs are generally produced from acid hydrolysis while CNFs are produced from mechanical fibrillation (generally assisted by other method), several studies have been conducted showing that the mechanisms for producing both materials are in fact more diverse. For example, several works show the application of cellulases for the production of CNCs combined with acid hydrolysis or even with no chemicals being used [1]. Table 1.2 provides reported data on the production of different types of CNMs from cellulosic and lignocellulosic sources using conventional technologies. Considering the different alternatives for cellulose extraction, the properties of CNMs do not depend only on factors such as plant species, growing conditions, and part of the plant. Pretreatment used for the lignocellulosic biomass processing has demonstrated to have a significant influence on the mechanical and thermal properties, crystallinity, morphology, and surface charge [6].

1.4.1. Chemical treatment

Among the different chemical treatments, acid hydrolysis is the easiest and oldest method to produce CNMs from cellulosic materials. During this method, the acid dissolves and removes the amorphous regions in cellulose, while the crystalline regions are retained. Different acids were investigated such as hydrochloric acid (HCl), sulfuric acid (H₂SO₄), hydrobromic acid (HBr), and phosphoric acid (H₃PO₄). However, H₂SO₄ is mainly used because it can strongly isolate CNCs from cellulose, as well as make the CNCs dispersed as a stable colloid system because of the esterification of the hydroxyl group by sulfate ions [46]. For acid hydrolysis method, the reaction time and temperature as well as the type of acid used and its concentration, influence the size and morphology of the extracted CNCs [47]. This method has several limitations such as high-water consumption due to washing steps, generation of acidic wastewater and effluents, relatively long processing times, high operational and maintenance costs, risk of equipment corrosion due to corrosiveness of chemicals, formation of inhibitors, and not being environmentally friendly [48].

Table 1.2. Reported data on different methods for the production of cellulose nanomaterials (CNMs).

CNM type	Source	Pretreatment	Preparation	Morphology and size	Properties	Reference
CNC	Pineapple leaf fibers	Combined methods	High-shear homogenization + Ultrasonication	Rod-like; W: 40-70 nm; L: 88-1100 nm	CrI: 62% T _{on} : 320°C	[31]
	Pulp and paper mills	Chemical	Ammonium persulphate	Rod-like; W: 10-20 nm; L: 150-500 nm	T _{on} : 250°C	[32]
	Lemon seeds	Chemical	TEMPO/NaBr/NaClO	Rod-like; W: 26-42 nm; L: 340-380 nm	CrI: 66% T _{on} : 215°C	[33]
			APS oxidation	Rod-like; W: 10-20 nm; L: 140-160 nm	CrI: 74% T _{on} : 220°C	
			Sulfuric acid hydrolysis	Rod-like; W: 26-42 nm; L: 340-380 nm	CrI: 70% T _{on} : 154°C	
	Eucalyptus cellulose Kraft pulp	Combined methods	Ball milling + Enzymatic hydrolysis + Ultrasonication	Rod-like; W: 24 nm; L: 294 nm	CrI: 78% T _{on} : 300°C	[34]
	Bleached sugarcane bagasse	Chemical	APS oxidation	Rod-like; W: 8-20 nm; L: 140-500 nm	CrI: 76%	[35]
	Cellulose powder	Mechanical	Ball milling + Magnetic stirrer	Fibrous network; W: 10–25 nm; L: several µm	CrI: 66% T _{max} : 335-365°C	[36]
	<i>Eucalyptus citriodora</i> waste	Combined methods	Ball milling + Ultrasonication	Fibrous; W: 100-200 nm	CrI: 77% T _{max} : 357°C ZP: -40-60 mV	[37]
CNF	Bleached bagasse Kraft pulp	Combined methods	Enzymatic hydrolysis + Grinding	Filamentous network; W: 9-26 nm; L: 298-4500 nm	CrI: 60% T _{on} : 314-330°C T _{max} : 347-357°C	[38]
	Bleached eucalyptus Kraft pulp	Enzymatic	Enzymatic hydrolysis	Fibrillar entangled network; W: 8-40 nm	CrI: 52-62% T _{on} : 230°C	[39]

(Continued on next page)

Table 1.2. (continued)

CNM type	Source	Pretreatment	Preparation	Morphology and size	Properties	Reference
CNF	Curaua	Combined methods	Enzymatic hydrolysis + Ultrasonication	Nanofibrous; W: 55-109 nm; L: 1280-4100 nm	CrI: 73-78%	[40]
	Sugarcane bagasse	Combined methods	Enzymatic hydrolysis + Ultrasonication	Nanofibrous; W: 30 nm; L: 255-872 nm	CrI: 70-72%	[41]
	Kenaf fibers	Mechanical	Ball milling	Web-like fibers; W: 21-30 nm	CrI: 65% T _{on} : 308°C T _{max} : 352°C	
	Wheat straw	Mechanical	Ball milling	Web-like fibers; W: 21-30 nm	CrI: 68% T _{on} : 309°C T _{max} : 353°C	
	Sugarcane bagasse	Mechanical	Ball milling	Rob-like fibrous; W: 50 nm; L: 2000 nm	CrI: 68% T _{on} : 299-364°C	[42]
	Bleached Kraft <i>Eucalyptus globulus</i> pulp	Chemical	TEMPO/NaBr/NaClO	W: 8-14 nm; L: 87-1343 nm	CrI: 50-52% T _{on} : 240-245°C	[43]
		Enzymatic	Enzymatic hydrolysis	W: 25-38 nm; L: 560-1010 nm	CrI: 53-64% T _{on} : 285-287°C	
BNC	<i>Acetobacter xylinum</i>	Combined methods	High-shear homogenization + Ultrasonication	nr	Improved tensile strength, thermal stability and water resistance of films	[44]
	<i>Komagataeibacter hansenii</i> strain CGMCC 3917	Combined method	Hydrochloric acid hydrolysis	Rob-like; W: 221 nm	CrI: 89% T _{max} : 335°C ZP: -39.5 mV	[45]

W: width; L: length; CrI: crystallinity index; T_{on}: onset degradation temperature; T_{max}: maximal weight loss temperature; nr: not reported; ZP: zeta potential.

TEMPO oxidation is another chemical method for the extraction of CNMs from cellulosic materials. TEMPO treatment is generally carried out in the presence of bleaching agents such as NaClO and catalysts such as sodium bromide (NaBr) under alkaline conditions (pH between 9 and 11). Researchers have reported the use of TEMPO oxidation treatment for the production of both CNCs and CNFs. For instance, Zhang et al. [35] prepared CNCs by TEMPO hydrolysis of bleached sugarcane bagasse pulp using TEMPO/NaBr/NaClO system in an aqueous medium at pH 10 for 5 h followed by washing, sonication, and centrifugation. More recently, Zhang et al. [33] obtained a CNCs using TEMPO hydrolysis of lemon seeds using TEMPO/NaBr/NaClO system in an aqueous medium at pH 10 followed by centrifugation, dialysis, and sonication, reaching an extraction yield of 52%. Besides, one of the main goals of using TEMPO for CNFs extraction is to reduce the energy required for mechanical disintegration by reducing the negative or positive charge on the surfaces of cellulose fiber and by improving the colloidal suspension's stability. It has been reported that CNFs isolated by TEMPO oxidation presents attractive properties such as excellent tensile, gas-barrier, and thermal properties [48]. In comparison with other pretreatments, TEMPO offer clear advantages, such as low energy consumption, high reaction rate, high conversion ratio, high selectivity, simple operation, relatively mild reaction conditions, and low cost as co-oxidant [48]. However, the use of toxic reagents represents one of the main drawbacks of TEMPO treatment [33].

In addition to TEMPO oxidation, ammonium persulfate (APS) oxidation is another chemical method used to produce CNMs. Because of its low toxicity and high solubility in water, APS has been recognized as a viable candidate to produce H_2O_2 and sulphate (SO_4^{2-}) free radicals at acidic conditions and high operating temperature, resulting effective to solubilize the cellulose amorphous regions. For example, Zhang et al. [33] employed the APS oxidation method to prepare CNCs from lemon seeds using 1 M APS solution under continuous stirring for 14 h at 70°C followed by centrifugation, dialysis, and sonication. One of the main disadvantages of this method is the longer reaction time, which limits its industrial-scale implementation. The concentration of APS as well as other processing conditions such as temperature and treatment time influence the extraction yield and characteristics of CNMs [35].

1.4.2. Mechanical treatment

Mechanical treatments, also known as physical treatments, usually refers to the use of different mechanical fibrillation equipment to decompose the fibers into their underlying nanoscale units by applying mechanical pressure and creating pure shear force. The procedure for isolating CNMs consists of disintegrating the cellulose fibers along their longitudinal axis. Regardless of the source, different mechanical methods are widely used because of their ease of use and low

environmental pollution, such as refining and homogenization, microfluidization, grinding, cryocrushing, ultrasonication, and steam explosion [49, 4]. All these mechanical treatments work under high shear forces which causes the cleavage of cellulose fibers and, thus, fibrillation. CNFs are mainly produced using mechanical treatment, resulting in particles having both crystalline and amorphous regions, and retaining degree of polymerization of the delaminated CNF filaments as much as possible.

High-pressure and high-shear homogenization refers to homogenization processes of cellulose suspension combining mechanical shear force and pressure action. The cellulosic suspension is passed through a tiny nozzle at very high pressure and high velocity which, along with shear and impact forces, influence the fluid to produce shear rates in the stream and disintegrates the cellulose fibers into CNMs, particularly CNFs. This procedure is repeated several times to increase the degree of fibrillation. The isolation of CNFs by homogenization can be used for a large-scale production since it is a rapid process with simple setup and no solvent requirements, which implies no waste-water generation. However, it has some disadvantages such as the high operational cost, extreme mechanical damage to the crystalline structure of CNF, and its large size distribution [48]. Besides, another drawback is the clogging issue because of its small orifice size, which could be overcome by introducing another mechanical treatment prior to the homogenization process to reduce the fiber size (e.g. refining). Refining in most cases is performed prior to homogenization, in which different refiners like PFI mills and disk refiners are used to refine cellulose pulp prior to mechanical disintegration [4]. For instance, in disk refiner, the diluted fiber suspension is passed through a gap which is between stator and rotor disks. The surfaces of these disks are fitted with grooves and bars against which the fiber suspension is exposed to sequential cyclic stresses. Since this mechanical process can cause irreversible alterations in cellulosic fibers and increases their bonding potentials, refining can also be considered as a method to produce CNFs from cellulosic fibers [50].

Microfluidization is another method similar to homogenization which is commonly employed for CNFs extraction. It consists of passing through a Z- or Y-shaped chamber the cellulosic suspension with very small channel sizes (200-400 μm) and by applying high pressure through the intensifiers pump [4]. Such a channel enables the cellulose fibers to intensively interact with the inside of the microfluidizer chamber, causing significant fiber size reduction. The resulting shear forces against the colliding suspension and the channel walls causes fibers delamination. Contrary to the homogenizer equipment, the clogging issue in microfluidizer can be overcome by reverse flow through the chamber, since the equipment has no in-line moving parts.

Grinding method is a novel mechanical method to prepare CNMs, which uses the friction generated by the equipment to decrease the size of cellulose [48]. In the grinding method, the

cellulosic slurry is passed through an ultrafine grinder in which the fibers are ground between a static and a rotating disc rotor. Based on the material, the distance of the discs can be adjusted. The shear forces produced during grinding cleaved the cell wall structure and bonds in the fibers, causing fiber delamination and CNFs production. Ball milling is a representative grinding method that has been recently applied to reduce the size of cellulose to CNMs. It is a highly energy-intensive grinding process, which is central in mineral processing industry to achieve particle size reduction and mineral liberation [51]. In this process, a cellulose suspension is kept in a hollow cylindrical jar that is partially filled with balls made from different materials (e.g., zirconia, ceramic, alumina, or metal). The high energy, which will be released by the collision and abrasion between the balls, the jar's walls, and the material, disintegrates the cellulose fibers. The ball milling conditions such as ball size, ball amount (ball to cellulose weight ratio), grinding time, and moisture content are factors of ball milling that affect CNMs extraction. One of the main limitations of ball milling is the high power and energy consumption, and the generation of a large amount of heat energy during processing.

Ultrasonication is a mechanical method commonly used to extract CNMs with the hydrodynamic forces of the ultrasound. During ultrasound treatment, the ultrasonic energy is absorbed by the liquid molecules, leading to the generation of mechanical oscillating power, which results in the formation, expansion, and implosion of microscopic gas bubbles [46]. It has been demonstrated that ultrasonication treatment usually delivers energy more efficiently, thus consuming less time and less energy than other mechanical methods such as homogenization and grinding. The cellulose concentration during treatment, processing time and ultrasonication power are factors that affect the CNMs extraction during the process. Even though mechanical methods are generally used for CNFs extraction, ultrasonication treatment is widely used for CNCs extraction in combination with acid hydrolysis. For instance, Gibril et al. [32] used ultrasonication to induce cavitation of cellulose in suspension prior to acid hydrolysis for CNCs extraction from dissolving wood pulp fibers. Besides, ultrasonication can be performed in combination with other mechanical method for the extraction of high purity CNFs. For instance, Mahardika et al. [31] reported the production of CNFs from pineapple leaf fibers combining ultrasonication with high shear homogenization. The high energy consumption is one of the main drawbacks of this method, along with the fact that huge amount of heat is generated during the ultrasonication process, which requires the use of ice-water bath or pool for controlling the heat transfer. However, it is an ecofriendly and cost-effective process and has proven to be a highly efficient technology in CNMs production.

Cryocrushing is another mechanical method used for the production of CNFs. In this process, cellulose fibers are kept in water, and cellulose absorbs water in their cavities. Then, water-soaked

cellulosic fibers are frozen in liquid nitrogen for water solidification and is subsequently crushed by high shear forces. The application of high impact forces to the frozen cellulosic fibers causes the rupture of the cell wall due to exerting pressure by ice crystals which leads to CNFs formation [52]. This process's drawbacks are high cost, high energy consumption, low recovery, and low uniformity of CNFs [53].

Steam explosion is a thermomechanical method commonly used for pretreatment of lignocellulosic biomass. In this process, the heat carried by the steam penetrates the sample by diffusion and the sudden release of pressure generates high shear forces that causes the cleavage the glycosidic and hydrogen bonds in cellulose fibers. This leads to the isolation of CNFs from cellulosic materials. Deepa et al. [54] and Cherian et al. [55] reported the isolation of CNFs from banana fibers and pineapple leaf, respectively, using steam explosion method.

Different mechanical methods have been employed independently and/or in combination to obtain CNMs (Table 1.2). Although these mechanical methods demonstrated to be efficient to extract CNMs, disadvantages such as high process cost, low efficiency, and the fact that extensive energy is required to disintegrate the highly ordered hydrogen bond network of cellulose to nanosized cellulose particles still limit its industrial implementation. For instance, it was reported typical energy consumption of around 20 kWh/kg for homogenizing, 4–15 kWh/kg for grinding, 3-6 kWh/kg for microfluidizing, and 40-48 kWh/kg for ultrasonication [56].

To overcome this, different pretreatments such as chemical and/or enzymatic are applied in combination with mechanical fibrillation based on the cellulose source, which normally decrease the energy demand and enhance CNFs properties by surface modifications. Examples of these pretreatment techniques are mild enzymatic or acid hydrolysis, phosphorylation, periodate oxidation, carboxymethylation, and TEMPO oxidation [3].

1.4.3. Combined treatment

The combination of types of treatments has shown several advantages in the production of CNMs such as shortening the reaction times, saving energy, and improving the CNMs properties. While mechanical methods can speed up the movement of molecules, provide energy, and increase the chance of molecular collisions to promote chemical reactions, chemical reactions can transform cellulose at the molecular level by promoting the partial hydrolysis of cellulose and introducing functional groups on the cellulose surface. Consequently, CNMs products obtained by combined treatment strategies generally show enhanced properties, such as smaller size, more homogeneous, and better dispersion. Examples of combined treatments to produce CNMs are presented in Table 1.2.

1.4.4. Emerging methods

Several emerging processing technologies have been recently investigated for CNMs production such as enzymatic pretreatment, deep eutectic solvent, microwaves, electron beam irradiation, plasma, and pulsed-electric field [48].

Deep eutectic solvents (DESs) are emerging green solvent which are prepared by mixing two or more substances at a proper ratio generally with moderate heating [48]. These substances can stabilize the interactions of hydrogen bonds and generate a eutectic mixture that has a lower melting point than the individual substances. Due to DESs beneficial characteristics such as simple preparation method, eco-friendly, recyclability, biodegradability, non-flammability, high thermal stability, relatively wide liquid range, and low vapor pressure, they can be used in various chemical reactions at near-ambient conditions. It was reported that DESs can disintegrate the recalcitrance structure of lignocellulosic biomass by cleaving the hydrogen bonding between the carbohydrates and lignin, and therefore, exposing cellulose. In addition, DESs can also be utilized for treatment of bleached cellulosic pulp for CNMs production, but a mechanical or chemical process is generally required following the DES pretreatment to convert the isolated cellulose into CNMs. The production of CNCs and CNFs with the assistance of DESs has been recently reported by many researchers. For example, Lim et al. [57] treated bleached rice straw pulp using DES (oxalic acid and choline chloride at a molar ratio of 1:1) at 80°C for 4 h under continuous stirring and obtained a CNC extraction yield of 55%, with higher thermal stability than CNCs produced by conventional acid methods. Furthermore, Liu et al. [58] extracted CNFs at high yield (84%) from bleached softwood Kraft pulp using DES (choline chloride and anhydrous citric acid at a molar ratio of 1:1) treatment at 90°C for 2 h followed by high-pressure homogenization. Even though DESs seem promising in CNMs synthesis, there are still difficulties in using them at an industrial scale that required further research.

Microwaves (MWs) are non-ionizing waves having a frequency in the range of 300 to 300 GHz and a wavelength from 1 mm to 1 m, which has been used widely in several areas because of its efficacy and easy operation. It is considered an alternative method to conventional heating and can be used to assist the chemical pretreatment of biomass prior to CNF extraction by mechanical disintegration. For instance, MW heating was used to assist dilute acid pretreatment combined with enzyme pretreatment to prepare CNCs from pure microcrystalline cellulose [48]. Also, MW was used to extract CNCs from cotton powder assisted by the APS method under pressurized conditions for 90 min, hydrolyzing the cellulosic amorphous regions, while the conventional heating required 16 h [59]. On the other hand, MW can be utilized in combination with other technologies to develop environmentally friendly processes for CNFs production with minimal use of chemicals. For instance, Liu et al. [60] produced high purity CNFs from wheat straw by an

eco-friendly method which consisted on steam explosion and MW-assisted alkaline hydrolysis process followed by microfluidization. MW main benefits such as short processing time, lower maintenance and operational costs than conventional heating, and environmentally friendly character make MW a promising emerging technology for CNMs production.

Electron beam irradiation (EBI) is generated using a linear accelerator which can be used both as a pretreatment method to isolate cellulose fibers from lignocellulosic feedstock and as a fibrillation method to reduce the cellulose fibers size to the nanometer range. The EBI treatment leads to the generation of free radicals either by cleaving glycosidic bonds or by removing hydrogen from a glucose moiety, which then induce the degradation of cellulose by oxidation or chain scission. It was reported to be effective in reducing the length and width of CNMs while improving the dispersion stability. EBI has also been used to produce CNMs from different cellulosic materials in combination with other chemical and/or mechanical methods. For example, Van Hai and Seo [61] utilized cotton linter and bleached softwood Kraft pulp to produce CNCs through EBI treatment followed by H_2SO_4 hydrolysis, whereas Le and Seo [62] prepared CNFs from cotton linter using EBI treatment followed by grinding with a super masscolloider. Recently, Kim et al. [63] showed the possibility of extracting CNFs from tall goldenrod plant pulps by using only EBI, without combining it with any other technology. Even though very limited research has been conducted on using EBI treatment in CNMs production, it has several beneficial aspects such as low environmental impact, lower energy consumption, short treatment time, and requirement of mild conditions [48]. However, many studies have indicated that using EBI treatment for CNMs extraction has several negative impacts, such as low yields, crystallinity, and thermal stability.

Solution plasma processing (SPP) or plasma is regarded as an advanced oxidation method because of its potential to generate highly active species, particularly hydroxyl radicals. Plasma is an ionized gas and can be described as a medium comprised of photons, metastable molecules, radicals, and charged particles such as free electrons and ions [64]. For its generation, an electric field is applied to electrodes with gas (e.g. CH_4 , NH_3 , N_2 , O_2 , Ar, and He) in between them, either at atmospheric or reduced pressure. The material and geometry of electrodes, electric power, and the nature of gases influence the characteristics of the generated plasma. The information regarding the use of plasma in CNMs production is very limited. Vizireanu et al. [65] prepared CNFs from commercially available microcrystalline cellulose by combining the submerged liquid plasma treatment with ultrasonication, which showed that this combined pretreatment resulted an effective green technology for cellulose defibrillation. Nevertheless, further research is required to determine the suitability and effectiveness of plasma technology to produce CNMs from various lignocellulosic feedstock.

Finally, the enzymatic pretreatment is an emerging route to obtain CNMs from lignocellulosic biomass. This method will be discussed in detail in the following section.

1.5. Enzymatic pretreatment of cellulose pulp

One of the most important methods for cellulose modification is enzymatic pretreatment. This biological method uses enzymes which play a variety of functions in the entire process, from modifying and degrading hemicellulose and lignin in the material to the most prevalent use of cellulases to yield CNMs. In enzyme-mediated pretreatment, the cellulose pulp results in various surface changes or disintegrations, depending on the enzymes used. The enzyme can be used in restrictive hydrolysis of cellulose or selective hydrolysis of selected compositions in the cellulosic fibers. Generally, a set of enzymes are used together for enzymatic pretreatment because one enzyme cannot degrade the cellulose fiber as it contains different compounds.

Enzymatic pretreatment has been used in combination with other techniques for CNMs production. For instance, enzymatic pretreatment of cellulose pulp is usually assisted with mechanical fibrillation such as ultrasonication, homogenization, extrusion and microfluidization for CNF extraction from cellulosic materials [66]. In the case of CNCs, a sonication phase following enzymatic pretreatment is included for a significant fragmentation of CNMs into nanoscaled-sized particles and efficient dispersion [67].

The enzymatic pretreatment of cellulose pulp for the production of CNMs offers several advantages compared to conventional methods. One of the main advantages is that the enzyme pretreatment can be conducted under milder conditions, including moderate pressure, temperature, and pH [48]. This offers benefits from an operational aspect, such as a safer operation, lower energy consumption, no requirement of corrosion-resistant processing equipment, and reduced cost. Also, the use of chemicals during the process may be reduced or avoided which reduces the quantity of effluents produced and, thus, ensures the environmental protection. The reduction or elimination of chemicals will allow the use of a final stream of sugars to produce other value-added products, such as biofuels, in accordance with a biorefinery approach [1]. Moreover, another advantage of the enzymatic pretreatment is the ability to avoid extensive pulp deconstruction by a proper control of the operational conditions. However, the high cost of enzymes remains a significant obstacle that needs to be addressed. As a result, cost-cutting measures aimed at the enzyme reuse, such as enzyme immobilization, as well as advances in production and purification of enzymes to overcome this hurdle [68].

1.5.1. Enzymes involved in pulp deconstruction

Even though different classes of enzymes have been employed for the extraction of CNMs from cellulose, cellulases account for the vast majority of these cases. Cellulases are a class of enzymes that directly catalyzes the degradation of cellulose into simpler sugars by working together. Their capability to target cellulose structure with great selectivity allows for a variety of cellulose modifications.

Cellulases are normally produced by cellulolytic organisms which includes many species of fungi, bacteria, protozoa, plant and animals, performing a fundamental role in several cellular physiological processes by regulating cellulose depolymerization. The wide variety of cellulase-producing species includes aerobic, anaerobic, mesophilic or thermophilic microorganisms, whereas the most studied cellulase producers includes those from the genera *Aspergillus*, *Trichoderma*, *Clostridium*, *Cellulomonas*, and *Thermomonospora* [1, 69]. Most commercial cellulase are being produced by fungi rather than bacteria. When compared to fungus, even though bacteria produce less cellulase, the bacterial cellulases are sometimes superior to that produced by fungus. One of the main advantages of using bacteria is that it grows more quickly and produces more recombinant enzymes. Furthermore, this type of microorganisms can endure harsh environmental conditions during bioconversion processes compared to fungi [67].

The cellulases preparations are mainly composed of three specific activities. Endo-(1,4)- β -D-glucanases (EGs) target loose chains that are found in the amorphous regions (less organized) of the cellulose supramolecular structure, which results in a decrease in viscosity that is directly associated to a reduction in cellulose degree of polymerization. EGs cleave random internal β -1,4-glycosidic bonds of the cellulose chains breaking cellulose into smaller lengths and forming new chain ends. Then, exo-(1,4)- β -D-glucanases or cellobiohydrolases (CBH) progressively hydrolyzes these cellulose chain ends yielding cellobiose units as the major product. While EGs have an affinity for the interior of the glucose chain, CBHs have an affinity for the ends of the glucose chain and attack the cellulose crystalline regions. Both EGs and CBHs are the main cellulolytic enzymes that typically carry out cellulose deconstruction. CBHs are the central catalysts behind fungal biomass degradation and, accordingly, are often the main enzyme secreted by many fungi, whereas EGs are the second primary enzyme in the arsenal of fungal cellulose degradation [70]. Finally, β -glucosidases (β Gs) catalyze the hydrolysis of cellobiose and other low molar mass cello-oligomers to glucose. Therefore, these enzymes cannot be classified as true cellulases, but they complete the conversion of cellulose to glucose. Fig. 1.3 presents an overall representation of the detailed mechanism involved in cellulose deconstruction by enzymes.

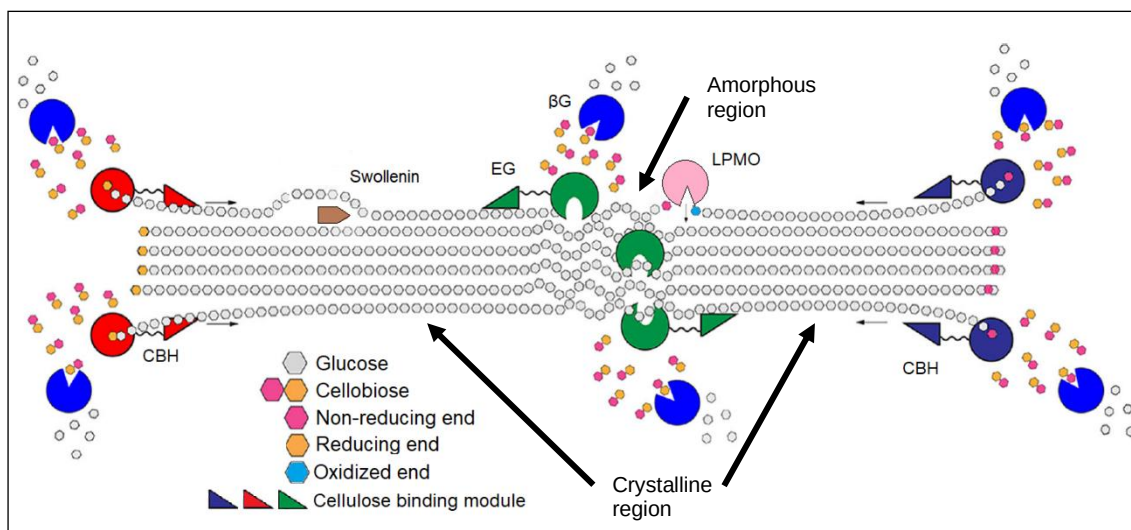


Figure 1.3. Schematic representation illustrating the synergism among enzymes during cellulose deconstruction (adapted from [69]).

All these enzymes act synergistically or cooperatively during cellulose deconstruction, which may be used for different technological applications such as the production of biofuels (bioethanol, biobutanol), amino acids or renewable hydrocarbons, among others. For instance, the cellulase system secreted by *T. reesei* contained at least four endoglucanases (EG I, II, III and IV) and two CBHs (I and II). These enzymes display an endo-exo synergism whereby the products generated by EG become the substrate for CBH attack. By this, CBH I releases cellobiose for reducing chain ends while CBH II exerts its activity on non-reducing chain ends [69]. Additionally, EGs act specifically on the cellulose amorphous regions whereas CBHs attack predominantly on its crystalline regions and the balance between these two action modes is a very important parameter for optimizing CNMs production by enzyme-mediated pretreatment.

The use of cellulases for fiber modification has increased considerably and now accounts for a significant share of the world enzyme market [69]. Cellulases are widely used in the textile industry, in the formulation of detergents, in pulp and paper industry, in animal feeds, in agriculture and food industry and in the production of biofuels such as ethanol. They are inducible enzymes which can act to a given extent and over specific cellulose sites, providing the desired level of modification over cellulose structure. For instance, regarding CNMs production, the internal amorphous regions in cellulose are the primary target for CNCs production, which are hydrolyzed by EGs resulting in smaller chains of crystalline cellulose. Instead, for CNFs production, since total removal of amorphous regions is not intended in this case, a deconstruction of cellulose chains to reduce the fibers length by the breakage of inter-fiber connections can be mainly achieved to enhance subsequent mechanical fibrillation. In both cases, EGs constitute a desirable mechanism to reduce the fiber length and, thus, the degree of polymerization, or to

increase crystallinity. On the other hand, the action of CBHs may result in undesirable fiber decomposition into cellobiose. Thus, it is very important to maintain an optimum enzyme mixture composition since it has an important role on the final extraction yield [1].

Monocomponent EGs have been employed in the production of both CNCs and CNFs from different lignocellulosic biomass. EG's efficiency is due to its mechanism of action which targets the amorphous regions of cellulose fibers by selectively disrupting accessible glycosidic bonds and forming new reducing and non-reducing ends. Because of its characteristics, EGs can stimulate specific alterations, which can help in the defibrillation process without affecting the crystalline portions. Besides, the wide utilization of EGs is due to their defibrillation efficiency, biosafety, commercial availability, and relatively low cost [39].

While the use of CBHs will intend to decrease the degree of polymerization of cellulose fibers, their extended action can be partially constrained by cellobiose accumulation through a mechanism of end-product inhibition. Thus, the CBHs may reduce the undesirable cellulose digestion to other monomers by yielding more cellobiose units, which would improve the production of CNMs by keeping low activity levels of β Gs [1].

An important question around the production of CNMs by enzyme-mediated pretreatment using cellulases is whether a complex cellulases cocktail or a monocomponent EG may be the suitable option for the extraction. Even though several studies report the use of monocomponent EG such as Novozyme 476, Fibercare R and Cellusoft L [1, 43, 71], the use of complex enzymes cocktails, such as Cellic[®] CTec, are reported for improving the deconstruction of cellulose for CNMs extraction [1, 72]. Since complex cellulases cocktails are intended to be used for biomass deconstruction to obtain maximum conversion of complex sugars into fermentable sugar [34], it is important to have a proper control on the pretreatment conditions to limit the degree of cellulose enzymatic degradation.

In addition to the typically used cellulases, the application of other enzymes can complement the process of CNMs production, by either providing a more purified cellulose material or assisting the action of cellulases. Recently, the use of xylanases in the enzymatic production of CNMs has been reported by some researchers [73, 74]. The glycosidic linkages of hemicelluloses can be hydrolyzed and, thus, the xylan chains from the cellulose surface can be peeled off by xylanases. By this, these enzymes can reduce the barrier formed by xylan polymer to cellulases action, which improves the CNMs extraction process. Furthermore, the use of xylanases allows xylans from the hemicellulose to be converted into renewable fuels and other value-added products. Thus, using xylanases not only enhances cellulosic fiber disintegration but also adds value to the overall CNMs extraction process by further valorization of the coproducts generated.

Moreover, xylanases synergistically work with cellulases to improve the properties of fiber by enhancing its porosity and swelling capacity [67]. Because of this, xylanases can be used in combination with cellulases in preparing a suitable enzyme mixture for the extraction of CNMs. The levels of xylanase employed will be case-specific, since it will depend on the source composition and the desired properties of the produced CNMs. Recently, Tong et al. [75] reported that the ratio of cellulases to xylanases could have an effect on CNMs morphology. However, it was reported that xylanases could be used alone to produce CNMs from certain types of cellulose sources, since the removal of xylan results sufficient to facilitate mechanical disintegration. For instance, Tibolla et al. [76] reported the production of CNFs from banana peel employing xylanases alone.

A more recently discovered class of enzymes that may assist the CNMs production are the lytic polysaccharide monooxygenases (LPMOs), which are also involved in deconstruction mechanism and has been increasingly used on cellulase cocktails for biomass deconstruction. They have now been classified under Auxiliary Activities families, and proteins of these families have been identified and characterized in a variety of fungi. LPMOs act on cellulose chains by initiating a very selective oxidative cleavage of glycosidic linkages at either C1 or C4 position, leading to the formation of oxidized glucose units (aldonic acids or gemdiols) in different positions. The formed oxidized units possess an ionizable carboxyl group that improves the cellulose electrostatic repulsion and, thus, enhances the fibrillation process by subsequent mechanical treatment. Also, they have been shown to have specificity toward crystalline (highly ordered) regions in cellulose. By cleaving and oxidizing cellulose chains in crystalline regions, LPMOs create additional chain ends, making hydrolysis of these crystalline regions more favorable for CBHs (Fig. 1.3). Moreover, strong synergistic interactions of LPMOs with cellulase enzymes toward deconstruction of cellulosic substrates have been shown, with only relatively small doses required to obtain enhanced conversion yields [71]. Even though major differences on the morphology of CNMs produced were not found when using LPMOs, it was shown that some properties were improved. For instance, it was found an increase in negative charges of cellulose fibers when LPMOs are used, possibly due to deposition of carboxylic acid or ketone structures from the oxidative cleavage [1]. This may contribute to a significant increase in zeta potential which improves cellulose nanofibrillation and suspension stability.

1.5.2. Factor affecting cellulose hydrolysis

Enzyme-mediated production of CNMs from lignocellulosic biomass can be influenced by several variables. Some of them directly influence the enzymes such as pH, temperature and binding affinity, while others influence the enzymes action over cellulose efficiency including

raw material composition, biomass pretreatment, reaction time, enzyme type, enzyme dosage, etc. These variables have a direct impact on the CNM extraction yield and characteristics and properties of the final CNMs.

Raw material composition and pretreatment

The composition of the raw material and particularly the proportion of each component (e.g. cellulose, hemicellulose, lignin) have a crucial role on the CNMs production, since it determines the type of pretreatment that should be carried out to obtain a highly cellulosic material for further enzymatic processing. Also, high lignin contents represent sometimes a particular obstacle, since cellulases have a high affinity to this component causing non-productive and irrevocable binding which leads to increased enzyme consumption and process cost. Moreover, since enzymatic pretreatment affects mostly cellulose, using a less purified raw material leads to CNMs production with more impurities. Another point to consider is the structural properties of the raw material and its influence on the way enzymes will act over cellulose. For instance, raw material with high crystallinity (e.g. tunicate, bacterial cellulose) have less amorphous regions that will be cleaved by enzymes action, which may results in the formation of larger CNCs [77].

Temperature and pH

Interactions between enzymes and substrate are generally a complex process controlled not only by the physical characteristics of both substrate and enzyme, but also the environmental parameters such as temperature, pH and surfactant. Cellulolytic and hemicellulolytic enzymes typically work best at mild temperature conditions (40 to 50°C) and pH levels in the range of 4.0 to 5.5, which are relatively gentle operating conditions compared to other chemical treatments. The pH range is very important when alkaline or acidic pretreatment methods for lignin or hemicellulose removal were performed prior to enzymatic pretreatment, since extensive washing would be required before enzymes addition.

Hydrolysis time and enzyme dosage

The hydrolysis time and enzyme dosage represent the most important factor for a controlled and selective production of CNMs. Based on the action mechanisms of EGs and CBHs, it would be expected that by using different concentration of enzymes different CNM products will result for the same hydrolysis time. For instance, contrary to the idea that EGs only attacks the internal cellulose amorphous regions, they may also attack crystalline regions under high concentration of enzymes. Recently, Tong et al. [75] showed that both hydrolysis time and enzyme concentration influence the morphology of CNCs, since rod-like CNCs were produced by lower enzyme levels, while spherical forms appeared by increasing enzyme concentration. One of the

main advantages of enzymatic pretreatment is the fact that allows a precise process control by controlling the enzyme dosage, enzyme cocktail composition and operational conditions.

1.6. Nanocellulose characterization

There are several tools for characterization of attributes and/or properties of CNMs. While the majority of these characterization methods are widely accepted, there remains unresolved challenges, particularly relating to quantification of the degree of fibrillation. Moreover, it is almost impossible to characterize the degree of fibrillation of CNMs, particularly CNFs, with a single method. The characterization techniques commonly employed to assess the quality of CNMs can be classified into direct and indirect methods, which will be discussed below.

1.6.1. Direct methods

Currently, the only method of measuring directly the size of materials is through visual observation through one of the several microscopy techniques such as optical microscopy (OM), scanning electron microscopy (SEM), field emission scanning electron microscopy (FESEM), transmission electron microscopy (TEM), atomic force microscopy (AFM), and fluorescence microscopy. SEM and TEM are widely used by many researchers to measure the dimensions of CNMs on a range of scales, particularly CNFs. With image analysis software (e.g. ImageJ), it is possible to obtain size distributions of CNMs from TEM images including mean size, standard deviation and estimation of polydispersity index (PDI). The PDI is a dimensionless parameter that refers to the broadness of the size distribution. Measurements by these methods are relatively straightforward, as two-dimensional images are obtained, and the fibrils' lengths, diameters (or widths) and aspect ratios (defined as length divided by width) can be measured both manually and by image analysis software. However, it may represent a time-consuming method, and since only a very small area of the material's sample is being focused using electron microscopy, this characterization is very unlikely to be representative of the whole sample. Also, the fibrils' length measurement does not represent an easy task for samples which are not well-dispersed into individual fibers, especially for CNFs with a network forming character.

1.6.2. Indirect methods

Dynamic light scattering

Dynamic light scattering (DLS) measures particle size in solutions or suspensions based on the assumption that all particles are spherical in morphology. DLS uses time-dependent fluctuations of the intensity of light scattered by particles in solution undergoing Brownian motion. These

fluctuations are related to the hydrodynamic radius (R_H) of the particles via the Stokes-Einstein equation under the assumption that they are spherical, which have a constant diffusion coefficient in all directions. The R_H is the radius of the hypothetical hard sphere that diffuses with the same speed as the particles assayed by DLS. However, R_H is a hypothetical measurement since hard spheres rarely exist in colloidal dispersions.

DLS represent a simple technique that offers both size and zeta potential determination as well as the fact that lesser volume of samples is required for DLS compared to other techniques (e.g. laser diffraction). However, DLS often do not correlate quite well with data obtained from TEM images, for instance, as the latter is a particle number-based technique and DLS is an intensity-based technique. Hence, the size obtained by DLS results usually bigger than TEM. Since DLS allows to measure a bigger number of particles in a sample (around millions) compared to TEM that allows to measure a few hundreds, DLS provides a more robust data on size distribution and PDI.

A common dilemma among researchers is whether express the particle size based on scattered light intensity, particle number or particle volume, since expressing the data on these parameters produce different apparent sizes and size distributions. DLS principles are based on measuring intensity of scattered light, so the R_H should always be deduced on intensity measurements while particle volume and number parameters should be used as supporting data [78].

Since CNMs have high aspect ratio, variable conformations (rod or fibrillar-like materials), and tendency to agglomeration, it is still a challenge to characterize CNFs employing DLS method. DLS cannot be directly linked to the particle length or cross-section. Instead, DLS gives a hydrodynamic “apparent particle size” that can be used to assess dispersion quality and/or state of aggregation. For particle size and size distribution measurements, a combination of light scattering technique and microscopy is recommended by researchers [79].

Laser diffraction

Laser diffraction (LD) represents a capable tool to determine the particle size of both cellulose materials in the micrometer and nanometer scales and, like DLS, LD is based on scattering of light. The light source used is usually a laser, so the technique is referred to as LD. Particles scatter light in a size-dependent way where large particles scatter light at small angles and small particles scatter light at large angles [78]. The angular scattering intensity is then analyzed to determine the size of the particles responsible for the scattering pattern, which is reported as a volume equivalent sphere diameter. LD measures particle size distributions by measuring the angular variation in intensity of the light scattered as the laser beam passes through a dispersed particulate sample. The results obtained from LD and DLS are not the same and differ depending on the

experimental conditions and types of material. LD is more suitable for bigger particles (e.g. $>1\ \mu\text{m}$) and in samples with impurities of larger sizes, while DLS provides better data for smaller materials (e.g. $<50\ \text{nm}$). Moreover, LD is becoming a standard particle sizing technique in many industry sectors because of its simplicity, rapid measurements, and better resolution when compared to traditional sizing techniques such as sieve analysis. Whereas the upper size detected is bigger in LD, it reaches its limits for small particles in the nanometer range ($< 100\ \text{nm}$) due to weak signals and low angular variance in scattering signal. In contrast, DLS presents a typical lower size range of $0.3\ \text{nm}$. Therefore, for bigger particles sizes ($> 10\ \mu\text{m}$) LD is more suitable whereas for smaller particle sizes ($< 40\ \text{nm}$) DLS provides better information.

Zeta potential

The zeta potential (ZP), also known as electrokinetic potential, is the potential at the slipping/shear plane of a colloid particle moving under electric field. The electric potential of a surface refers to the amount of work that needs to be done to bring a unit positive charge from infinity to the surface without any acceleration. The ZP reflects the potential difference between the electric double layer (EDL) of electrophoretically mobile particles and the layer of dispersant around them at the slipping plane. The EDL refers to the adsorbed double layer that develops on the surface when a charged particle is dispersed [78].

One of the most popular uses of ZP data is to correlate it with colloid stability. It was conventionally defined as highly unstable, relatively stable, moderately stable and highly stable dispersions with absolute ZP values in the ranges $0\text{--}10\ \text{mV}$, $10\text{--}20\ \text{mV}$, $20\text{--}30\ \text{mV}$, and $> 30\ \text{mV}$, respectively [78]. However, since ZP provides information on the electrostatic repulsive forces and not on the attractive van der Waals forces, it is not uncommon to come across stable colloids with low ZP and vice versa. As well as DLS measurement, ZP is based on light scattering, so only clear samples can be subjected to ZP analysis.

Viscosity

Viscosity measurement of CNM suspensions in water has been widely used by many researchers to qualitatively determine the degree of cellulose fibrillation. As the size of the fibrils generally decreases during mechanical processing, the degree of fibrillation increases. The smaller or more highly fibrillated CNFs progressively increases fibril–fibril interactions, which results in greater suspension viscosity [79]. Even though attempts have been made to fit models to viscoelastic data to correlate viscosity and fibrillation extent, no widely accepted relationship between viscoelastic data and CNM dimensions or CNF degree of fibrillation has been established.

Transmittance or turbidity

The transmittance of light through a CNM suspension in water is related to the particle or fibril dimensions, surface area and interparticle interactions at a given concentration. A higher transmittance generally correlates to a more highly fibrillated material. While transmittance can be directly correlated to the degree of fibrillation, it is possible to inversely correlate turbidity to the degree of fibrillation. The turbidity of a suspension is a measurement of the light scattered at 90° to the incident light, and it determines the number of particles in a suspension. Both transmittance and turbidity of a suspension can be used to qualitatively assess the fibrillation of CNFs, but results are also affected by any agglomeration of fibrils in the sample. Transmittance and turbidity values are commonly measured using UV–Vis spectrophotometric method. Recently, more specialized and automated equipment has become available that combines transmittance measurements with optical image analyses of residual microscale fibers after fibrillation to provide an estimation of the degree of fibrillation [79].

Water retention value

Water retention value (WRV) is a widely technique used in the pulp and paper industry to assess the degree of fibrillation by determining the amount of water retained by the fibers under specific conditions. Even though this method was developed for pulp fibers, which have diameters of several microns, it has been used by researchers to measure the water retention capability of micro- and nanofibers. WRV can be qualitatively correlated with the degree of fibrillation on the basis that the greater the amount of water retained by the fibers (thus, the higher WRV), the higher the fibrillation extent. One of the main drawbacks of the WRV technique is the fact that is primary used for larger fibers, so its efficacy with nanomaterials is sometimes problematic, since the drainage rate is greatly reduced (close to near-zero) as the nanofiber size decreases.

Specific surface area

Since the surface area of fibers increases substantially when they are disaggregated into micro- and nanoscale particles, the surface area determination can be used as an indirect method to estimate the degree of fibrillation. Commonly, UV-Vis spectroscopy coupled with dyeing procedure with specific compounds (such as water, dyes, gas, enzymes, or charged species) and subsequent centrifugation is used for specific surface area measurements. Others adsorption-based methods include Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) analyses employing nitrogen gas adsorption and desorption [56]. It should be mentioned that the surface area determination gives no indication of the dimensions or aspect ratio of the CNMs. Moreover, surface area measurements are often difficult to interpret since they rely on the

adsorption of compounds onto the surface, which will depend on the physical size of the molecules and its affinity to adsorb to cellulose fibers/microfibers/nanofibers [56].

1.6.3. Other properties

The cellulose crystallinity degree (CrI), defined as the fraction of cellulose with crystalline nature, and the degree of polymerization (DP), defined as the number of repeating monomer units in the polymer molecule, influence the physical properties of CNMs. Generally, a high DP of cellulose leads to the formation of nanocellulosic films with improved tensile strength. Also, the aspect ratio of CNMs, which is defined as the length divided by the width, presents an important role in determining their reinforcing capacity since it affects the ability of fibers to keep the film network, particularly for CNFs. Also, the introduction of anionic charged carboxyl groups by the different treatments that could induce the formation of additional hydrogen bonds to those from the original material contributes to more inter-fiber bonding and, thus, a stronger network [1]. For instance, Tarrés et al. [43] found strength differences on nanopapers obtained from CNFs produced by different treatments which may probably be due to differences in the CNF intrinsic properties such as specific surface, fibrillation extent, swelling capacity and ability to form three-dimension network.

The thermal properties of CNMs are important since the processing temperature of most composite materials are usually above 200°C [1]. It has been shown that the particle size and the pretreatment used to obtain the cellulosic material affect the thermal properties, since the degradation temperature depends on the structure and chemical composition. Generally, CNCs presents reduced thermal stability compared to CNFs with decomposition typically starting at temperatures in the range of 200 to 300°C, due to the introduction of charged sulfate groups. This limits their applications which requires high processing temperatures. Also, it has been reported that CNCs produced by enzymatic pretreatment with higher crystallinity presents higher thermal stability [34]. Instead, CNFs starts to decompose at higher temperature (around 310-350°C), which offer superior thermal stability among CNMs.

The optical properties of CNMs are also related to different characteristics such as morphology, size, surface charge, and structure. It was reported that CNFs present great optical properties because the size of the CNFs results lower than the visible light wavelength [1]. Nanocellulosic films prepared with CNFs result smoother and less porous than the traditional cellulosic papers, and contain certain level of transparency depending on the CNF dimensions. On the other hand, transparent nanocellulosic films can also be produced with CNCs, but in this case they are usually more fragile or brittle than those prepared with CNFs due to their crystalline nature [80].

1.7. Cellulose nanomaterials market and emerging applications

Nanocellulose has an enormous potential application in biomedical, food industry, composites and coating, cosmetics and health care, electronics and optoelectronic devices, and water treatments due to a range of unique advantages like as biodegradability, non-toxic, biocompatibility, good mechanical properties, high water absorption and holding ability [5,13]. Moreover, the nanoscale dimensions, high aspect ratio and outstanding reinforcing potential of these nanomaterials have increased the demand of CNMs to be used in nanocomposites. The market is expected to grow from 297 million USD in 2020 to 783 million USD by 2025 [1]. Worldwide CNMs markets and production capacity are presented in Table 1.3. Nowadays, the pulp and paper industry represent the majority of the market, as CNMs (particularly CNFs) are used as additives in papermaking to produce lighter and stronger paper and provide improved properties such as less porosity and higher printing quality. The addition of CNFs in the mass of the paper improves the mechanical properties (strength and breaking length) of the fibers without causing structural damage, as can occur when conventional mechanical refining techniques are used. Another way of incorporating CNFs into papermaking is by surface addition, which results in improved mechanical properties as a function of the CNF morphology. The addition of CNFs to paper makes it possible to provide special barrier and/or superficial properties which allow, for example, to prevent some gases or liquids from penetrating into the heart of the paper. Fig. 1.4 shows the wider applicability of CNMs in versatile industries.

Table 1.3. Status of worldwide CNM production [67].

Country	Product	Company name	Source and method	Capacity
Canada	CNC	Alberta Innovates (AITF)	Microcrystalline cellulose and bleached Kraft pulp; acid hydrolysis	20 kg/day
	CNC	Blue Goose Biorefineries Inc.	Wood, cereal straws, and grasses; oxidative and nanocatalytic processing	10 kg/day
	CNC	CelluForce	Kraft pulp; bleaching and acid hydrolysis	1 ton/day
	CNC	FP Innovations	Bleached chemical pulp; acid hydrolysis	2 ton/day
	Filament formed cellulose, wet fluff rolls or forms	Performance BioFilaments Inc.	Kraft pulp; mechanical treatments	21 tons/ day
	Cellulose filaments	Kruger Bioproducts Inc. FILOCELL	Kraft pulp; mechanical treatments	5 tons/ day
China	CNC, CNF	Tianjin Haojia Cellulose Co., Ltd.	Cotton or Kraft pulp; mechanical treatments and combined acid and enzymatic hydrolysis	Pilot-scale plant
Finland	Biofibrils™.	UPM-Kymmene Ltd	Wood fibres; mechanical treatment	Pilot-scale plant
	CNF	VTT -Aalto U, UPM	Birch fibril pulp/mechanical treatment	Pilot-scale plant
India	CNF, CNC	Central Institute for Research on Cotton Technology (ICAR-CIRCOT)	Cotton linters, sugar cane bagasse, other agro-residues; novel chemico-mechanical, microbial, and enzymatic hydrolysis	10 kg/day (pilot plant)
Japan	CNF	Chuetsu Pulp and Paper	Kraft pulp, bamboo, wood; counter collision technique	50 ton/year
	CNF	Dai-ichi Kogyo Seiyaku Co., Ltd.	TEMPO oxidation	50 ton/year
	CNF	Daio Paper	Mechanical treatment	150 tons/day
	CNF	Nippon Paper Industries	Wood pulp; TEMPO oxidation, carboxymethylation, mechanical treatments	>0.1 ton/day
	CNF	Seiko PMC	Wood pulp; mechanical treatment and hydrophobization	30 tons/year
Sweden	CNC	MoRe Research Institute	Paper industry sludge; controlled acid hydrolysis and sonication	0.1 ton/day
Switzerland	CNF	Swiss Federal Laboratories, Empa	Wood and other lignocelluloses residue; enzymatic pretreatments and microfluidization	15 kg/day
UK	CNF	CelluComp	Waste streams of root vegetables/ proprietary technology	Small plant
	CNF	Sappi in partnership with Edinburgh Napier University	Wood fibers; CNFs surface modified for hydrophobic/hydrophilic nature	8 tons/year
USA	CNF, CNC	American Process Inc. (AVAPCO)	Wood chips, agricultural residue; mechanical treatments	0.5 ton/day
	CNF	University of Maine	Wood pulp/mass collider grinder	1 ton/week
	CNF, CNC	USDA-Forest Products Laboratory	Wood pulp/sulfuric acid hydrolysis	50 kg/week

Recently, CNMs have attracted interest as potential fillers in nanocomposites. Gradually, the packaging industry is undergoing a revolution, replacing traditional petroleum-based plastics with biodegradable polymer materials. However, the poor mechanical, thermal and barrier properties are not yet at the level of the materials currently used, which has delayed and limited the introduction of biopolymers in the market. One simple and low-cost solution that can be an efficient way to enhance these properties is blending the polymer matrix with nanofillers [3].

In the food industry, CNFs have been used in nanocomposites as a reinforcing material, for coating technologies and food packaging. CNCs are used as a natural emulsifying and stabilizing ingredient in meals including salad dressing, ice cream, bread, and milk products to replace high heat energy materials. BNC has a wide range of uses in the culinary industry: texture modifiers, frostings, desserts, thickeners, low-calorie addition, stabilizer, frozen dairy products, among others [67]. Additionally, CNMs as a dietary fiber, can be used in functional foods to reduce the risk of chronic diseases such as diabetes, obesity, cardiovascular disease, and diverticulitis. It also fosters beneficial physiological effects, such as laxation and attenuation of blood cholesterol and glucose, and has been explored to produce reduced-fat foods to treat weight disorders [67].

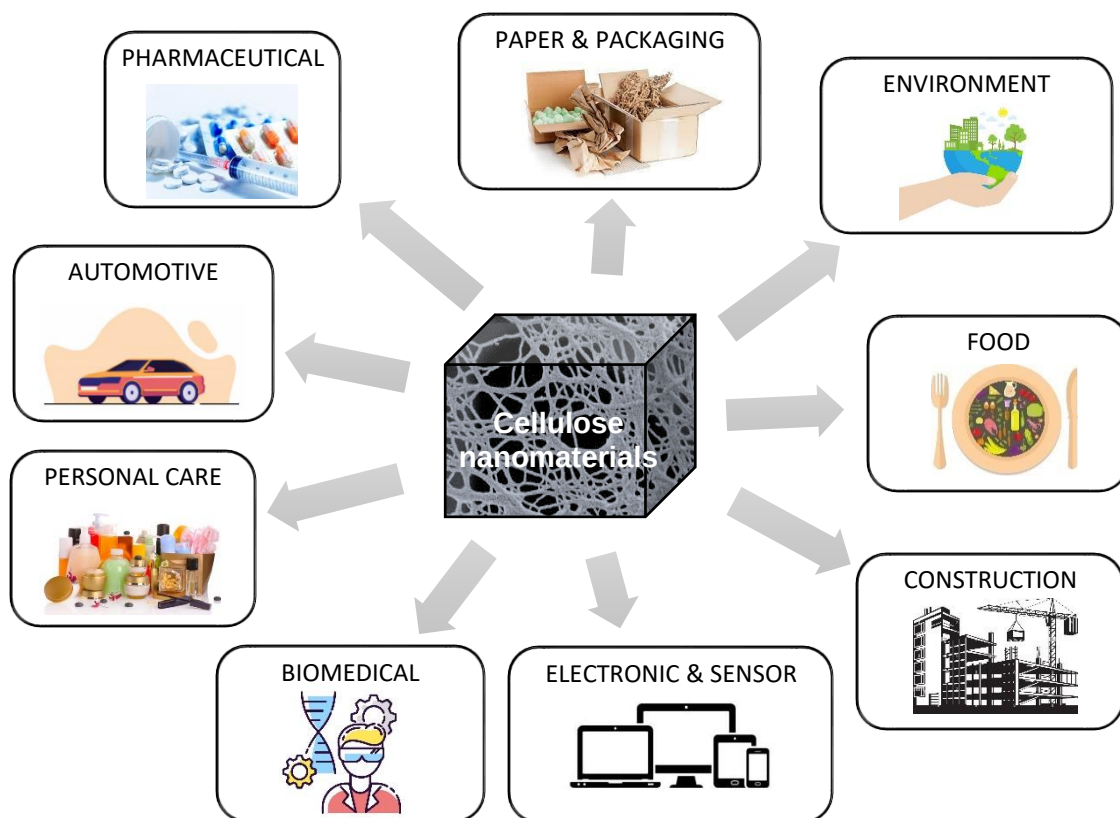


Figure 1.4. Potential applications of cellulose nanomaterials in different types of fields.

In the biomedical field, CNC and CNF hydrogels, gel, membranes, and films have been used for many biomedical applications, such as drug carriers, wound dressing, drug delivery, and tissue

engineering, due to their biocompatibility, biodegradability, mechanical properties, and the advantages of a nanostructure. Moreover, CNMs have antimicrobial activity, wound healing properties, and a non-toxic nature which provides a suitable environment for the wound healing process for burnings and wounds [67]. Besides, much attention has been paid to the 3D printing technique for fabricating implants and scaffolds for tissue engineering applications.

In electronic devices applications, CNMs are not directly used to fabricate electrical devices, such as batteries and supercapacitors, since they are not good electrically conductors. Instead, they are mixed with conductive materials (e.g. conductive polymers, metallic particles, conductive carbon materials) given that conductivity is essential for some core components of an energy device. These composite materials will take advantage of both materials, and therefore, the combination of CNM properties and the new flexible and sustainable electronics will be able to meet the most demanding needs for energy applications. Bionanocomposites made in their majority of CNFs, also called nanopaper, demonstrated great potential as a substrate in printed electronics due to the flexibility, thermal stability, and biodegradability, pointing out the transparent nanocellulose substrates as a good alternative to the traditional plastic and glass substrates. CNMs have been used as a flexible and transparent substrate for the development of flexible electronic devices (e.g. solar cells, sensors, and flexible displays).

CNMs have also been applied in environmental remediation as a new generation of nanostructured adsorbents for different classes of pollutants due to their large surface area, vast surface hydroxyl groups, and their easy functionalization [1, 81]. Generally, CNM-based adsorbents (e.g. aerogels, filtration membranes, porous beads, and flocculants) need to be subject to a surface functionalization process to be able to enhance their adsorption capacity and adsorb a specific class of pollutant. NC-based adsorbents, such as, have been used for removal of air contaminants, heavy metal ions (e.g. Cu^{2+} , Pb^{2+} , Hg^{2+}), organic pollutants (e.g. pesticides), industrial chemicals, dyes, and oils in water purification systems and wastewater treatment [1].

1.8. Integrating the production of nanocellulose with other value-added products

Even though CNMs are considered a high-value product with outstanding properties, their production can be associated with other compounds through an integrated coproduction process to increase the viability of the process. Currently, the worldwide production of CNMs is attributed to the pulp and paper industry as a coproduct to improve its economic efficiency. Moreover, this integration strategy can also be relevant to the emerging concept of modern biorefinery, which aims to achieve an integral valorization of the cellulosic source. Nevertheless, literature reports on efficient integration of CNMs with other value-added products is still scarce.

Hydrolytic enzymes employed during enzyme-mediated pretreatment of cellulosic sources for CNMs extraction, such as cellulases and xylanases, are known to decompose both cellulose and hemicellulose in cell walls to its constituent sugars, apart from causing fiber disintegration. In this sense, CNMs would be produced as the main product, while generating a secondary stream with the released sugars. These released sugars, mostly fermentable sugars, are separated from the main process and could be converted into different compounds by fermentation. However, a careful selection of the enzyme mixture used for CNMs extraction results relevant for an optimum scenario, since carbohydrate conversion into cello-oligomers and xylo-oligomers is not desirable.

In a different approach, the solid residues obtained from the enzymatic processing of lignocellulosic biomass to produce other value-added products (e.g. biofuels, organic acids), which is usually enriched in recalcitrant cellulose (mainly crystalline regions), could be used to produce CNCs [1]. This approach is also aligned with the biorefinery concept, which involves the coproduction of more than one product, each of which have different values and market sizes.

Typical modern biorefinery coproducts include, along with bioethanol and biobutanol as biofuels, chemicals such as levulinic acid, succinic acid, hydroxymethylfurfural, furfural, lactic acid, xylitol, and sorbitol. Due to their potential use, these chemical products are the focus of extensive research since the number of publications dealing with the subject has steadily increased over the last decades (Fig. 1.5). Based on the number of publications available in the literature in the period 2000-2018, the most heavily studied bioproducts in decreasing order are bioethanol, biobutanol, and furfural. The other products have been studied less extensively but with commercial promise, potentially providing new avenues for future work [82].

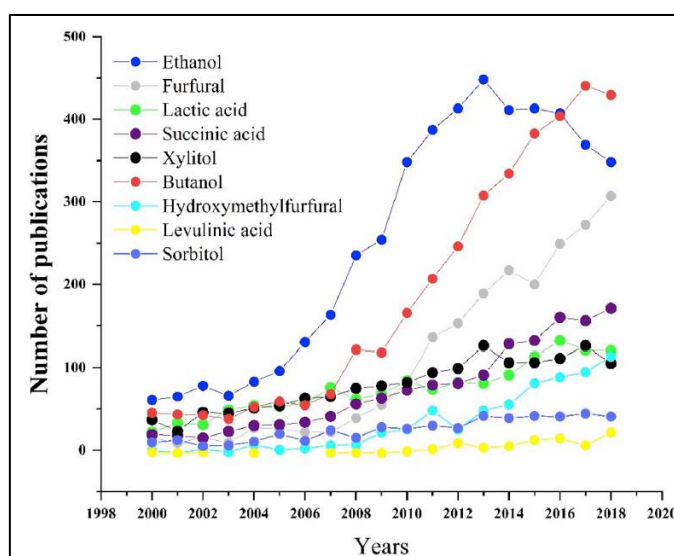


Figure 1.5. Literature related to the production of biofuels and chemicals in the period 2000-2018 [82].

1.8.1. Biobutanol

Biofuels, particularly bioethanol and biobutanol, are alternative fuels made from renewable sources such as plant resources. Biofuels are commonly advocated as a cost-effective and environmentally friendly alternative to petroleum and other fossil fuels, particularly within the context of rising petroleum prices and increased concern of global warming issues related to fossil fuels [24]. Besides, the production of biofuels from different carbon sources is emerging as a promising sustainable energy resource and may offer long-term environmental and economic benefits in contrast to fossil fuels, provided that the production of biofuels comes from renewable sources.

Butanol is a very promising and competitive alternative to fossil fuels because it has several advantages among other clean fuels, particularly bioethanol [83]. Its low corrosion index, high energy content, low viscosity, and low freezing point all make it economically attractive [84]. Also, it can be blended with gasoline at a higher proportion compared to ethanol. Butanol can be applied in the production of antibiotics, vitamins and cosmetics [82], as well as used as a solvent, precursor for paints, and polymers formulation [85].

Industrially, butanol is mainly produced by petrochemical synthesis through the Oxo process. However, biobutanol, as well as bioethanol, can be produced by biological synthesis from first generation (sugarcane, beet, and starchy plants) and second generation (lignocellulosic biomass, residues, and agricultural by-products) sources. The combined use of enzymes and microorganisms make possible the biological synthesis of biobutanol from different lignocellulosic biomass (e.g. wheat straw, wood, corn fiber, barley straw, bagasse sorghum, etc.). Enzymatic reaction releases the sugars present in the lignocellulosic biomass matrix, which are subsequently converted to biobutanol by fermentation.

The microorganisms responsible for producing biobutanol are the anaerobic *Clostridium* type strains, which have proven to be efficient [29, 86, 87]. These butanol-producing strains can metabolize released sugars such as xylose together with glucose which can be obtained from lignocellulosic biomass after pretreatment and enzymatic hydrolysis. Among the most commonly used microorganisms for biobutanol synthesis are *C. acetobutylicum* and *C. beijerinckii* through the so-called ABE (acetone-butanol-ethanol) and IBE (isopropanol-butanol-ethanol) fermentations [85, 88]. The catabolism involves a first acetogenic step in which acetic and butyric acids are produced along with carbon dioxide (CO₂) and hydrogen (H₂), followed by a solventogenic step in which butanol is produced along with other solvents (e.g. acetone, isopropanol, ethanol) from the acids.

Biobutanol fermentation is much less efficient compared to bioethanol fermentation. Since biobutanol selectivity can be reduced by the formation of undesirable coproducts such as acetone and ethanol, great amounts of energy are necessary for product recovery. This, together with the cost of substrates, make the biobutanol microbial synthesis non-sustainable. For this reason, research efforts have been focused on decreasing the cost of the raw material and improving biobutanol selectivity by further reducing the generation of coproducts. For instance, Chua et al. [89] aimed to improve the conversion of sugars into biobutanol and acetone with negligible amounts of ethanol as coproduct. Consequently, the separation of these two components became simpler and minimized the energy consumption in the separation and purification processes. In other words, the high yield and productivity of biobutanol, while minimizing the generation of coproducts and solid residues is relevant to the economy of the process.

Chapter 2. Objectives

The primary objective of this work is to evaluate the integrated production of cellulose nanofibers (CNFs) and biobutanol from the cellulosic fraction of eucalyptus wood, which represents a promising lignocellulosic material, with special emphasis on enzymatic pretreatment of the raw material and fermentation. To accomplish this primary objective, the following specific objectives are defined below:

- To evaluate the physicochemical properties of eucalyptus pulp provided by a local pulp mill and determine its applicability for CNFs and biobutanol coproduction.
- To assess the enzyme-mediated pretreatment of eucalyptus pulp employing catalytic and non-catalytic proteins that improve cellulose accessibility and fiber susceptibility for further processing.
- To investigate fiber changes occurring during enzyme-mediated pretreatment of eucalyptus pulp by physicochemical, morphological, and thermal analysis.
- To investigate the isolation of CNFs from enzyme-treated eucalyptus pulp by ultrasonication and study the effect of its process conditions on CNF extraction yield and properties.
- To assess ball milling treatment as an alternative method to extract CNFs from enzyme-treated eucalyptus pulp and study the effect of its process conditions on CNF extraction yield and properties.
- To propose an integrated process that enables the coproduction of biobutanol by microbial fermentation and CNFs by mechanical treatment from eucalyptus pulp, attending to maximize the use of the raw material.

Chapter 3. Materials and methods

3.1. Raw materials, enzymes, and microorganisms

3.1.1. Bleached Eucalyptus Kraft Pulp (BEKP)

Bleached eucalyptus Kraft pulp (BEKP) was used as raw material, which was provided by a commercial local pulp mill (UPM Fray Bentos, Uruguay). The BEKP was industrially obtained by treating eucalyptus wood chips through the Kraft process, followed by a bleaching process performed in a series of stages involving bleaching agents (ClO_2 , H_2O_2 , O_2) and alkaline extractions. The BEKP was provided as dry sheets (66.5 cm x 44.5 cm x 0.2 cm; $\sim 370 \text{ g}_{\text{BEKP}}/\text{sheet}$ on wet basis; $\sim 9\%$ moisture content), which were rewetted and resuspended for conditioning before use (Fig. 3.1). Briefly, the sheets were torn into small pieces and soaked in distilled water for around 5 min before stirring by a lab-scale disintegrator for 15 min at a solid consistency of 2% (w/w). The resulting BEKP slurry was then vacuum-filtered through a Büchner funnel to a moisture content of $\sim 75\%$ and the wet BEKP stored at 4°C until its use. Glucan, xylan, and lignin contents of BEKP were $77.3 \pm 0.6\%$, $17.6 \pm 0.1\%$, and $0.7 \pm 0.1\%$, respectively.

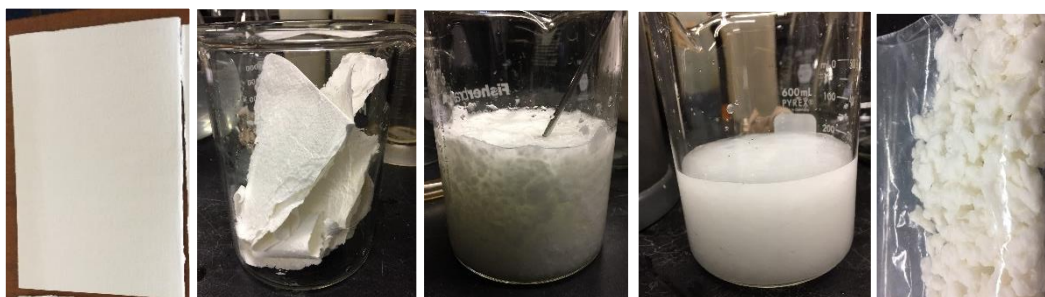


Figure 3.1. Raw material conditioning: (a) dry sheets of BEKP, (b) dry sheets torn into small pieces before rewetting, (c) soaking of dry sheets in distilled water, (d) BEKP slurry after rewetting and resuspension, and (e) wet BEKP.

3.1.2. Enzymes and non-catalytic protein

The cellulase complex Cellic[®] CTec3 (Novozymes, Davis California) with a filter paper activity of $231 \pm 5 \text{ FPU/mL}$ and a total protein content of $279 \pm 1 \text{ mg/mL}$ was used in this study, which was produced from a genetically modified strain of *Trichoderma reesei*. The xylanase specific activity of the cellulase complex resulted of $19 \pm 5 \text{ U/mg}_{\text{protein}}$. The endoglucanase monocomponent used was the endo-1,4- β -D-glucanase (Megazyme Ltd.) with a specific activity of $1,200 \text{ U/mL}$, which was purified from *Aspergillus niger*. The xylanase complex Cellic[®] HTec (Novozymes, Davis California) with a protein content of $53 \pm 10 \text{ mg/mL}$ and specific xylanase

activity of 211 ± 31 U/mg_{protein} was used in this study. Swollenin, expressed in *Trichoderma reesei*, was used as non-catalytic protein, which was provided by VTT Technical Research Center of Finland and produced as described by Gourlay et al. [90].

3.1.3. *Clostridium* sp. strains

The butanol producers *Clostridium* spp. strains used in this work (*Clostridium beijerinckii* DSM 6422 and *Clostridium beijerinckii* DSM 6423) were obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The lyophilized strains were activated following the supplier's instructions and maintained in stock cultures prepared in reinforced clostridial medium (RCM), containing peptone 10 g/L, beef extract 10 g/L, glucose 5 g/L, sodium chloride 5 g/L, yeast extract 3 g/L, sodium acetate 3 g/L, soluble starch 0.5 g/L and L-cysteine-HCl 0.5 g/L, at 4°C under anaerobic conditions.

3.2. Enzyme activity and protein content assays

The cellulase activity of Cellic® CTec3 was measured following the filter paper activity protocol of NREL [91]. The endoglucanase activity of endo-1,4-β-D-glucanase and xylanase activity of Cellic® HTec was measured using 0.7 % (by mass) solutions of carboxymethyl cellulose (90 kDa average molecular weight, 0.7 carboxymethyl groups per anhydroglucose unit; Sigma) and birchwood xylan (Sigma), respectively, in 100 μL reactions with 50 mM sodium acetate buffer pH 5 on microplates at 50°C. Appropriately diluted enzyme samples were incubated with substrate for 30 min and stopped by addition of a dinitrosalicylic acid (DNS) reagent solution, prepared as previously described [91]. The samples were incubated at 105°C for 30 minutes to allow reaction of DNS with liberated saccharides, allowed to cool to room temperature, and then the color produced was measured at 540 nm on a microplate spectrophotometer. A standard curve was prepared with serial dilutions of glucose and xylose treated in the same manner with DNS reagent, from which the activity of the enzymes was extrapolated using the measured absorbances.

The protein content of catalytic and non-catalytic proteins was determined according to the ninhydrin assay using bovine serum albumin (BSA) as protein standard [92]. Briefly, 40 μL of diluted samples were mixed with 1 N HCl in 1.5-mL screw cap centrifuge tubes. The tubes were incubated overnight at 105°C. After incubation, the tubes were cooled down for 30 min and briefly centrifuged, before 2% ninhydrin reagent (Sigma) was added. The mixture was then heated at 100°C for 20 min with subsequent cooling at room temperature for 10 min. Finally, 50% (v/v) of ethanol was added into the tubes and the absorbance was measured at 570 nm using Cary 60 UV–Vis spectrophotometer.

3.3. Enzymatic hydrolysis of BEKP

The enzymatic hydrolysis of BEKP was performed in duplicate at 50°C and 200 rpm in acetate buffer (50 mM, pH 4.8) using enzymes and/or non-catalytic protein preparations accordingly (Fig. 3.2). Before enzyme addition, the slurries were equilibrated at 50°C and 200 rpm for 1 h in an orbital shaker for pulp disintegration. Enzymatic hydrolysis assays were carried out at a solid concentration range of 2% to 16% (w/w) and an enzyme loading range of 1 mg_{protein}/g_{dry pulp} to 7 mg_{protein}/g_{dry pulp}. After enzymatic hydrolysis, the enzyme was deactivated by heating the slurries at 100°C for 10 min. The liquid (enzymatic hydrolysate) and solid (cellulose fibers) fractions were then separated by centrifugation (4,200 *g* for 15 min). The enzymatic hydrolysates were stored at -20°C for further analysis. The cellulose fibers were resuspended in distilled water and washed three times by centrifugation (4,200 *g* for 15 min) before storage at 4°C for further processing. Solid yield (SY, wt%) was calculated based on the amount of oven-dried mass of BEKP and cellulose fibers recovered after enzymatic pretreatment. Cellulose and xylan hydrolysis efficiency were calculated based on the chemical composition of enzymatic hydrolysates according to Eq. 3.1 and Eq. 3.2, respectively, as follows:

$$\text{Cellulose hydrolysis (\%)} = \frac{[\text{glucose}]}{\frac{[\text{cellulose}]}{100} \cdot f_c \cdot \frac{[\text{solid}]}{100} \cdot \rho} \cdot 100 \quad (3.1)$$

$$\text{Xylan hydrolysis (\%)} = \frac{[\text{xylose}]}{\frac{[\text{xylan}]}{100} \cdot f_x \cdot \frac{[\text{solid}]}{100} \cdot \rho} \cdot 100 \quad (3.2)$$

where $[\text{glucose}]$ and $[\text{xylose}]$ denotes the glucose and xylose concentrations (g/L) in the liquid fractions both in monomeric and oligomeric forms (expressed as equivalent monomers). $[\text{Cellulose}]$ and $[\text{xylan}]$ denotes the cellulose and xylan contents in BEKP (expressed as g of cellulose or xylan per 100 g of dry BEKP). ρ denotes the density of the liquid fraction (1000 g/L on average). f_c and f_x denotes the stoichiometric factors of glucan to glucose conversion (180/162) and xylan to xylose conversion (150/132), respectively. $[\text{Solid}]$ denotes the solid concentration at the beginning of the enzymatic hydrolysis (expressed as g of dry BEKP per 100 g of liquid).



Figure 3.2. Enzymatic hydrolysis assays of BEKP performed at 50°C in orbital shaker.

3.4. Biobutanol fermentation

3.4.1. Inoculum development

For fermentation inoculum, a pre-culture was prepared by inoculating 2 mL of the stock culture into a serum bottle containing 40 mL of the RCM medium, which was then incubated in orbital shaker until active growth was observed by increased turbidity and vigorous gassing (20–24 h). Fermentation inoculum was prepared in 250-mL bottles with 100 mL of inoculum medium containing 30 g/L glucose, 1 g/L yeast extract and 1% (v/v) of filter sterilized P2 stock solutions. The P2 stock solutions corresponded to buffer (K_2HPO_4 50 g/L, KH_2PO_4 50 g/L, ammonium acetate 220 g/L), vitamin (p-amino benzoic acid 0.1 g/L, thiamine 0.1 g/L, biotin 0.001 g/L), and mineral ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 20 g/L, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 1 g/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 1 g/L, NaCl 1 g/L) solutions. The pH of the inoculum medium was adjusted to 6.0 ± 0.1 , and then swept with O_2 -free N_2 over the headspace of the bottles, followed by sterilization at 121°C for 15 min. Filter-sterilized P2 stock solutions were then added at 1% (v/v) after bottles were cooled down to room temperature, followed by inoculation with 10% (v/v) highly-active cells grown in the pre-culture. Fermentation inoculum was incubated at the cultivation temperature recommended by the supplier for each microorganism (35°C for both *C. beijerinckii* DSM 6422 and DSM 6423) and 150 rpm until a late exponential phase of growth (16–20 h).

3.4.2. Fermentations in semi-synthetic and enzymatic hydrolysate medium

Biobutanol fermentations were performed in duplicate in 100-mL bottles containing 40 mL of enzymatic hydrolysate supplemented with yeast extract (1 g/L) under anaerobic conditions. The pH was adjusted to 6.0 ± 0.1 and the medium was swept with O_2 -free N_2 over the headspace of the bottles, followed by sterilization at 121°C for 15 min. Filter-sterilized P2 stock solutions were then added at 1% (v/v) after bottles were cooled down to room temperature. The bottles were inoculated with 10% (v/v) highly-active cells from the fermentation inoculum. Control fermentations were performed using semi-synthetic media with a mixture of glucose and xylose at the same concentrations to those found in the enzymatic hydrolysates (S1: 37 g/L glucose + 13 g/L xylose; S2: 26 g/L glucose + 10 g/L xylose; S3: 15 g/L glucose + 5 g/L xylose). All fermentation experiments were conducted in duplicate at 150 rpm and 35°C with orbital agitation for 72 h (Fig. 3.3). Samples were taken at regular intervals for sugars, products, and optical density (OD) analysis. Dry cell weight (X) was determined by OD at 600 nm considering that one unit of $\text{OD}_{600 \text{ nm}}$ corresponded to 0.33 ± 0.02 g/L of X and 0.38 ± 0.03 g/L of X for *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423, respectively.



Figure 3.3. Biobutanol fermentation assays of BEKP performed in (a) orbital shaker using (b) enzymatic hydrolysate and (c) semi-synthetic media.

3.5. Mechanical treatments for CNF extraction

3.5.1. Ultrasonication

The enzyme-treated BEKP samples were redispersed in deionized water to reach solid consistencies of 0.07% to 0.5% (w/w). The resulting suspensions were subjected to ultrasonication (50–90% amplitude) with a Q700 sonicator (QSonica, Newtown, CT, USA) operating at 20–25 kHz frequency and equipped with a 0.5-inch flat tipped titanium probe (Fig. 3.4). The suspensions were cooled in an ice bath during ultrasonication to avoid overheating, and temperature was kept below 30°C. The ultrasonication was performed with 5 s pulse-on time followed by 5 s of pulse-off time for 60 and 120 min when corresponded. The resulting preparations were centrifuged (4,200 *g* for 30 min) in order to separate the micro/nanofibrils.

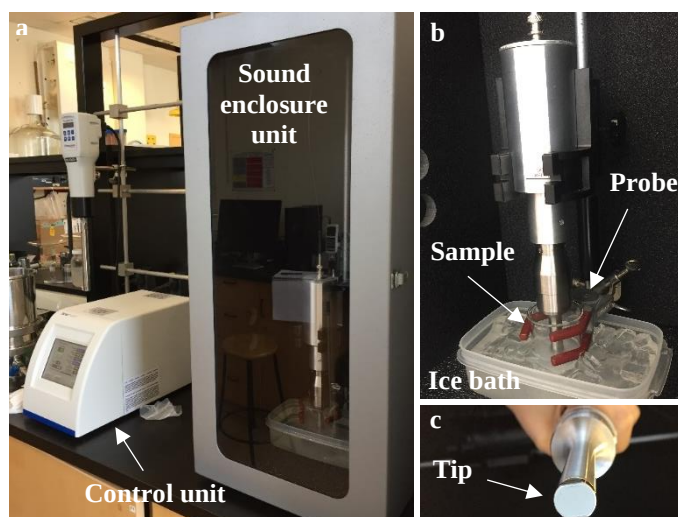


Figure 3.4. Ultrasonication equipment: (a) handheld ultrasonic system with its control unit mounted inside a sound enclosure unit, (b) ultrasonication probe and ice bath for sample cooling, and (c) titanium flat tip.

3.5.2. Ball milling

Ball milling was performed at room temperature in a laboratory ball mill (MM 400, Retsch) for cellulose nanofibers (CNF) extraction (Fig. 3.5). For this, 10 mL of 1% (w/w) enzyme-treated BEKP suspension was loaded onto a 25-mL zirconia grinding jar containing zirconia balls of 0.5 mm and 3 mm in diameter with a ball to solid ratio (BSR) ranging from 40 g_{balls}/g_{dry solid} to 200 g_{balls}/g_{dry solid}. The weight ratio of the balls with 0.5 mm and 3 mm diameters was 7:3. The ball milling was performed at a frequency of 10 Hz and 20 Hz at room temperature for milling times from 15 min to 180 min. After the milling process, balls separation was performed by mesh filtration. Since the slurry was gel-like and sticky, distilled water was added to the slurry until a consistency of 0.2% (w/w) to facilitate balls separation.

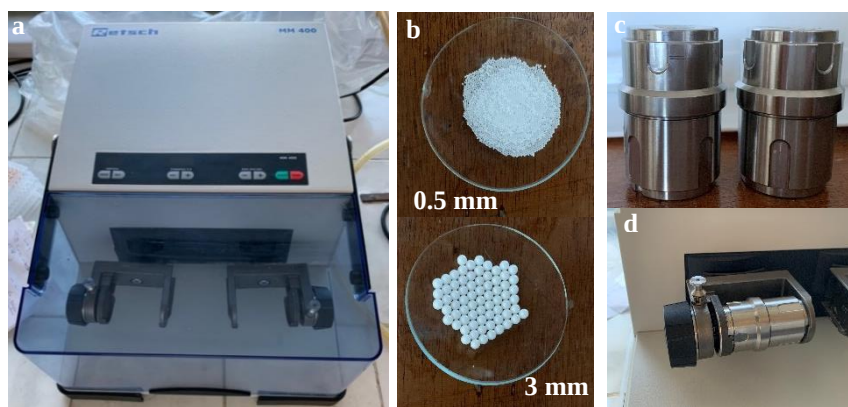


Figure 3.5. Ball milling equipment: (a) laboratory ball mill, (b) zirconia balls, (c) 25-mL zirconia grinding jars, and (d) grinding jar mounted on equipment.

3.6. Determination of CNF yield

Suspensions obtained after mechanical treatment were diluted to 0.1% w/w consistency, and then centrifuged at 5,000 *g* for 30 min to separate the nanofibrillated material (which remains in the supernatant fraction) from the unfibrillated or partially fibrillated one (which settles down). For ball milled suspensions, homogenization using an Ultra-turrax system (16,000 rpm for 5 min) was performed after dilution to improve the dispersion of the suspension. Both supernatant fraction and the material that settled down were dried to a constant weight at 105°C. The CNF yield was calculated using either Eq. 3.3 or Eq. 3.4 as follows:

$$CNF \text{ yield (\%)} = \frac{M_{CNF}}{M_F} \cdot 100 \quad (3.3)$$

where M_{CNF} denotes the mass of the dried CNF in the supernatant, and M_F denotes the mass (oven-dry basis) of the pulp before mechanical treatment.

$$CNF\ yield\ (\%) = 100 - \frac{M_{UNF}}{M_F} \cdot 100 \quad (3.4)$$

where M_{UNF} denotes the mass of the dried unfibrillated or partially fibrillated material that settled down.

3.7. Analytical methods

A summary of the analytical methods employed for the characterization of the different types of materials used in this study is presented in Appendix 1. The analytical methods are thoroughly described below.

3.7.1. Chemical characterization of cellulose fibers, enzymatic hydrolysates and fermentation samples

The chemical composition of untreated and enzyme-treated BEKP was analyzed according to NREL/TP-510-42618 protocol for biomass [93]. The chemical analysis of enzymatic hydrolysates was performed according to NREL protocol (NREL/TP-510-42623) for liquid fractions [94]. Acid soluble lignin was determined by measuring absorbance at 205 nm using Cary 60 UV-Vis spectrophotometer. Monomeric sugars were quantified by high performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) using a refractive index detector (RID) and an Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) operated at 45°C. The mobile phase used was 5 mM H₂SO₄ at a flow rate of 0.6 mL/min. A post-hydrolysis step in dilute acid (4% w/w H₂SO₄) was performed at 121°C for 1 h to determine the total oligomeric sugars, following NREL/TP-510-42623 protocol [94]. Oligomeric sugars content was determined based on the difference between the amount of sugars obtained after the post-hydrolysis stage and that present in the liquid fractions.

The concentrations of butanol, acetone, isopropanol, ethanol, acetic acid, and butyric acid in fermentation samples were determined by gas chromatography (GC, Shimadzu GC-2010) equipped with a flame ionization detector and a fused silica column (RTX®-Wax, 30 m long, 0.5 µm film thickness and 0.32 mm ID, Restek).

3.7.2. Water retention value

The water retention value (WRV) was measured in duplicate using the TAPPI Useful Method-256. Briefly, 0.5 g (oven-dry basis) of the pulp was soaked in 50 mL of water overnight prior to filtration through a 200-mesh screen. The resulting pulp pad was centrifuged at 900 g for 30 min,

and finally oven-dried at 105°C overnight. WRV represents the percentage of retained water of the dried substrate, according to Eq. 3.5 as follows:

$$WRV = \frac{W_w - W_d}{W_d} \quad (3.5)$$

where W_w and W_d denote the weight of the wet sample after centrifugation and the dried pulp, respectively.

3.7.3. Cellulose accessibility

The cellulose accessibility was assessed using Direct Blue (DB) as dye following a modified version of the Simons' staining procedure [95]. Briefly, 10 mg (oven-dry basis) of substrates were mixed with 1 M phosphate buffered saline solution (PBS, pH 6) and increasing DB dye concentrations in 1.5-mL screw cap centrifuge tubes. The tubes were kept overnight at 70°C in an orbital shaker at 180 rpm. After incubation, the tubes were centrifuged at 4,200 g for 10 min and the absorbance of the supernatant was read at 455 nm on a Cary 60 UV–Vis spectrophotometer. The amount of DB adsorbed on the fiber was calculated by the difference between the concentration of DB added initially and the concentration of DB in the supernatant, according to Beer-Lambert Law. The extinction coefficient was calculated by preparing a standard curve of DB dye and determining the slope of their absorbance at 455 nm.

3.7.4. Degree of polymerization

The degree of polymerization (DP) of BEKP and enzyme-treated BEKP was determined by viscosity (25°C) of fiber solution in 0.5 M cupriethylenediamine (CED) solution using Ubbelohde viscometer according to TAPPI standard method T230. The DP was determined using Eq. 3.6 as follows [96, 97]:

$$DP^{0.905} = 0.75 [\eta] \quad (3.6)$$

where η denotes the intrinsic viscosity expressed in cm^3/g and calculated according to ASTM D1795-13.

3.7.5. Crystallinity index

BEKP and enzyme-treated BEKP were analyzed using X-ray diffractometer (MiniFlex600, Rigaku). The samples were dried and ground into powders for X-ray diffraction (XRD) test. The diffracted intensity of $\text{CuK}\alpha$ radiation (40 kV and 15 mA) was measured in a 2θ range of 5° to 60° with increments of 0.05° , at a scanning rate of $4^\circ/\text{min}$. The relative crystallinity index (CrI)

was calculated using the Segal et al. [98] method, as the difference between the maximum intensity of the peak located at $2\theta = 22\text{--}23^\circ$, and the minimum intensity located between the major peaks at $2\theta = 18^\circ\text{--}19^\circ$ divided by the intensity of the highest peak.

3.7.6. Thermogravimetric analysis

The thermal properties of BEKP, enzyme-treated BEKP and CNF samples were studied using thermogravimetric analysis (TGA) and derivative thermogravimetric analysis (DTG) with a thermogravimetric analyzer (TGA 4000, PerkinElmer, USA). The samples were analyzed under a nitrogen atmosphere with a gas flow of 20 mL/min by heating the material from 35°C to 600°C at a heating rate of 10°C/min. The thermal decomposition onset temperature (T_{on}), which represents the temperature of the beginning of the degradation, and the maximal weight loss temperature (T_{max}), which represents the temperature of the maximum degradation rate, were estimated from TGA and DTG curves according to literature [99].

3.7.7. Particle size distribution

Number-based fiber dimensions of BEKP and enzyme-treated BEKP were determined optically using a Hi-Resolution fiber quality analyzer (FQA, LDA02-series, OpTest Equipment Inc, Hawkesbury, Canada) that uses a sheath flow cell to hydrodynamically orient fibers into a thin plane before passing through a charge-coupled device image sensor where fibers are measured by circular polarized light. About 10,000 fibers were collected and measured at 20-40 events/s to calculate the fiber length distribution and the length-weighted average (L_w) in the range of 0.07 to 10.0 mm. Fines content, defined as fiber lengths in the range of 0.07 to 0.20 mm, was measured. Volume-based fiber dimensions of enzyme-treated BEKP was also evaluated using a laser diffraction (LD) particle size analyzer (Mastersizer Hydro 2000G/S, Malvern Instruments Limited, Worcestershire, UK). The sample suspensions were sonicated for 10 s while stirred in the dispersion unit prior to LD analysis. A refractive index of 1.63 was used for the measurements.

3.7.8. Dynamic light scattering and zeta potential

Dynamic light scattering (DLS) and zeta potential (ZP) analysis of CNF suspensions were performed using a Malvern Zetasizer Nano-ZS (Malvern Instruments Inc.). The CNF suspensions were previously diluted to 0.01% (w/w) and then sonicated for 5 min prior to analysis. A refractive index of 1.63 was used for the measurements.

3.7.9. Transmittance of CNF suspensions

The light transmittance of CNF suspensions (0.1% w/w) from BEKP and enzyme-treated BEKP was measured in the range of 300 to 800 nm using Genesys 10S UV-Vis spectrophotometer.

3.7.10. Aspect ratio

The relative aspect ratios of the ball milled CNF samples were calculated from gel point concentrations, as determined by sedimentation experiments. Briefly, a series of diluted CNF suspensions of 0.15, 0.1, 0.05, 0.025 and 0.01% (1.5, 1.0, 0.5, 0.25 and 0.1 kg/m³) were prepared and then allowed to settle for 48 h. Photographs were taken before and after sedimentation to determine the sedimentation ratio (Hs/Ho) as the ratio of sediment height (Hs) to initial suspension height (Ho) using ImageJ software. An estimated gel point concentration was calculated by plotting fiber concentration against sedimentation ratio and determining the derivative of the trendline equation, following the methodology used by other authors [100, 101]. The aspect ratio was calculated based on the Crowding Number theory described by Martinez et al. [102] using Eq. 3.7, which describes the gel point and aspect ratio of cellulose pulp fibers assuming a density of fibers around 1,500 kg/m³ [100].

$$\text{Aspect ratio} = 6.0 \times \frac{gp^{-0.5}}{1000} \quad (3.7)$$

where *gp* is the gel point concentration given in kg/m³.

3.7.11. Conductometric titration

Conductometric titration was used to determine the acidic groups content of BEKP, enzyme-treated BEKP and CNF samples following the method described by Katz and Beatson [103]. Briefly, 50 mL of 0.001 M NaCl solution was added to 0.25 g (oven-dry basis) of sample and the pH of the suspension was adjusted to 3.0 ± 0.1 with 0.1 M HCl. The suspension was then titrated with 0.05 M NaOH, and the conductivity values of the suspension during titration were recorded. The acidic groups content was then determined from the titration curve (conductivity vs NaOH amount) from the region between the intersection of the decreasing and increasing phases with the steady phase (see Appendix 9).

3.7.12. Optical microscopy

Optical microscopy (OM) of BEKP and enzyme-treated BEKP was performed using an OLYMPUS CX21 microscope. Digital pictures were taken with a Dino-Eye eyepiece microscopy

camera using 4x and 10x objectives. Fiber suspensions of 0.5% (w/w) were firstly stained with Direct Blue dye and then loaded onto glass slides with a glass cover slip for observation. ImageJ software was used for image analysis. Width and length distributions of samples were studied by measuring over 100 individual fibers.

3.7.13. Scanning electron microscopy and energy dispersive X-ray spectroscopy

The surface morphology of BEKP, enzyme-treated BEKP and ball milled CNF samples were investigated by scanning electron microscopy (SEM). SEM micrographs were captured using a Joel jsm-5900LV SEM operated at an acceleration voltage of 20 kV. For observations, samples were coated with gold on a Denton Vacuum Desk II coater at 40 mA for 120 s. Energy dispersive X-ray spectroscopy (EDS) was performed for elemental concentrations, which was collected from points, areas or as maps in SEM of ball milled CNF samples using Thermo System 7.

3.7.14. Transmission electron microscopy

The morphology of CNF samples was assessed by transmission electron microscopy (TEM). TEM images were captured using a Jeol JEM 1010 TEM operated at 100 kV, and the samples were prepared as follows: 10 μ L of 0.01% (w/w) CNF suspension was deposited onto carbon-coated copper grid with formvar/carbon film, excess liquid was removed with filter paper, and after ambient drying 10 μ L of 2% uranyl acetate solution was deposited on the TEM grid to negatively stain the sample. The widths of CNF from TEM images were measured using ImageJ software from over 100 individual CNF.

3.7.15. Atomic force microscopy

The morphology of CNF samples was studied by atomic force microscopy (AFM) using Multimode AFM Nanoscope-III from Veeco Instruments (Santa Barbara, CA, USA) with the PeakForce tapping mode. A freshly cleaved mica was chemically modified with a cationic compound (3-aminopropyl)triethoxysilane (APTES) according to previous studies [104]. Briefly, a drop of 0.1% (w/v) of APTES solution was placed on a cleaved mica surface for 30 s and then thoroughly rinsed with nanopure water. The samples were prepared by depositing a drop of diluted 0.01% (w/w) CNF suspension on the modified mica surface and leaving it to dry for at least 1 h. AFM images were taken at a scan rate of 0.7 Hz using a RTESPA-150 cantilever probes with a nominal spring constant of 7 N/m. AFM images were analyzed using NanoScope Software 8.10 (Veeco, Santa Barbara, CA, USA).

3.7.16. Contamination analysis of CNF samples

Eventual contamination of CNF samples during ultrasonication was assessed by X-ray fluorescence spectrometry (XRF) using Niton XL2 GOLDD XRF analyzer (Fisher Scientific). This method determines the elemental composition of the CNF samples and allows to identify the nature of the eventual contamination. Eventual contamination of CNF samples during ball milling treatment was assessed by XRF and the loss on ignition (LOI) method. The LOI method was used to estimate the inorganic content of the CNF samples based on gravimetric weight change associated with high temperature oxidation of organic matter in the sample. Briefly, samples were burned at 1000°C during 1 h using an Atec furnace model HFA10 (Montevideo, Uruguay) and inorganic ashes were determined.

3.8. Statistical analysis

Data was presented as the mean $\pm$ standard deviation of replicates. Analysis of variance (ANOVA) was performed to determine statistical differences using InfoStat software (student version 2013, Universidad Nacional de Córdoba, Argentina, <http://www.infostat.com.ar>). Differences between means were studied according to Tukey test and were considered significant when $p \leq 0.05$.

Chapter 4. Results and discussion

4.1. The use of cellulase, xylanase and non-catalytic protein for enzyme-mediated pretreatment of BEKP

4.1.1. Background

Most of the reported work on CNM production from cellulosic materials through enzymatic pretreatment were focused mainly on the use of cellulase enzymes, that catalyze the hydrolysis of cellulose fibers and makes fibers processing much easier. Endoglucanases are the type of enzymes with the highest interest because they break the cellulose polymer into smaller length polymers by acting on the amorphous, or less organized, part of the cellulose [1]. However, it has been shown a high degree of synergism between exoglucanases and endoglucanases, which could enhance cellulose hydrolysis and further CNM extraction [105]. It was also demonstrated that exoglucanases can hydrolyze microcrystalline cellulose by peeling the cellulose chains from the microcrystalline structure [106]. Recently, it was found that the combination of cellulolytic enzymes (cellulases) with accessory enzymes such as hemicellulases (e.g. xylanases), laccases, and lytic polysaccharide monooxygenases (LPMO) could improve cellulose accessibility during biomass deconstruction, without directly hydrolyzing cellulose [74, 71, 107]. For instance, it was shown that the synergism between cellulases and xylanases increases cellulose hydrolysis efficiency by improving cellulose accessibility, not only due to xylan removal but also to changes in fiber morphology such as increasing fiber porosity and fiber swelling [73]. Even though several studies have been conducted in the past years to produce nanocellulose from different cellulosic substrates through an enzyme-mediated pretreatment, the selection of an effective and low-cost enzyme cocktail still remains a challenge.

Moreover, it has been reported that some non-catalytic proteins (e.g. swollenin) enhances the cellulolytic/hemicellulolytic activity by their effects on the dispersion of the cellulose microfibrils and loosening of the cellulose macrofibers, causing a decrease in cellulose crystallinity and increase in cellulose accessibility [108]. This type of non-catalytic proteins is generally required at a very low amount and not present in base enzyme preparations. Swollenin represents one of these types of proteins, which was first isolated and characterized from the cellulolytic fungus *Trichoderma reesei*. Swollenin has been shown to facilitate delamination or fibrillation of substrates, or splitting of microfibrils, resulting in a greater exposure of new crystalline regions of cellulose without producing detectable amounts of reducing sugars [109, 110]. Recently, studies on the supplementation of fungal swollenin into a cellulolytic enzyme preparation for

improving enzymatic hydrolysis showed an increase in both cellulose and xylan hydrolysis of lignocellulosic pretreated substrates such as corn stover [90, 111].

In the following section, the enzymatic pretreatment of BEKP consisting of xylanases as hemicellulolytic enzymes and swollenin as non-catalytic protein with cellulase complex was assessed to evaluate the partial removal of hemicellulose from BEKP and catalyze the hydrolysis of cellulose fibers. The obtained cellulose fibers were characterized to quantify fiber changes during enzyme-mediated pretreatment.

4.1.2. Effect of solid concentration on hydrolysis rate and sugar release during cellulase-mediated pretreatment

Initially, enzymatic pretreatment experiments were performed using the cellulase complex Cellic® CTec3 (CTec3) at an enzyme loading of 5 FPU/g_{solid} (7.0 mg_{protein}/g_{solid}) and different solid concentrations (2, 4 and 8% w/v) for 24 h (Fig. 4.1.1). Low and relatively high solid concentrations were assessed to evaluate possible effects due to mass transfer limitations and the recovery of released sugars at an increased concentration.

As it was expected, higher glucose and xylose concentrations were obtained with increasing solid concentration, reaching 45 g/L and 13 g/L, respectively, for 8% solid concentration at 24 h. However, similar glucose and xylose concentrations were obtained during the first 4 h of hydrolysis when the solid concentration was increased from 4% to 8%, indicating similar enzymatic hydrolysis rates. This suggests possible mass transfer limitations at the initial stages of hydrolysis due to the higher solids content. In order to determine if mass transfer or other limitations affected the hydrolysis performance, results were analyzed in terms of cellulose and xylan hydrolysis (Fig. 4.1.1c and d). It can be observed that higher cellulose (33%) and xylan (52%) hydrolysis was reached at 4% solid concentration, but further increasing solid concentration up to 8% w/v negatively affected the hydrolysis rates (15% and 30% for cellulose and xylan hydrolysis, respectively). Similar results in terms of hydrolysis rates were observed when the solid concentration decreased to 2% (29% and 49% for cellulose and xylan hydrolysis, respectively) compared to 4% during the first 4 h of hydrolysis. However, lower glucose (5.0 g/L) and xylose (2.1 g/L) concentrations were reached in this case. Consequently, considering that after 24 h of hydrolysis significant cellulose degradation was achieved due to glucose release, further enzymatic pretreatment experiments were performed at 4% solid concentration for 4 h that minimizes cellulose loss and increases the released glucose (11.7 g/L) and xylose (4.1 g/L) concentrations.

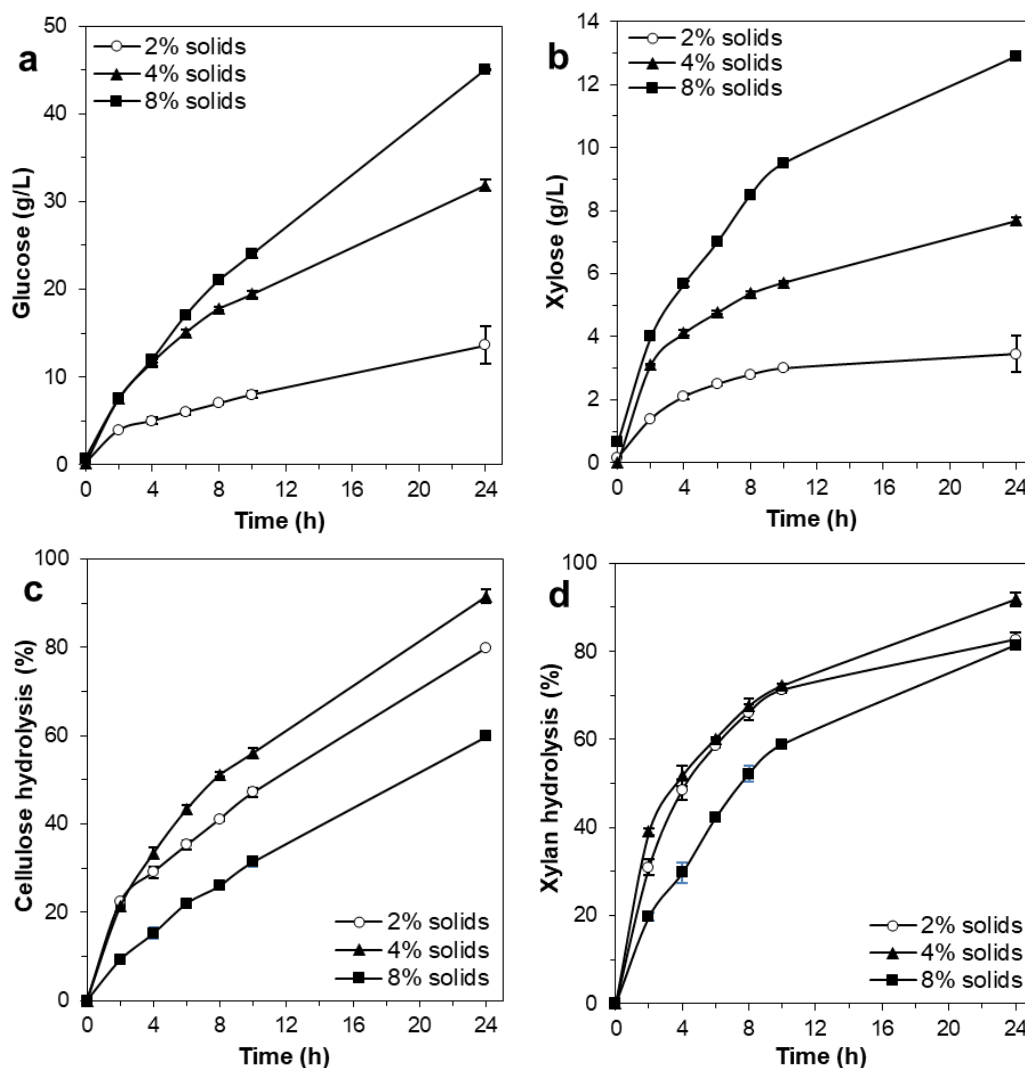


Figure 4.1.1. (a) Glucose, (b) xylose, (c) cellulose hydrolysis, and (d) xylan hydrolysis profiles during 24 h enzymatic hydrolysis of BEKP at 5 FPU/g_{solid} and different solid concentrations (2%, 4% and 8%).

4.1.3. Evaluating the xylanase combination in cellulase-mediated pretreatment

Cellulase pretreatment of BEKP resulted in relatively low hemicellulose removal after 4 h, considering that only 50% of the original xylan was effectively hydrolyzed. Based on the hypothesis that the hydrolysis and removal of hemicellulose amorphous parts from pulp fibers may increase the accessibility of cellulose to enzyme attack by exposing a greater amount of cellulose, and thus, further break down and open up the fiber structure, the supplementation of hemicellulolytic enzymes was then assessed. Also, it has been previously demonstrated the synergistic effect between cellulases and xylanases to open up the cellulose fiber structure in order to enhance cellulose accessibility for further processing [73].

In this context, the cellulase pretreatment was then evaluated using the xylanase complex Cellic® HTec (HTec) commonly used for biomass deconstruction in combination with CTec3

using BEKP as substrate. In brief, the xylanase complex was added by replacing a portion of cellulase complex with xylanase enzyme at cellulase to xylanase ratios (C:X) of 1:0, 0:1, 1:1, 2:1, and 1:2 (expressed on enzyme mass basis) to avoid an increase in the total protein loading (Fig. 4.1.2). However, as the protein contents of CTec3 and HTec were different, the total protein loading of these experiments varied in the range of 1.4 to 7.0 mg_{protein}/g_{solid} (Table 4.1.1). The highest degree of synergism between the enzymes was observed at a C:X ratio of 1:1, which resulted in a substantial increase in both cellulose and xylan hydrolysis (43% cellulose and 76% xylan hydrolysis at 4 h).

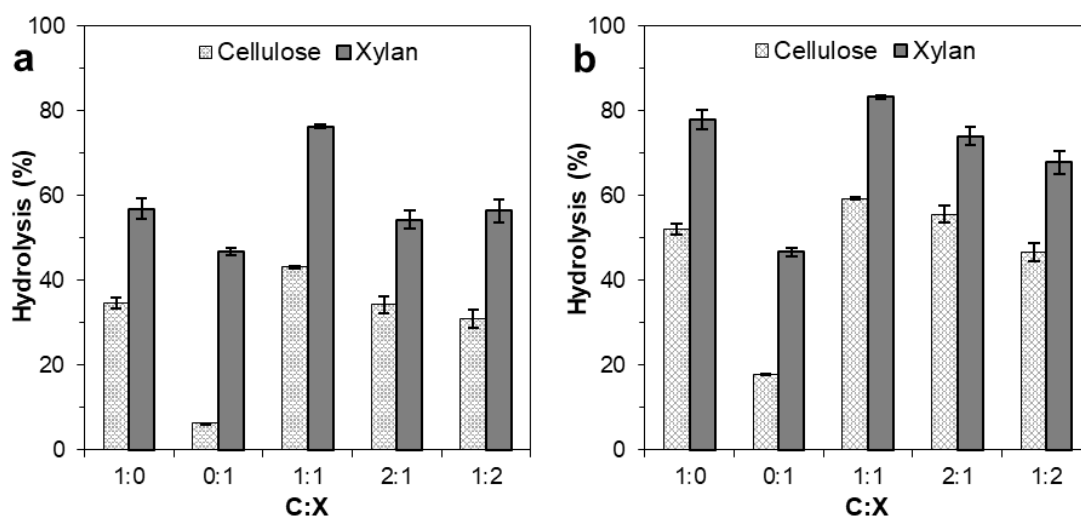


Figure 4.1.2. Cellulose and xylan hydrolysis extents during (a) 4 h and (b) 8 h enzymatic hydrolysis of BEKP at different cellulase to xylanase ratios (C:X) of 1:0, 0:1, 1:1, 2:1 and 1:2.

In order to limit the amount of cellulose loss during enzymatic pretreatment, the amount of enzyme loading was decreased up to 1.0 mg_{protein}/g_{BEKP} while keeping a C:X ratio of 1:1. Even though the cellulosic fraction in BEKP was hydrolyzed at lower total protein loadings (20%), it was observed a significant reduction (33-67%) on the hydrolysis rates compared to previous experiments performed at a total protein loading of 4.2 mg_{protein}/g_{BEKP}. (Fig. 4.1.3). Moreover, decreasing the total protein loading from 4.2 mg_{protein}/g_{BEKP} to 3.0 mg_{protein}/g_{BEKP} significantly reduced the xylan removal (from 76% to 49% at 4h) during enzymatic hydrolysis, which could have a negative effect on enhancing cellulose accessibility. Based on these results, further experiments were performed at an enzyme loading of 4.2 mg_{protein}/g_{BEKP} in order not to compromise cellulose fiber changes due to the extensive hemicellulose removal, since it was reported that hemicellulose acts as a physical barrier to prevent cellulose microfibrils aggregation [73].

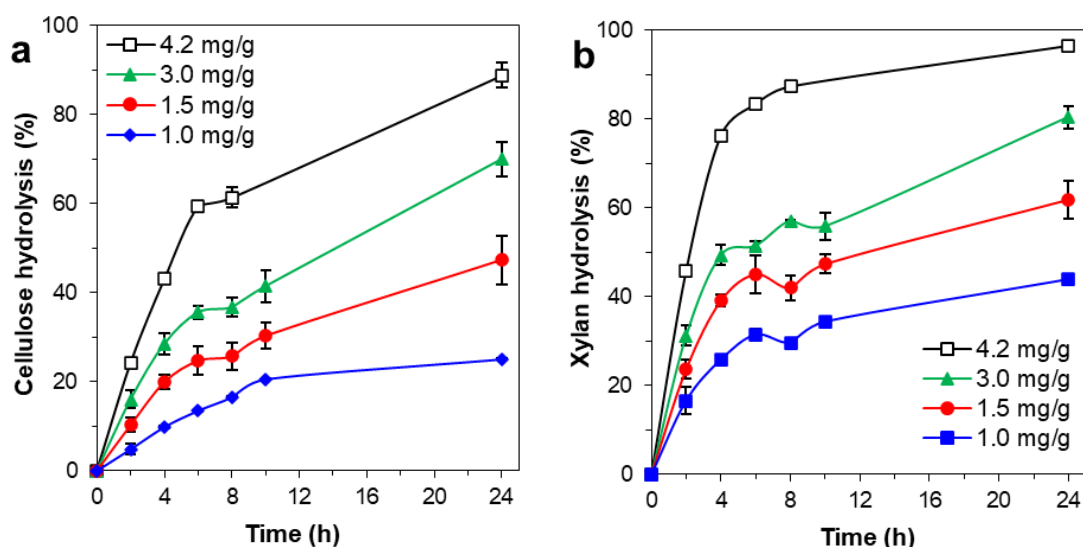


Figure 4.1.3. (a) Cellulose and (b) xylan hydrolysis extents during 24 h enzymatic hydrolysis of BEKP performed at C:X of 1:1 and different enzyme loadings (4.2, 3.0, 1.5 and 1.0 mg_{protein}/g_{BEKP}).

4.1.4. Evaluating non-catalytic protein supplementation to enzyme-mediated pretreatment

Based on the hypothesis that swollenin as non-catalytic protein can alter the hemicellulose structure of pulp fibers and increase cellulose accessibility by loosening/opening up the cellulose fibers, swollenin was assessed in this study in combination with cellulase and xylanase at a C:X ratio of 1:1 and dosage of 4.2 mg_{protein}/g_{BEKP} (Fig. 4.1.4). Moreover, it has been previously reported by other authors that swollenin has strong synergism with xylan degrading enzymes [109, 90]. According to the results obtained, swollenin was able to disrupt the relatively loosely ordered xylan structure during cellulase and xylanase enzymatic pretreatment, since an increased xylan hydrolysis (85%) was reached compared to enzymatic pretreatment without swollenin supplementation (76%). Moreover, no significant increase on cellulose hydrolysis was observed at 4 h (from 43% to 46%) with the use of swollenin, which suggests that swollenin does not cause cellulose degradation but has a significant effect on the hemicellulose component.

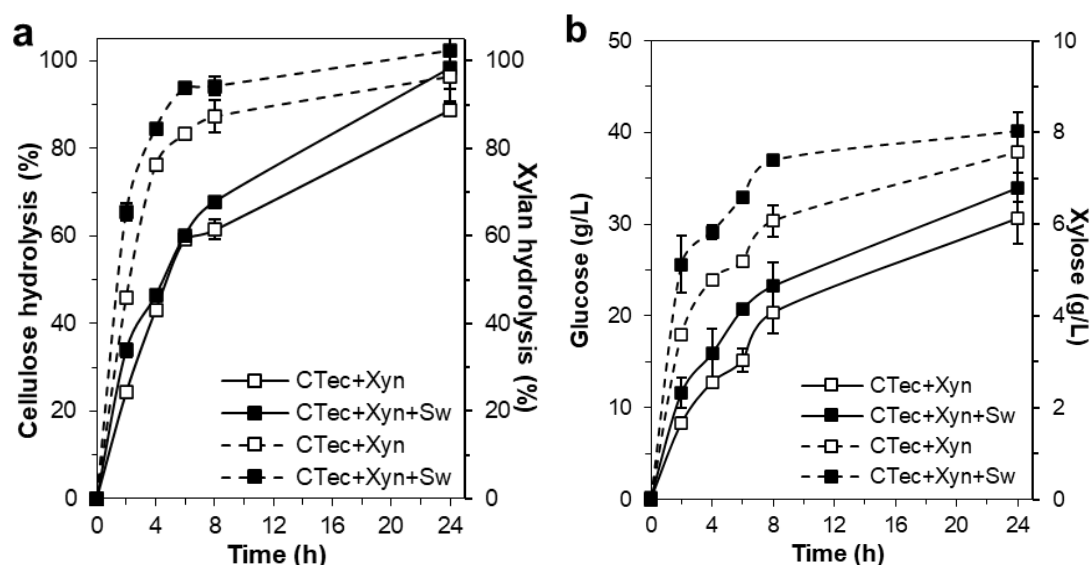


Figure 4.1.4. (a) Cellulose and xylan hydrolysis, and (b) glucose and xylose concentration profiles during 24 h enzymatic hydrolysis of BEKP performed at C:X of 1:1 and enzyme loading of 4.2 mg_{protein}/g_{BEKP} with and without swollenin supplementation. (Note: CTec: CTec3; Xyn: HTec; Sw: swollenin; solid lines: cellulose, glucose; dashed lines: xylan, xylose).

Table 4.1.1. Summary of xylanase and swollenin supplementation effect on cellulose and xylan hydrolysis to enzyme-mediated pretreatment of BEKP at 4% solid concentration after 4 h and 8 h.

C:X	Swollenin addition	Total protein loading (mg _{protein} /g _{BEKP})	Cellulose hydrolysis (%)		Xylan hydrolysis (%)	
			4 h	8 h	4 h	8 h
1:0	No	7.0	33.4 ± 1.3	52.0 ± 1.5	52.3 ± 1.5	77.9 ± 1.6
0:1	No	1.4	6.3 ± 0.1	17.8 ± 0.4	46.8 ± 1.0	46.7 ± 0.9
2:1	No	5.2	34.2 ± 1.9	55.6 ± 2.0	54.3 ± 2.1	74.0 ± 2.0
1:2	No	3.2	30.9 ± 2.2	46.8 ± 2.0	56.4 ± 2.8	67.8 ± 2.5
1:1	No	4.2	43.1 ± 0.2	59.3 ± 0.8	76.2 ± 0.4	83.3 ± 0.5
1:1	No	3.0	28.5 ± 2.4	36.8 ± 2.0	49.3 ± 2.2	57.1 ± 0.2
1:1	No	1.5	19.9 ± 1.6	25.7 ± 3.0	39.1 ± 1.3	42.0 ± 2.8
1:1	No	1.0	9.8 ± 0.2	16.5 ± 0.1	25.8 ± 0.1	29.6 ± 0.4
1:1	Yes	8.4	46.3 ± 1.6	67.8 ± 2.3	84.6 ± 2.0	94.3 ± 3.7

C:X: cellulase to xylanase ratio on mass basis

Considering that an enhanced cellulose and xylan hydrolysis after 4 h (Table 4.1.1) was reached by xylanase and swollenin supplementation, the liquid fractions were further analyzed for cellulose-derived and xylan-derived saccharides to evaluate the potential valorization of this stream (Fig. 4.1.4b). Both cellulase (CTec3) and xylanase (HTec) preparations showed good performance for xylan degradation, since considerable amounts of xylose (32-74%) and soluble xylooligomers (6-15%) were released from the original xylan present in the BEKP (Fig. 4.1.5). A higher proportion of xylooligomers than of xylose was released when xylanase was employed

alone (32%) and in combination with cellulase (20%) during enzymatic pretreatment, compared to cellulase pretreatment (10%). This suggests that the xylanase complex used in this study may lack of β -xylosidase activity to further convert xylooligomers to xylose by hydrolysis. However, the use of swollenin in combination with cellulase and xylanase enhanced xylan hydrolysis, while decreasing the proportion of xylooligomers in the liquid fraction to 12% after 4 h hydrolysis. This allows to get released sugars in a suitable way which represents a possible feedstock to produce other value-added chemicals [112, 72].

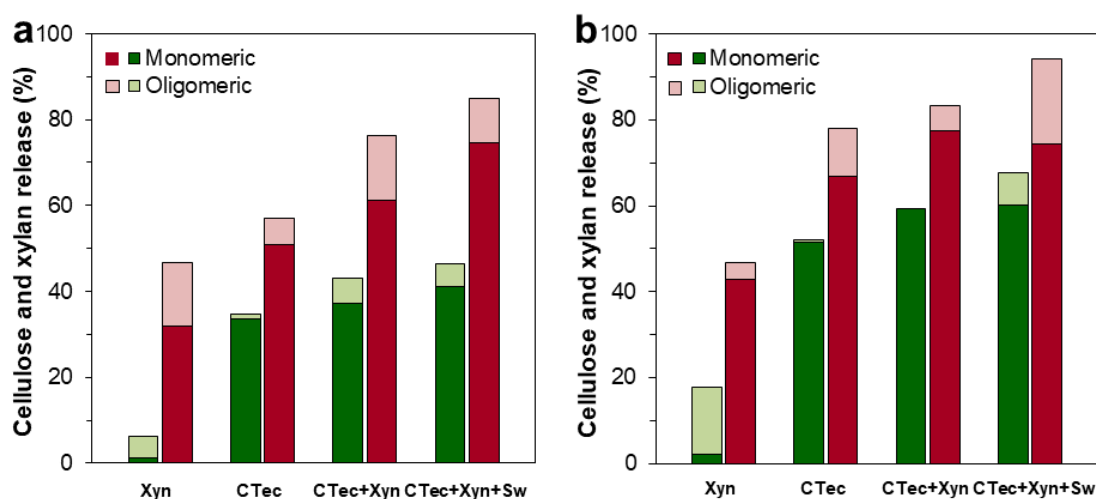


Figure 4.1.5. Cellulose (green and pastel green) and xylan (dark and pastel red) release in monomeric and oligomeric forms for (a) 4 h and (b) 8 h enzymatic hydrolysis of BEKP performed at C:X of 1:1 and enzyme loading of 4.2 mg_{protein}/g_{BEKP}. (Note: CTec: CTec3; Xyn: HTec; Sw: swollenin.)

4.1.5. Quantifying cellulose fibers changes during enzyme-mediated pretreatment

Cellulosic fiber characteristics such as cellulose accessibility, fiber swelling and degree of polymerization (DP) were first assessed for the enzyme-treated BEKP after 4 h and 8 h pretreatment (Fig. 4.1.6). The cellulase pretreatment of BEKP has shown to significantly reduce the DP (from 1218 to 465). This was expected considering that cellulases could randomly cleave the glycosidic linkages in both the disorganized (also known as amorphous) and organized (also known as crystalline) regions of the cellulose fiber, which induces the cellulose DP reduction [113]. Moreover, since xylan plays an important role in protecting cellulose chains against degradation [114], xylan removal by cellulase pretreatment may have caused cellulose becomes more susceptible to depolymerization. DP reductions with enzyme pretreatment was also reported by other authors [115, 73, 116]. On the other hand, even though xylan removal was increased during cellulase and xylanase enzymatic pretreatment, no further DP reduction was observed in this case. The greater exposure of cellulose due to extensive xylan removal could have caused a higher cellulose depolymerization and, thus, lower DP values. On the other hand, considering that

xylans are categorized as short chain polysaccharides [115], removal of these low molecular weight carbohydrates may lead to an increase in the DP extent [114]. This effect may have offset the DP reduction by cellulose depolymerization occurring when xylan is removed and, thus, DP remained almost unchanged. On the other hand, xylanase pretreatment of BEKP resulted in lower DP variations (from 1218 to 735) compared to cellulase pretreatment (Fig. 4.1.6a). This was also shown by Zhou et al. [107] and reflects the difference in the catalytic activity of the enzymes used.

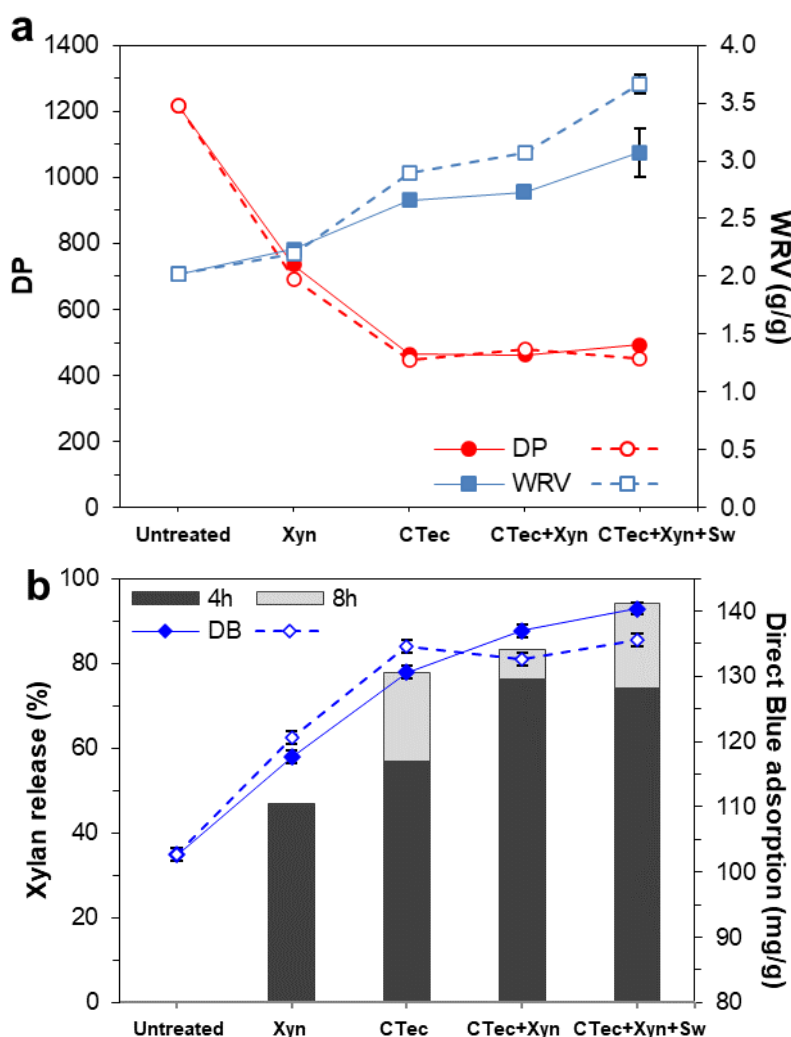


Figure 4.1.6. Characteristics of untreated and enzyme-treated BEKP: (a) water retention value (WRV) and degree of polymerization (DP), and (b) total xylan release and Direct Blue (DB) adsorption. (Note: CTec: CTec3; Xyn: HTec; Sw: swollenin; solid lines: 4 h hydrolysis; dashed lines: 8 h hydrolysis.)

The water retention value (WRV), which can be an indicator of overall fiber accessibility, showed a contrary trend to the observed for DP. Moreover, results showed that the enzymatic pretreatment may have increased the cellulose accessibility, considering the significant increase in the DB adsorption and the WRV observed (Fig. 4.1.6). Even though the supplementation of xylanases to cellulase pretreatment had little effect on the fiber swelling according to WRV (Fig. 4.1.6a), the

synergism between cellulases and xylanases further improved the cellulose accessibility of enzyme-treated BEKP according to DB adsorption (Fig. 4.1.6b). The improvement achieved on the cellulose accessibility indicated that the fiber structure was further opened up, which could be beneficial for subsequent cellulose nanofibrillation. The partial replacement of cellulase by xylanase had very little effect on the cellulose DP of the enzyme-treated BEKP obtained (Fig. 4.1.6a). This indicates that the combination of these enzymes was suitable for keeping cellulose properties while improving cellulose accessibility, without causing further cellulose degradation.

On the other hand, the addition of swollenin improved the cellulose accessibility and fiber swelling (Fig. 4.1.6). It was apparent that the increased xylan hydrolysis could have facilitated the enzyme access to the cellulosic component of BEKP thus opening up the biomass structure and cellulose accessibility. Also, an increased WRV achieved with the use of swollenin might suggest exposure of greater amount of cellulose due to xylan removal that enhanced water retention, since it is likely that xylan acts as a physical barrier possibly coating cellulose microfibrils and/or limiting cellulose fiber swelling. However, it was less able to alter the more highly-ordered cellulose structure, since the degree of polymerization (DP) kept almost unchanged and no significant cellulose degradation occurred (Fig. 4.1.6a).

Since it has been shown that hemicellulose contributes to fiber strength and its solubilization influences pulp fiber properties, fiber dimensions and fiber size distribution of BEKP and enzyme-treated BEKP samples were also investigated. As it was likely that the different enzyme-mediated pretreatments resulted in different particle size distributions, particle size analysis of the untreated and enzyme-treated BEKP substrates was performed in two ways. The first method used was the number-based fiber quality analysis (FQA, Fig. 4.1.7), and the second method used was the volume-based laser diffraction analysis (LD, Fig. 4.1.8).

FQA is a method developed for the pulp and paper industry to evaluate changes at the fiber level. This method can determine the true dimensions of particles (e.g. width and length) by using a charge-coupled device camera using circular polarized light. According to FQA results, BEKP was 15.1 μm in mean width and 1.02 mm in length, containing 25% of fines (fibers with length ranging from 0.07 mm to 0.2 mm). After enzymatic pretreatment, significant fiber fragmentation was observed using cellulase alone or in combination with xylanase and swollenin (Fig. 4.1.7). When xylanase was used alone during enzymatic pretreatment of BEKP, the fiber length decreased up to 0.64 mm, with a fines content of 51%. However, cellulase pretreatment significantly increased the level of fibrillation, as the fines content increased up to 87% and 65% when used alone or in combination with xylanase, respectively. Due to the high fines content of the enzyme-treated BEKP and the fact that FQA method is limited to particles greater than 72 μm

in length, the size distributions of enzyme-treated BEKP samples that undergo extensive particle fragmentation cannot be fully measured using this technique. Because of this, the size distribution after enzymatic pretreatment using cellulase enzyme was then determined by LD analysis (Fig. 4.1.8), since this method allows the measurement of smaller particle sizes (down to the submicron range).

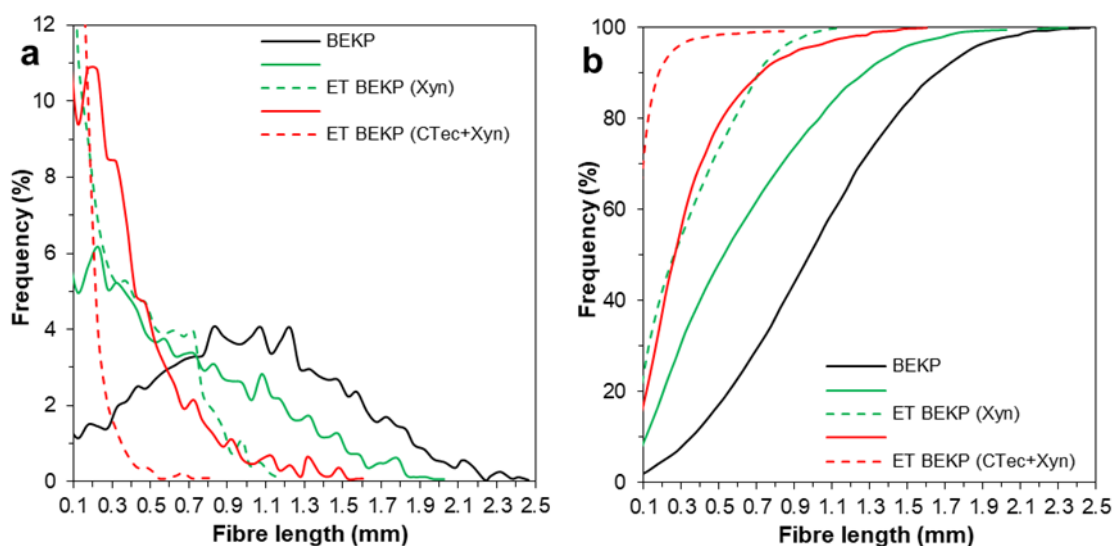


Figure 4.1.7. Size distribution of BEKP and enzyme-treated (ET) BEKP samples through fiber quality analysis: (a) probability density function, and (b) cumulative distribution function. (Note: CTec: CTec3; Xyn: HTec; Sw:swollenin; solid lines: 4 h hydrolysis; dashed lines: 8 h hydrolysis.)

Although microscopy analysis (e.g. SEM, TEM) could be used to determine size dimension and distribution of samples with high fiber fragmentation, the accuracy of microscopy measurements is limited to the small fraction of sample analyzed relative to the whole sample. Thus, LD was used for a more complete evaluation of the size distribution of enzyme-treated BEKP samples. LD is a well-known method which determined the hydrodynamic “apparent particle size” of cellulose micro/nanomaterials based on the scattering of a laser beam in particulate suspensions and subsequent interpretation of the light scattering data according to Mie theory [117, 79]. This method provides equivalent sphere diameters. However, since rod-like fibers are treated as spherical particles, LD measurements does not give absolute values for size distribution [118]. Instead, LD gives a hydrodynamic “apparent size” distribution that can be used to compare between samples that were prepared under different pretreatment conditions. According to LD results, fibers fractionation was apparent during cellulase pretreatment of BEKP, reaching an average hydrodynamic size of 28.6 μm . However, there were still some large fibers in the range of 200 to 800 μm , which should be fractionated during subsequent mechanical treatment. When cellulase was used in combination with xylanase during enzymatic pretreatment, the average

hydrodynamic size remained relatively constant (27.8 μm) and no significant changes was observed compared to cellulase pretreatment (Fig. 4.1.8).

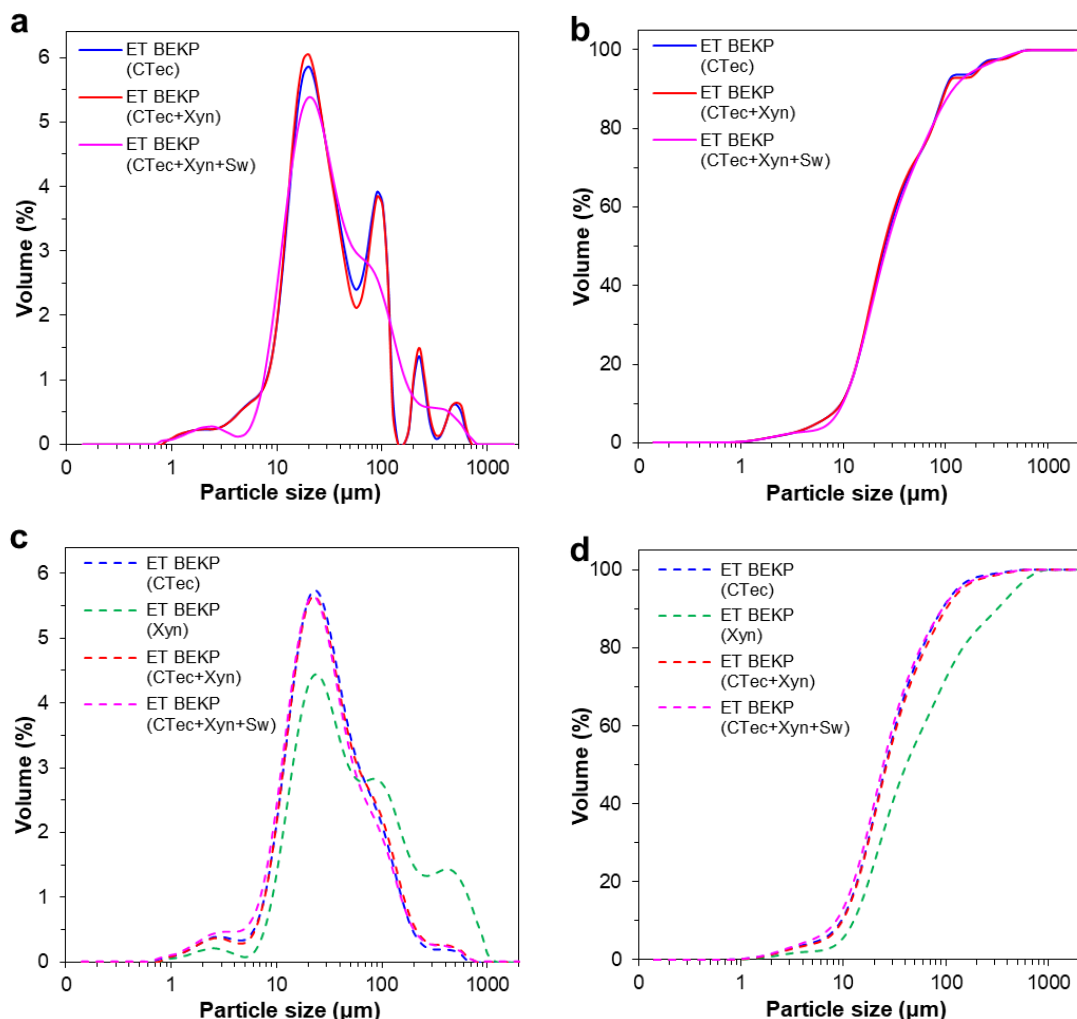


Figure 4.1.8. Size distribution of enzyme-treated (ET) BEKP samples through laser diffraction analysis: (a, c) probability density function, and (b, d) cumulative distribution function. (Note: CTec: CTec3; Xyn: HTec; Sw: swollenin; solid lines: 4 h hydrolysis; dashed lines: 8 h hydrolysis.)

On the other hand, the addition of swollenin during enzymatic pretreatment allowed to achieve a more uniform hydrodynamic size distribution of the fibers, without significantly affecting the average hydrodynamic size (29.8 μm). This may be explained by the increased fiber swelling observed with the use of swollenin during enzymatic pretreatment, which enhanced fiber fragmentation and, thus, less larger fibers in the range of 60 to 800 μm were observed. It should be noted that in all cases between 80 and 90% of the fibers were less than 100 μm size on average, which is in the microfiber range. Also, considerable size reduction after enzymatic pretreatment of BEKP was reached, which implies that fibers became smaller due to increased degree of microfibrillation and thus, greater exposure of surface area on the fibrils occurred. This correlates

quite well with the increased WRV of enzyme-treated BEKP samples related to both fibrils and microfibrils surface area.

X-ray diffraction (XRD) was used in this study to evaluate changes in the relative crystallinity index (CrI) of BEKP by enzymatic pretreatment since it is widely used to investigate the crystal structure of cellulosic materials [79]. CrI is based on the Segal method [98] which, despite some objections and fault found by other authors [119, 120], it is commonly used to analyze XRD spectra to determine CrI due to its simplicity. XRD spectra and CrI of BEKP and enzyme-treated BEKP samples are presented in Fig. 4.1.9. The XRD spectra for all the samples showed characteristic peaks for cellulose I β at $2\theta = 15.0^\circ$, 16.5° , 22.6° , and 34.8° corresponding to the (110), (102), (200) and (040) crystallographic planes of the monocyclic cellulose I β lattice, respectively [121].

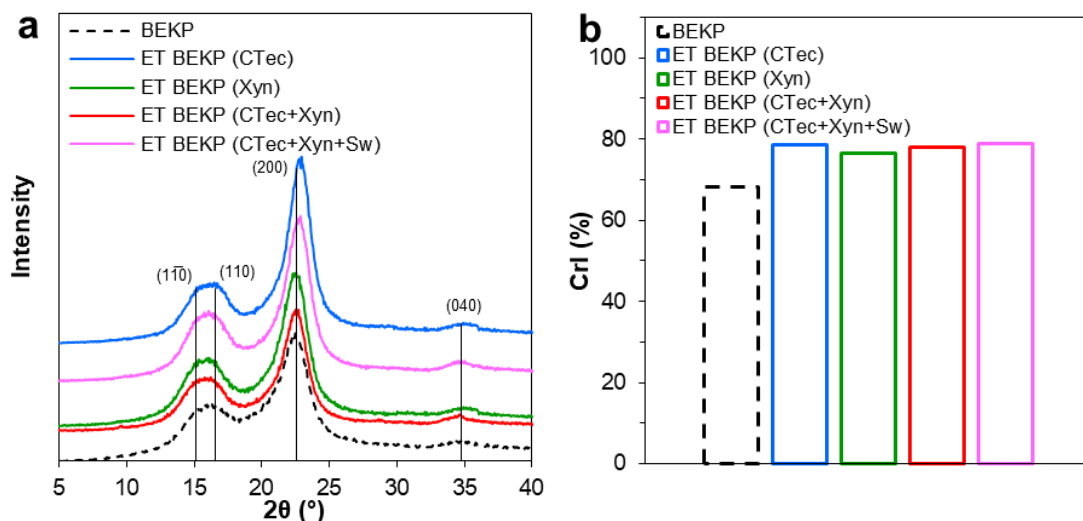


Figure 4.1.9. (a) XRD spectra and (b) crystallinity index (CrI) of BEKP and enzyme-treated (ET) BEKP. (Note: CTec: CTec3; Xyn: HTec; Sw:swollenin; pretreatment conditions: 4% solid, 4 h.)

The CrI of BEKP was 68%, which increased to values in the range of 76 to 79% after enzymatic pretreatment. These higher CrI values for the enzyme-treated BEKP samples (12–16% increase) compared to BEKP was mainly due to the removal of cellulose disordered regions and amorphous hemicellulose during enzymatic pretreatment. Even though the CrI of the enzyme-treated BEKP samples resulted higher than the CrI of BEKP, differences in the CrI values were observed for the different enzyme-treated samples. These differences may be related to the hemicellulose removal during enzymatic pretreatment under the different conditions evaluated, since it was previously shown that decreasing hemicellulose content in pulps increases crystallinity, possibly by the apparent partial recrystallization of amorphous cellulose regions to crystalline, or partial co-crystallization of crystallites in adjacent fibers [122]. However, it should be noted that further increasing hemicellulose removal (e.g. by swollenin addition) did not increase CrI of enzyme-

treated BEKP. This may be explained by the possible presence of grooves in cellulose structure due to high hemicellulose extensive removal, which was previously reported to result in lower CrI values [122]. No further changes were observed on XRD spectra and, thus, CrI values when enzymatic pretreatment was performed for 8 h (Table 4.1.2).

Finally, thermal properties of BEKP and enzyme-treated BEKP samples was investigated through sample mass change using thermogravimetric analysis (TGA). Fig. 4.1.10 shows the TGA and derivative thermogravimetric (DTG) curves of the different samples. According to the TGA curves, the material weight loss during sample heating can be divided into three regions of temperature degradation. The first region starts below 150 °C and is attributed to water evaporation on the sample's surface. The second region starts above 200 °C until 300 °C and represents the thermal decomposition of hemicelluloses, which result more susceptible to thermal degradation than cellulose and lignin mainly due to the presence of acetyl groups. The third and major weight loss happens at high temperatures (310–390 °C) due to cellulose thermal decomposition. Thus, a dominant peak is observed in the DTG curves at maximum weight loss. On the other hand, small portions of lignin in the biomass decompose over a broader degradation temperature range (250–700 °C) than cellulose and hemicellulose components due to its aromatic ring structure. Table 4.1.2 summarizes the thermal properties of the different samples. An increase on the T_{on} and T_{max} was observed for the enzyme-treated BEKP samples compared to BEKP, probably due to the removal of amorphous hemicellulose and non-crystalline cellulose regions during enzymatic pretreatment. Among the different enzyme-treated BEKP samples, it can be observed that higher T_{on} values (340–342 °C) were obtained for samples with higher xylan release (76–85%) during enzymatic pretreatment, which demonstrates that the removal of non-cellulosic components (e.g. xylan) helps to increase the thermal stability.

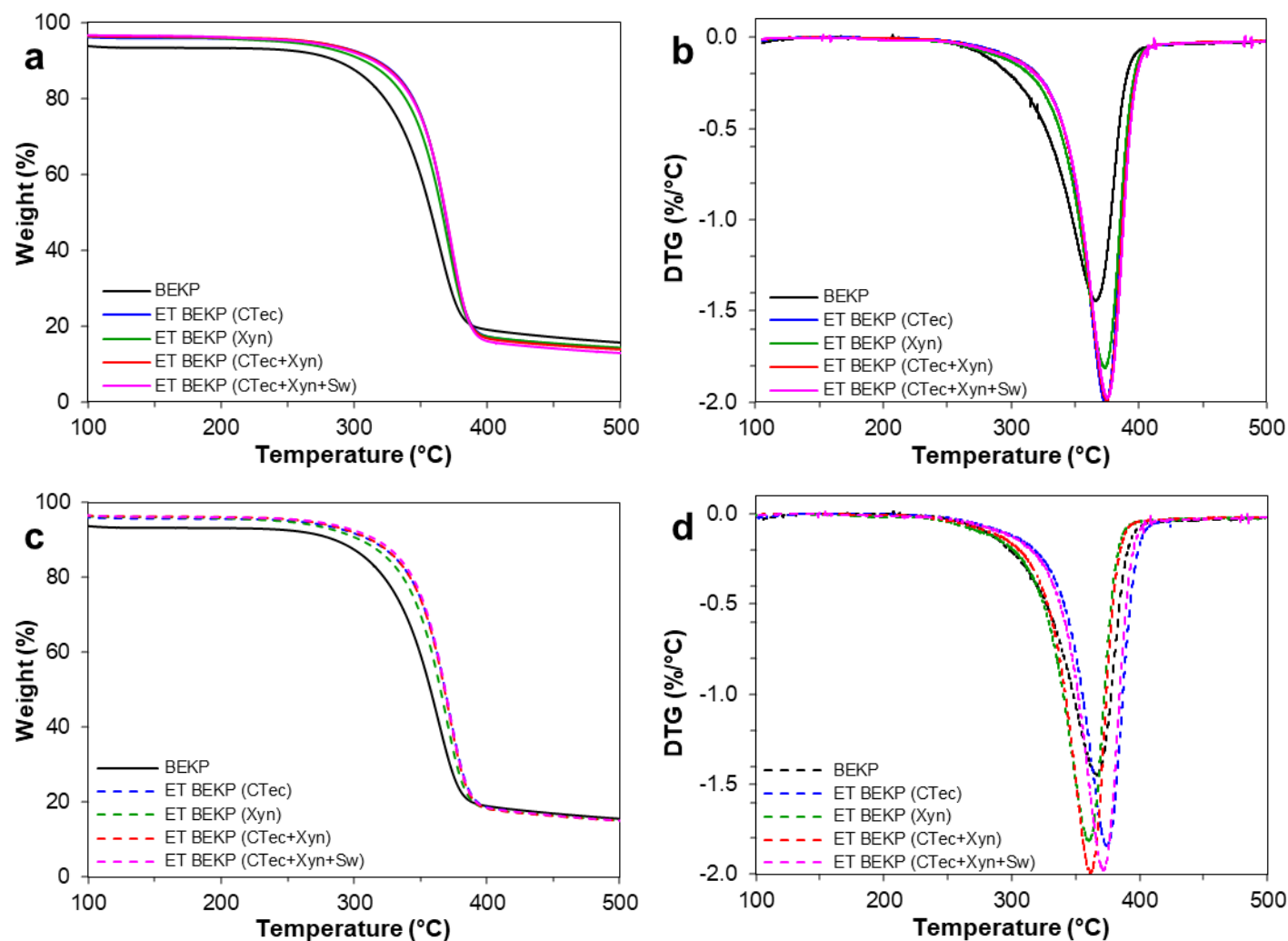


Figure 4.1.10. (a, c) Thermogravimetric analysis and (b, d) derivative thermogravimetric (DTG) curves of BEKP and enzyme-treated (ET) BEKP under different enzymatic pretreatment conditions. (Note: CTec: CTec3, Xyn: HTec; Sw: swollenin; solid lines: 4 h hydrolysis; dashed lines: 8 h hydrolysis.)

Table 4.1.2. Summary of xylanase and swollenin supplementation effect on pretreatment parameters and cellulose fibers properties to cellulase-mediated pretreatment of BEKP.

Sample	BEKP	ET BEKP (CTec)		ET BEKP (Xyn)		ET BEKP (CTec+Xyn)		ET BEKP (CTec+Xyn+Sw)	
Time (h)	-	4	8	4	8	4	8	4	8
SY (%)	na	61.2 ± 0.1	44.5 ± 1.8	82.9 ± 4.6	75.0 ± 2.6	56.1 ± 3.8	37.7 ± 2.1	53.2 ± 5.0	40.5 ± 6.1
Cellulose hydrolysis (%)	na	34.7 ± 1.3	52.0 ± 0.5	6.3 ± 0.2	17.8 ± 0.2	43.1 ± 0.2	59.3 ± 0.8	46.3 ± 1.6	67.8 ± 2.3
Xylan hydrolysis (%)	na	56.9 ± 2.3	77.9 ± 1.8	46.8 ± 1.0	46.7 ± 1.3	76.2 ± 0.4	83.3 ± 0.5	84.6 ± 2.0	94.3 ± 3.7
<i>Physicochemical analysis</i>									
DP	1218 ± 4	465 ± 6	466 ± 3	735 ± 1	693 ± 2	462 ± 1	479 ± 4	492 ± 4	450 ± 1
WRV (g/g) ^a	2.02 ± 0.05	2.66 ± 0.03	2.89 ± 0.03	2.24 ± 0.01	2.20 ± 0.01	2.72 ± 0.04	3.06 ± 0.04	3.07 ± 0.21	3.66 ± 0.08
DB adsorption (mg/g)	103 ± 1	131 ± 1	135 ± 1	118 ± 1	121 ± 1	137 ± 1	133 ± 2	140 ± 1	136 ± 2
CrI (%)	68.2 ± 2.7	78.5 ± 3.1	77.3 ± 3.1	76.5 ± 3.0	78.2 ± 3.2	77.9 ± 3.1	77.3 ± 2.9	78.8 ± 3.2	78.2 ± 3.1
<i>Morphological analysis</i>									
Lw (mm)	1.02 ± 0.45	0.20 ± 0.09	0.17 ± 0.07	0.52 ± 0.10	0.36 ± 0.15	0.37 ± 0.17	0.13 ± 0.05	0.35 ± 0.14	0.12 ± 0.05
Fines content (%)	n.a.	87.1	96.1	31.2	73.1	64.7	96.7	74.2	95.5
Average hydrodynamic size (µm)	n.a.	28.6	25.8	n.a.	40.9	27.8	26.3	29.8	24.3
<i>Thermal analysis</i>									
T _{on} (°C)	314	335	335	330	322	340	325	342	335
T _{max} (°C)	365	374	374	372	360	375	362	375	370
Residue (%)	13.8	12.3	19.8	12.2	13.2	12.0	13.2	11.2	13.3

ET: enzyme-treated; DB: Direct Blue; na: not applicable; Lw: length-weighted average.

^aExpressed as g of bound water per g of dry solid.

4.1.6. Assessing the use of endoglucanases for enzyme-mediated pretreatment of BEKP

Several enzymes have been assessed to facilitate fibrillation of cellulose fibers for CNF extraction, such as enzymatic cocktails, xylanases, endoglucanases, lytic polysaccharide monooxygenase, among others [68, 1]. However, monocomponent endoglucanases (EG) as cellulolytic enzymes have been explored more widely over the years due to their effectiveness in fiber defibrillation since EG preferentially attacks the less ordered regions of cellulose fibers, breaking accessible glycosidic bonds and introducing new reducing and non-reducing ends [39]. Moreover, it has been reported that EG has the potential to promote specific fiber modifications which can facilitate the defibrillation process without compromising the cellulose crystalline regions. Thus, the mechanical properties could be preserved. Even though enzymatic pretreatment of cellulosic materials with monocomponent endoglucanases has been reported to be beneficial to maximize the extraction of CNF, the processing conditions differ greatly in the literature. It is not completely clear the effect that variables such as reaction time (ranging from few minutes to up to 72 h) and enzyme loading (ranging from 1 to 350 U/g_{fiber}) have on fiber modifications for CNF extraction and CNF properties [39]. Even though it depends on the type and amount of enzyme used, prolonged reaction times (48 h-120 h) are generally used for CNCs extraction from cellulosic materials instead of CNFs [1]. In this part of the study, the endoglucanase (EG) pretreatment of BEKP was compared to the cellulase pretreatment of BEKP, which was previously discussed. A preliminary evaluation of the effect of enzyme loading and reaction time on cellulose and xylan hydrolysis efficiency was first assessed during EG pretreatment. Table 4.1.3 presents the conditions evaluated for the enzymatic pretreatment, which were selected based on previous reported data on EG pretreatment [1, 123].

Table 4.1.3. Endoglucanase (EG) pretreatment conditions evaluated at a solid concentration of 4%.

Sample code	Enzymatic hydrolysis conditions	
	EG loading (U/g _{BEKP})	Hydrolysis time (h)
EG30-8	30	8
EG30-24	30	24
EG100-8	100	8
EG100-24	100	24
EG170-8	170	8
EG170-24	170	24
EG240-8	240	8
EG240-24	240	24

Interestingly, the enzymatic hydrolysis of BEKP into soluble sugars resulted limited during EG pretreatment of BEKP, even at the highest severity in terms of enzyme loading (240 U/g_{BEKP}) and reaction time (24 h). As can be seen in Fig. 4.1.11 and Table 4.1.4, the highest released sugars yield was reached under this condition and resulted of 3% for both cellulose and xylan fractions. Consequently, high solid yields (97-99%) were achieved for all the conditions evaluated, which resulted much higher than those achieved employing cellulase complex mixture (Table 4.1.2). This implies that a higher or almost complete fiber recovery can be achieved employing EG enzymes from BEKP, which could benefit CNF extraction yields by further fiber processing. It was also reported by other authors [38] that by selecting the appropriate cocktail or monocomponent enzyme, it is possible to benefit the formation of CNF by subsequent mechanical treatment without hydrolyzing cellulose to soluble sugars.

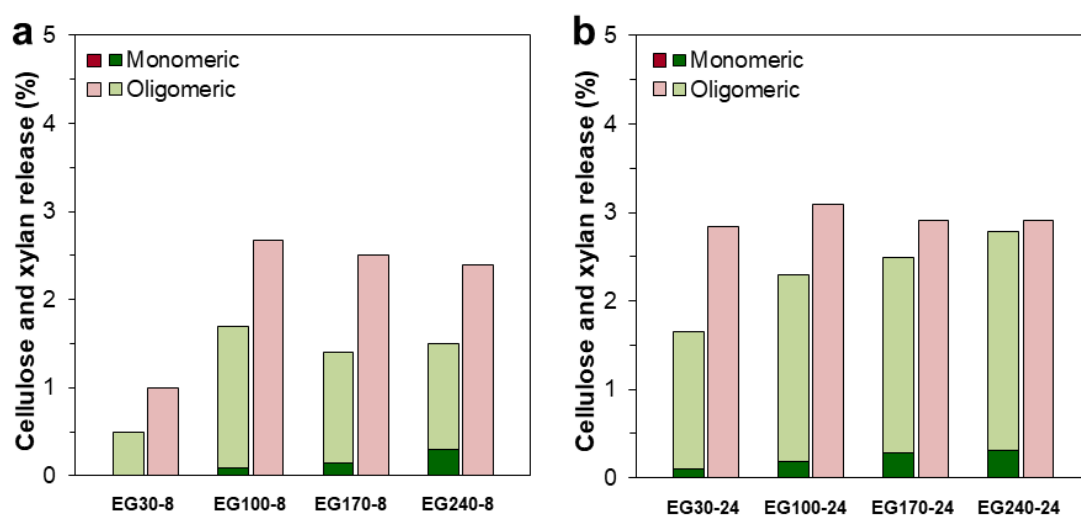


Figure 4.1.11. Cellulose (green and pastel green) and xylan (dark and pastel red) release in monomeric and oligomeric forms for (a) 8 h and (b) 24 h endoglucanase (EG) pretreatment of BEKP performed at a solid concentration of 4%.

Even though differences on the solid yields resulted not significant among the EG pretreatment conditions evaluated, variations in the severity of the pretreatment led to fiber changes and modifications. Therefore, to evaluate the EG pretreatment efficiency, cellulosic fiber changes of BEKP after EG pretreatment were investigated such as fiber swelling by WRV, cellulose accessibility by DB adsorption, DP, and morphological analysis. The effect of enzyme loading and reaction time on fiber characteristics are presented in Fig. 4.1.12. Despite the limited cellulose and xylan hydrolysis previously discussed, a considerable DP reduction (up to 40%) was determined for the EG-treated BEKP. Both enzyme loading and reaction time had a positive effect on cellulose depolymerization according to DP values. This demonstrates the catalytic potential of EG monocomponent enzymes which can reach and break accessible glycosidic bonds in BEKP fibers, causing depolymerization of cellulose chains.

Table 4.1.4. Solid yields, and characterization of cellulose fibers obtained after endoglucanase (EG) pretreatment of BEKP under the different conditions evaluated.

Sample	SY (%)	Cellulose hydrolysis (%)	Xylan hydrolysis (%)	Cellulose- derived sugars (g/L) ^a	Xylan-derived sugars (g/L) ^a	WRV (g/g)	DB adsorption (g/g _{dry solid})	DP
BEKP	na	na	na	na	na	2.71 ± 0.04	87 ± 1	1196 ± 5
EG30-8	99.4 ± 0.2	0.5 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	2.75 ± 0.03	96 ± 1	1071 ± 10
EG30-24	98.1 ± 0.2	1.6 ± 0.1	2.8 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	2.90 ± 0.02	119 ± 1	930 ± 13
EG100-8	98.1 ± 0.2	1.7 ± 0.1	2.7 ± 0.1	0.6 ± 0.1	0.2 ± 0.1	3.01 ± 0.03	101 ± 1	911 ± 9
EG100-24	97.6 ± 0.2	2.3 ± 0.2	3.1 ± 0.6	0.8 ± 0.1	0.2 ± 0.1	3.11 ± 0.04	126 ± 1	829 ± 16
EG170-8	98.5 ± 0.3	1.4 ± 0.1	2.0 ± 0.2	0.5 ± 0.1	0.1 ± 0.1	3.00 ± 0.10	109 ± 1	880 ± 8
EG170-24	97.4 ± 0.1	2.5 ± 0.2	2.9 ± 0.1	0.9 ± 0.1	0.2 ± 0.1	3.23 ± 0.11	126 ± 1	756 ± 15
EG240-8	98.6 ± 0.6	1.5 ± 0.4	2.4 ± 0.4	0.5 ± 0.1	0.2 ± 0.1	3.06 ± 0.10	120 ± 1	840 ± 10
EG240-24	97.2 ± 0.1	2.8 ± 0.1	2.9 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	3.34 ± 0.04	126 ± 1	719 ± 14

Enzymatic pretreatment performed at a solid concentration of 4%.

SY: solid yield; WRV: water retention value; DB: Direct Blue; DP: degree of polymerization; na: not applicable.

^aConcentration of total sugars (both in monomeric and oligomeric forms) in liquid fractions after EG pretreatment.

Low enzyme loadings (30 U/g_{BEKP}) for short reaction times (8 h) may not be enough for the EG enzyme to produce fibers modifications that would facilitate subsequent fiber fibrillation for CNF extraction, since the DP reduction resulted relatively low (from 1196 to 1071). However, by prolonging the reaction time from 8 h to 24 h, it was possible to reach a decrease in cellulose DP by 22%. Considering that only a very limited fraction of cellulose in the BEKP is accessible to the enzymes, longer reaction times allowed to achieve greater interactions between cellulose and EG enzyme, leading to an efficient activity of the enzyme over the cellulose chain. Thus, greater reduction of DP was expected by extending the reaction time.

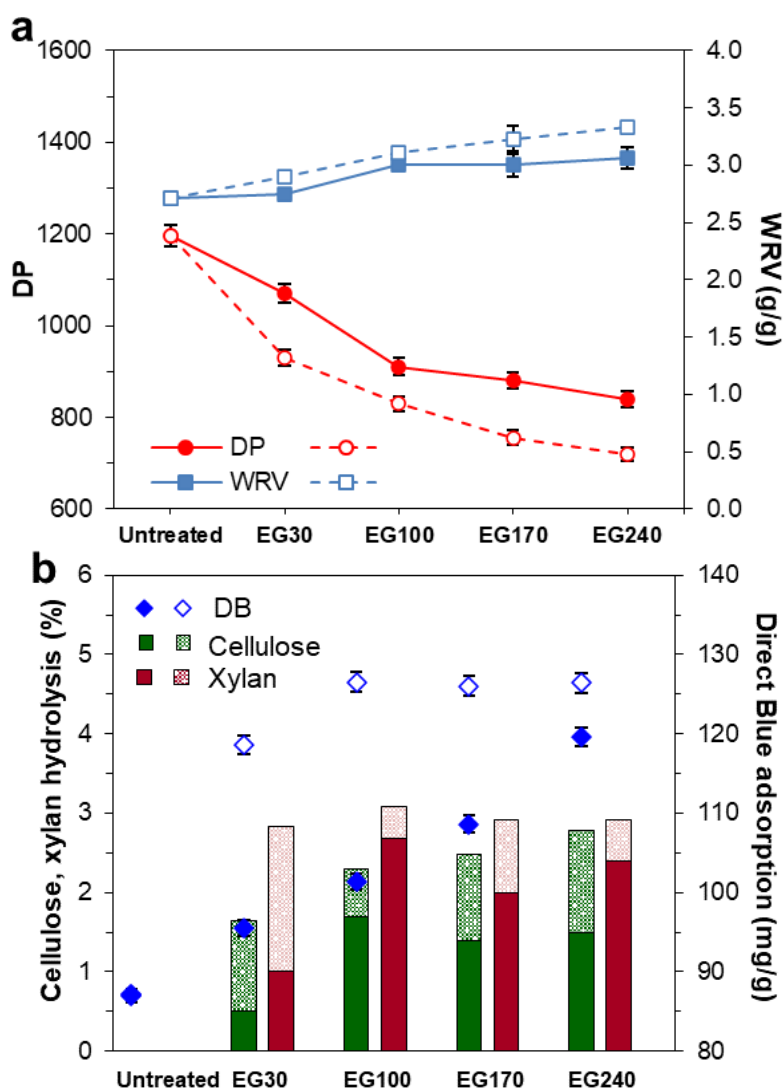


Figure 4.1.12. Characteristics of untreated and endoglucanase-treated (EG) BEKP: (a) water retention value (WRV) and degree of polymerization (DP), and (b) cellulose and xylan hydrolysis and Direct Blue (DB) adsorption after 8 h (solid lines, solid fill) and 24 h hydrolysis (dashed lines and pattern fill).

On the other hand, no significant difference was observed in terms of carbohydrate release (for both cellulose and xylan) when working at enzyme loading in the range of 100 to 240 g/g_{BEKP} for 8 h and 24 h according to Fig. 4.1.12b. However, DP reductions were observed when both enzyme

loadings and reaction times were increased. This DP reductions show that it may be occurring the opening up of the fiber structure and fiber shortening during EG pretreatment rather than fiber hydrolysis to monosaccharides and/or oligosaccharides. Moreover, when the EG pretreatment of BEKP was performed for 8 h, an increase in cellulose accessibility with enzyme loading was observed according to DB adsorption analysis. An approximate 10% increase was observed by further increasing the enzyme loading, reaching a maximum value of 120 g/g_{solid} for the maximum enzyme loading of 240 U/g_{BEKP}. This may indicate an enhanced opening up of the fiber microstructure with the enzyme loading and, thus, further improvements on cellulose accessibility by employing higher amounts of EG. However, when the EG pretreatment was performed for 24 h, a sharp increase in cellulose accessibility was achieved even working at the lowest enzyme loading of 30 U/g_{BEKP} (119 g/g_{solid}). A maximum value of 126 g/g_{solid} was reached after 24 h pretreatment for an enzyme loading of 100 U/g_{BEKP}, and further improving the enzyme loading up to 240 U/g_{BEKP} did not improved the DB adsorption.

The swelling ability of EG-treated BEKP samples was evaluated by WRV determination, and results are presented in Fig. 4.1.12a. The WRV was increased during EG pretreatment (from 2.71 to 3.34), which means that more water accessible cavities were established on the cellulosic fibers during EG-mediated pretreatment. This indicates that a better fiber swelling was achieved after EG pretreatment probably due to the weakening of the binding forces between fibrils. An improvement on WRV value at a lesser extent was observed for the lower enzyme loading (30 U/g_{BEKP}) but was further enhanced by prolonging reaction time to 24 h.

Moreover, the supplementation of xylanase to EG-mediated pretreatment of BEKP was then assessed since it was previously discussed their synergistic effect to open up the cellulosic fiber structure. The xylanase complex (HTec) was added by replacing a portion of EG enzyme with xylanase at an EG:X ratio of 1:1 (expressed on enzyme mass basis), as it was previously discussed in Section 4.1.3. Table 4.1.5 presents the results obtained in terms of carbohydrate hydrolysis and fiber characterization.

According to the results obtained, the addition of xylanase resulted in a substantial increase in both cellulose (45%) and xylan (73%) hydrolysis compared to EG pretreatment without supplementation (1.5% cellulose, 2.4% xylan). An abrupt decrease in the solid yield (57%) was achieved due to extensive carbohydrate decomposition, with the consequent release of both glucose (1.7 g/L) and xylose (5.0 g/L) sugars. It should be noted that cellulose release was mostly in oligomeric form (89%), particularly a considerable amount of cellobiose (14.2 g/L) was detected. This was expected given that both EG and xylanase preparation lack of β -glucosidase activity to further convert oligosaccharides to glucose.

Table 4.1.5. Effect of xylanase supplementation to BEKP endoglucanase (EG) pretreatment at 4% solid concentration on cellulose and xylan hydrolysis, and fiber characterization.

Sample	EG240	EG120+Xyn
EG:X ratio	na	1:1
Time (h)	8	8
SY (%)	98.6 ± 0.6	57.2 ± 5.1
Cellulose hydrolysis (%)	1.5 ± 0.4	44.7 ± 1.0
Monomeric	0.3 ± 0.1	4.8 ± 0.1
Oligomeric	1.2 ± 0.4	39.9 ± 0.9
Xylan hydrolysis (%)	2.4 ± 0.4	73.3 ± 1.5
Monomeric	<0.1	60.5 ± 1.0
Oligomeric	2.4 ± 0.4	12.8 ± 0.5
<i>Physicochemical and morphological analysis of EG-treated BEKP</i>		
DP	840 ± 10	484 ± 10
WRV (g/g)	3.06 ± 0.10	3.36 ± 0.21
DB adsorption (mg/g)	120 ± 1	130 ± 1
Average size (µm)	62.3	24.1
<i>Analysis of liquid fractions</i>		
Cellulose-derived sugars (g/L) ^a	0.5 ± 0.1 (<0.1)	15.8 ± 1.0 (1.7 ± 0.1)
Xylan-derived sugars (g/L) ^a	0.2 ± 0.1 (<0.1)	6.0 ± 1.1 (5.0 ± 0.9)

na: not applicable; nd: not detected.

^aValues in brackets correspond to sugars concentration in monomeric form.

The DP was significantly decreased (from 1116 to 484) after EG pretreatment with xylanase supplementation compared to EG pretreatment without xylanase (from 1116 to 840), which demonstrated the extensive fiber depolymerization in this case. Also, this may indicate an enhanced fiber fragmentation of the material during EG-mediated pretreatment. According to LD analysis (Fig. 4.1.13), fiber fractionation was significant when EG pretreatment was performed in combination with xylanase since the average hydrodynamic size decreased to around 24.1 µm. An average hydrodynamic size of around 62.3 µm was obtained for EG-mediated pretreatment without xylanase supplementation, with many large fibers in the range of 200 to 1000 µm and still some large fibers above 1 mm. However, a more uniform hydrodynamic size distribution of the fibers was obtained with xylanase supplementation in the micrometer range, which enhanced BEKP fiber fragmentation. Also, less larger fibers in the range of 200 to 600 µm were observed in this case, and around 90% of the fibers were less than 100 µm size on average. It was apparent that the supplementation of xylanase allowed to improve the degree of microfibrillation during EG-mediated pretreatment of BEKP by fiber size reduction and depolymerization, causing a greater exposure of surface area.

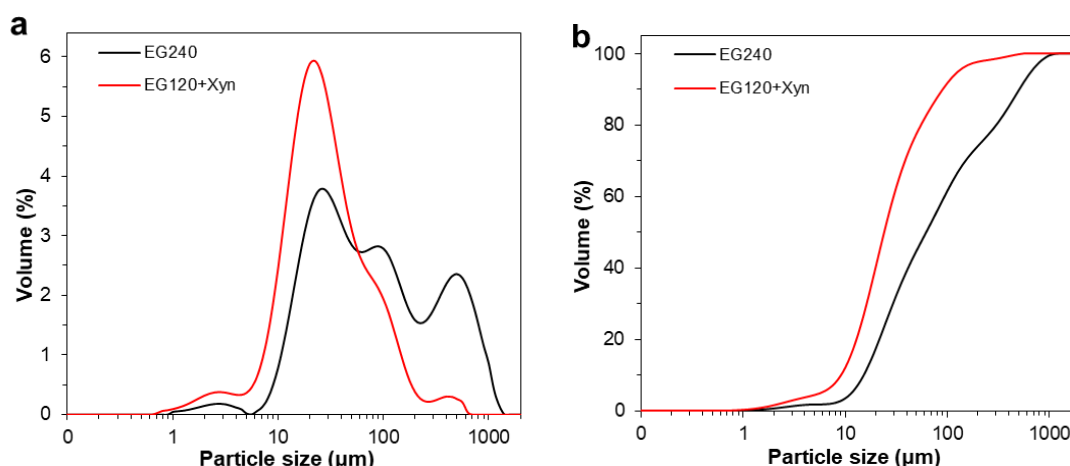


Figure 4.1.13. Size distribution of endoglucanase-treated BEKP without (EG240) and with xylanase supplementation (EG120+Xyn) through laser diffraction analysis: (a) probability density function, and (b) cumulative distribution function.

Finally, optical microscopy (OM) and image analysis were performed to get morphological information of the fibers and microfibrils obtained after enzymatic pretreatment employing either the cellulase complex mixture or the EG monocomponent with the supplementation of xylanase as auxiliary enzyme. It was previously reported by other authors [124] that this analysis allowed to get a morphological characterization of cellulose microfibrils suspensions and to estimate the extent of peeling/fibrillation and fiber shortening produced by enzymes. Fig. 4.1.14 shows the modification in the fiber morphology with the enzymatic pretreatment through OM images. The microscopic images for the enzyme-treated samples showed a reduced fiber length compared to the initial BEKP, which clearly reflect the breakage of the fibers due to enzyme catalytic activity. The enzymes act mostly in the amorphous regions of cellulose by cutting the cellulose chains and, thus, reducing the fiber length. This fiber length reduction was intensified along with the severity of the enzymatic pretreatment in terms of carbohydrate decomposition. Consequently, the initial aspect ratio of BEKP which was around 53, was altered after enzymatic pretreatment according to width distributions presented in Appendix A3. These aspect ratio alterations may be caused by both fibers breakage and/or peeling of the primary fibers, which consists on the separation of microfibrils from the primary structure of cellulose. For instance, when the EG monocomponent was employed (sample EG240, Fig. 4.1.14b), even though the average fiber length (1.61 mm) was not significantly different from the BEKP (1.73 mm), the number of larger fibers was significantly decreased, and a wider and more uniform fiber length distribution was obtained compared to BEKP. This effect may be probably due to the shortening of the fibers caused by the enzyme catalytic activity. Also, a slight decrease in the diameter of the fibers (27.2 μm) can be seen compared to BEKP (32.6 μm), which produced a slight increase in the aspect ratio (from 53 to 59). This increase may indicate that the effect of fiber fibrillation in this case counteracted the breakage of fibers.

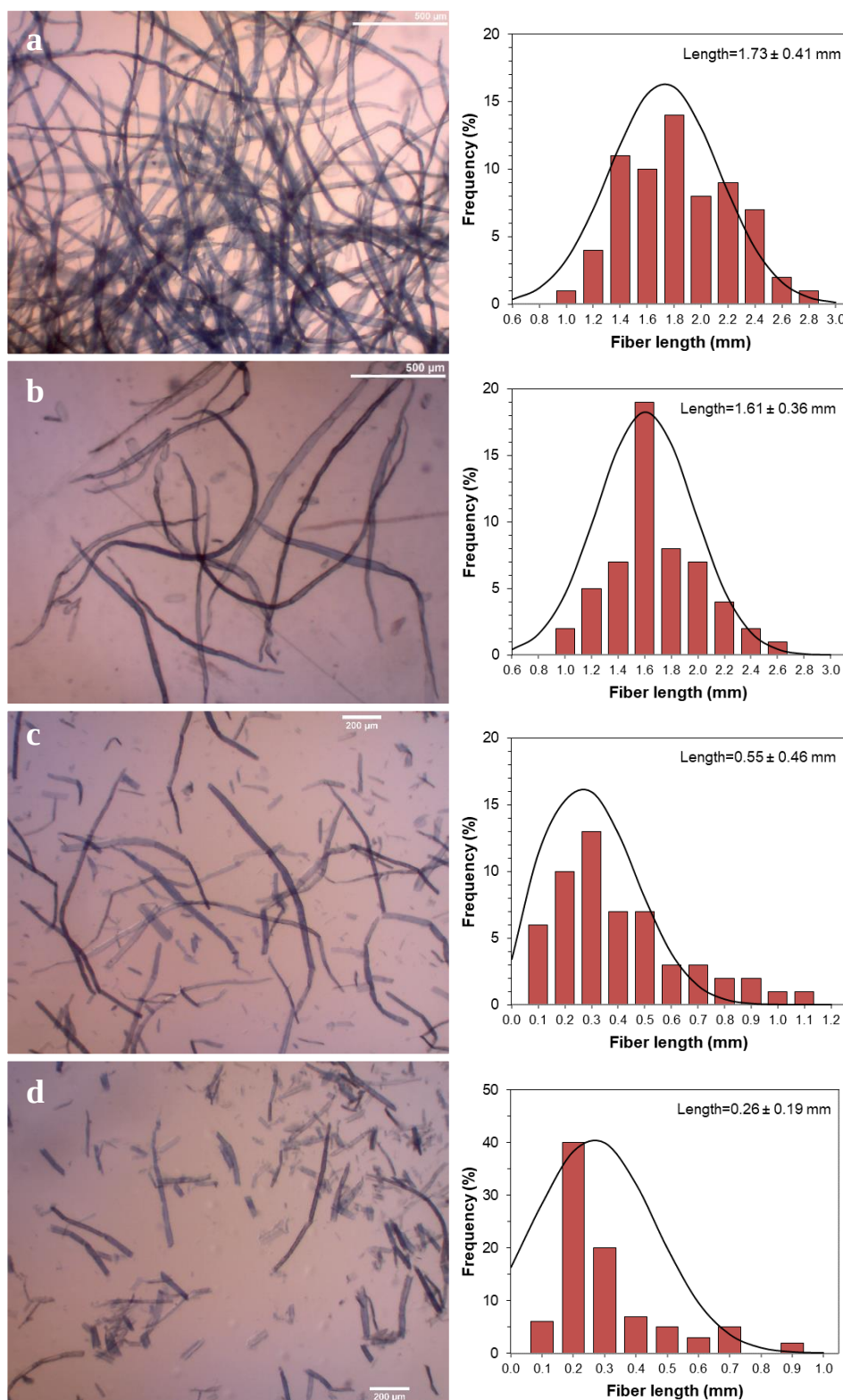


Figure 4.1.14. Optical microscopy images showing the morphology and fiber length distributions for samples: (a) untreated BEKP, (b) endoglucanase-treated at 240 U/g_{BEKP} for 8 h (EG240), (c) endoglucanase-treated at 120 U/g_{BEKP} for 8 h with xylanase supplementation (EG120+Xyn), and (d) cellulase-treated BEKP at 4.6 FPU/g_{BEKP} for 8 h with xylanase supplementation (ET BEKP(CTec+Xyn)). The average length and standard deviation are labeled in the figures.

However, it was observed by OM a reduction on the fiber length (0.55 mm) when xylanase was employed during EG pretreatment of BEKP (sample EG120+Xyn, Fig. 4.1.14c). Also, a significant fiber length reduction (0.26 mm) was observed when the cellulase complex mixture was employed during enzymatic pretreatment with xylanase supplementation (sample CTec+Xyn). Even though in both cases a decrease in the fiber width was observed (from 32.6 μm to 21.8-26.6 μm), the number of fiber breakages induced by enzyme activity likely prevailed over fiber fibrillation, since the aspect ratio was significantly reduced (from 53 to 12-21). Microscopic images also showed some microfibrils with a width of a few microns (10-15 μm) completely separated (Fig. 4.1.14c and Fig. 4.1.14d), which may be coming from the peeling of primary fibers.

These observations resulted in accordance with the fact that the supplementation of xylanase to either EG or cellulase pretreatment enhanced fiber fragmentation and size reduction. The xylanolytic activity of the mixture caused an improved cellulose accessibility due to xylan hydrolysis, and an improved catalytic activity of cellulase preparations due to the synergism effect of cellulase and xylanase enzymes.

4.1.7. Summary

BEKP resulted a promising raw material for the production of CNMs due to its high cellulose content (77%) and low lignin content (<1%). Moreover, it presented a relatively high hemicellulose content (mainly xylan) (18%).

The cellulase complex Cellic® CTec3 resulted effective for promoting the cellulose accessibility during enzyme-mediated pretreatment of BEKP, achieving improvements of up to 50% compared to the untreated BEKP. In addition, the combination of cellulase complex with auxiliary proteins such as catalytic xylanase complex Cellic® HTec and/or non-catalytic expansin-like swollenin, allowed to further improve the cellulose accessibility by opening up the cellulose fibers due to two main effects: i) the hydrolysis of hemicellulose (mainly xylan) due to the xylanolytic activity of the enzyme complex; ii) the breakage of hydrogen bonds between cellulose fibers and hemicellulose, which enhances the opening up of the fibers and, therefore, the enzymes accessibility to cellulose for hydrolysis.

The hydrolysis percentages achieved using CTec3 and HTec preparations were high (up to 46% cellulose and 85% xylan), even for relatively short reaction times (4 h). Additionally, the degree of polymerization of BEKP was reduced by 50-75% after enzyme-mediated pretreatment, which implies a significant shortening of the length of the cellulose chains and, thus, a decrease in the molecular weight distribution. Moreover, swollenin as a non-catalytic protein was able to disrupt

the fiber network of BEKP by swelling, which enhanced the opening up of the pulp fibers and further improved the accessibility of enzymes to cellulose and xylan.

Physicochemical characteristics of enzyme-treated BEKP demonstrated the synergism between cellulase and xylanase enzymes that enhanced the fiber fragmentation induced by catalytic cellulose and xylan hydrolysis. Consequently, liquid fractions rich in sugars were obtained (20-40 g/L glucose, 6-13 g/L xylose), which must be further valorized by conversion to value-added chemicals for an integral exploitation of the raw material.

On the other hand, the EG pretreatment of BEKP resulted with negligible hydrolytic effects for carbohydrate decomposition (< 5%) since very low amounts of cellulose-derived and xylan-derived sugars were achieved (< 1 g/L). Thus, fiber recoveries of above 95% were reached. There was a reduction in the DP of BEKP of around 30%, lower than that achieved when using CTec3 complex. Even though depolymerization and fiber fragmentation during EG pretreatment occurred at a lesser extent compared to cellulase pretreatment with CTec3, it was found that EG action improved cellulose accessibility in pulp fibers by 30-40%. Also, it was observed an improved cellulose-water interaction that led to greater fiber swelling, which indicated that EG pretreatment allowed to relieve intrinsic recalcitrance of fibers in BEKP. Microscopy analysis showed that the enzyme-mediated pretreatment caused a predominant reduction in fiber length and size, which could enhance subsequent defibrillation by mechanical treatment for CNF extraction.

Results presented in this section showed how beneficial the addition of xylanases and swollenin to cellulase pretreatment can be in enhancing major fiber characteristics such as fiber swelling and overall cellulose accessibility, which could facilitate cellulase-mediated pretreatment of BEKP for further CNF extraction. Also, based on the results achieved, it can be concluded that by selecting the appropriate enzyme complex or monocomponent, it is possible to benefit the extraction of CNF by mechanical treatment without hydrolyzing cellulose to soluble sugars. Besides, the enzyme-mediated pretreatment of BEKP could afford significant amount of sugars released as a coproduct, which can have further biotechnological applications through the production of value-added chemicals.

4.2. Isolation and characterization of CNF from BEKP using ultrasonication

4.2.1. Background

Several methods have been recommended to produce CNM, but mechanical treatments are typically employed for CNF production from cellulosic materials, which includes grinding, refining, high pressure homogenization, microfluidization, ultrasonication and ball milling [125]. These treatments operate under high shear force which induce fiber fibrillation by facilitating the cleavage of cellulose fibers. However, the high energy consumption associated to these treatments along with the heterogeneity degree in fibrillation still represent the limiting factors on the implementation of these methods. To overcome this, the combination of enzymatic or chemical pretreatments are currently being proposed to facilitate mechanical treatment by removing the non-cellulosic components and, thus, enhancing cellulose accessibility.

Ultrasonication, which consists of the exposure of the suspension to ultrasonic waves, is one of the mechanical methods used to produce CNFs and it was shown to be very effective for cellulose depolymerization [31]. During the ultrasonication process, highly intensive waves are produced by the formation, expansion, and implosion of microscopic gas bubbles when ultrasonic energy is absorbed by molecules, creating hydrodynamic forces that are used for cellulose nanofibers liberation from the material [126]. Process optimization using ultrasonication for cellulose fibrillation has previously been reported. Mainly, the effect of temperature, suspension concentration, sonication power and time on fibrillation efficiency was evaluated [127]. However, these conditions greatly depend on the type of cellulosic material used, for example, suspension concentration depends on the size of the fibers, since longer size fiber needs to be treated at lower concentrations. On the other hand, few studies have been reported on combined enzymatic and ultrasonication pretreatment for CNFs production. Filson et al. [19] obtained CNFs from enzyme-treated recycled pulp by sonication and studied the effect of enzymatic pretreatment conditions on the CNF extraction yields. Also, Tsukamoto et al. [128] proposed a method to isolate CNFs from enzymatic hydrolysis residues of citrus processing waste using an ultrasonic processor. De Campos et al. [40] produced CNFs from dewaxed and bleached curauá and sugarcane bagasse using enzymes under different enzymatic pretreatment conditions, followed by ultrasonication.

In the following section, the ultrasonication treatment was assessed as a mechanical method for cellulose nanofibrillation and, thus, CNF extraction from enzyme-treated BEKP. The effect of ultrasonication process conditions such as suspension consistency, sonication intensity and treatment time on the CNF extraction yields was first investigated. Upon selection of the working condition for the ultrasonication method that maximizes CNF extraction yield, the CNF extraction from different enzyme-treated BEKP samples that were presented in the previous section was

assessed. The effect of the enzyme-mediated pretreatment of BEKP on the CNF extraction yields and CNF properties was investigated. Finally, nanocellulosic films of CNF were prepared by casting, and some of their properties were investigated.

4.2.2. Evaluating the ultrasonication conditions to maximize CNF extraction yield

The extraction of CNF from BEKP was performed by enzyme-mediated ultrasonication treatment, being the ultrasonication step important for fiber defibrillation. Process conditions such as suspension consistency, ultrasonication intensity (amplitude) and time were evaluated during ultrasonication. Fig. 4.2.1 and Table 4.2.1 present the results obtained which were analyzed in terms of CNF extraction yields when varying the suspension consistency in the range of 0.1 to 0.5%, the treatment time in the range of 30 to 120 min, and the ultrasonication intensity (as ultrasonication amplitude) in the range of 50 to 90%. It can be observed that relatively high CNF extraction yields of 61-93% were reached for enzyme-treated BEKP samples. This indicates that cavitation by ultrasound hydrodynamic forces was effective for opening the microfiber structure, releasing the nanofibrils from the fiber cell wall.

Table 4.2.1. CNF extraction yields and transmittance values of 0.1% CNF suspensions obtained under the different ultrasonication conditions evaluated from enzyme-treated BEKP.

Substrate	Suspension consistency (%)	Amplitude (%)	Time (min)	CNF yield (%)	Transmittance at 800 nm (%)
Enzyme-treated BEKP ^a	0.07	90	30	54.1 ± 2.9	75.2 ± 1.5
			60	78.9 ± 4.9	85.2 ± 2.0
	0.15	90	60	92.7 ± 2.0	89.6 ± 1.8
	0.20	90	60	93.0 ± 2.7	95.4 ± 1.5
	0.30	50	60	61.3 ± 4.4	82.8 ± 2.0
		70	60	74.5 ± 1.4	81.5 ± 1.2
		90	60	92.7 ± 2.0	93.7 ± 1.1
	0.50	90	120	86.5 ± 4.0	92.4 ± 1.8
			60	77.0 ± 3.0	89.7 ± 1.6

^aEnzymatic pretreatment condition: 4% solids, 4 h, CTec3 at 4.2 mg_{protein}/g_{BEKP}.

To evaluate the effect of suspension consistency and treatment time on CNF extraction, the ultrasonication was performed at maximum amplitude (90%). The CNF extraction yield was increased from 79% to 93% by increasing the suspension consistency from 0.07% to 0.15% when ultrasonication treatment was performed for 60 min (Fig. 4.2.1a). While increasing the fibers concentration in the suspension, the hitting and crashing effects among the fibers that are accelerated by the microbubbles aquatic force became significant, enhancing the disintegration

of the fibers and the degree of nanofibrillation. No significant difference was observed in CNF extraction yields when increasing suspension consistency up to 0.3%. However, CNF yield was significantly reduced to 77% when the suspension consistency was increased up to 0.5%, which suggests that further increasing solid concentration negatively affect the cellulose nanofibrillation. This could be due to poor agitation and inadequate stirring at high solid concentrations by the microbubbles force generated during ultrasonication, so the fibers have a lower chance of passing the probe tip. A similar effect was also reported by Wang and Cheng [127] with increasing concentrations of cellulose suspensions.

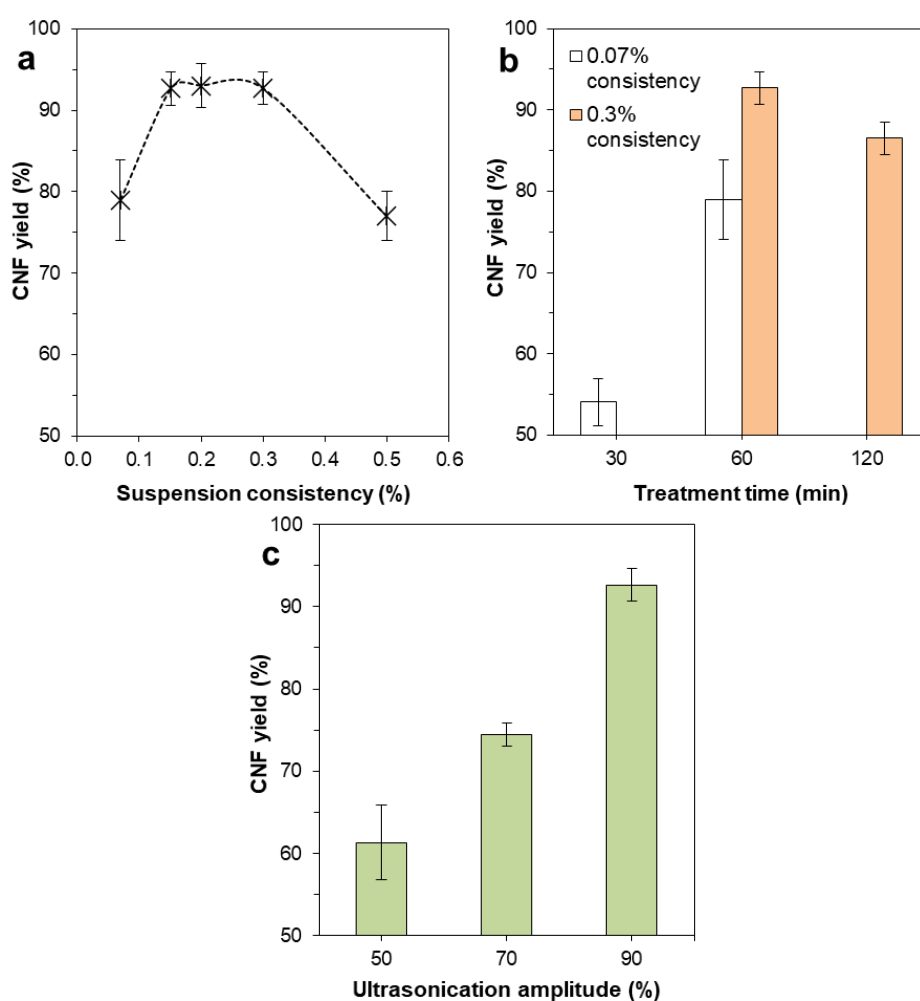


Figure 4.2.1. Effect of ultrasonication process conditions on CNF extraction yield for different conditions of: (a) suspension consistency at 90% amplitude for 60 min and, (b) treatment time at 90% amplitude, and (c) ultrasonication amplitude at 0.3% suspension consistency for 60 min.

Based on this finding, a suspension consistency of 0.3% was selected for further experiments, which allowed to obtain higher concentrations of CNF in supernatants, without negatively affecting the CNF extraction performance. Under this condition, extending the ultrasonication process time from 60 min to 120 min was then assessed to evaluate improvements in the CNF extraction performance due to a longer exposure time (Fig. 4.2.1b). However, no significant

difference was observed when the treatment time was increased to 120 min. It was also demonstrated that 30 min treatment was not enough for an efficient CNF extraction from the substrate when working at a low suspension consistency (Fig. 4.2.1b), even though the ultrasonication was performed at the maximum intensity (90% amplitude). Because of this, the treatment time was set at 60 min and the effect of ultrasonication amplitude on CNF extraction yield was then studied to see whether it was possible to perform the CNF extraction at lower treatment severities.

Regarding the ultrasonication intensity, CNF extraction yields changed greatly depending on the ultrasonication amplitude used (Fig. 4.2.1c and Table 4.2.1). It can be observed that the higher the ultrasonication amplitude used (90%), the higher the CNF extraction yield achieved (93%). When an ultrasonication amplitude of 50% was used, the CNF extraction yield resulted of only 61%, which increased to 74% by increasing the amplitude to 70%. It was demonstrated that working at higher operating amplitude facilitated cellulose disintegration and nanofibrillation, which led to almost complete conversion from microfibrils to nanofibrils in enzyme-treated BEKP. Based on the CNF extraction yields achieved for the different conditions evaluated, the process condition selected for the ultrasonication method that allows to achieve a maximum CNF extraction yield was 0.3% suspension consistency, 90% ultrasonication amplitude, and 60 min treatment time.

4.2.3. Evaluating the effect of enzymatic pretreatment on CNF extraction

Once the working condition for the ultrasonication method was selected, the degree of nanofibrillation in terms of CNF extraction yield was compared among different enzyme-treated BEKP samples (Table 4.2.2). The cellulose nanofibrillation degree achieved (93-98%) using enzyme-treated BEKP substrates resulted higher compared to the untreated BEKP substrate (33%). This demonstrated that enzymatic pretreatment using cellulases and/or xylanases effectively enhanced cellulose nanofibrillation during the subsequent ultrasonication process, enhancing the extraction of CNFs from BEKP. However, the relatively high CNF yield (33%) obtained from untreated BEKP proved the efficiency of the ultrasonication method for the extraction of CNFs from this type of cellulosic material, even though eucalyptus pulp presents shorter fibers compared to other woody materials (e.g. Pinus pulp) which could facilitate fiber disintegration.

It was presented and discussed in Section 4.1 that conditions during enzymatic pretreatment may cause fiber changes that could benefit the subsequent mechanical processing of the pretreated samples. Results showed that the nanofibrillation degree was increased when cellulase pretreatment was supplemented with xylanase enzyme, which allowed to achieve the highest CNF

extraction yield (97%). This correlates quite well with the increased cellulose accessibility and fiber swelling of the substrates previously discussed (Fig. 4.1.6 in Section 4.1.5). Even though it was previously demonstrated that swollenin addition during cellulase pretreatment enhanced cellulose accessibility by fiber swelling, no significant difference in cellulose nanofibrillation was observed with the addition of swollenin during enzymatic pretreatment, which also allowed to achieve almost complete conversion of microfibers to nanofibers (96%). This was expected due to the increased cellulose accessibility and fiber swelling observed for this enzyme-treated sample, which was previously discussed in Section 4.1.5. Moreover, these results demonstrated that the xylan removal achieved using swollenin during enzymatic pretreatment facilitated the initial fibrillation to separate cellulose fibrils, but had no significant effect on nanofibrillation through subsequent ultrasonication. However, it should be noted that, despite the extensive xylan removal under this condition (85%), no negative effect was observed on cellulose nanofibrillation in this study. It should also be mentioned that by extending the enzymatic pretreatment time from 4 h to 8 h, no significant improvement on CNF extraction yields was achieved for cellulase pretreatment substrates by ultrasonication (Table 4.2.2).

Table 4.2.2. Effect of enzymatic pretreatment on CNF extraction yields by ultrasonication.

Substrate	Enzymatic pretreatment conditions	CNF sample	CNF yield (%)
BEKP	na	US(BEKP)	33.1 ± 0.1
ET BEKP	4% solids, 4 h	US(C-4h)	92.7 ± 2.0
(CTec)	4% solids, 8 h	US(C-8h)	95.0 ± 2.5
ET BEKP	4% solids, 4 h	US(C+X-4h)	97.3 ± 1.1
(CTec+Xyn)	4% solids, 8 h	US(C+X-8h)	93.8 ± 5.1
ET BEKP	4% solids, 4 h	US(X-4h)	73.0 ± 1.1
(Xyn)	4% solids, 8 h	US(X-8h)	81.3 ± 0.3
ET BEKP	4% solids, 4 h	US(C+X+S-4h)	95.9 ± 0.2
(CTec+Xyn+Sw)	4% solids, 8 h	US(C+X+S-8h)	98.5 ± 1.5

Ultrasonication conditions: 0.3% suspension consistency, 60 min, 90% amplitude.

US:ultrasonication; ET: enzyme-treated; C: CTec; X: Xyn; S: Sw.

On the other hand, lower cellulose nanofibrillation was obtained using enzyme-treated BEKP with xylanase alone (73%), probably due to the lower cellulose accessibility of the substrate after enzymatic pretreatment for 4 h (Fig. 4.1.6 in Section 4.1.5). By extending the enzymatic pretreatment from 4 h to 8 h, it was possible to further improve the nanofibrillation of enzyme-treated BEKP by ultrasonication and, thus, the CNF extraction yield (81%). This improvement on CNF extraction yield may be caused by the increased cellulose accessibility and decreased DP

previously found for the substrate (Fig. 4.1.6 in Section 4.1.5) when enzymatic pretreatment with xylanases alone was performed for longer reaction times (8 h).

4.2.4. Characterization of CNF suspensions

Fig. 4.2.2 shows the physical appearance of the different CNF suspensions obtained after ultrasonication from the enzyme-pretreated substrates presented in Table 4.2.2. All the CNF suspensions were shown to be completely dispersed and stable for several weeks, since no phase separation was observed.

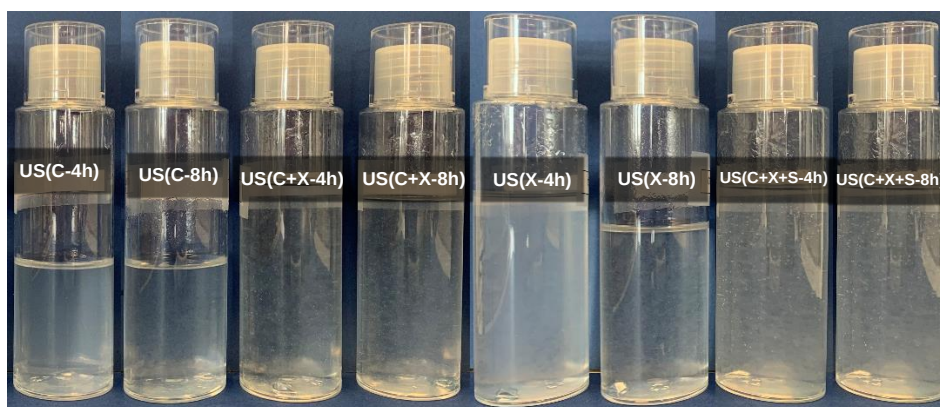


Figure 4.2.2. Visual aspects of 0.1% CNF suspensions after ultrasonication treatment at 0.3% suspension consistency and 90% amplitude for 60 min.

Recently, turbidity measured as the amount of light transmitted has been proposed as a quick method to assess the dispersion of CNMs [79]. Thus, lower turbidity of suspensions means more fibrillated CNFs. Because of this, light transmittance was determined by spectrometry to evaluate the opacity and ensure uniform nanofibrils morphology of CNF suspensions obtained after ultrasonication. Table 4.2.3 presents the results obtained for the different CNF samples, and UV-Vis transmittance spectra are presented in Appendix A4 (Fig. A4.1). Transmittance values of over 80% at 800 nm demonstrates CNF suspensions well dispersed in water and with nano-properties (Table 4.2.3). As expected, the use of xylanase enzymes with or without swollenin supplementation during cellulase pretreatment improved the transparency of CNF suspensions after ultrasonication (89–90% at 800 nm), which demonstrates an improved cellulose nanofibrillation. However, lower transparency values (82% at 800 nm) were obtained by using xylanase enzymes alone during enzymatic pretreatment, which suggests lower cellulose nanofibrillation degree. These results correlate quite well with the CNF yields previously discussed (Table 4.2.2). Also, it was observed that transparency values improved when enzymatic pretreatment was extended from 4 h to 8 h. For instance, an improvement of up to 12% was achieved when using the BEKP substrate pretreated with xylanase alone, which corresponds to sample US(X-8h) in Table 4.2.2, allowing to reach transmittance values above 90%.

Table 4.2.3. Effect of enzymatic pretreatment on CNF size determined by dynamic light scattering analysis, and zeta potential.

CNF sample	Average size (nm)	PDI ^a	Zeta potential (mV)	Transmittance at 800 nm (%)
US(C-4h)	550 ± 64	0.73 ± 0.16	-20.3 ± 1.1	89.8 ± 2.0
US(C-8h)	531 ± 93	0.56 ± 0.07	-16.6 ± 1.6	95.6 ± 1.5
US(C+X-4h)	838 ± 134	0.79 ± 0.19	-17.8 ± 2.0	89.5 ± 2.0
US(C+X-8h)	754 ± 38	0.67 ± 0.13	-17.5 ± 4.9	96.4 ± 1.5
US(X-4h)	2613 ± 500	0.97 ± 0.06	-5.9 ± 0.7	82.2 ± 1.0
US(X-8h)	992 ± 158	0.84 ± 0.14	-15.4 ± 1.2	92.0 ± 1.2
US(C+X+S-4h)	670 ± 27	0.75 ± 0.20	-20.7 ± 3.1	89.0 ± 1.0
US(C+X+S-8h)	627 ± 31	0.74 ± 0.04	-20.8 ± 0.06	96.2 ± 1.0

Ultrasonication conditions: 0.3% suspension consistency, 60 min, 90% amplitude.

US:ultrasonication; C: CTec; X: Xyn; S: Sw.

^aPDI: polydispersity index (values < 0.7 are considered homogeneous).

DLS analysis was performed on CNF suspensions to investigate the statistical distribution of the nanofibers present in the samples (Table 4.2.3). The determination of the average hydrodynamic size and the PDI of CNF size distribution gives an indication of the presence of large and/or agglomerate particles as well as the degree of homogeneity of the suspension. According to the results obtained, almost all samples resulted in the nanometer scale with average sizes ranging from 531 nm to 992 nm. Ultrasonication caused significant fiber size reduction and efficient nanofibrillation, which results in CNF extraction at high yields. Differences on the average size indicates that enzymatic pretreatment conditions influenced fiber fragmentation and/or fibrillation through fiber alterations. Employing cellulases alone or in combination with xylanases for enzyme-mediated pretreatment of BEKP favored the extraction of CNF, since average sizes of around 530 nm were reached. Also, relatively low PDI values (0.56-0.79) were achieved under these conditions, which indicates that ultrasonication exhibited homogeneous dispersion, mainly due to disruption of hydrogen bonds. However, the only sample which resulted in an average size in the micrometer scale (2.6 µm) was US(X-4h), which was obtained from substrate ET BEKP (Xyn) (Table 4.2.2). The highest PDI value (0.97) achieved for this sample may indicate the presence of larger particles which were not completely fibrillated during ultrasonication. Despite of this, a CNF yield of 73% was achieved in this case, and its size distribution confirms that there are particles in the nanometer scale (Fig. 4.2.3).

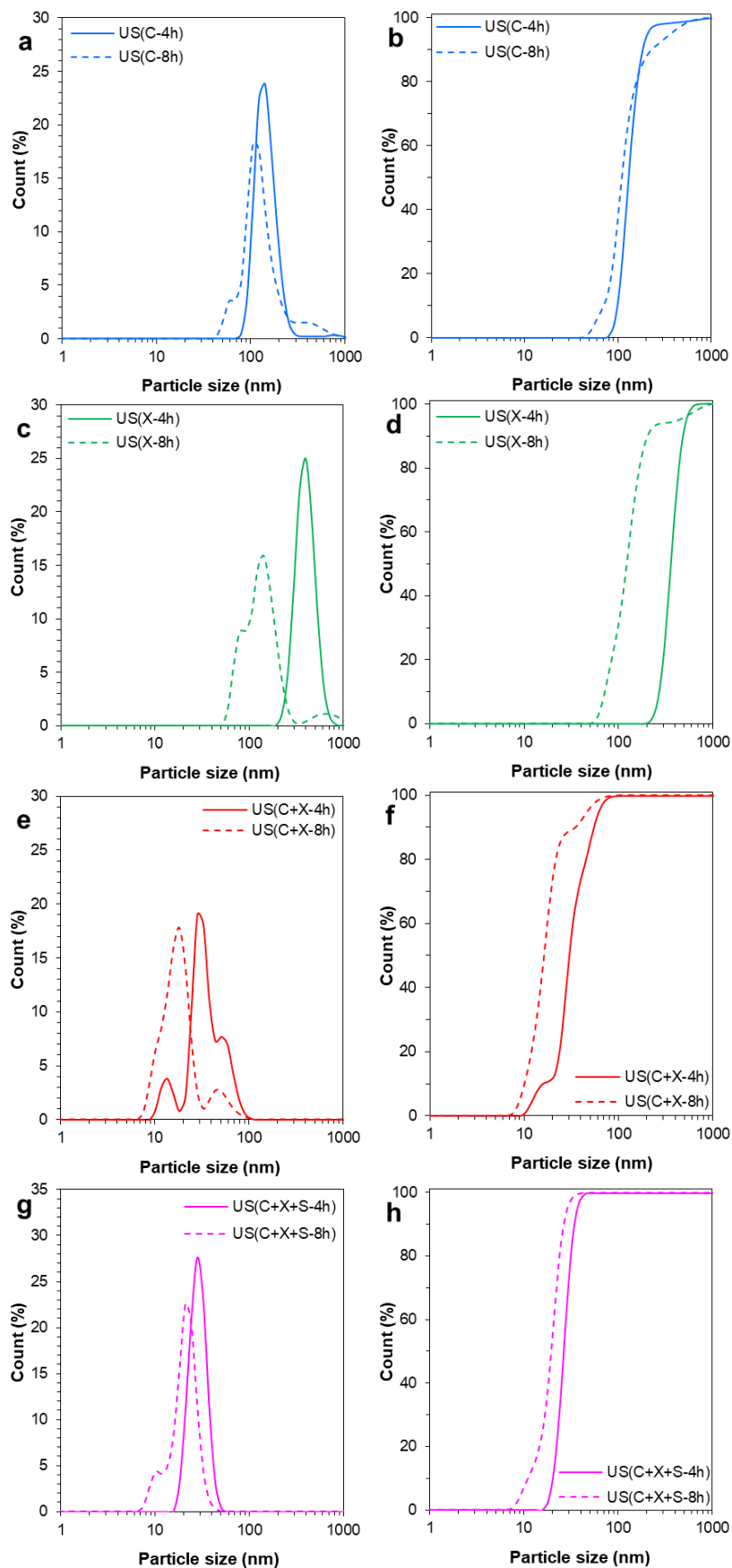


Figure 4.2.3. Size distributions of CNF samples through dynamic light scattering analysis: (a, c, e, g) probability density function, and (b, d, f, h) cumulative distribution function.

It should be mentioned that for all the enzymatic pretreatment conditions evaluated, extending the hydrolysis time from 4 h to 8 h provided higher fiber fragmentation and CNF extraction, since decreases of up to 62% on the average size was observed. The PDI also decreased, which indicates a greater CNF size homogeneity. According to size distributions presented in Fig. 4.2.3, a wide size dispersion was observed for samples US(C-8h), US(X-8h), US(C+X-4h), US(C+X-8h) and US(C+X+S-8h), which may be caused by agglomeration of nanomaterials.

Zeta potential (ZP) analysis is commonly reported within the literature and used to assess colloidal stability [79]. ZP determinations were performed to determine the surface charge and evaluate the electrostatic stability of the ultrasonicated CNF suspensions (Table 4.2.3). Generally, suspensions with absolute ZP values above 20 mV are considered colloidally stable [79]. Also, it was reported that absolute ZP values below 15 mV represent high tendency to agglomeration, and absolute ZP values above 30 mV are considerable desirable for good colloidal stability due to high electrostatic charges. Most of the CNF suspensions presented ZP values in the range of -21 mV to -15 mV, which indicates a partial colloidal stability due to the low presence of hydroxyl groups. The sample US(X-4h) was the only one that presented an absolute ZP value (-5.9 mV) below 15 mV, which indicates high tendency to agglomeration and flocculation. The lowest ZP value obtained for this sample was probably because of the larger average size (2.6 μm) which means lower surface area and, thus, lower availability of hydroxyl groups. This poor superficial charge results in high tendency to flocculation, which was also visible by eye (Fig. 4.2.2). The absence of strong charges on the fibers surface tends to cause a wide size dispersion, mainly because of the agglomeration of the nanomaterials. Despite of this, no phase separation was observed for any of the CNF suspension samples.

4.2.5. Assessing the morphology and size of CNF by microscopy analysis

To complement the DLS analysis, morphological analysis was then conducted. The morphology of selected CNF samples prepared through enzymatic and ultrasonication pretreatment was analyzed by AFM (Fig. 4.2.4). AFM observations revealed networked and ribbon-like CNF samples, with a quite uniform size distribution. Though the fibers were entangled by the long lengths, they were well dispersed into almost elementary fibril levels. The diameter of the nanofibrils was estimated from the height profile and resulted in the range of 3 to 10 nm for individual non-aggregated nanofibrils (Fig. 4.2.4). This indicates efficient fibrillation and separation of CNF through the combination of enzymatic and mechanical pretreatment. Long et al. [73] also produced CNFs from BEKP by enzyme-mediated pretreatment followed by ultrasonication. However, the diameters of CNFs obtained in this study (3–10 nm) were smaller than the nanocellulose products reported in their study (about 50–150 nm), which indicates

improved cellulose nanofibrillation. Also, Baati et al. [129] reported CNFs production with a fiber size in the range of 2 to 5 nm after using a twinscrew extruder for fibers disintegration, which were chemically pretreated (TEMPO-Mediated oxidation) prior to mechanical pretreatment. In their study, smaller CNF sizes were mainly due to the stronger pretreatment which facilitated cellulose disintegration and further nanofibrillation, with a quite uniform width distribution.

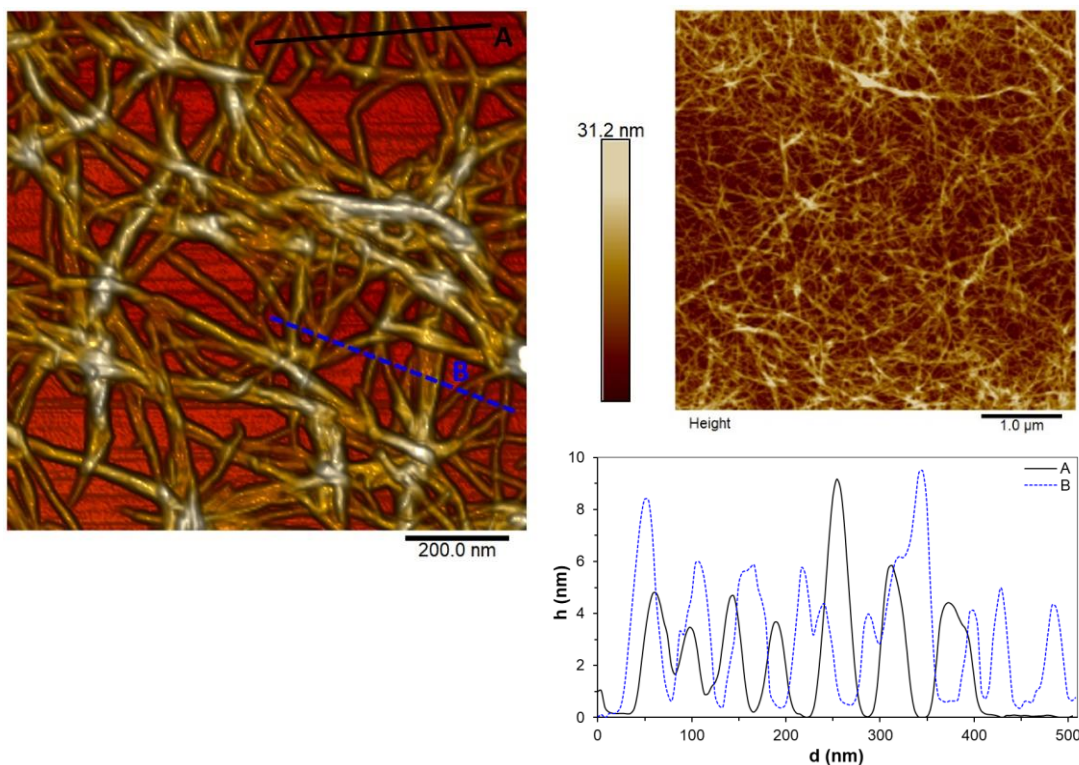


Figure 4.2.4. AFM images of CNF extracted by ultrasonication from enzyme-treated BEKP, and cross-section profile analysis (the arrows A and B mark the points used for height measurements).

4.2.6. Preparation and characterization of CNF films

Nanocellulosic films were then prepared by simple casting evaporation at 105°C of the different CNF suspensions obtained after ultrasonication treatment. After casting, the visual appearance of the films is presented in Fig. 4.2.5. All the nanocellulosic films produced were highly transparent and flexible. The flexibility of the films can be attributed to the fact that CNF has flexible fibril structure. As can be seen in Fig. 4.2.5, the lettering “Universidad de la República Uruguay” printed on the underlying sheets was clearly visible through the nanocellulosic films, without distance between the films and the underlying sheets.

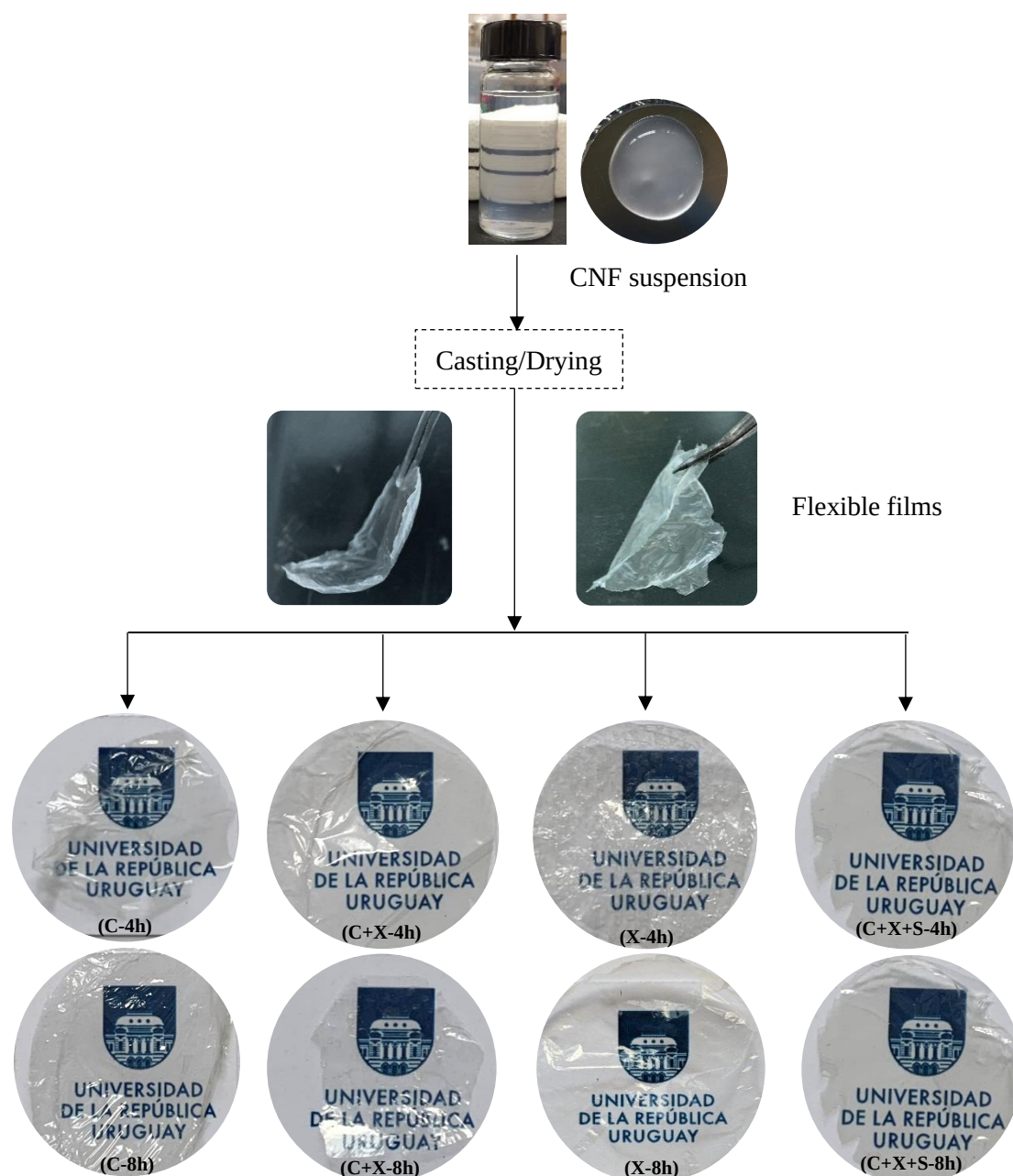


Figure 4.2.5. Visual aspects of the nanocellulosic films prepared by casting evaporation from 0.1% CNF suspensions obtained after ultrasonication treatment.

The crystalline behavior of CNF films obtained after ultrasonication was investigated using XRD. Fig. 4.2.6a shows XRD pattern for sample US(C+X-4h) and compares it with XRD pattern for untreated BEKP and enzyme-treated BEKP (sample ET BEKP CTec+Xyn)). The XRD spectra for all the samples presented a similar trend exhibiting the principal crystal planes (110), (102), and (200) at 2θ approximately 15° , 16° , and 22° , respectively, as it was already discussed in Section 4.1.5 for the enzyme-treated BEKP samples. The small and broad peak at around 2θ of 35° represents the contribution of crystal plane (040). However, XRD measurements for the nanocellulosic films showed that CNF obtained after ultrasonication resulted in lower CrI values

(67–76%) compared to that of the enzyme-treated BEKP samples. This indicates breakage of the intermolecular hydrogen bonds between cellulose chains during mechanical treatment, which caused damage or peeling of the cellulose crystalline structure. This was also observed by other authors, who reported a certain degree of damage to the CNF crystalline region when ultrasonication pretreatment was performed as CNF extraction method [130].

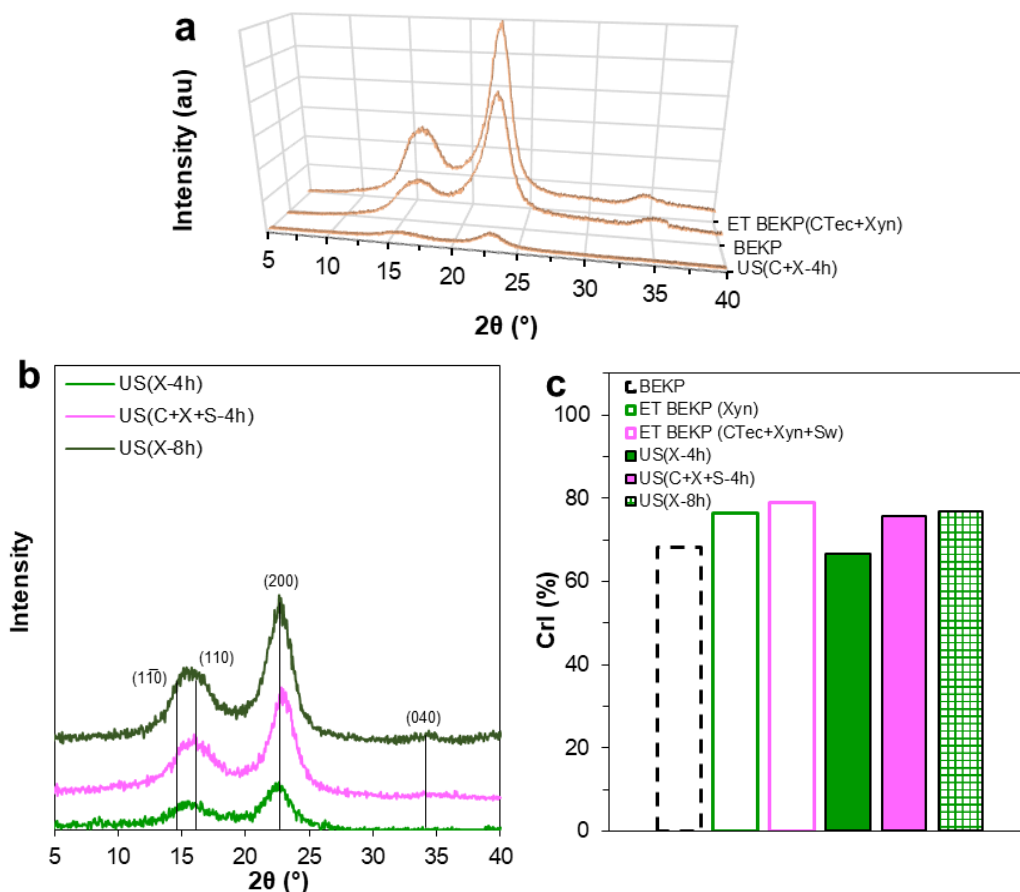


Figure 4.2.6. (a, b) XRD patterns and (c) CrI of CNF samples obtained under different enzymatic pretreatment conditions. (Note: CTec: CTec3; Xyn: HTec; Sw: swollenin.)

The impact of ultrasonication treatment on the thermal stability of BEKP cellulose was investigated by thermogravimetric analysis (TGA) of CNF films. Fig. 4.2.7 shows the TGA and derivative thermogravimetric (DTG) curves of the different samples. According to the TGA curves, samples exhibited the three main weight-loss regions during heating, which were previously discussed in Section 4.1.5. Table 4.2.4 summarizes the thermal properties of the different samples. After ultrasonication treatment, the main thermal decomposition range of the BEKP cellulose decreased, as well as the temperature at which the maximum weight loss occurred decreased ($T_{\max}$) compared to the enzyme-treated BEKP samples (Table 4.1.2 in Section 4.1.5).

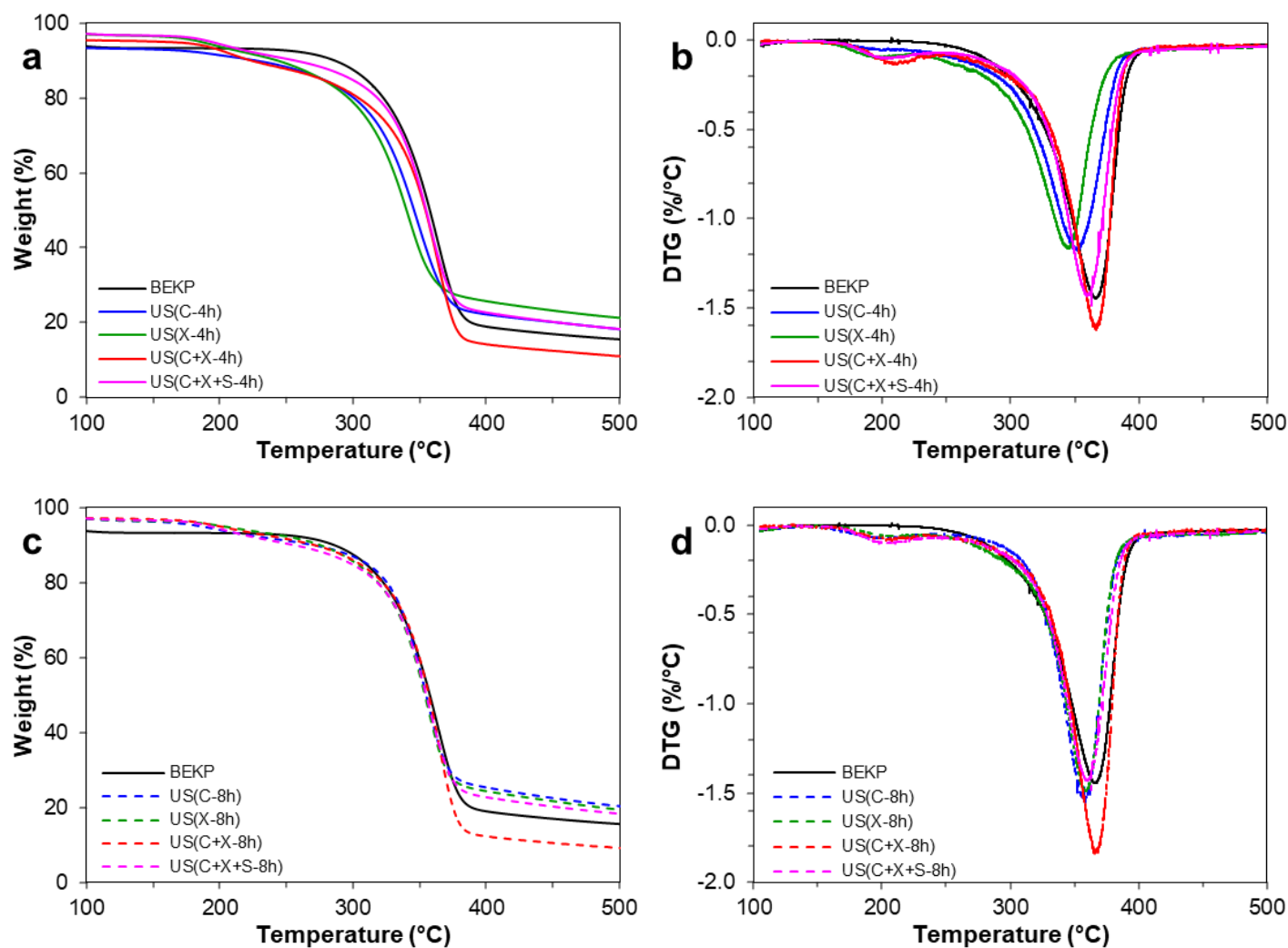


Figure 4.2.7. (a, c) Thermogravimetric analysis and (b, d) derivative thermogravimetric (DTG) curves of CNF produced from enzyme-treated BEKP under different enzymatic pretreatment conditions. (Note: CTec3: CTec3, Xyn: HTec; Sw: swollenin; solid lines: 4 h hydrolysis; dashed lines: 8 h hydrolysis.)

On the other hand, the obtained CNF samples had a lower T_{on} and T_{max} , due to damages in cellulose crystalline region that occurred during ultrasonication, as it was already observed by XRD analysis. Moreover, several factors such as size reduction, degree of polymerization decrease, and specific surface area increase that occurred during ultrasonication may decrease the thermal stability of the samples. However, the thermal stability of CNF samples obtained in this study resulted better than that previously reported for CNFs produced by TEMPO-mediation oxidation or acid hydrolysis [131, 132].

Table 4.2.4. Thermal properties of CNF extracted from enzyme-treated BEKP by ultrasonication.

CNF Sample	T_{on} (°C)	T_{max} (°C)	Residue (%)
US(C-4h)	300	350	14.9
US(C-8h)	318	358	17.7
US(X-4h)	292	345	19.0
US(X-8h)	318	358	16.8
US(C+X-4h)	324	368	11.1
US(C+X-8h)	325	370	10.0
US(C+X+S-4h)	320	360	16.0
US(C+X+S-8h)	320	358	15.5

According to TGA measurements, the amount of residue resulted higher for some conditions compared to the enzyme-treated substrate before ultrasonication (Table 4.1.2 in Section 4.1.5). During ultrasonication, wear of the titanium tip was observed, and CNF suspensions had a relatively dark grey color after treatment. This grey color indicated the presence of titanium particles, which were easily removed from the CNF suspension by dilution and centrifugation. These titanium particles were removed from the ultrasonicated samples as they changed the appearance of the samples and may affect the samples' characterization. The visual appearance of CNF suspensions after titanium particles removal was illustrated in Fig. 4.2.2. XRF analysis was performed on nanocellulosic films and results showed that titanium was not detected in the supernatant fraction of ultrasonicated suspensions. This may indicate that titanium particles were completely removed by centrifugation, or its content was too low to be detected by the equipment.

4.2.7. Summary

The ultrasonication treatment resulted effective for the nanofibrillation of enzyme-pretreated BEKP, allowing to achieve CNF extraction yields in the range of 61 to 93%. The improved cellulose nanofibrillation degree achieved using enzyme-treated BEKP substrates compared to the untreated BEKP (33%) demonstrates that enzyme-mediated pretreatment using cellulases and/or xylanases effectively enhanced the extraction of CNF from BEKP during the subsequent

mechanical treatment. Cavitation caused by ultrasonic hydrodynamic forces was effective in opening the structure of cellulose microfibrils and releasing the nanofibrils from the cell wall of the cellulosic material.

Regarding the ultrasonication process working conditions, the ultrasonication amplitude turned out to be the most significant variable in the isolation of CNF. Working at higher operating amplitude (90%) facilitated cellulose disintegration, which led to almost complete conversion from microfibrils to nanofibrils (CNF extraction yields up to 98%). Moreover, the relatively high CNF extraction yield (33%) achieved for the untreated BEKP at the highest ultrasonication intensity demonstrates the efficiency of the ultrasonication process for cellulose nanofibrillation. Ultrasound waves in ultrasonication generated a good defibrillation of enzyme-treated cellulose fibers, which allowed to obtain greater uniformity of CNF sizes, smaller diameters, good stability and transmittance of suspensions, and high CNF extraction yields.

Physicochemical and microscopic characterization of the obtained CNF suspensions was performed as well as for the nanocellulosic films produced by casting. CNF suspensions resulted with good stability and dispersion despite the relatively poor surface charge (around -20 mV). The size distribution obtained by DLS analysis revealed uniform nanometer ranges of CNF, with average sizes ranging from 531 nm to 992 nm. AFM observations revealed ribbon-like and entangled CNFs with diameters in the range of 3 nm to 10 nm, which were well dispersed into almost elementary fibril levels.

The nanocellulosic films obtained by casting evaporation resulted highly transparent and flexible, which results as attractive properties for potential applications to electronic devices, for instance. XRD and TGA analysis performed to nanocellulosic films showed that ultrasonication caused damages in the cellulose crystalline region that decreases the crystallinity degree and thermal stability of CNF. However, the thermal stability of the CNF samples obtained in this study resulted better than those CNFs produced through the chemical pathway reported in the literature.

Titanium contamination during ultrasonication treatment, which results to be one major challenge for this mechanical method for CNF extraction, was confirmed by XRF. These titanium particles, which were easily removed from CNF suspension by centrifugation, were mostly associated to the wear of the titanium tip during ultrasonication,.

Results presented in this section demonstrated that enzyme-mediated pretreatment followed by ultrasonication constitutes a potential strategy to isolate nanofibrils from different cellulosic materials.

4.3. Exploring the extraction of CNF from enzyme-treated BEKP by ball milling

4.3.1. Background

Ultrasonication treatment resulted effective to produce CNF at high yields from BEKP after enzymatic pretreatment, as it was discussed in previous Section 4.2. However, low sample concentrations needed to be processed for an effective treatment implies that CNF suspensions are obtained at relatively low consistency (~0.3%). Because of this, and with the aim to find an environmental process with high yields and low costs, ball milling was studied as an alternative mechanical treatment for CNF extraction from enzyme-treated BEKP. While the ultrasonication equipment applies the use of oscillating power to create hydrodynamic forces of ultrasound, the ball mill involves friction and shear forces that can convert particles from micrometer to nanometer scale. The movement of the milling jar creates the force and induce high shear forces among balls and balls with the jar's surface, causing the breakage of materials into smaller sizes with large surface areas. However, there are several variable factors which affects the characteristics of the ball milled products. Type of ball, number and size of balls, milling time, suspension consistency, state of milling (dry or wet), and frequency or angular velocity of milling jar are among significant factors to be considered during ball milling treatment.

Since the early 2010s, ball milling was studied for the extraction of CNM from different cellulosic feedstocks [46]. Baheti et al. [133] compared the performance of ball milling of jute fiber wastes to produce CNM in a dry and wet state (with deionized water). In the dry state, the raw material was stuck and aggregated on the milling balls and jar wall which affected the disintegration of the material and, thus, the size distribution of the milled product. They did not find this phenomenon in the wet state ball milling, in which they obtained a narrow particle size distribution of the CNM products in the range of 100 to 1000 nm. They concluded that the addition of liquid (water or other solvent) during ball milling protects agglomeration of the materials inside the milling jar and enables a more uniform size reduction during treatment. Also, the liquid medium partly preserved the elementary fibrils from the violent shear and impact force generated from balls friction and collision that damages and crushes the fibers. However, it was also reported that the ball milling conditions should be carefully adjusted to obtain product sizes in the nanometer scale (smaller than 100 nm). Zhang et al. [134] studied the ball milling of northern bleached softwood Kraft pulp to produce CNM and found that the size of the balls results important for controlling the nanomaterial product size and properties. Also, they reported that the selection of the ball to solid ratio and the milling time is critical for controlling the balls collisions to produce CNM of fibrous morphology within 100 nm of diameter.

Nuruddin et al. [41] extracted CNF with the size of 8-100 nm in diameter from kenaf fibers and wheat straw by ball milling after 2 h treatment, which demonstrated that ball milling can reduce both length and diameter of the material. Moreover, Sofla et al. [42] extracted CNF from delignified sugarcane bagasse, and those CNF ranged in size from micrometer to nanometer scale and presented higher thermal stability compared to CNC extracted by acid hydrolysis. More recently, Zeng et al. [135] isolated CNF from bleached softwood Kraft pulp using different mechanical treatments including ball milling, and evaluated its combination with ultrasonication to obtain more uniform size distributions of CNF and higher aspect ratios.

In this context, ball milling treatment represent an attractive alternative method for the isolation of CNF from cellulosic materials owing to its simple use, economical equipment and applicability to most types of biomass. Also, it could produce CNM in large quantities at room temperature and pressure by carefully controlling the processing conditions. In the following section, ball milling in a wet state was assessed as a mechanical method for the extraction of CNF from enzyme-treated BEKP. The ball milled products were characterized to study the effects of the variable factors ball to solid ratio (BSR), milling time and milling frequency on the CNF extraction yield, suspensions properties (transmittance, sedimentation), and size and morphology. Crystallinity and thermal analysis of ball milled products were also performed.

4.3.2. Preliminary evaluation of ball to solid ratio effect during ball milling treatment on CNF extraction

Since previous studies regarding ball milling treatment were scarce, treatments were initially conducted for 90 min and 180 min at maximum frequency (20 Hz) varying the BSR in the range of 40 to 200 $\text{g}_{\text{balls}}/\text{g}_{\text{solid}}$ for a preliminary evaluation of the effect of ball milling conditions on CNF extraction. Fig. 4.3.1 shows the gel-like aspect of the ball milled products obtained. The stability of the CNF suspensions was first assessed by sedimentation studies, and a preliminary evaluation of the CNF size was performed by DLS analysis. For the sedimentation tests, the CNF suspensions obtained after ball milling treatment were diluted to 0.2% consistency (Fig. 4.3.1) and allowed to settle down for 7 days (168 h) at room temperature ($\sim 21^{\circ}\text{C}$). Images of phase separation and sedimentation for the conditions evaluated are presented in Appendix A6.



Figure 4.3.1. Visual appearance of (a) 1% CNF suspension after ball milling treatment performed at 20 Hz and BSR of 80 g/g for 90 min, and (b) 0.2% CNF suspension after dilution.

Fig. 4.3.2 presents the sedimentation ratios (H_s/H_o) and Table 4.3.1 presents the average sizes determined by DLS analysis for the preliminary conditions evaluated. The sedimentation tests of these preliminary studies showed less stability of the CNF suspensions with increasing BSR. For a milling time of 90 min, higher H_s/H_o values were obtained for samples BM40-90, BM80-90 and BM120-90 (0.90) compared to samples BM160-90 (0.60), BM200-180 (0.55) and enzyme-treated BEKP (0.10). Even though a rapid phase separation was observed for sample BM120-90, the H_s/H_o value achieved after 72 h remained the same as samples BM40-90 and BM80-90. However, it should be noted that significant differences on suspensions sedimentation were observed for samples before (untreated) and after ball milling treatment. An instant phase separation was observed for the untreated sample (without ball milling treatment), which indicates that changes are occurring during ball milling treatment of enzyme-treated BEKP (Appendix A6).

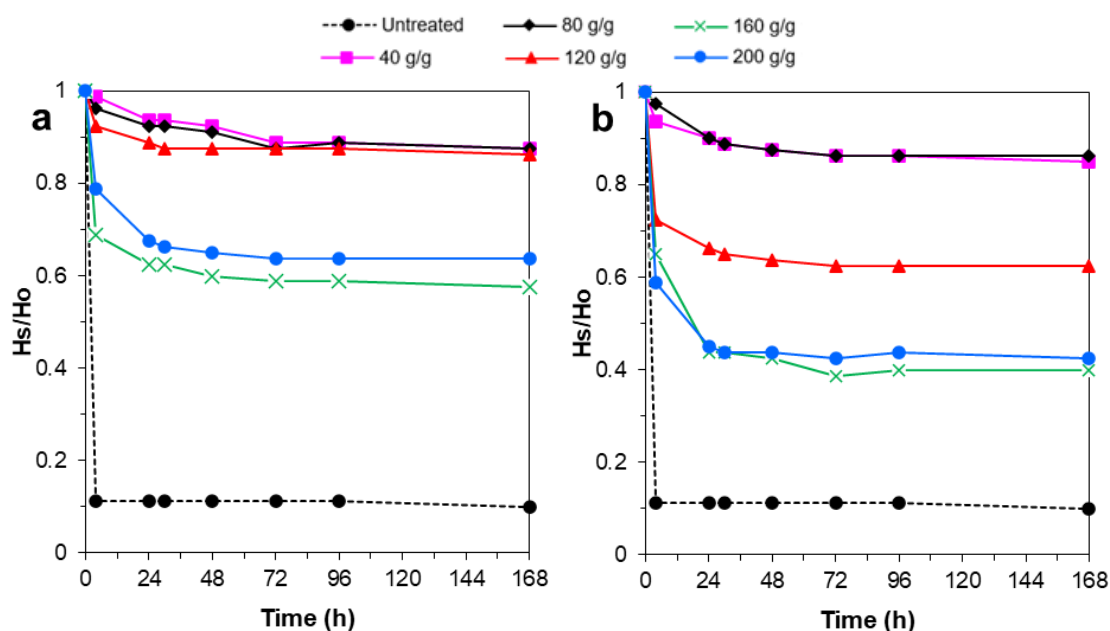


Figure 4.3.2. Effect of ball to solid ratio (BSR) on CNF suspension sedimentation ratio (H_s/H_o) after ball milling treatment performed at 20 Hz for (a) 90 min, and (b) 180 min.

On the other hand, a decrease of suspension stability was found with increasing milling times for samples obtained at a BSR higher than 80 g/g (samples BM120-180, BM160-180 and BM200-180). Interestingly, no significant differences on suspension sedimentation were found for samples BM40-180 and BM80-180, which were obtained at a lower BSR (40 g/g and 80 g/g, respectively). For samples BM160-180 and BM200-180, a greater phase separation was observed reaching Hs/Ho values of 0.40-0.45, which shows a poorer stability of CNF suspensions compared to samples BM160-90 and BM200-90. The same effect was observed for sample BM120-180, in which in this case the suspension stability greatly decreased with milling time, since Hs/Ho values of 0.90 and 0.55 were reached for 90 and 180 min, respectively.

Table 4.3.1. Effect of ball milling conditions on CNF size distribution and zeta potential.

Substrate	Ball milling conditions		CNF sample	Average size (nm)	PDI ^b	Zeta potential (mV)
	BSR (g/g)	Time (min)				
Enzyme-treated BEKP ^a	40	90	BM40-90	4929 ± 775	0.95 ± 0.14	-10.2 ± 0.7
		180	BM40-180	2578 ± 1003	0.85 ± 0.16	-7.0 ± 1.5
	80	90	BM80-90	1116 ± 87	0.76 ± 0.12	-10.5 ± 1.6
		180	BM80-180	702 ± 46	0.39 ± 0.07	-8.6 ± 2.6
	120	90	BM120-90	1490 ± 175	0.62 ± 0.16	-13.7 ± 0.8
		180	BM120-180	695 ± 16	0.58 ± 0.03	-12.2 ± 0.4
	160	90	BM160-90	1869 ± 157	0.70 ± 0.09	-10.6 ± 1.3
		180	BM160-180	1704 ± 344	0.81 ± 0.18	-10.1 ± 1.0
	200	90	BM200-90	1805 ± 424	0.84 ± 0.16	-13.5 ± 0.6
		180	BM200-180	1244 ± 34	0.66 ± 0.10	-8.2 ± 1.8

^aEnzymatic pretreatment condition: 4% solids, 4 h, CTec3 and HTec at 4.2 mg_{protein}/g_{BEKP}.

^bPDI: polydispersity index (values < 0.7 are considered homogeneous).

To better understand the CNF extraction process during ball milling treatment of enzyme-treated BEKP, size evaluation of ball milled samples was performed by DLS analysis. As it was already discussed in Section 4.2.4, the determination of the average hydrodynamic size and the PDI gives an indication of the presence of large particles or agglomerates and the degree of size homogeneity in the sample, respectively. According to DLS analysis, average hydrodynamic sizes resulted in the range of 700 nm to 4.9 µm for all the conditions evaluated (Table 4.3.1). Considering that average hydrodynamic sizes higher than 25 µm were determined for the enzyme-treated materials before mechanical treatment (see Fig. A10.4 in Appendix A10), DLS results demonstrated that ball milling treatment effectively contributes to fiber size reduction and CNF extraction. This considerable fiber fragmentation results from the high shear forces produced from the collisions between the balls of different sizes and the friction between the balls and the jar's wall.

However, the ball milling treatment performed at a BSR of 40 g/g (samples BM40-90 and BM40-180) was not enough to completely break the cellulose microfibrils into nanostructures, since average sizes (4.9 μm and 2.6 μm , respectively) of the samples obtained were not in the nanometer scale (Table 4.3.1). Besides, CNF suspension resulted heterogeneous since a non-uniform size distribution was obtained for these samples (Fig. 4.3.3), with minimum and maximum sizes of around 50 nm and above 1 μm . This indicates that some microfibrils have better nanofibrillation while others remain in the micrometer scale which could be due to an uneven milling process, in which some fibrils were not ground enough, and other fibrils were ground too much. Similar results were also reported by other authors [133, 42]. For instance, Sofla et al. [42] extracted CNF from sugarcane bagasse by ball milling (BSR 80 g/g, 0.5 mm zirconia balls, 1 h) and achieved CNF structures with sizes of 10 μm but also structures of 1 μm , which was due to an uneven breakage of the fibers. Previously, Baheti et al. [133] studied the CNM extraction from jute fibers by ball milling (BSR 10 g/g, 3 mm and 10 mm zirconia balls, 3 h) and reported the formation of fibrous materials that coated the balls and decreased the impact force of the balls and the fibers, which resulted in some structures remaining as longer fibers in the micrometer scale.

On the other hand, DLS analysis showed a greater reduction in material size with increasing BSR and milling time, reaching average sizes in the nanometer scale (around 700 nm) for BSR between 80 g/g and 120 g/g (Table 4.3.1). Incrementing the ball milling time increases the collision incidents with balls and CNF suspension in the milling jar, which contributes to higher fiber fragmentation. For instance, for a BSR of 80 g/g, it was observed that as milling time increased from 90 min to 180 min, the bimodality in size distribution reduced giving a narrower size distribution (Fig. 4.3.3). A better size distribution was also obtained for a BSR of 120 g/g when milling time was increased from 90 min to 180 min, even though a bimodal distribution was observed in this case. However, further increasing the BSR from 120 g/g to 200 g/g did not cause a significant decrease on the average size. One possible explanation to this could be the fact that overloading the jar with balls may not cause enough treatment intensity due to poor movements of the balls in the milling jar and, thus, inefficient collisions among balls and materials caused poor fiber fragmentation.

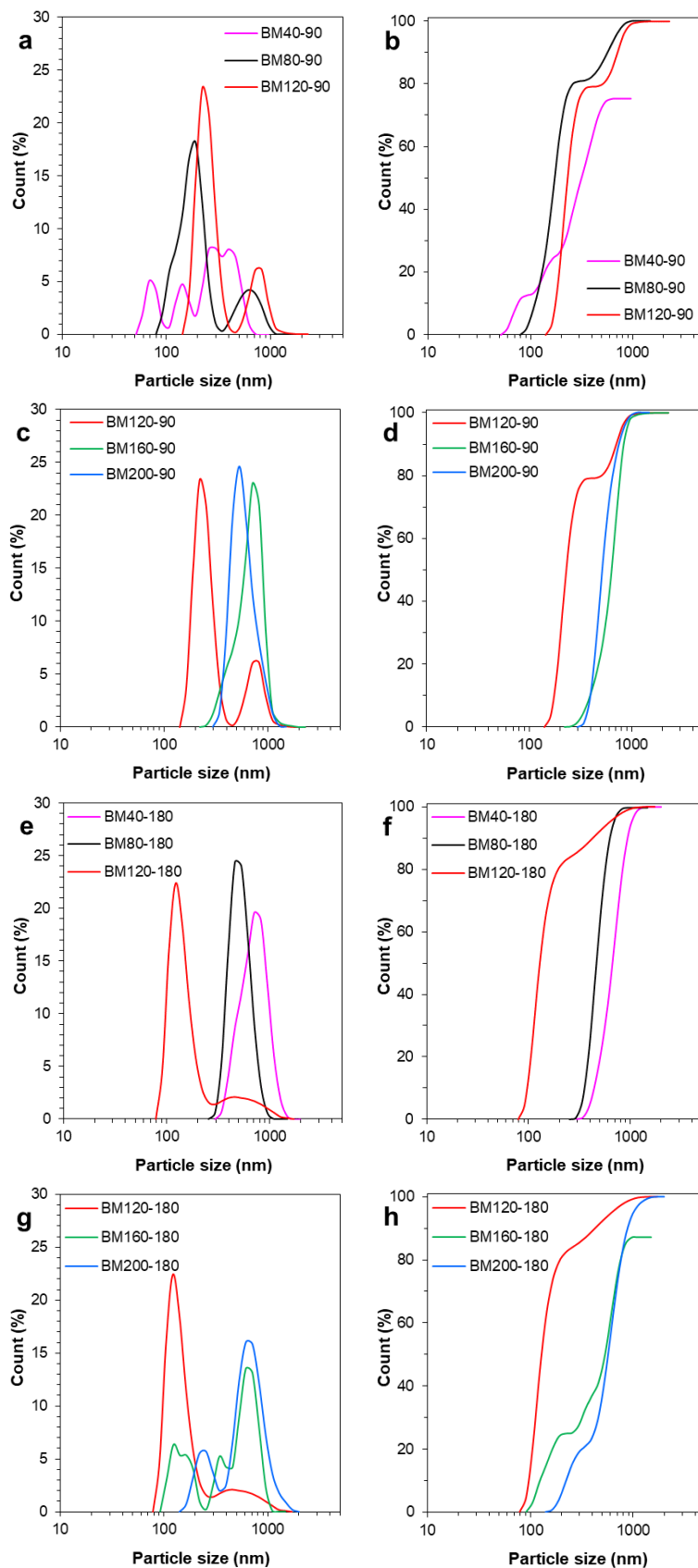


Figure 4.3.3. Effect of ball to solid ratio (BSR) on CNF size distributions after ball milling treatment performed at 20 Hz for (a-d) 90 min and (e-h) 180 min through dynamic light scattering analysis: (left) probability density function, and (right) cumulative distribution function.

Results showed that CNF samples obtained by ball milling treatment presented higher dispersibility compared to ultrasonicated samples, since PDI values in the range of 0.4 to 0.9 were obtained in this case (Table 4.3.1). Also, it should be mentioned that wide size distributions were obtained for ball milled CNF samples (Fig. 4.3.3) compared to the ultrasonicated ones (Fig. 4.2.3). This may indicate that samples contain higher particle sizes since it was demonstrated in Section 4.2 that ultrasonication promotes a better disintegration of the cellulose fibers compared to ball milling.

To complement the DLS and sedimentations analysis, UV-Vis spectroscopy was then performed to evaluate the effect of BSR and milling time on the transmittance of CNF suspensions, since it represents a simple way to get an approximate idea of the fibrillation extent and the dispersibility of CNF. Fig. 4.3.4 presents the transmittance spectra of the different CNF suspensions and the effect of BSR and milling time on transmittance values at 800 nm.

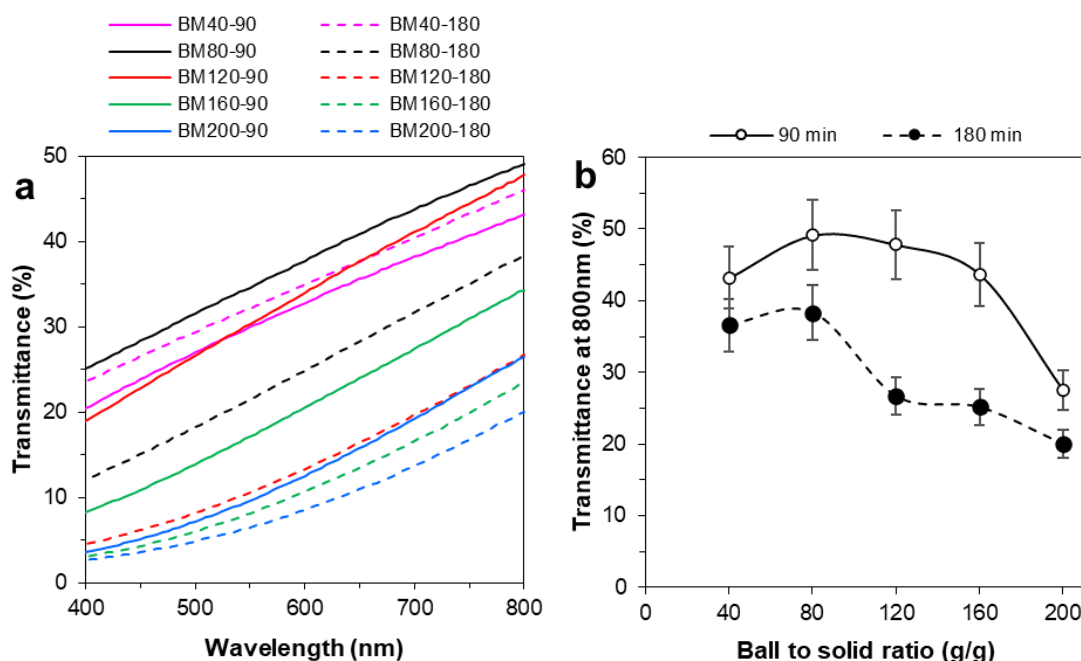


Figure 4.3.4. (a) UV-Vis transmittance spectra of 0.1% CNF suspensions, and (b) the effect of ball to solid ratio (BSR) on 0.1% CNF suspension transmittance at 800 nm after ball milling treatment performed at 20 Hz for 90 min and 180 min.

It was observed that while increasing the milling time, transmittance values abruptly decreased, especially for BSR higher than 80 g/g. In general, it is expected that the transmittance of CNF increases by increasing intensity of the mechanical treatment in terms of number of balls (expressed as BSR), milling frequency, and/or milling time. This is because higher fiber disintegration and greater nanofibrillation is expected with higher treatment severity. However, according to Fig 4.3.4, it was observed that increasing milling time from 90 min to 180 min negatively affects the transmittance of the CNF suspensions. For instance, samples BM80-90 and

BM120-90 had the highest transmittance values at 800 nm (almost 50%), which means that these samples had the smallest size and relatively uniform size distributions compared to the others. However, this does not correlate quite well with the fact that the smallest sizes were determined for samples BM80-180 and BM120-180 according to DLS results. Lower transmittance values were obtained when milling was performed for 180 min for all the BSR conditions evaluated (Fig. 4.3.4).

Since DLS analysis showed that CNF average sizes decreased with milling time (Table 4.3.1), one possible explanation for this contrary trend could be an eventual contamination of CNF samples during ball milling treatment. The presence of impurities may bring down the transmittance of the ball milled product by scattering visible light. Even though the material of the equipment (balls and jar) employed was high zirconia (ZrO_2), the violent shear and impact forces generated from the friction between the balls and the jar's wall and balls collision causes mainly wear of the balls. This phenomenon may result not easy to avoid when working at harsh conditions in terms of milling intensity (milling frequency and time) and number of balls.

Meanwhile, the transmittance of CNFs produced by ball milling (20-50%) resulted lower compared to the transmittance of CNFs produced by ultrasonication (82-96%). This could be explained by the fact that planetary ball milling was more gentle and uneven compared to ultrasonication, which allowed to achieve an almost complete nanofibrillation and dispersion of the CNF.

Additionally, ZP was measured to evaluate the electrostatic stability of CNF (Table 4.3.1). CNF suspensions presented ZP values in the range of -14 mV to -8 mV (Table 4.3.1). Since all absolute values resulted less than 20 mV, the CNF suspensions obtained by ball milling are considered colloidally unstable. In fact, these absolute ZP values below 15 mV represent high tendency to agglomeration, which was visible by eye since phase separation was observed for all the samples after several hours (Appendix A6). This poor superficial charge may probably occur due to the larger particles compared to the ultrasonicated CNF suspensions, which mean lower surface area and lower availability of free hydroxyl groups. Also, these facts are consistent with high PDI values, which interferes with heterogeneity of particle size and agglomeration in suspension.

In this part of the study, it was found that BSR is an important variable to control the number of collision events between the milling balls and the fibers. If the BSR is too low, the number of collision events results too small and, thus, the fiber nanofibrillation. Using optimized milling time may effectively reduce the eventual contamination in the CNF suspensions, possibly from both the milling balls and the jar's wall. Due to these results, ball milling treatment was then performed with a minimal BSR of 80 g/g and the performance of ball milling treatment at shorter milling times and lower frequency was evaluated.

4.3.3. Evaluating the effect of milling time and frequency on CNF extraction

The reduction of ball milling treatment intensity in terms of milling time and frequency was then evaluated with the aim of reducing the energy requirements of the process and possible inorganic contamination. For this, ball milling treatment was performed for shorter milling times (15 min, 30 min and 45 min) and lower milling frequency (10 Hz). Fig. 4.3.5 shows the physical appearance of the 0.1% CNF suspensions obtained by ball milling treatment.

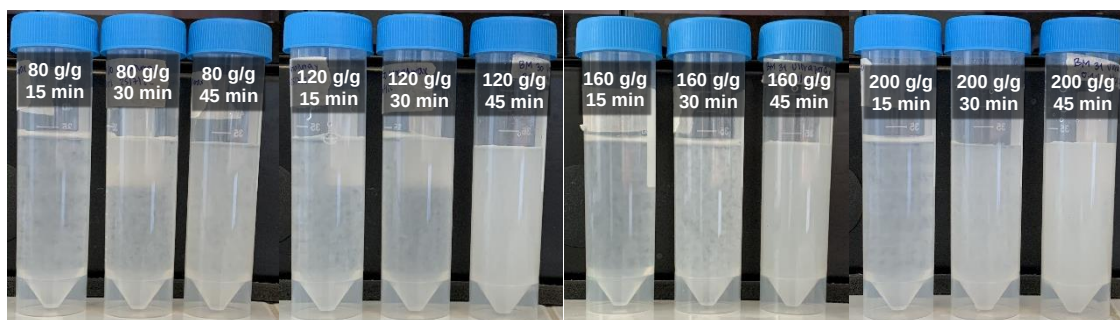


Figure 4.3.5. Visual aspects of 0.1% CNF suspensions obtained after ball milling treatment performed at 20 Hz under the different BSR (80, 120, 160 and 200 g/g) and reaction time (15, 30 and 45 min) conditions.

To investigate the milling treatment performance, results were evaluated in terms of CNF extraction yield for the different BSR conditions (80 g/g, 120 g/g, 160 g/g, and 200 g/g), which are presented in Fig. 4.3.6. A maximum CNF extraction yield of around 35% was achieved at a BSR of 80 g/g after 30 min treatment. No significant differences were achieved on CNF extraction yield for BSR values in the range of 80 to 160 g/g after 45 min treatment, while a relatively lower CNF extraction yield was achieved for a BSR of 200 g/g (30%). However, results showed that the fiber size reduction and/or nanofibrillation occurred at a faster extent for a BSR of 80 g/g, since higher extraction yields were obtained at shorter reaction times. For instance, after 15 min treatment, the CNF extraction yield for a BSR of 80 g/g resulted above 30%, while for a BSR of 120 g/g and 160 g/g the extraction yield was around 25% and only 20% for a BSR of 200 g/g. This reinforces the fact that better ball milling performance in terms of CNF extraction yield could not necessarily be achieved by increasing the number of balls in the milling jar. Overloading the jar with balls could not cause enough treatment intensity due to poor movements of the balls in the milling jar. This could mean that either longer reaction times are needed to reach sufficient collisions among balls and materials or that poor movements cause inefficient collisions and poor extraction performance. When milling treatment was performed at a maximum BSR of 200 g/g, the highest CNF extraction yield achieved under this condition was almost 30% after 30 min of treatment, and was not further improved by prolonging the milling treatment up to 180 min.

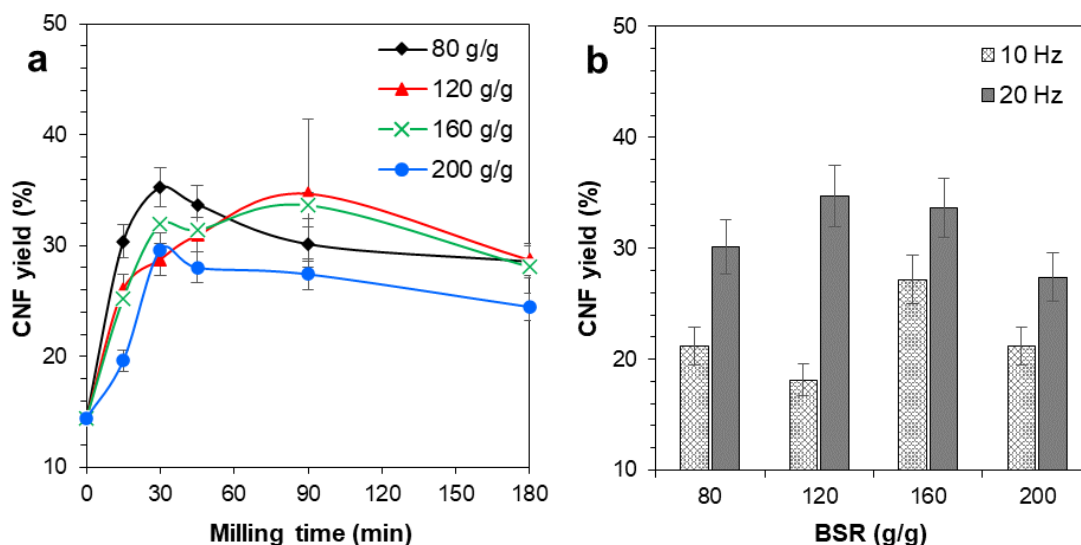


Figure 4.3.6. Effect of ball milling conditions on CNF extraction yield: (a) ball to solid ratio (BSR), and milling time at 20 Hz, and (b) milling frequency for 90 min milling time.

Regarding the effect of milling frequency on CNF extraction performance, it can be observed a drastic decrease (40% on average) on the CNF extraction yield while working at lower BSR conditions (80 g/g and 120 g/g) when the frequency was reduced to 10 Hz. This may probably be a consequence of inefficient collisions due to fewer balls in the container and, thus, poor shear and impact forces that causes fiber fragmentation and fibrillation were generated. In contrast, a lower decrease (20% on average) on the CNF extraction performance was observed for BSR values of 160 g/g and 200 g/g at 10 Hz. In this case, results may indicate that the effect of a higher number of balls in the container and relatively long treatment time (90 min) surpasses the effect of the reduction on the severity of the treatment due to the lower milling frequency. Based on this, performing the milling treatment at lower frequency (10 Hz) implies that ball milling should be performed at higher BSR to achieved sufficient shear and impact forms for an effective fiber fragmentation and nanofibrillation. However, even employing a minimal BSR of 160 g/g, the CNF extraction yield achieved results lower (28%) compared to the extraction yield achieved when working at 20 Hz and BSR of 80 g/g (35%).

Transmittance values of CNF suspensions at 800 nm was also investigated to get an approximate idea of the fibrillation extent and the dispersibility of the CNF suspensions obtained at shorter milling times and lower frequency. Results are presented in Fig. 4.3.7. A contrary trend was observed for transmittance values by increasing the BSR at 10 Hz compared to 20 Hz milling frequency. It is observed that increasing the BSR, transmittance values increased, especially for BSR higher than 120 g/g. This correlate quite well with the fact that higher CNF extraction yields were achieved when working at a BSR of 160 g/g and 200 g/g. In general, it is expected that the transmittance of CNF increases by increasing intensity of the mechanical treatment in terms of

BSR, milling frequency, and/or milling time. However, according to Fig 4.3.7, when a BSR of 200 g/g was used, it was observed that increasing the milling frequency from 10 Hz to 20 Hz negatively affects the transmittance of the CNF suspensions. The same trend was observed for longer treatment times in the previous section. To get a better understanding of these results, eventual contamination of the CNF samples was assessed.

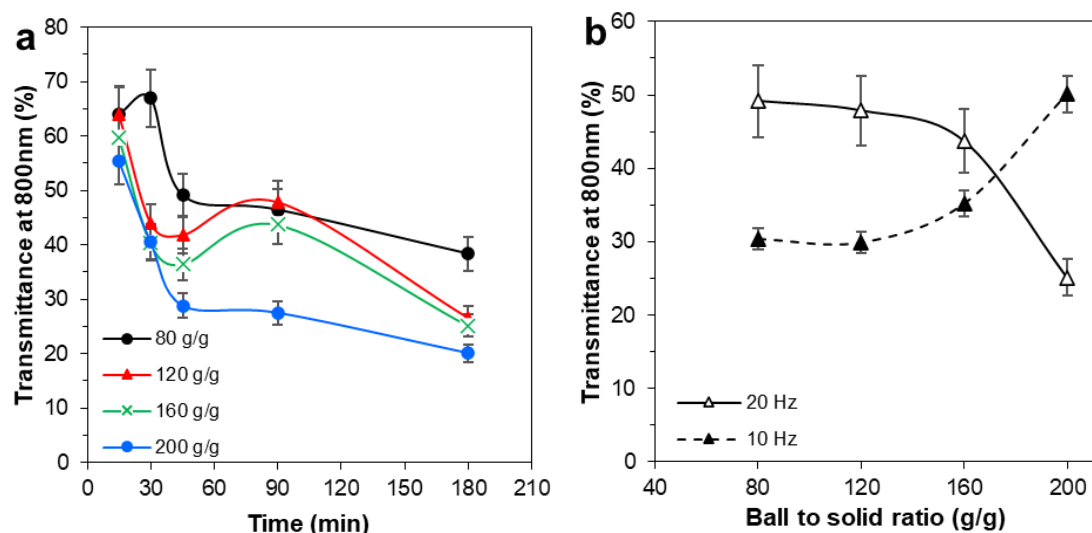


Figure 4.3.7. Effect of (a) milling time and (b) milling frequency on 0.1% CNF suspension transmittance at 800 nm after ball milling treatment for ball to solid ratio (BSR) in the range of 80 to 200 g/g.

Firstly, XRF analysis was performed to determine the nature of the inorganic contaminants present in the CNF samples after ball milling treatment. It was observed the presence of Zr in relatively high amounts (2.7-8.8%) which may be associated to the balls and jar's wall composition (ZrO_2). Additionally, EDS in SEM analysis of ball milled products also confirmed the presence of Zr in CNF samples, which results are presented in Appendix 7. Then, loss on ignition (LOI) method was employed for the estimation of the inorganic contamination and impurities content in CNF samples. Fig. 4.3.8 shows the results obtained in terms of inorganic impurities content in CNF suspensions obtained under the different treatment conditions (BSR, milling time, and milling frequency) evaluated. It should be mentioned that no inorganic impurities were found in the enzyme-treated BEKP samples prior to ball milling treatment. Consequently, the inorganic contaminants determined in CNF suspensions as milled products were introduced during the milling treatment process. This phenomenon may result not easy to avoid when working at the conditions evaluated, and achieved a maximum value of around 7 mg/100 g_{suspension} for prolonged milling times (180 min) and high milling frequency (20 Hz). Also, it can be observed that while increasing the severity of the milling process, the inorganic contamination of the CNF suspension greatly increased.

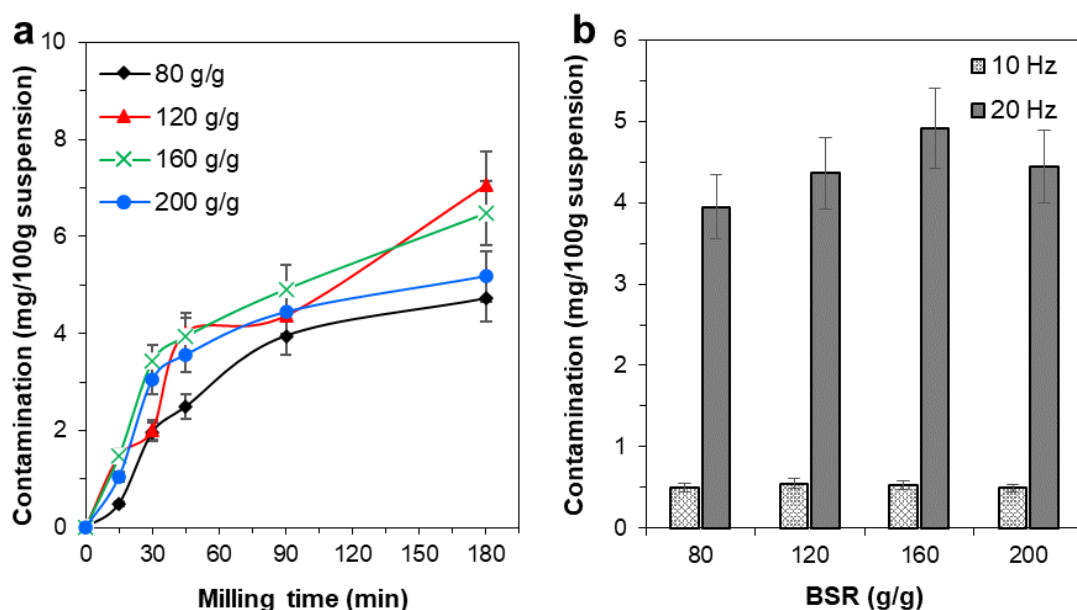


Figure 4.3.8. (a) Effect of milling time on contamination of 0.1% CNF suspension at 20 Hz, and (b) effect of ball to solid ratio (BSR) on contamination of CNF suspension for 90 min milling process.

For longer milling times, it was observed that contamination impurities content in CNF suspension was maximum when ball milling was performed at a BSR of 120 g/g (7.0 mg/100 g_{suspension}) and 160 g/g (6.5 mg/100 g_{suspension}). This may be explained by the fact that frictions between the balls and the jar walls occurred at a lesser extent for a BSR of 80 g/g because of the lower number of balls. On the other hand, a higher BSR value (200 g/g) may be causing overloading of the jar and poor movements of the balls, so the frictions that causes mainly wear of the balls in this case decreased. Inorganic contamination resulted almost negligible (0.5 mg/100 g_{suspension}) when milling treatment was performed at low frequency (10 Hz). However, inorganic impurities are less under this treatment condition at the expense of getting a poor milling performance and, thus, reaching lower CNF extraction yields.

Based on these findings, balance must be done between fiber fragmentation/nanofibrillation by size distribution and CNF extraction yield, and level of contamination during ball milling to select the milling treatment working condition. It was found that the BSR and milling frequency are important variables to control the number of collision events between the milling balls and the fibers. Working at high milling frequency (20 Hz) allowed to achieve higher fiber fragmentation and CNF extraction yield, and working at low BSR (80 g/g) allowed to mitigate the inorganic contamination during the milling process. Moreover, shorter milling times (30 min) allowed to further reduce the inorganic impurities (from 4 mg/100 g_{suspension} to 2 mg/100 g_{suspension}) without compromising the CNF extraction performance.

4.3.4. Evaluating the effect of enzymatic pretreatment on CNF extraction

Once the working condition for the ball milling treatment was selected (20 Hz, 80 g/g, 30 min), the CNF extraction performance was compared among different enzyme-treated BEKP samples (Table 4.3.2). The results obtained after performing the CNF extraction by ball milling from these substrates showed that the nanofibrillation degree was increased when cellulase cocktail was employed for enzymatic pretreatment of BEKP instead of the EG monocomponent, which allowed to achieve a higher CNF extraction yield (35%). Considering the different fiber properties discussed in Section 4.1.5 and Section 4.1.6 and how fiber changes occurred during enzymatic pretreatment may affect subsequent mechanical processing of the substrates, the results obtained were expected. By comparing the fiber characteristics of both enzyme-treated BEKP substrates, it was concluded that cellulase pretreatment allowed to relieve intrinsic recalcitrance of fibers in BEKP at a greater extent compared to EG pretreatment. This was demonstrated by the relatively low CNF extraction yield achieved for the EG-treated BEKP substrate by ball milling treatment, which showed that these pretreated fibers did not achieve an opening up enough of the microfiber structure to ensure an efficient nanofibrillation of the material.

Table 4.3.2. Effect of enzymatic pretreatment on CNF extraction from BEKP by ball milling performed at 20 Hz and BSR of 80 g/g for 30 min.

Substrate	Cellulase-treated BEKP		EG-treated BEKP
Enzymatic pretreatment	4% solids, CTec3 and HTec at mass ratio of 1:1, 4 h	4% solids, EG, 24 h	4% solids, EG and HTec at mass ratio of 1:1, 8 h
Sample	BM(C+X)	BM(EG)	BM(EG+X)
CNF yield (%)	35.2 ± 1.8	13.7 ± 1.1	15.8 ± 1.2
Transmittance at 800 nm (%)	66.9 ± 4.5	28.8 ± 1.5	34.7 ± 2.0
Average size (nm)	1652 ± 596	8930 ± 471 ^c	3280 ± 1141
ZP (mV)	-13.7 ± 0.8	-10.0 ± 0.5	-10.4 ± 0.5
SY (%) ^a	56.1 ± 3.8	97.2 ± 0.1	57.2 ± 5.1
Overall CNF yield (%) ^b	19.7 ± 2.9	13.3 ± 1.0	9.0 ± 0.9

Ball milling conditions: 20 Hz, 80 g/g, 30 min.

^aSY: solid yield; solids recovered after enzymatic pretreatment for CNF extraction.

^bCalculated based on the amount of solids recovered after enzymatic pretreatment (solid yield, SY) and the amount of CNF extracted by ball milling (CNF yield). Expressed as g of CNF extracted per 100 g of BEKP.

^cThis value resulted close to the upper limit of the equipment detection range (10,000 nm).

On the other hand, even though the CNF extraction yield for sample BM(C+X) resulted higher compared to samples BM(EG) and BM(EG+X), the overall CNF extraction yields resulted comparable among the different samples. The overall CNF extraction yield refers to the amount

of CNF effectively extracted by the combined methods of enzymatic pretreatment and ball milling from the original BEKP. Relatively low overall CNF yields were achieved for samples BM(C+X) (20%) and BM(EG+X) (13%) because of the lower solid recoveries obtained in these cases as a consequence of the considerable loss of carbohydrate fraction during enzymatic hydrolysis. The carbohydrate fraction released mostly as soluble sugars in a separate liquid stream can be converted to value-added chemicals for further valorization and, thus, an integral use of the raw material. Instead, for sample BM(EG), the fiber recovery was almost complete after enzymatic pretreatment (97%), which allowed to maximize the conversion of this material to CNF.

It should be mentioned that a faster phase separation and sedimentation was observed for samples BM(EG) and BM(EG+X), which may indicate the presence of larger particles possibly due to a poor fiber fragmentation and size reduction. This can be confirmed by the higher average sizes determined by DLS (9 μm and 3 μm , respectively) and the lower transmittance values determined for these samples (29% and 35%, respectively), which showed that nanofibrillation occurred at a lesser extent compared to sample BM(C+X).

Nanocellulosic films of ball milled products described in Table 4.3.2 were prepared by simple casting evaporation at 105°C of the different CNF suspensions. After casting, the visual appearance of the films is presented in Fig. 4.3.9. The nanocellulosic films obtained from milled enzyme-treated BEKP were not highly transparent as those obtained from ultrasonicated enzyme-treated BEKP (Fig. 4.2.4). For instance, it can be observed in Fig. 4.3.9 that nanocellulosic film from sample BM(C+X) resulted of greater transparency compared to the other films, since the lettering “Universidad de la República Uruguay” printed on the underlying sheets was visually much clearer through the nanocellulosic films, without distance between the films and the underlying sheets. However, the nanocellulosic film formed from sample BM(EG+X) was barely transparent since the lettering printed on the underlying sheets were hardly visible in this case. Despite of this, it had a rather smooth surface which was comparable to the nanocellulosic film from sample BM(C+X). It was already mentioned in Section 4.2.6 that the transparency of nanocellulosic films could be attributed to the degree of nanofibrillation of the sample, which in this case resulted higher for sample BM(C+X) (Table 4.3.2). This correlates quite well with the higher transparency of the film.

On the other hand, the film formed from sample BM(EG) was not transparent and was also hazy. This film contained a rough surface, which resulted completely different to the films formed from samples BM(C+X) and BM(EG+X). Despite that the CNF extraction yields from the EG-pretreated BEKP substrates resulted low (around 15%), the nanocellulosic films formed using the different samples were flexible, as can be seen in Fig. 4.3.9. The flexibility of these films can be attributed to the fact that CNF has flexible fibril structure.

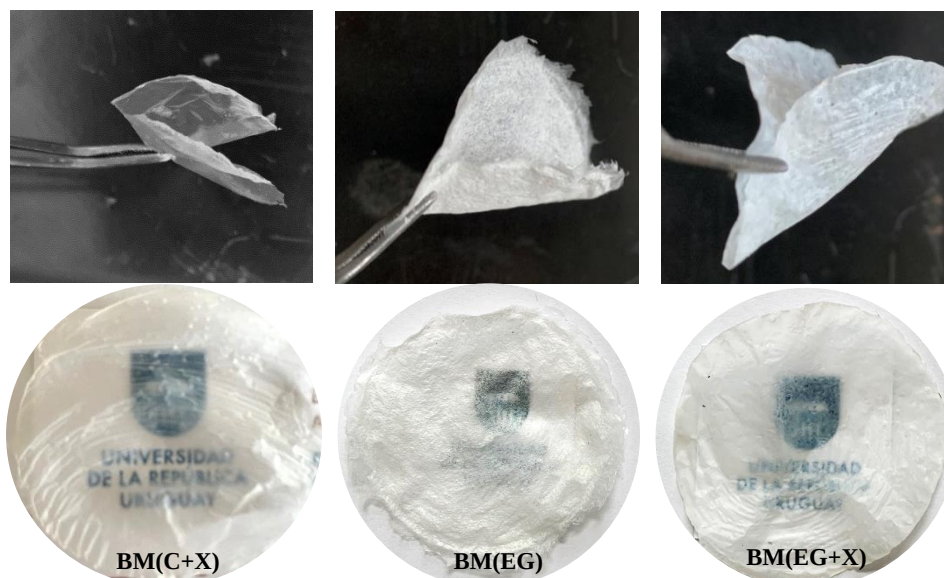


Figure 4.3.9. Visual appearance of nanocellulosic films obtained from cellulase-pretreated BEKP and endoglucanase-pretreated BEKP substrates after ball milling treatment performed at 20 Hz and BSR of 80 g/g for 30 min.

Nanocellulosic films were analyzed by SEM to investigate the morphological and disintegration characteristics of the cellulose micro- or nanofibers upon ball milling treatment and enzymatic pretreatment with the different enzymes. Fig. 4.3.10a shows the shape and size of the original cellulose fibers in the BEKP with diameters of around 20 μm , which was also assessed by OM and presented in Appendix A3 ($32.6 \pm 8.6 \mu\text{m}$). It was not possible to observe either micro- or nanofibers in sample BM(C+X), probably because the diameters of the CNF were below the detection limit of the equipment (Fig. 4.3.10e and f), and this characterization should be performed by TEM or AFM. Instead, it was observed a highly entangled cellulose network with microfibrils of different sizes (up to 15 μm) for sample BM(EG) (Fig. 4.3.10c and d). Since higher sizes and greater heterogeneity was observed for sample BM(EG) compared to sample BM(C+X), SEM analysis proved a drastic reduction on fiber size due to ball milling treatment of cellulase-treated BEKP compared to EG-treated BEKP. Thus, sample BM(C+X) showed better cellulose nanofibrillation with greater fiber size reduction and homogeneity, which demonstrated that the type of enzyme used could control the morphology of the extracted CNFs.

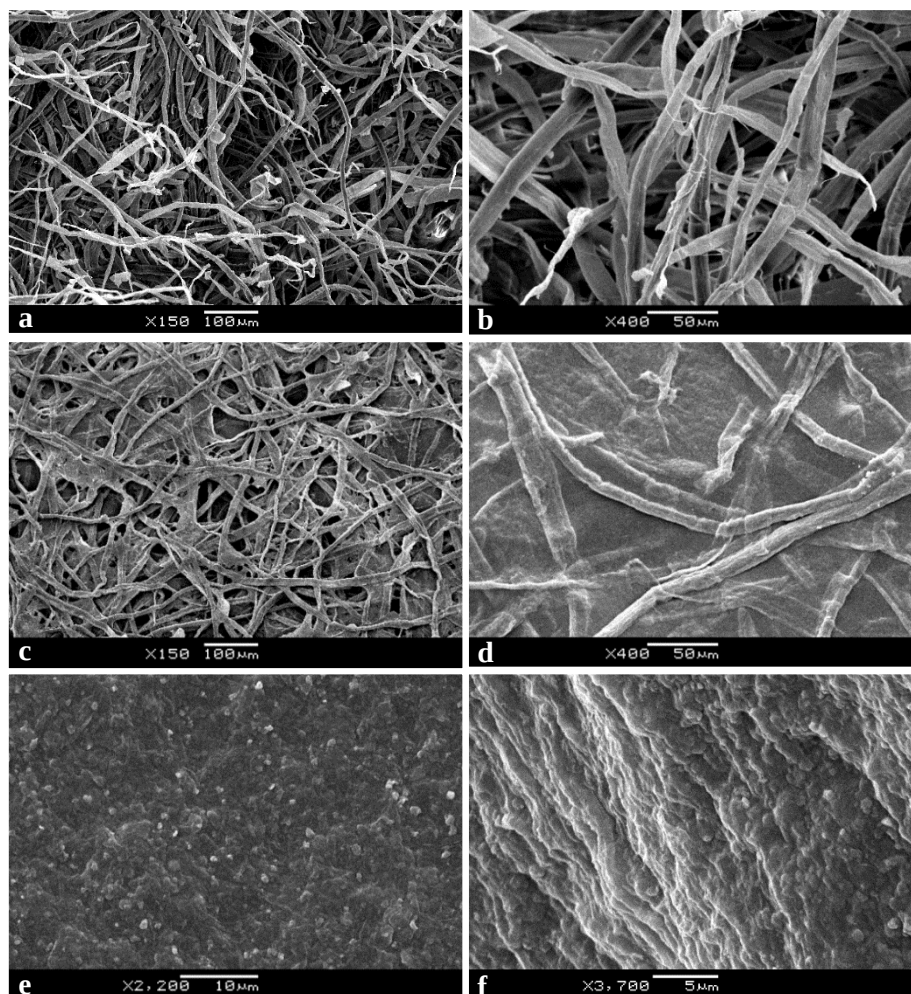


Figure 4.3.10. SEM images of (a, b) untreated BEKP and (c, d) ball milled CNF sample BM(EG) at x150 and x400 magnification, and (e, f) ball milled CNF sample BM(C+X) at high magnifications.

4.3.5. Evaluating sequential ball milling treatment to improve CNF yield

Finally, ball milling treatment was performed in 5 stages in order to evaluate further improvements on the CNF extraction yield by successive extractions. All the ball milling treatment stages were performed at the same conditions: BSR of 80 g/g, milling time of 30 min and milling frequency of 20 Hz. The cellulase-treated BEKP substrate described in Table 4.3.2 was used as initial solid for the sequential ball milling treatment based on the results previously discussed. The sequential ball milling treatment was performed in duplicate to ensure reproducibility. Fig. 4.3.10 presents a schematic illustration of the sequential ball milling treatment of cellulase-treated BEKP for CNF extraction.

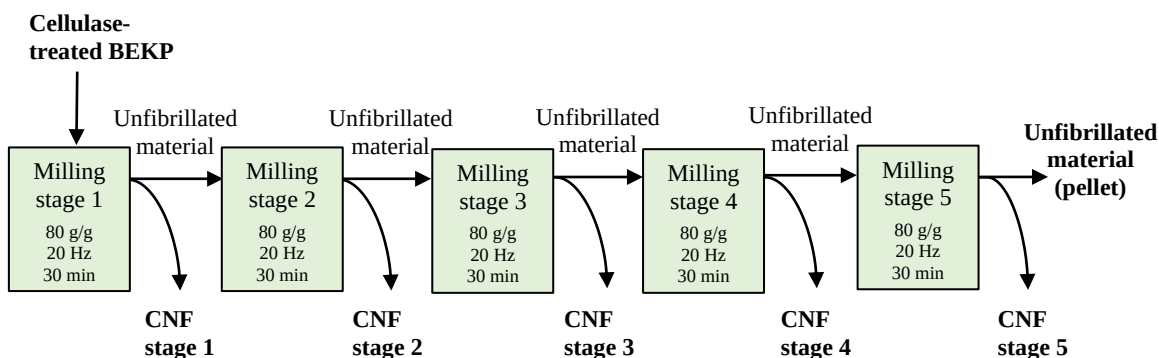


Figure 4.3.11. Schematic representation of the sequential ball milling treatment performed to cellulase-treated BEKP at 20 Hz and BSR of 80 g/g for 30 min for CNF extraction.

After each milling stage, the CNF and unfibrillated fractions were separated by centrifugation. The unfibrillated fraction recovered as sediment (pellet) after centrifugation was then ball milled at the subsequent milling stage. The extracted CNF was recovered in the supernatant fraction after centrifugation. Finally, the extracted CNF fractions obtained from each milling stage were mixed and vacuum-concentrated to reach approximately 0.1% (w/w) consistency. Fig. 4.3.12 shows the visual appearance of the supernatant's mixture obtained before and after vacuum concentration.

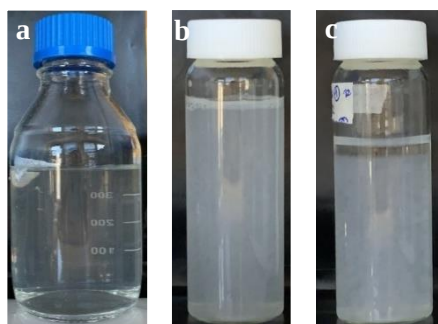


Figure 4.3.12. Visual appearance of supernatants mixture obtained after the sequential ball milling treatment: (a) diluted supernatants from stages 1 to 5 before vacuum concentration, (b) vacuum-concentrated supernatants, and (c) vacuum-concentrated supernatants allowed to settle down for 15 days.

Results in terms of CNF extraction yield are presented in Fig. 4.3.13. The sequential ball milling treatment allowed to reach a CNF extraction yield of 48%, which corresponds to an increase of 36% on the CNF extraction performance without the sequential treatment (Table 4.3.2). After each milling stage, CNF extraction yields in the range of 2 to 5% were obtained, which yielded the cumulative CNF extraction of 48%. This increase on the extraction performance after each milling stage resulted relatively low, even though a considerable increase on the overall CNF yield was achieved by the sequential treatment. One possible explanation to this phenomenon could be linked with viscosity changes occurring during the grinding process, and its effect on the grinding efficiency. For instance, it was reported by some researchers that the efficiency of wet grinding processes is strongly influenced by the solid concentration [136, 137].

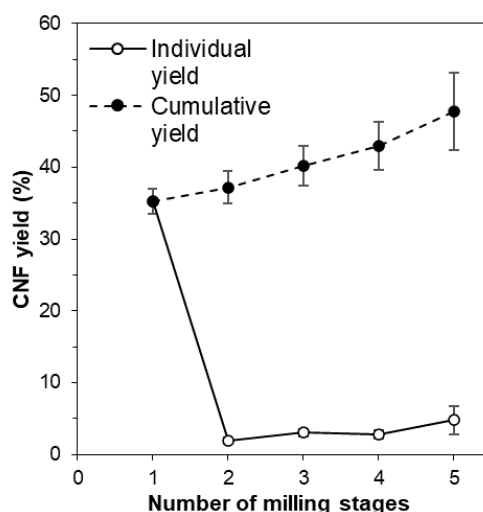


Figure 4.3.13. Effect of sequential ball milling treatment on CNF extraction yield performed at 20 Hz and BSR of 80 g/g for 30 min.

The question of interest is how the viscosity affects the extraction efficiency during the milling process. It should be noted that the CNF suspensions obtained after ball milling treatment, which had a concentration of around 1%, displayed a gel-like behavior. This gel-like behavior could be due to the high aspect ratio of the nanofibril gelation network obtained by disintegration, fragmentation, and fibrillation of cellulose microfibrils which caused material size reduction. An increase on the viscosity may be caused by the produced surface area because of this fiber disintegration.

In addition to solid concentration, other main parameters can also affect the viscosity of a suspension such as the particle size, the particle size distribution, and the nature of the interparticle forces [137]. In wet grinding processes, a critical viscosity exists where the grinding efficiency starts to decrease due to the high solids content and excessive fines. If the viscosity becomes too high, the velocity of the grinding media is strongly damped and the transferred energy from the balls is no longer enough to trigger the fibers breakage. In this case, a viscous dampening-related milling limit is reached. This milling limit does not allow a further decrease in particle size with milling time and stages, resulting in a reduced grinding efficiency. It has been reported by some authors [137] that this phenomenon becomes more relevant especially for ultrafine grinding applications, where strong attractive particle interactions affect the suspension rheology.

The rheology of concentrated suspensions is a matter of considerable complexity, especially in the case of wet grinding, as the rheological properties of the suspension vary considerably with grinding time [138]. From a rheological point of view, a concentrated suspension could also demonstrate a high viscosity due to the presence of flocculated particles. For instance, fine particles of sizes below 1 μm are generally attracted to one another by van der Waals forces to form aggregates or flocculated particles. These interactions become relevant for smaller sizes and

increasing collision rate of the particles due to the smaller interparticle distances. To enhance the grinding process, one possible solution could be the addition of a small amount of a water-soluble surface-active polymer to stabilize the particles against agglomeration. By changing the nature of the interparticle forces to be entirely repulsive it could be possible to avoid the flocculation of the fine particles and remain as discrete entities. For example, sodium polyacrylate (SPA) is a surfactant applied in the paper industry for decreasing the slurry viscosity of coatings [136]. Surfactant increases the particle charge, which could be determined by ZP analysis, and decreases the apparent viscosity. Since solid concentration was not a milling parameter evaluated in this study, the rheological behavior of the CNF suspensions obtained by ball milling should be addressed in depth for a better understanding of this phenomenon.

DLS of the concentrated CNF suspension recovered after the sequential milling treatment was assessed to evaluate the size reduction and product uniformity. Results are presented in Fig. 4.3.14 and compared to CNF suspension without sequential milling treatment, corresponded to sample BM80-30. The size distribution showed that all particles are within the nanoscale range, with average hydrodynamic sizes in the range of 40 to 500 nm. This demonstrated that the CNF separation from unfibrillated material by centrifugation was successful. Besides, a bimodal distribution was observed in this case, with a minimum particle size of around 40 nm. If compared to sample BM80-30 which was not subjected to sequential milling treatment, this bimodal distribution resulted narrower. However, it indicates that the sample contains particles with higher sizes due to an uneven breakage of the microfibers during ball milling treatment. This demonstrated the heterogeneity of ball milled products that was previously discussed in Section 4.3.2 and also reported by other researchers [42, 133].

The ZP value of the concentrated CNF suspension resulted of -10.9 ± 0.3 mV, which means that the CNF tends to agglomerate. The tendency to agglomeration was visible by eye since phase separation was observed in Fig. 4.3.12c.

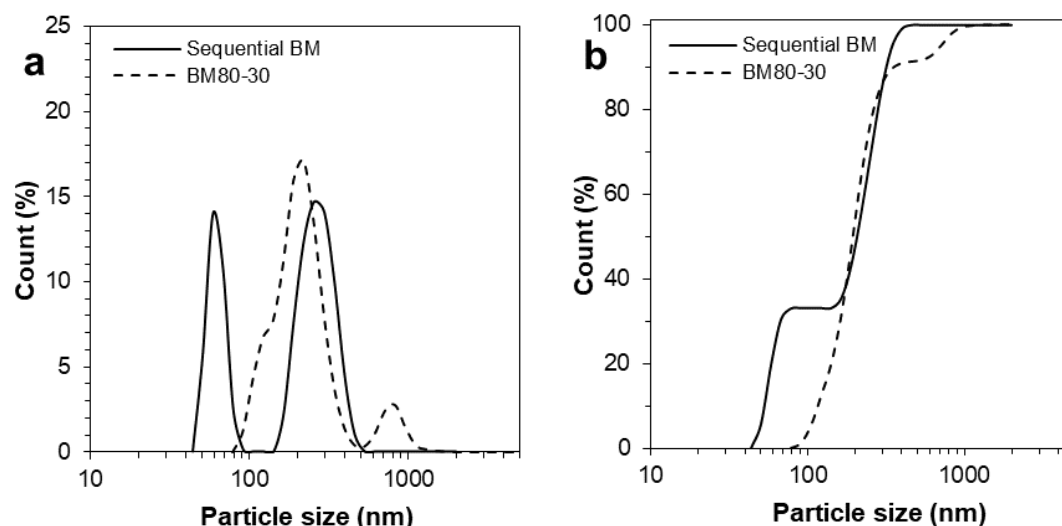


Figure 4.3.14. Effect of sequential ball milling treatment performed at 20 Hz and BSR of 80 g/g for 30 min on CNF size distributions through dynamic light scattering analysis: (a) probability density function, and (b) cumulative distribution function.

4.3.6. Crystallinity and thermal properties of CNF extracted by ball milling treatment

The impact of ball milling treatment on the thermal stability of BEKP cellulose was investigated by thermogravimetric analysis (TGA) of nanocellulosic films. Fig. 4.3.15 shows the TGA and derivative thermogravimetric (DTG) curves of sample BM80-30. TGA curves exhibited the three main weight-loss regions during heating which were previously discussed in Section 4.1.5. The thermal analysis showed that the thermal degradation phenomenon of cellulose was not affected by ball milling treatment, since the main thermal properties of CNF obtained after treatment did not change (Table 4.3.3). It is inferred that the CNF produced by ball milling method retained the thermal stability of the cellulose-treated BEKP microfibers. In contrast, the CNF sample obtained by ultrasonication presented a lower T_{on} , which may be due to damages in cellulose crystalline region that occurred during ultrasonication. These results may indicate that ball milling treatment did not have a negative effect on the crystallites of cellulose compared to ultrasonication treatment. However, the lower degradation temperature of ultrasonicated sample could be due to the smaller CNF dimensions compared to the ball milled sample, which leads to higher specific area. The onset temperature occurred at 324°C which results lower than 335°C (Table 4.3.3). The higher residual mass at 500°C of ball milled CNF compared to both the untreated and cellulase-treated BEKP could be attributed to inorganic contamination introduced during the mechanical method, which was discussed in Section 4.3.3.

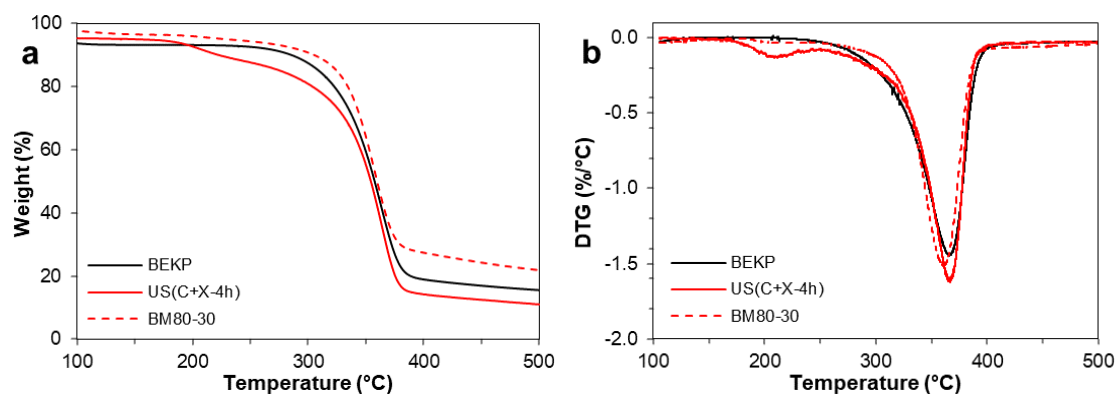


Figure 4.3.15. (a) Thermogravimetric analysis and (b) derivative thermogravimetric (DTG) curves of CNF produced by ball milling treatment and its comparison to ultrasonicated CNF.

XRD analysis on nanocellulosic films obtained by ball milling treatment was performed to assess crystallinity properties. XRD pattern for sample BM80-30 is presented in Appendix A8 and compared to XRD pattern for cellulase-treated BEKP and ultrasonicated CNF (sample US(C+X-4h)). The XRD spectra presented a similar trend exhibiting the principal crystal planes (110), (102), and (200) at 2θ approximately 15° , 16° , and 22° , respectively, as it was already discussed in Section 4.2.6 for the ultrasonicated CNF samples. Regarding crystallinity, results showed that ball milling treatment did not result destructive on cellulose crystallinity compared to ultrasonication treatment which led to a drop in the CrI of cellulase-treated BEKP fibers from 77.9 to 75.7% (Table 4.3.3). This confirms the gentler nature of ball milling treatment compared to ultrasonication as mechanical methods. In fact, the CrI of the ball milled product was slightly increased after milling treatment (81%), possibly due to the rupture of some cellulose amorphous regions due to the shear forces.

Table 4.3.3. Crystallinity and thermal properties of ball milled and ultrasonicated CNF products compared to untreated and enzyme-treated BEKP.

Sample	CrI (%)	T_{on} (°C)	T_{max} (°C)	Residue (%)
BEKP	72.0 ± 3.6^a	314	365	13.8
Cellulase-treated BEKP ^b	77.9 ± 3.1	340	375	12.0
BM80-30	81.0 ± 4.0	335	360	19.8
US(C+X-4h)	75.7 ± 2.7	324	368	11.1

^aAverage value of two determinations: 68.2 ± 2.7 and 75.7 ± 2.3

^bEnzymatic pretreatment: 4% solids, 4 h, CTec3 and HTec at 4.2 mg_{protein}/g_{BEKP}.

4.3.7. Summary

Ball milling treatment allowed to extract CNM from enzyme-treated BEKP, resulting in an attractive alternative mechanical method for CNF extraction. It also represents an industrially

applicable process since it can produce CNM in large quantity, which is more advantageous when compared to other alternative methods.

Ball milling treatment results attractive for CNF extraction since it allows working with higher sample consistencies (1% w/w) compared to ultrasonication (0.3% w/w). Therefore, CNF suspensions in higher concentrations can be obtained after ball milling treatment of enzyme-treated BEKP, which displays a gel-like behavior. However, the rheology in this case turns to be more complex. The high solids content and the presence of small particles may cause the viscosity of the slurry to increase, which negatively affects the energy transfer that triggers the fibers breakage. Also, high viscosity could also be caused by the formation of aggregates or flocculated particles from fine particles and their interparticle forces. This limits the milling efficiency and, thus, a complete conversion from microfibers to nanofibers was not reached. CNF extraction yields achieved by ball milling (35%) resulted lower compared to ultrasonication (97%). When performing the milling treatment in a sequential configuration, this extraction yield was improved up to 48%. By performing the ball milling in 5 stages, it was possible to obtain a CNF suspension with greater uniformity of CNF sizes. Further research should be performed for a better understanding of this phenomenon.

Inorganic contamination during milling treatment results to be one major challenge for this mechanical method for CNF extraction. XRF and EDS in SEM confirmed the presence of Zr which may be associated to the balls and jar's wall composition (ZrO_2). This inorganic contamination introduced from the milling equipment to the CNF suspension conditions the working conditions, especially at high severity. The operational conditions should be carefully selected by balancing the CNF extraction yield and size distribution with the level of contamination. It was found that the BSR and milling frequency are important variables to control the number of collision events between the milling balls and the fibers. Working at high milling frequency (20 Hz) allowed to achieve higher fiber fragmentation and CNF extraction yield, while working at low BSR (80 g/g) allowed to mitigate the inorganic contamination during the milling process. Also, milling time turned out to be a significant variable to avoid or diminish the inorganic contamination.

The relatively poor surface charge of ball milled CNF suspensions (ZP absolute values in the range of 7 to 20 mV) resulted in unstable dispersions, which could be due to the higher particle sizes compared to ultrasonicated CNF suspensions. However, ball milled products presented enhanced thermal properties and crystallinity compared to ultrasonicated CNF.

Results presented in this section demonstrated that ball milling represents a promising alternative mechanical method for the extraction of CNF from enzyme-pretreated cellulosic materials, with potential for industrial applications.

4.4. Integrating the coproduction of CNF and biobutanol from BEKP using an environmentally friendly process

4.4.1. Background

In modern biorefineries from lignocellulosic biomass, integrating the coproduction of biofuels and/or value-added chemicals from the different biomass components has become a target to reach economic self-sustainability as well as get an efficient exploitation of the natural resources. For instance, second generation biofuels plants commonly coproduce power and biogas using the solid residues obtained during the cellulosic biofuel production and waste treatment processes [139]. Recently, Borregaard company operates one of the most advanced and sustainable biorefineries using renewable raw materials, particularly wood, to produce advanced and environmentally friendly biochemicals that replace oil-based products [140]. By using the different components in the woody feedstock and integrating the different production processes, bioethanol, biopolymers, cellulose fibrils and biovanillin are being coproduced for a wide range of applications from biofuels, agriculture, construction, to pharmaceutical and cosmetic, and food [141].

Biofuels and/or value-added chemicals production strategies by fermentation from lignocellulosic biomass consist mainly of the usage of both hexose and pentose sugars extracted from the biomass carbohydrate components. Hydrolysis of lignocellulosic biomass breaks down the cellulose fraction into glucose and the hemicellulose fraction into pentoses (e.g. xylose, arabinose) and hexoses (e.g. glucose, galactose, mannose). Since these sugars can be fermented to biofuels or other value-added chemicals using various microorganisms, the integration of CNM production along with coproducts implies that the recovery of fermentable sugars during CNM extraction should be addressed. Despite of this, reported data on efficient integration of CNM production with other value-added chemicals are still scarce. Generally, endoglucanases are the hydrolytic cellulases mostly employed for CNM production to avoid excessive cellulose depolymerization, since they attack the amorphous cellulose chains to produce chain ends [123, 39]. Thus, enzymatic pretreatment results in low soluble sugars release, which was already discussed in Section 4.1.6. If the integrated production of CNM and biofuels or value-added chemicals is intended, low levels of soluble sugars are not desired. To overcome this, the introduction of a combined cellulase-cocktail containing endoglucanases along with exoglucanases/cellobiohydrolases and β -glucosidase/cellobiases followed by mechanical treatment is proposed, in which the enzymes mixture can degrade amorphous and crystalline regions without significantly compromising the cellulose fraction before the cellulose is nanofibrillated by mechanical shear. A suitable control of the enzyme loading, operational conditions, and cellulase-cocktails composition could allow a

high process control of the degradation level of the different solid components, especially the cellulose fraction. This would prevent an extensive cellulose hydrolysis which could negatively affect the CNM extraction yield, and also enable the possibility to recover a separate sugar stream that could be valorized.

Biobutanol represents an important liquid biofuel which can be produced from biomass-derived sugar processing. In addition to its role as an alternative to fossil fuels, butanol serves as an important platform chemical with applications such as a solvent, precursor for paints, and polymers formulation, among others [85]. It can be produced by fermentation from both pentose and hexose sugars through ABE or IBE fermentation, in which butanol and coproducts (acetone, isopropanol and ethanol) are simultaneously produced. The most commonly wild type microorganisms employed for biobutanol fermentation include *Clostridium beijerinckii* strains [88]. Since these strains may produce low butanol concentration and yields because of butanol inhibition and coproducts formation, biobutanol production must be integrated into existing biomass conversion processes and equipment which allows the coproduction of value-added chemicals to improve the overall economics of the process and help achieve sustainability over time.

It was already discussed in Sections 4.1.2, 4.1.3 and 4.1.4 that cellulase-mediated pretreatment of BEKP generated a separate sugars stream with concentrations up to 30 g/L, which should be further valorized for a complete exploitation of the raw material. In this context, the following section presents the evaluation of integrating the extraction of CNF through enzyme-mediated pretreatment employing cellulases and xylanases with the production of biobutanol from BEKP (Fig. 4.4.1). The fermentable sugars stream obtained as coproduct was further valorized by fermentation for biobutanol production using *Clostridium* strains. Since high fermentable sugars concentrations are desirable for a promising biochemical platform process converting cellulosic feedstocks into value-added chemicals, enzymatic pretreatment of BEKP should be performed at high solid concentrations. Because of this, solid concentrations of 10% and 16% were assessed in this part of the study. For the extraction of CNF from enzyme-treated cellulose fibers, ball milling (20 Hz, BSR of 80 g/g, 90 min) was employed as mechanical treatment because of its ease of use, relatively inexpensive equipment, and applicability to different types of materials. Considering that CNF applications strongly depend on their properties, the effects of high solid concentrations and reaction time for enzymatic pretreatment on the main properties of the extracted CNF were investigated. This section highlights the promising opportunity to produce value-added chemicals from the sugars generated during CNF extraction by enzyme-mediated pretreatment from cellulosic materials.

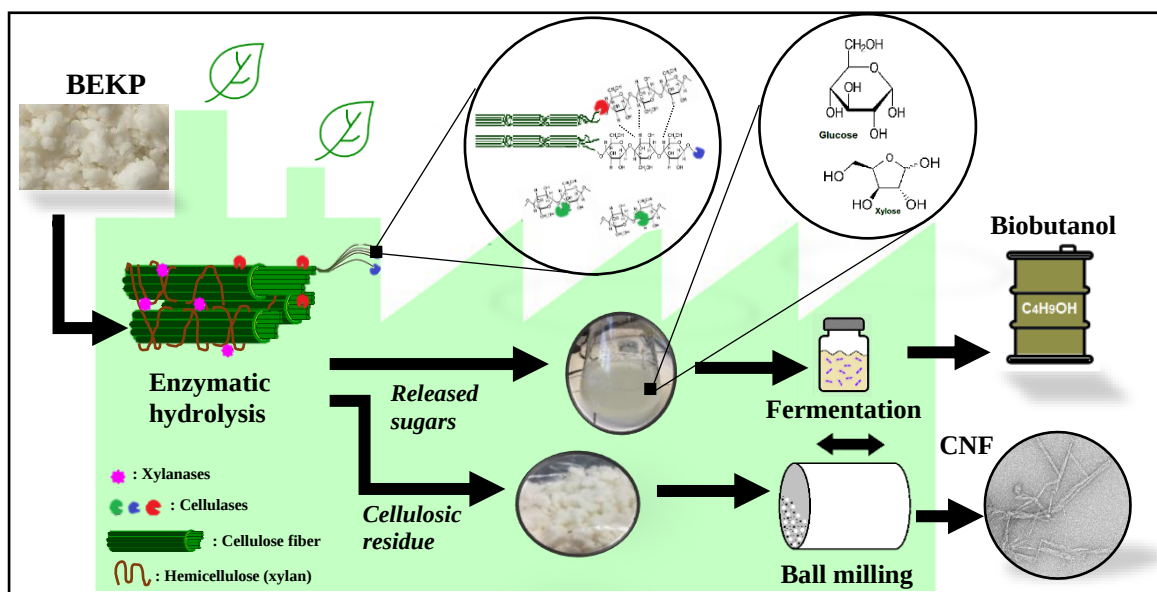


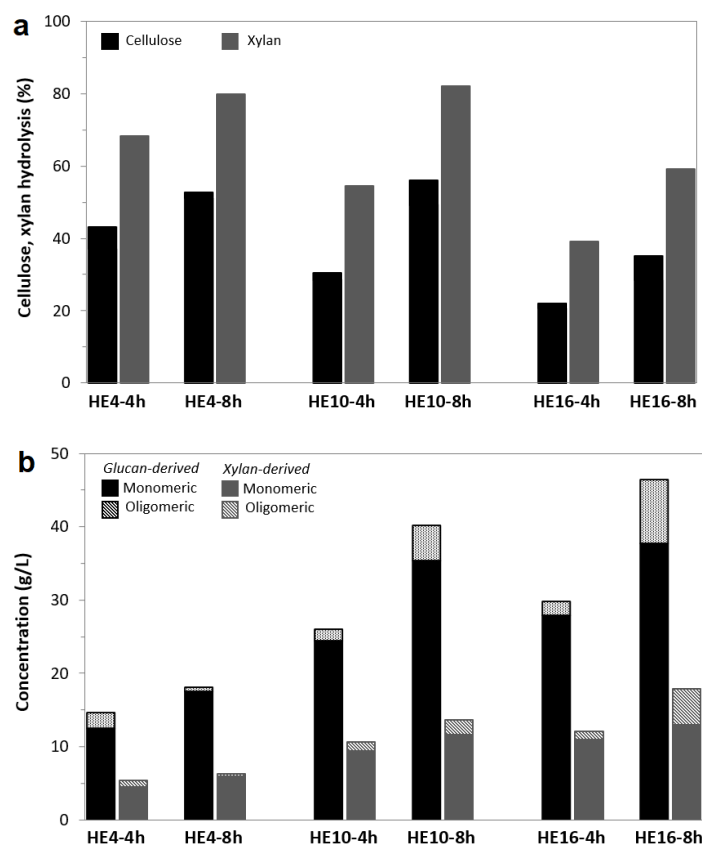
Figure 4.4.1. Schematic representation of the integrated production of CNF and biobutanol from BEKP by enzyme-mediated ball milling treatment.

4.4.2. Effect of high solid concentration on hydrolysis rate and sugar release during enzyme-mediated pretreatment

In this part of the study, the enzymatic cocktail used was prepared by employing equal amounts of cellulase complex Cellic® CTec3 and xylanase complex Cellic® HTec on mass basis (ratio of 1:1) at an enzyme loading of 4.6 FPU/g_{solid} (total protein content of 4.2 mg_{protein}/g_{solid}). Different conditions in terms of solid concentration (4%, 10%, and 16%) and hydrolysis time (4 h and 8 h) were evaluated for enzymatic pretreatment of BEKP (Table 4.4.1). Fig. 4.4.2 shows the results obtained after enzymatic pretreatment in terms of glucose and xylose yields and concentrations under the conditions evaluated. It was clearly observed the tendency of conversion yield decrease with increasing solid concentrations, which was also reported in several previous studies [142, 112, 143]. However, this may be alleviated with an increased reaction time. For instance, higher cellulose and xylan conversions of the cellulose-rich BEKP were reached at 4% solid concentration when hydrolysis was performed for 4 h (43% and 68%, respectively) and 8 h (53% and 80%, respectively). Even though lower cellulose and xylose conversions were achieved at 10% solid concentration after 4 h of hydrolysis (30% and 55%, respectively), these results were further increased when hydrolysis was performed for 8 h (56% and 82%, respectively). Thus, comparable results were achieved both at 4% and 10% solid concentrations when performing enzymatic hydrolysis for 8 h. On the other hand, enzymatic hydrolysis at higher solid concentration (16% w/w) for 8 h resulted in cellulose and xylan conversions of 35% and 59%, respectively, which represents a decrease of 26% to 37% on conversion rates.

Table 4.4.1. Enzymatic pretreatment conditions evaluated using an enzyme loading of 4.6 FPU/g_{solid}.

Sample code	Enzymatic hydrolysis conditions	
	Solid concentration (wt%)	Hydrolysis time (h)
HE4-4h	4.0	4
HE4-8h	4.0	8
HE10-4h	10.0	4
HE10-8h	10.0	8
HE16-4h	16.0	4
HE16-8h	16.0	8

**Figure 4.4.2.** (a) Cellulose (as glucan) and xylan hydrolysis efficiency, and (b) glucan-derived and xylan-derived sugars concentrations in monomeric and oligomeric forms obtained after enzymatic pretreatment of BEKP under the different conditions evaluated.

Despite cellulose and xylan conversion decrease with higher solid concentrations, higher glucan-derived and xylan-derived sugars concentrations were achieved under these conditions. For instance, glucose (15-20 g/L) and xylose concentrations (5 g/L) reached at 4% solid concentration resulted two-fold lower than glucose (30-45 g/L) and xylose (10-15 g/L) concentrations achieved at 16% solid concentration. However, by increasing the solid concentration from 10% to 16% only an increase of 20% was observed on the glucose and xylose concentrations, while an increase of 120% was observed by increasing the solid concentration from 4% to 10% (Fig. 4.4.2). This

may be explained by the significant reduction on the carbohydrate conversion rates by further increasing the solid concentration of the slurry.

High monosaccharides (e.g. glucose, xylose) concentrations are desirable for a biochemical platform process converting cellulosic feedstocks into high value-added chemicals. Because of this, enzymatic hydrolysates with a total sugar (glucose and xylose) concentration higher than 20 g/L were subsequently utilized to produce biobutanol by *Clostridium* fermentation. It was observed that the amount of sugars in oligomeric form which were released during enzymatic pretreatment was relatively low (<30%) in all cases (Fig. 4.4.2). This could be due to the efficient performance of the enzyme preparations used in this study that were specifically developed for biomass deconstruction into monomeric sugars.

4.4.3. Assessing cellulose fibers changes after enzyme-mediated pretreatment at high solid concentration

The solid residues (cellulose fibers) recovered after enzymatic pretreatment were analyzed to assess the modification to the cellulose fibers in the original BEKP (Table 4.4.2). The cellulosic substrates were characterized for cellulose accessibility using the WRV and DB adsorption assays. At fiber level, the overall porosity was assessed by the WRV, which provides a relative indication of the fiber swelling capability by estimating the amount of water that can be retained by the inner pores of a cellulosic substrate. As fibers swell, they are more easily defibrillated, exposing greater surface area [144]. The accessible surface area of cellulose was estimated by DB adsorption assays. Both the WRV and DB adsorption values of the different cellulose fibers recovered increased, compared to the control BEKP (2.71 and 87.0, respectively), indicating an enhanced overall cellulose accessibility after the enzymatic pretreatment stage. During enzymatic hydrolysis, highly ordered regions of microfibrils are believed to be delaminated and disrupted, causing the increase of porosity as enzyme action further opened up their structure [145]. Samples HE4-4h (4.16), HE4-8h (4.29) and HE10-8h (4.03) had the highest WRV, which may indicate a greater exposure of cellulose chains that enhanced water retention. Also, the highest DB adsorption values were reached for samples HE4-4h (130) and HE4-8h (123), which remained almost unchanged, and sample HE10-8h (122). These results corresponded quite well with the higher sugar conversion rates achieved under these conditions (4% and 10% solid concentrations, 8 h hydrolysis, Fig. 4.4.2). The enhanced DB adsorption and water retention could be due to a reduction in the fiber aggregation degree caused by the enzymes, which leads to an increase of the external surface area and, thus, a greater exposure of cellulose chains. Also, since amorphous regions occur between microfibrils, cellulase pretreatment may lead to swelling of the cellulose fibers which may cause an increase in accessibility to solvents and reagents, thus improving

overall cellulose accessibility [146, 143]. It was also reported by other authors that enzyme-mediated pretreatment increases external surface area through external fibrillation, which increases fiber water retention [147]. According to these results, a higher fiber fragmentation was expected for samples HE4-4h, HE4-8h, and HE10-8h, which could be related to the greater xylan hydrolysis under these conditions (Fig. 4.4.2), since the decrease in the xylan content on the fibers surface could have facilitated the accessibility of the enzyme to cellulose [143].

On the other hand, even though enzyme-mediated pretreatment contributes to the fibers fibrillation, it was previously reported that the enhanced fiber swelling can be related to increased concentration of acidic groups in the pulp [144]. These acidic groups, which are associated with the carboxyl groups of the cellulosic fibers, causes electrostatic repulsion between the negatively charged carboxylate anions of fibers and, thus, fiber swelling [148]. The acidic groups of the cellulose fibers were determined by conductometric titration measurement (see titration curves in Appendix A9). As presented in Table 4.4.1, the acidic groups content and WRV values show the same changing trend, which was also reported by other authors [144, 149]. The increase in the WRV could be attributed to the introduction of acidic groups by enzymatic pretreatment, since the swelling of the fibers can be enhanced with increasing fiber charge.

The DP is an important parameter which evaluates the length of cellulose chains, as it is defined as the number of times that the monomeric unit that forms the polymer chain repeats [145]. Table 4.4.2 presents the effect of enzymatic hydrolysis conditions on cellulose DP from the different cellulosic residues. Enzymatic hydrolysis resulted in a significant reduction (>50%) in DP for all the conditions evaluated. This drastic decrease in DP was expected since it is widely reported that enzymatic hydrolysis using cellulases causes shortening in the length of cellulose chains and, thus, a decrease on the molecular weight distribution of cellulose. For instance, Ramos et al. [150] reported DP values in the range of 268 to 220 after enzymatic hydrolysis of peroxide pretreated eucalyptus, achieving a 40% DP reduction after 10 h. Moreover, it was possible to determine the CrI of cellulose fibers from XRD analysis. During enzymatic hydrolysis, amorphous or disordered segments in cellulose is generally believed to be hydrolyzed at a faster rate than crystalline or ordered segments in cellulose. Because of this, it was expected an increase on CrI after enzymatic pretreatment. A slight increase (2-5%) was observed compared to the control BEKP (CrI 75.7%) after 8 h of enzymatic hydrolysis for the conditions evaluated (Table 4.4.1). Considering that cellulose hydrolysis extent was apparent for all the conditions evaluated according to conversion rates (Fig. 4.4.2), this result may indicate that both amorphous and crystalline domains were hydrolyzed at similar rates which did not cause a significant change in the CrI.

Table 4.4.2. Solid, glucose, and xylose yields, and characterization of cellulose fibers obtained after enzymatic pretreatment of BEKP under the different conditions evaluated.

Sample	SY (%)	Glucose yield (g/100 g _{BEKP})	Xylose yield (g/100 g _{BEKP})	WRV (g/g)	DB adsorption (g/g _{dry solid})	AG (mmol/ kg _{dry solid})	DP	CrI
BEKP	na	na	na	2.71 ± 0.04	87 ± 1	44.2 ± 1.3	1196 ± 5	75.7 ± 0.5
HE4-4h	52.2 ± 4.6	31.9 ± 2.0	11.6 ± 0.3	4.16 ± 0.64	130 ± 2	65.4 ± 1.6	332 ± 9	nd
HE4-8h	42.8 ± 2.4	43.9 ± 1.6	15.3 ± 1.2	4.29 ± 0.37	123 ± 1	77.3 ± 1.9	288 ± 3	79.2 ± 0.8
HE10-4h	65.0 ± 3.2	24.5 ± 1.5	9.7 ± 0.3	3.48 ± 0.05	108 ± 1	58.8 ± 1.4	345 ± 3	nd
HE10-8h	39.2 ± 2.0	42.3 ± 2.5	14.0 ± 0.4	4.03 ± 0.20	122 ± 2	75.2 ± 1.8	258 ± 2	78.2 ± 0.8
HE16-4h	74.9 ± 3.7	17.7 ± 1.1	7.1 ± 0.2	3.49 ± 0.04	89 ± 1	48.7 ± 1.2	524 ± 6	nd
HE16-8h	60.4 ± 3.0	24.5 ± 1.5	8.6 ± 0.5	3.58 ± 0.04	98 ± 1	66.2 ± 1.6	458 ± 2	77.4 ± 0.7

Enzymatic pretreatment performed using an enzyme loading of 4.6 FPU/g_{solid}.

SY: solid yield; WRV: water retention value; DB: Direct Blue; AG: acidic groups; DP: degree of polymerization; CrI: crystallinity index; na: not applicable; nd: not determined.

Average values of duplicates and standard deviation are presented.

4.4.4. Extraction and characterization of CNF suspensions

Table 4.4.3 presents the main characterization results for the CNF suspensions obtained after ball milling from each experimental condition employed during enzymatic hydrolysis. One of the main challenges when working with fibrillated cellulosic materials is that the particle size and aspect ratio of the fibrils are difficult to determine accurately using traditional microscopy, since the fibrils form a complex and entangled network. Because of this, DLS method was used for a more complete evaluation of the size distribution of CNF samples, while a relatively simple gel point determination technique was proposed to estimate the aspect ratio of the fibrils. Even though several studies used this method to quantify the approximate relative aspect ratios of micro- and nano-fibrillated cellulose fibers [100, 151, 152], the calculated aspect ratio should not be considered as the true average aspect ratio of a sample, but as relative values to compare between samples that were produced under different pretreatment conditions. Also, DLS method should be used in combination with other methods when comparing different samples.

Table 4.4.3. Characterization of CNF suspensions extracted from enzyme-treated BEKP by ball milling treatment performed at 20 Hz and BSR of 80 g/g for 90 min.

CNF sample	Average size (nm)	PDI ^a	Zeta Potential (mV)	Aspect ratio	Transmittance at 800 nm (%)
BM(HE4-4h)	1116 ± 87	0.76 ± 0.11	-10.5 ± 1.6	222	39.7
BM(HE4-8h)	535 ± 19	0.35 ± 0.05	-15.5 ± 0.6	220	50.0
BM(HE10-4h)	1321 ± 301	0.78 ± 0.23	-14.8 ± 0.3	189	39.2
BM(HE10-8h)	942 ± 162	0.43 ± 0.13	-12.3 ± 0.4	227	49.1
BM(HE16-8h)	2435 ± 209	0.92 ± 0.09	-9.5 ± 0.2	144	33.8

PDI: polydispersity index

^aValues < 0.7 are considered homogeneous

The determination of the average hydrodynamic size and the PDI gives an indication of the presence of large particles or agglomerates and the degree of size homogeneity in the sample, respectively. According to DLS analysis of the ball milled samples, average hydrodynamic sizes resulted in the range of 500 nm to 2.4 µm for all the conditions evaluated (Fig. A10.1 in Appendix A10). Considering that average hydrodynamic sizes higher than 25 µm were determined for the enzyme-treated materials before mechanical treatment (see Fig. A10.4 in Appendix A10), DLS results demonstrated that ball milling treatment contributes to fiber size reduction and CNF extraction. This considerable fiber fragmentation results from the high shear forces produced from the collisions between the balls of different sizes and the friction between the balls and the jar's wall. Differences on the average hydrodynamic sizes of the CNF samples

indicates that both solid concentration (4%, 10% and 16%) and reaction time (4 h and 8 h) conditions during enzymatic hydrolysis influenced subsequent fiber fragmentation and/or fibrillation by ball milling through fiber structure alterations. It can be observed that the lowest fiber fragmentation and highest PDI value was obtained for sample BM(HE16-8h). This indicates the presence of larger particles which could be due to incomplete fiber fragmentation, and, thus, low cellulose nanofibrillation degree. On the other hand, it can be observed that fiber surface changes that occurred during enzymatic hydrolysis performed at 4% and 10% solid concentrations, which corresponds to samples BM(HE4-4h), BM(HE4-8h), BM(HE10-4h), and BM(HE10-8h), enhanced fiber size reduction by subsequent ball milling. Extending the enzymatic hydrolysis time from 4 h to 8 h provided higher fiber fragmentation (average sizes in the nanoscale range -500 to 900 nm-) which favored the extraction of CNF, since decreases of 30% to 50% on the average size was observed. The PDI also decreased (0.35-0.43), indicating a greater CNF size homogeneity.

The determination of the CNF aspect ratio gives an indication of the separation and breakage extent of cellulosic fibers by estimating the ratio of the longest side to the shortest side. Even though the breakage of fibers is more apparent during ball milling treatment, enzymes act mostly in the amorphous regions of cellulose during enzymatic pretreatment, which cuts the cellulose chains and reduces their length. Because of this, even though the applied ball milling conditions were the same for all the samples, the aspect ratio values resulted different (Table 4.4.2). The aspect ratio values presented a more pronounced increase for samples BM(HE4-4h), BM(HE4-8h), and BM(HE10-8h), which reached values higher than 220, probably because of a decrease in the diameter of the microfiber due to peeling. Also, this could be associated to a better fibrillation of these cellulose fibers through ball milling treatment, which has been previously explained by the easier fragmentation due to fiber alterations during enzymatic hydrolysis. No significant difference was observed between samples BM(HE4-4h) and BM(HE4-8h) in terms of aspect ratio value, even though DLS results showed an increased fiber fragmentation for sample BM(HE4-8h) given the lower average hydrodynamic size (535 nm). This could be due to the fact that during ball milling, not only are microfibers separated but also shortened, which means that the length and the diameter of the extracted CNF decreased in a similar proportion resulting in a similar aspect ratio. On the other hand, it can be observed by comparing samples BM(HE10-4h) and BM(HE10-8h) that the aspect ratio was subsequently increased with the enzymatic hydrolysis time because of a predominant fibrillation effect, reaching a value of 227. Thus, these results may indicate that cellulosic samples HE4-4h, HE4-8h and HE10-8h resulted more processable during ball milling treatment, since the aspect ratio values of samples BM(HE4-4h), BM(HE4-8h) and BM(HE10-8h) resulted higher compared to samples BM(HE10-4h) and BM(HE16-8h). The lower aspect ratio values of samples BM(HE10-4h) and BM(HE16-8h) could be attributed to the

higher hemicellulose content in these samples, since it was reported by other authors that hemicellulose could apply a strong holding of fiber bundles, preventing them from becoming smaller after ball milling [143, 152]. Thus, it may be possible that a greater removal of hemicellulose during enzymatic hydrolysis facilitated the peeling of the cellulosic fibers during ball milling.

Zeta potential (ZP) analysis was performed for the CNF samples based on the tracking of the moving rate of negatively or positively charged particles across an electric field. The ZP of the CNF suspensions varied from -15 mV to -10 mV (Table 4.4.3), which indicates that they form an unstable colloidal suspension. Usually, absolute ZP values less than 15 mV represents the onset of agglomeration, and absolute ZP values greater than 30 mV are considered desirable for good colloidal stability [153]. However, the ZP values obtained were expected, because unlike other methods such as acid hydrolysis, enzymatic pretreatment does not incorporate negatively charged groups on the cellulose surface. The produced CNF has only a weak charge of around -15 mV, originated from its inherent hydroxyl groups. The absence of strong charges on the cellulose surface favors aggregation of the CNF, which may induce agglomeration of the CNM, resulting in a wide size dispersion (Table 4.4.2). The presence of acidic groups was estimated by conductometric titration for sample BM(HE10-8h) on 209 mmol/kg_{dry solid} (see titration curve in Appendix A9). Even though the acidic groups content resulted higher than the 75 mmol/kg_{dry solid} (sample HE10-8h in Table 4.4.2) for enzyme-treated BEKP, the surface charge density resulted much lower compared to CNF extracted by chemical methods. For instance, it was reported surface charge densities of around 1,290 mmol/kg for TEMPO-oxidized CNF, which can improve its dispersibility by the introduction of electrostatic repulsive forces [154]. These findings result in agreement with previous studies using enzymatic pretreatment for the extraction of CNM (nanocrystals or nanofibers) [153, 34]. However, it should be noted that materials with lower negative ZP values were reported more suitable for biomedical and related applications [34].

Spectroscopic methods have been employed as an indirect method to evaluate the fibrillation extent by measuring the transmittance or turbidity of the suspension [79]. The increase of transmittance at a specific wavelength through CNF suspension indicates the presence of smaller and/or more homogeneous materials in the nanoscale range. Moreover, it was previously reported a good correlation between transmittance and CNF gravimetric yield determined by centrifugation method. Thus, it is possible to correlate transmittance directly to the degree of fibrillation [148]. However, it is difficult to use this rapid measurement alone to determine fiber disintegration since nanomaterials are released simultaneously with detectable particles when mechanical disintegration is performed [148]. UV-Vis transmittance spectra (Fig. A10.2, Appendix A10) and transmittance values at 800 nm (Table 4.4.3) are presented for the CNF

samples obtained. These values ranged from 34% to 50%, achieving the highest value for samples BM(HE4-8h) and BM(HE10-8h). Transmittance results correspond quite well with the DLS analysis, which indicated that a higher fiber fragmentation was achieved for these samples considering the lower average hydrodynamic sizes (500-1000 nm) and PDI values (0.3-0.4) compared to the other samples. Also, results showed that extending the enzymatic hydrolysis time from 4 h to 8 h allowed to achieve a better fibrillation extent during ball milling process.

The crystallinity of CNF samples BM(HE4-8h) and BM(HE10-8h) was not significantly affected by ball milling treatment (CrI 81-83%) in comparison to the cellulosic samples before ball milling (76-79%). This shows that the ball milling had little effect on the crystalline structure of CNF. Moreover, the morphology of the CNF extracted from enzyme-treated BEKP observed in TEM micrographs showed entangled networks with fibril diameters in the nanoscale range (Fig. 4.4.3). TEM images were also used to analyze the width distribution of the CNF using Image J software. The CNF sample extracted by ball milling after 8 h of enzymatic hydrolysis (sample BM(HE10-8h)) presented an average width of 8.0 ± 2.0 nm (Fig. 4.4.3c). TEM analysis demonstrates that the pretreatment conditions used in this study were effective to provide a significant reduction in the diameter of the untreated BEKP (around $30 \mu\text{m}$, see Appendix A3).

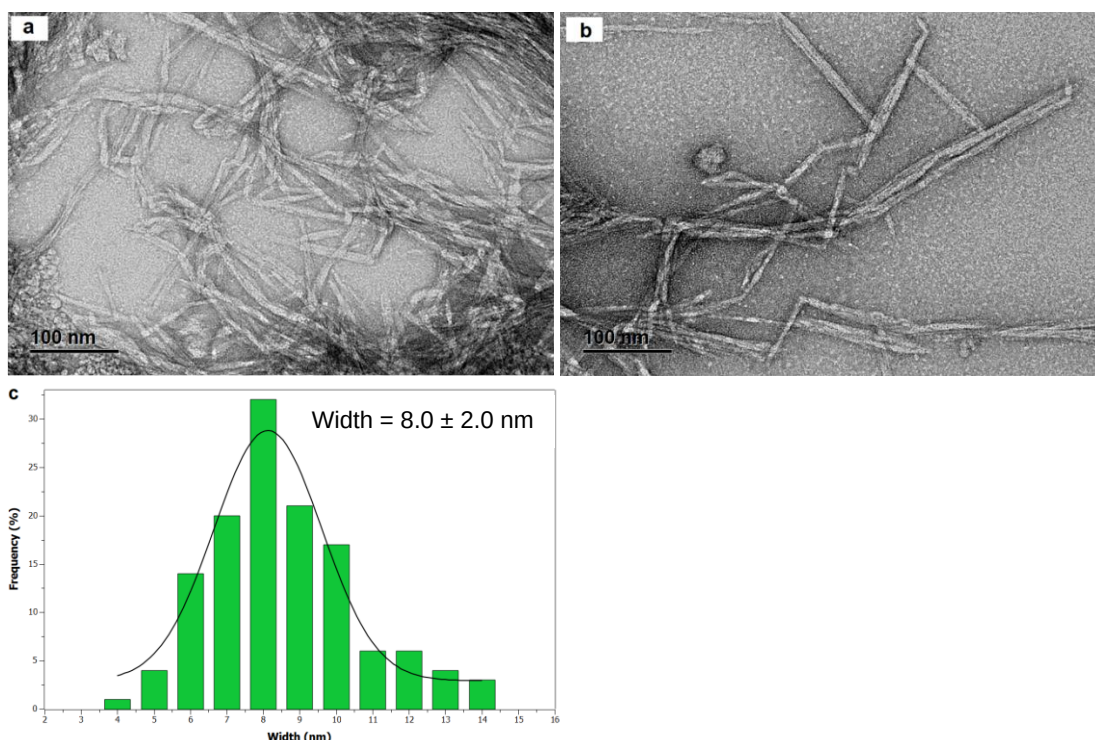


Figure 4.4.3. (a, b) TEM images and (c) width distribution of CNF extracted from enzyme-treated BEKP (sample HE10-8h). Average width and standard deviation are presented in the figure.

4.4.5. Evaluating the fermentability of enzymatic hydrolysates for biobutanol production by *Clostridium* sp.

Enzymatic hydrolysates from enzyme-mediated pretreatment of BEKP were used as substrate for biobutanol fermentation by *Clostridium beijerinckii* DSM 6422 and DSM 6423 for ABE and IBE fermentations, respectively. Glucose and xylose consumption, biomass growth, and butanol and solvents production profiles during fermentation in all experiments are presented in Fig 4.4.4.

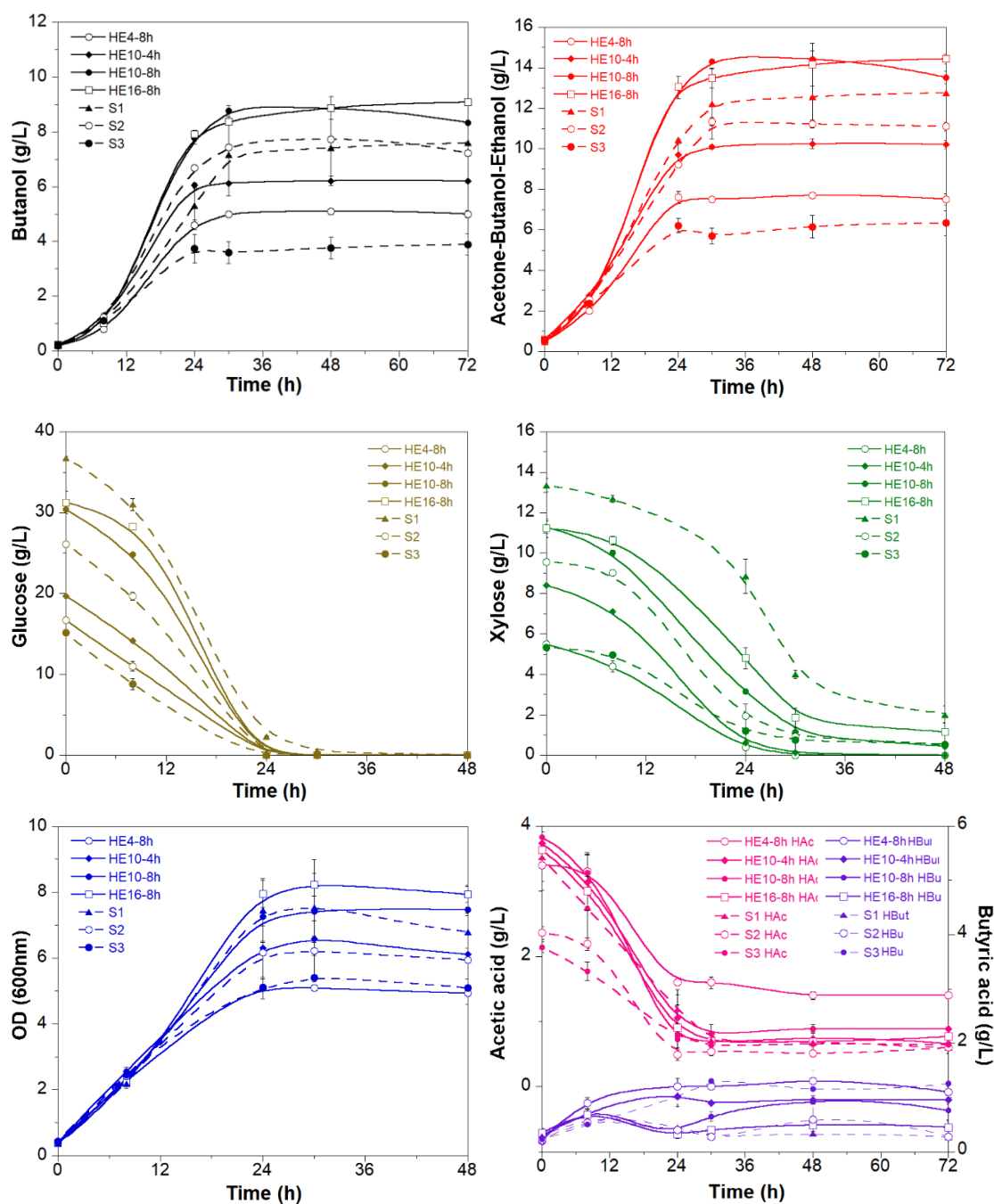


Figure 4.4.4. Butanol, total ABE, glucose, xylose, OD at 600 nm, and acetic (HAc) and butyric (HBu) acids profiles for ABE fermentation of enzymatic hydrolysates (HE4-8h, HE10-4h, HE10-8h, HE16-8h) and semi-synthetic media (S1, S2 and S3).

For ABE fermentation, it can be observed that both glucose and xylose were almost completely consumed within 24-48 h of incubation. It should be noted that lower initial sugars concentrations (20-35 g/L) required less than 24 h for complete consumption, while higher sugars concentrations (50 g/L) required up to 48 h for complete consumption. Higher amounts of butanol and solvents (acetone and ethanol) were produced when the medium contained higher initial concentration of sugars. The highest butanol concentration of 8.9 g/L (14 g/L total solvents) was obtained after 48 h in the enzymatic hydrolysates HE10-8h and HE16-8h with higher initial sugar concentrations (41-43 g/L). Acetone and ethanol were also produced during fermentation in lower concentrations, up to 5.3 g/L and 0.35 g/L, respectively. Regarding biomass growth, biomass concentrations of 1.4-2.4 g/L were achieved after 24 h of cultivation for all the experiments, with the highest growth (2.3 g/L) observed in samples with high initial concentrations of glucose and xylose. Table 4.4.4 presents the overall parameters determined after 24 h and 48 h when corresponded, for each experiment.

Table 4.4.4. Butanol fermentations of hydrolysates obtained from enzymatic pretreatment and semi-synthetic media by *C. beijerinckii* DSM 6422.

Parameter	Enzymatic hydrolysate				Semi-synthetic medium		
	HE4-8h	HE10-4h	HE10-8h	HE16-8h	S1	S2	S3
t _F (h)	24	24	48	48	48	24	24
G _i (g/L)	16.7	19.7	30.4	31.3	36.8	26.1	15.2
X _i (g/L)	5.5	8.4	11.3	11.2	13.4	9.6	5.3
C _G (%)	100	100	100	100	92	87	100
C _X (%)	93	93	96	90	31	46	78
B (g/L)	4.6	6.1	8.9	8.9	7.4	6.7	3.7
AE (g/L)	3.0	3.6	5.6	5.3	5.2	2.6	2.5
ABE (g/L)	7.6	9.7	14.5	14.2	12.6	9.3	6.2
HAc (g/L)	1.6	1.1	0.7	0.7	0.7	0.5	0.8
HBu (g/L)	1.2	1.0	0.9	0.5	0.3	0.4	1.0
ΔX (g/L)	1.6	1.9	2.2	2.3	2.3	1.8	1.3
Y _{B/S} (g/g)	0.20	0.21	0.21	0.21	0.19	0.19	0.18
Y _{ABE/S} (g/g)	0.32	0.33	0.34	0.33	0.32	0.28	0.29
Q _B (g/Lh)	0.19	0.25	0.18	0.18	0.15	0.22	0.16
Q _{ABE} (g/Lh)	0.32	0.40	0.30	0.29	0.26	0.32	0.26
Butanol:ABE ^a	0.55	0.56	0.55	0.56	0.53	0.67	0.54

S1, S2, S3: semi-synthetic medium 1, 2 and 3, respectively; t_F: fermentation time; G_i: initial glucose concentration; X_i: initial xylose concentration; C_G: glucose consumption; C_X: xylose consumption; B: butanol; AE: acetone and ethanol; HAc: acetic acid; HBu: butyric acid; ΔX: overall biomass growth; Y_{B/G}: overall sugars to butanol conversion yield; Y_{ABE/G}: overall sugars to ABE conversion yield; Q_B: overall butanol productivity; Q_{ABE}: overall ABE productivity

^aProduced butanol to ABE molar ratio

One of the main advantages of *Clostridium* strains are their ability to ferment both hexoses (e.g. glucose) and pentoses (e.g. xylose) during fermentation of lignocellulosic-derived materials [29]. In this study, high consumption rate of both glucose (up to 100%) and xylose (up to 96%) were achieved for all the conditions evaluated using enzymatic hydrolysates. Moreover, these consumption rates resulted higher compared to those achieved in the experiments using semi-synthetic media (87-100% for glucose, 31-78% for xylose). It should be noted that a higher concentration of initial acetic acid was observed in the enzymatic hydrolysate samples (up to 4 g/L) than in the semi-synthetic media (up to 2.5 g/L), which may be possibly due to the acetate buffer used during enzymatic pretreatment. According to previous studies [29], the higher acetate concentration in the enzymatic hydrolysates could have enhanced butanol fermentation performance and, thus, sugars consumption, improving overall butanol yields (0.20-0.21 g/g).

For IBE fermentation, the same trend was observed, the highest butanol concentration reached corresponded to the highest initial sugar concentration (Fig 4.4.5). A maximum butanol concentration of 6.9 g/L (12 g/L total solvents) was achieved in this case after 48 h in the enzymatic hydrolysate (HE16-8h) with an initial sugar concentration of 46 g/L. Isopropanol (up to 5.5 g/L) was produced in this case instead of acetone, along with low concentrations of ethanol (up to 0.2 g/L). Regarding biomass growth, biomass concentrations of 1.4-3.0 g/L were achieved after 24 h of cultivation for all the experiments, with the highest growth (2.9 g/L) observed in samples with high initial concentrations of glucose and xylose. Table 4.4.5 presents the overall parameters determined after 48 h for each experiment. In this case, high consumptions were not achieved in all the experiments. Only in experiments with relatively low initial sugar concentration (20-28 g/L) was achieved high consumption rates of glucose (92-100%) and xylose (81-100%). When initial sugar concentration was further increased (35-50 g/L), residual amounts of both glucose and xylose sugars remained unconverted (up to 27% for glucose and 56% for xylose). It should be noted that the amount of unconverted sugars resulted lower in the enzymatic hydrolysate samples rather than in the semi-synthetic media, probably due to the enhanced butanol fermentation performance at higher initial acetate concentration [29]. Incomplete sugar conversion to butanol and coproducts in IBE fermentation was also observed in our previous studies [87, 29] and others [155] IBE fermentation studies. Even though incomplete sugar consumption was not achieved at higher sugars concentration, higher butanol yields (0.20-0.21 g/g) were reached in this case.

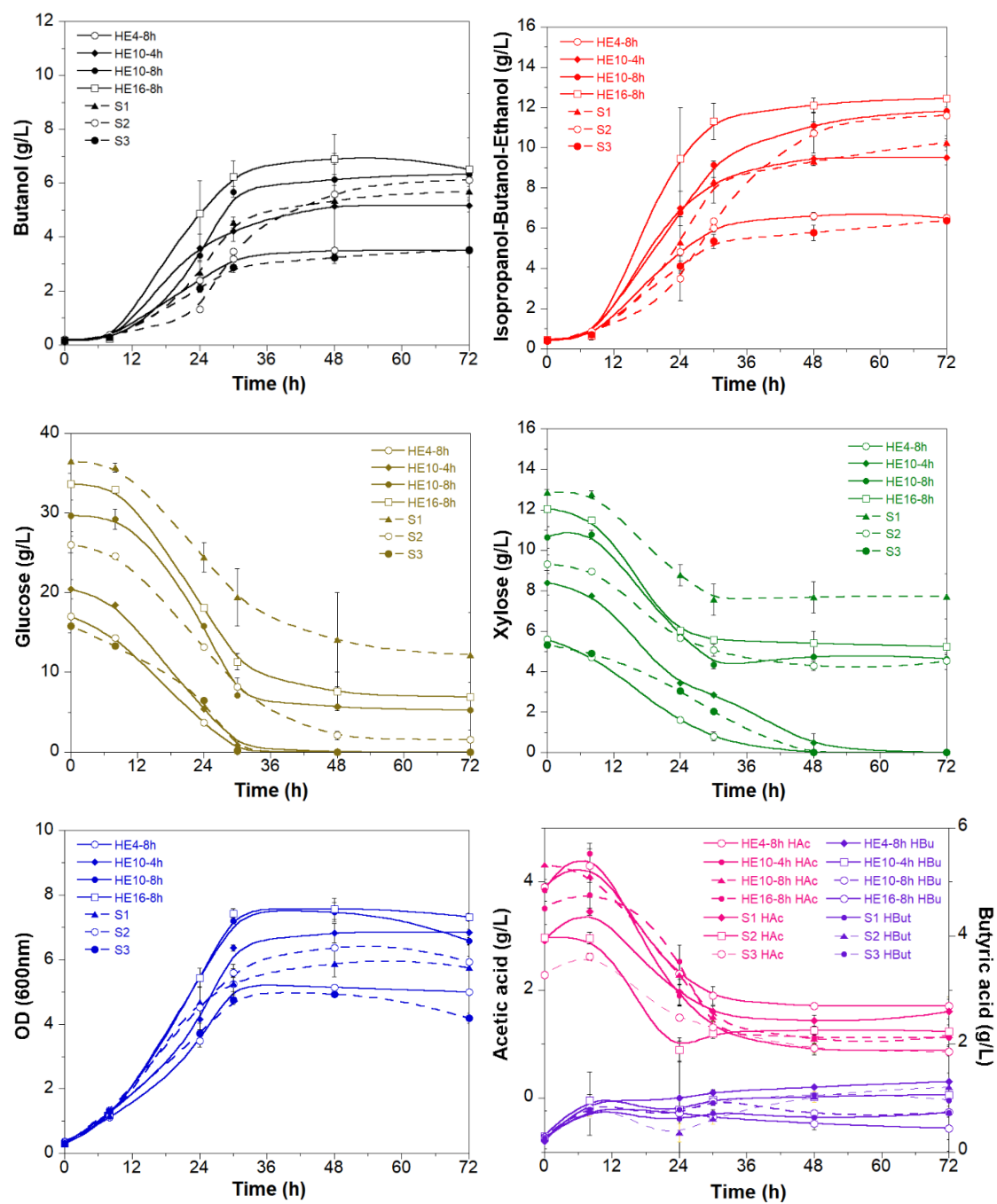


Figure 4.4.5. Butanol, total IBE, glucose, xylose, acids (acetic and butyric), OD at 600 nm, and acetic (HAc) and butyric (HBut) acids profiles for IBE fermentation of enzymatic hydrolysates (HE4-8h, HE10-4h, HE10-8h, HE16-8h) and semi-synthetic media (S1, S2 and S3).

Table 4.4.5. Biobutanol fermentation of hydrolysates obtained from enzymatic pretreatment and semi-synthetic media by *C. beijerinckii* DSM 6423.

Parameter	Enzymatic hydrolysate				Semi-synthetic medium		
	HE4-8h	HE10-4h	HE10-8h	HE16-8h	S1	S2	S3
t _F (h)	48	48	48	48	48	48	48
G _i (g/L)	17.0	20.4	29.7	33.7	36.5	26.0	15.8
X _i (g/L)	5.6	8.4	10.6	12.1	12.9	9.3	5.3
C _G (%)	100	100	81	77	73	92	100
C _X (%)	81	94	56	55	44	54	100
B (g/L)	3.6	5.1	6.1	6.9	5.4	5.6	3.2
IE (g/L)	3.0	4.4	4.6	4.9	3.9	5.1	3.6
IBE (g/L)	6.6	9.5	10.7	11.8	9.3	10.7	5.8
HAc (g/L)	1.7	0.9	1.1	1.1	1.4	1.2	0.9
HBu (g/L)	1.2	1.0	0.7	0.5	0.6	1.0	1.1
ΔX (g/L)	1.0	2.2	2.7	2.9	2.3	1.9	1.3
Y _{B/S} (g/g)	0.15	0.18	0.20	0.21	0.16	0.19	0.15
Y _{IBE/S} (g/g)	0.27	0.32	0.35	0.36	0.28	0.35	0.25
Q _B (g/Lh)	0.07	0.11	0.13	0.14	0.11	0.12	0.07
Q _{IBE} (g/Lh)	0.14	0.20	0.23	0.25	0.19	0.22	0.12
Butanol:IBE ^a	0.48	0.49	0.50	0.51	0.52	0.47	0.50

S1, S2, S3: semi-synthetic medium 1, 2 and 3, respectively; t_F: fermentation time; G_i: initial glucose concentration; X_i: initial xylose concentration; C_G: glucose consumption; C_X: xylose consumption; B: butanol; IE: isopropanol and ethanol; HAc: acetic acid; HBu: butyric acid; ΔX: overall biomass growth; Y_{B/G}: overall sugars to butanol conversion yield; Y_{IBE/G}: overall sugars to IBE conversion yield; Q_B: overall butanol productivity; Q_{IBE}: overall IBE productivity

^aProduced butanol to IBE molar ratio

A proposed process which intends to produce biofuels and/or commodity chemicals must result in products at high titers and yields to be cost-competitive. In this part of the study, both ABE and IBE fermentations allowed to obtained high overall sugars to butanol yield (0.21 g/g) at high initial sugar concentration (up to 50 g/L), even though incomplete sugar conversion to product was obtained in IBE fermentation at this condition. However, it was reported by other authors that IBE mixture represents more attractive than ABE for biofuel application, for instance, owing to acetone corrosiveness to rubber or plastic engine parts and isopropanol higher energy density and octane number [84].

4.4.6. Overall mass balance for the coproduction process

Fig. 4.4.6 presents the overall mass balance for the proposed CNF and biobutanol coproduction process from BEKP including enzymatic pretreatment, ball milling treatment, and fermentation

stages. Considering the BEKP composition, 61% of it was recovered in the form of fermentable sugars (70% w/w glucose and 30% w/w xylose) at relatively high concentration in the enzymatic hydrolysate after enzyme-mediated pretreatment. After the fermentation step, it was possible to completely convert these sugars into butanol and value-added coproducts (acetone or isopropanol, and ethanol). Additionally, 39.2% of BEKP was effectively extracted in the form of CNF from cellulose fibers after enzymatic hydrolysis and ball milling. Thus, for an initial 100 kg of BEKP, the proposed integrated process allows to recover 39.2 g of CNF, and 19 kg of ABE mixture (62% w/w butanol and 35% w/w acetone) or 15 kg of IBE mixture (57% w/w butanol and 40% w/w isopropanol) depending on the *Clostridium* strain used (Fig. 4.4.6). The product yields achieved in this study resulted comparable or even higher to those reported by other authors using both cellulose and hemicellulose fractions in lignocellulosic materials (Table 4.4.6). For example, Bondancia et al. [72] used BEKP as cellulosic feedstock and reported a recovery of 37% of the initial BEKP as CNC and 39% as glucose for ethanol production. Also, Wang et al. [125] used bleached hardwood Kraft pulp (BHKP) for CNC extraction by acid hydrolysis and reported a recovery of approximately 9% and 91% of the initial BHKP as CNC and glucose for ethanol fermentation, respectively. Both studies reached bioethanol production yields in the range of 25 to 34 kg of ethanol per 100 kg of cellulosic material, while this study achieved biobutanol production yields in the range of 8.4 to 11.7 kg of butanol per 100 kg of BEKP. This was expected considering the higher production yields of ethanol fermentation compared to butanol fermentation.

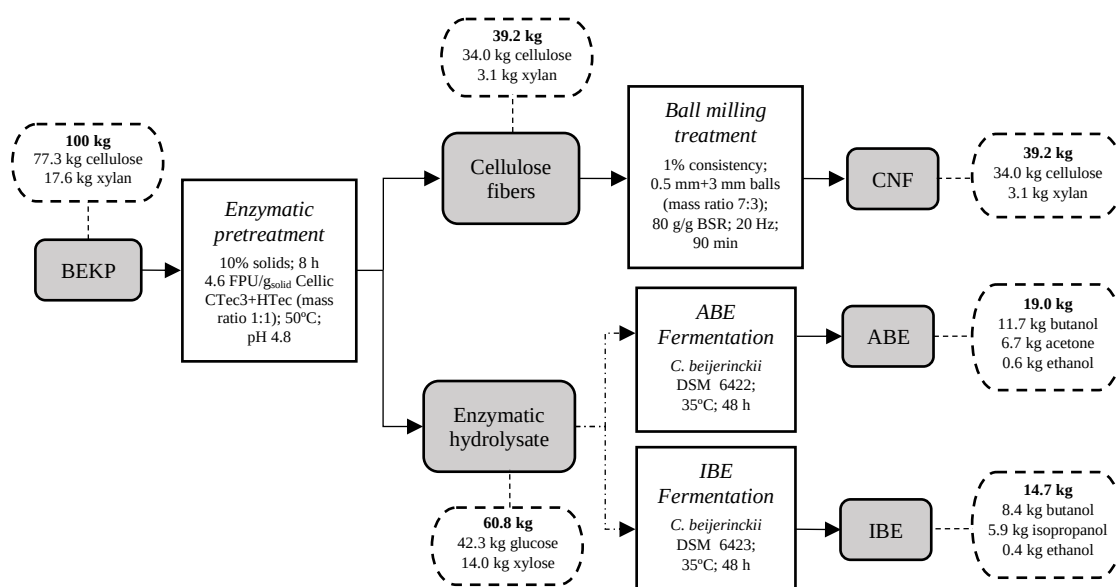


Figure 4.4.6. Mass balance for the proposed biobutanol and cellulose nanofibers (CNF) coproduction process from an initial dry bleached eucalyptus kraft pulp (BEKP) of 100 kg.

Table 4.4.6. Literature review of enzyme-mediated biorefinery processes that coproduce cellulose nanomaterials (CNMs) and biofuels or value-added chemicals.

Raw Material	Composition RM (wt%)	Enzyme pretreatment of cellulose-rich pulp	Pretreatment for CNM production	Overall CNM yield (wt% RM)	Overall co-products yield (wt% RM)	Microorganism for fermentation	Reference
Bleached hardwood Kraft pulp	Cellulose: 79.1 ^a Xylan: 21.2 ^a Lignin: 4.0 ^a	Non-commercial NS 51129 (Novozymes®) 15 FPU/g; 50°C, pH 4.8; 10% solids; 8 h	Acid hydrolysis: 64 wt% H ₂ SO ₄ , 45°C, 2 h	CNC: 8.9	Ethanol: 24.8 ^b	Modified <i>Saccharomyces cerevisiae</i> Y128	[125]
Sugarcane bagasse	Glucan: 42.4 Xylan: 22.1 Lignin: 22.1	Cellic CTec2 (Novozymes®) 15 mg/g _{solid} ; 50°C; pH 4.8; 17.5% solids; 72 h	Disc ultra-refining: 1% w/w, 1600 rpm, 100 µm distance grinding discs, 1-13 cycles	CNF: 26.1	Ethanol: 9.4 XOS: 6.6 LNP: 11.6	<i>Saccharomyces cerevisiae</i> MH36 (Mangrove Jack)	[156]
Bleached eucalyptus Kraft pulp	Cellulose: 75.6 Hemicellulose: 14.6 Lignin: 6.7 Ash: 1.1	Cellic CTec3 (Novozymes®) 10 mg/g _{solid} ; 50°C; pH 5; 20% solids; 24 h	Enzymatic hydrolysis: Cellic CTec3 10 mg/g _{solid} ; 35°C; pH 5; 20% solids; 120 h	CNC: 36.9 ^b	Ethanol: 19.4 ^b	<i>Saccharomyces cerevisiae</i> (Fleischmann)	[72]
Douglas-fir wood chips	Glucan: 44.6 Xylan/mannan: 16.7 Lignin: 32.0 Others: 4.9	Cellic CTec2 + Cellic HTec2 (Novozymes®) 5.5 g/100g _{solid} ; 50°C; pH 4.8-5.3; 19% solids; 8 h	Acid hydrolysis: 64 wt% H ₂ SO ₄ , 44°C, 30 min	CNC: 5.6	Glucose: 12.1 Xylose/Mannose: 4.0	na	[157]
Bleached softwood Kraft pulp	Glucan: 79.2 Xylan: 15.3 Lignin: 1.2	<i>Caldicellulosiruptor bescii</i> DSM 6725 7.5 mg/g _{solid} ; 75°C; 1% solids; 72 h	na	CNM: 42	Glucose: 44	na	[105]
Bleached eucalyptus Kraft pulp	Glucan: 77.3 Xylan: 17.6 Lignin: 0.7	Cellic CTec3 + Cellic HTec2 (Novozymes®) 4.2 mg/g _{solid} ; 50°C; pH 4.8; 10% solids; 8 h	Ball milling: 1% w/w, 20 Hz, 80g _{balls} /g _{solid} , 1.5 h	CNF: 39.2	Butanol: 11.7 AE: 7.3 Butanol: 8.4 IE: 6.3	<i>Clostridium beijerinckii</i> DSM 6422 <i>Clostridium beijerinckii</i> DSM 6423	This study

RM: raw material; CNC: cellulose nanocrystals; XOS: xylo-oligosaccharides; LNP: lignin nanoparticles; AE: acetone and ethanol; IE: isopropanol and ethanol; na: not applicable

^aSource: Beyene et al. [158]; ^bCalculated from data reported by the authors.

On the other hand, the integrated coproduction of CNM and biofuels or other chemicals were also studied using lignocellulosic materials. For instance, Pereira et al. [156] using sugarcane bagasse reported recovery of 30% of the initial xylan as XOS, 52% of the initial lignin as lignin nanoparticles (LNP), and approximately 38% of the initial cellulose as sugars for ethanol production [156]. Du et al. [157] using Douglas-fir wood chips recovered 12% of the initial cellulose as CNC, 24% as glucose, and 21% of the initial xylan/mannan as xylose/mannose [157]. This demonstrates that the coproduction strategy proposed in this study has the potential to improve productivity and enable maximal value recovery from cellulosic materials.

4.4.7. Summary

Eucalyptus cellulose fibers were partially hydrolyzed by cellulase and xylanases cocktails at high solid concentrations and low enzyme loading. The enzymatic cocktail employed during enzymatic pretreatment made cellulose fibers more swollen and accessible by breaking the cellulose network, which caused fiber surface changes that influenced subsequent fiber size reduction. As it was already mentioned, the use of hydrolytic enzymes to produce CNM allows the coproduction of sugars that could be further converted to biofuels and/or value-added chemicals for an integral use and exploitation of the raw material. In this part of the study, the by-product streams containing relatively high amount of sugars (50 g/L) were recovered and completely fermented into biobutanol and coproducts (acetone and isopropanol) using native *Clostridium beijerinckii* reaching concentrations close to 15 g/L. The enzymatic pretreatment of the cellulose fibers and subsequent mechanical defibrillation by ball milling allowed to obtain CNF with a width of 4-14 nm and an aspect ratio of 220-230. Differences on CNF size distribution indicated that process conditions during enzymatic pretreatment such as solid concentration and hydrolysis time influenced fiber fragmentation and/or fibrillation by ball milling through fiber structure alterations.

The results presented in this section demonstrated the feasibility of coproducing CNF and biobutanol as high value-added products from eucalyptus pulp by enzyme fractionation. This highlights the promising opportunity to produce value-added bioproducts from the sugars generated during enzyme-mediated pretreatment of cellulosic materials for CNM extraction. These findings serve as an important starting point for future studies and contributes to the knowledge regarding the coproduction of biomaterials and value-added chemicals in a lignocellulosic biorefinery.

Chapter 5. Conclusions and future work

The major outcomes of this research are presented in this chapter, along with future work directions based on the research of this thesis.

5.1. Conclusions

The main goal of this work was to research on the integrated production of CNF and biobutanol from the cellulosic fraction of eucalyptus wood through a biochemical approach and under a biorefinery concept. This work aimed to provide insight into the pretreatment of the raw material employing enzymes in combination with a mechanical treatment for CNF isolation, and fermentation of soluble sugars to biobutanol. It also assessed how fiber changes occurring during enzymatic pretreatment was influenced by the operational conditions and the enzymes employed. In particular, a major goal was to better understand how enzymatic pretreatment of eucalyptus pulp takes place in order to be able to facilitate mechanical extraction of CNF from cellulosic substrates.

Some of the main findings and specific contributions of this study to the field are listed below:

- Local eucalyptus cellulose Kraft pulp used as raw material has shown to be a promising cellulosic material for the isolation of CNF due to its high cellulose content (77%) and low lignin (<1%) content. Also, the relatively high hemicellulose (as xylan) content represents an interesting carbohydrate source for its bioconversion to value-added coproducts and its integration to the CNF extraction process.
- The enzymatic pretreatment using both cellulase complex and the monocomponent EG enzyme was effective in improving the accessibility of cellulose in eucalyptus pulp for subsequent CNF extraction by mechanical methods. The addition of auxiliary proteins, either with catalytic or non-catalytic properties, to the enzyme cocktail allowed to work with lower cellulase loadings and achieve comparable results in terms of cellulose accessibility, depolymerization and average fiber size reduction to those achieved using only cellulases.

Physicochemical characteristics demonstrated that the synergism effect between xylanase complex and swollenin during cellulase pretreatment can greatly open up the eucalyptus pulp fibers by selective removal of hemicellulose and fiber morphology changes, increasing cellulose accessibility without compromising cellulose properties.

Microscopy analysis confirmed fiber length and size reduction of eucalyptus pulp caused by enzyme-mediated pretreatment, which could enhance mechanical defibrillation for CNF isolation.

The enzymatic pretreatment by cellulase complex caused a significant shortening of the cellulose chains' length of eucalyptus pulp, as well as hydrolysis of cellulose and hemicellulose polymers (mainly xylan), with the consequent release of soluble sugars. Given the composition of the cellulase complex, the cooperative action of EG, CBHs and β Gs resulted in cellulose fiber depolymerization and decomposition to glucose. In contrast, the EG pretreatment caused negligible carbohydrate hydrolysis, which allowed to achieve almost complete solid recoveries for CNF isolation. In this case, depolymerization and fiber fragmentation occurred at a lesser extent compared to cellulase pretreatment, since EG enzymes targets amorphous cellulose regions by selective disruption of glycosidic bonds, resulting in smaller cellulose chains. Due to the lack of CBHs and β Gs, the decomposition of cellulose to glucose did not occur.

Based on these results, it can be concluded that by selecting the appropriate enzyme complex or monocomponent, it is possible to benefit the extraction of CNF by mechanical treatment without hydrolyzing cellulose to soluble sugars. Also, the ratio between CNF and fermentable sugars can be tuned by controlling the enzymatic pretreatment conditions such as solid concentration and reaction time, which have shown to have an important effect on this ratio. Besides, the cellulase-mediated pretreatment of cellulose pulp afforded significant amount of sugars released as a coproduct, which can be easily recovered in a separate liquid fraction for further biotechnological applications through the production of value-added chemicals.

- The ultrasonication treatment allowed to achieve a complete nanofibrillation of enzyme-treated eucalyptus cellulose microfibers, which demonstrated that enzyme-mediated pretreatment enhanced the isolation of CNF from eucalyptus pulp. Regarding the process conditions, the ultrasonication amplitude turned out to be the most significant variable in the CNF isolation.

Physicochemical and microscopic characterization showed that CNF of uniform nanometer sizes, small diameters, and good stability and transmittance were obtained, which demonstrated that the ultrasound waves in ultrasonication generated a good defibrillation of enzyme-treated cellulose fibers. However, differences obtained in the CNF size distribution showed that the enzymatic pretreatment conditions, such as the type of enzyme and the reaction time, influenced the fragmentation and/or fibrillation of the fiber through fiber structure alterations. Despite the relatively poor surface charge, homogenous and stable CNF

suspensions were obtained by ultrasonication. Ribbon-like CNFs with diameters of 3–10 nm were well dispersed into almost elementary fibril levels. Also, highly transparent and flexible nanocellulosic films were produced by ultrasonicated CNF suspensions, which results as attractive properties for potential applications to electronic devices, for example.

Crystallinity and thermal analysis showed that ultrasonication caused damages to the crystalline structure of enzyme-treated fibers, but better properties were achieved in this case compared to conventional acid extraction methods. These results demonstrated that ultrasonication combined with enzymatic pretreatment constitutes an effective strategy to isolate nanofibers from different cellulosic materials with attractive properties.

- Ball milling as a mechanical treatment resulted a promising alternative method for the extraction of CNF from enzyme-treated eucalyptus pulp. A great advantage of this treatment compared to ultrasonication is the ability to obtain CNF suspensions of greater consistency, and its feasibility for large-scale application. However, rheology in this case turned out to be more complex since working at higher solid concentrations did not allow to achieve complete nanofibrillation of the cellulosic material.

Sequential operating strategies (in stages) were evaluated and resulted promising for improving the CNF extraction efficiency, but further research should be performed to better understand the phenomenon occurring.

Regarding the process conditions, the BSR and milling frequency turned out to be important variables to control the number of collision events between the milling balls and the fibers for an efficient isolation of CNF. Also, enzymatic pretreatment conditions, such as the type of enzyme, solids concentration and reaction time, influenced the fiber fragmentation by ball milling through alterations in the fiber structure.

One of the main challenges of ball milling method is the inorganic contamination introduced from the milling equipment. In this study, it was demonstrated that the influence of milling conditions is critical in controlling the extent of inorganic contamination during this mechanical treatment. Working conditions should be carefully selected by balancing the CNF extraction yield and size distribution with the level of contamination.

Morphological, thermal, optical and crystallinity analysis of ball milled CNF suspensions were effectively obtained. CNFs with diameters of 4–14 nm and aspect ratios of 220–230 with enhanced CNF thermal properties were obtained by milling treatment compared to ultrasonication. Also, crystallinity analysis did not show damages in the crystalline structure by milling treatment. Even though unstable CNF suspensions due to the poor surface charge were obtained, flexible nanocellulosic films were produced by ball milled products.

- The by-product stream generated during the enzyme-mediated pretreatment of cellulose pulp for CNF extraction, which contained relatively high amount of sugars, was effectively converted into biobutanol through ABE and IBE fermentation. Along with biobutanol, coproducts such as acetone and isopropanol were produced using native *Clostridium beijerinckii* microorganisms, which were capable of metabolizing both glucose and xylose. These sugars were derived from the cellulosic and hemicellulosic fractions of the raw material, respectively. The feasibility of coproducing CNF and biobutanol as high value-added products from eucalyptus cellulose pulp was demonstrated. Production yields of biobutanol and coproducts of 15-19 kg per 100 kg of raw material processed (mixture containing 57-62% w/w of butanol) and CNF extraction yield of 39 kg per 100 kg of raw material processed were achieved under the proposed integrated process.

In summary, this work serves as an important starting point for future studies and contributes to the knowledge regarding the coproduction of biomaterials and value-added chemicals in a lignocellulosic biorefinery.

5.2. Possible future work

Based on this research work, suggested future studies in the following topics are listed below:

- Enzymatic pretreatment of eucalyptus cellulose pulp for CNF extraction was shown in this study to be highly dependent on the nature of the enzymes employed. One of the main drawbacks of enzyme-mediated pretreatment of lignocellulosic materials is the cost of enzymes. Alternatives to the use of commercially available enzymes should be assessed. For instance, it would be beneficial to formulate an enzyme mixture by screening a series of enzymes with various functionalities for their propensity to produce CNMs. Thus, studies should be focused on the economic viability of enzymes production for CNMs application.
- While ball milling treatment proved to be an adequate and promising process for CNF extraction from cellulosic materials, improvements in equipment efficiency and capacity are required for a viable commercial process. Rheological studies should be performed on ball milled products to better understand the phenomenon occurring during high solid concentration to achieve higher CNF extraction yields. Also, future work would need to evaluate the use of dispersants to allow efficient fiber disintegration into nanostructures at relatively high solid concentration during milling process.

- The evaluation of the scale-up of the ball milling treatment would be of interest to add more value to the proposed process, as well as evaluating both the enzymatic pretreatment and fermentation stages at a larger scale (e.g. bioreactor).
- Considering that the costs involved in CNMs production makes their large-scale operation difficult, evaluate if the combination of pretreatments proposed in this study contributes to decrease the energy consumption and total cost of the process. Also, evaluate the environmental impact related to others production methods. For this, techno-economic and life-cycle assessments would be of interest (TEA and LCA, respectively). Further examination of the process design along with enzymatic and mechanical treatments optimization could likely prove essential in facilitating cost-effective biorefining of lignocellulosic biomass at industrially conditions.
- Perform in-depth characterization of the nanocellulosic films produced to evaluate potential applications. For example, the study of the mechanical properties of the nanocellulosic films.
- Evaluate the applicability of the proposed integrated process to other cellulosic or lignocellulosic materials under a biorefinery concept that allows a complete valorization of the raw material.

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Appendix A1. Summary of analytical methods used for materials' characterizations

Method	Abbreviation	Parameters	Type of material	Samples
High performance liquid chromatography	HPLC	Glucose and xylose concentrations	Liquid fractions	Liquid fractions from chemical characterization assays Liquid fractions from enzymatic pretreatment (enzymatic hydrolysates) and fermentation experiments
Gas chromatography	GC	Butanol, acetone, isopropanol, ethanol, acetic acid, and butyric acid concentrations	Liquid fractions	Liquid fractions from fermentation experiments
Water retention value	WRV	-	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw) HE4-4h / HE4-8h / HE10-4h / HE10-8h / HE16-4h / HE16-8h
			EG-treated raw material	EG30-8 / EG30-24 / EG100-8 / EG100-24 / EG170-8 / EG170-24 / EG240-8 / EG240-24 / EG240 / EG120+Xyn
Direct Blue adsorption	DB adsorption	-	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw)
			EG-treated raw material	EG30-8 / EG30-24 / EG100-8 / EG100-24 / EG170-8 / EG170-24 / EG240-8 / EG240-24 / EG240 / EG120+Xyn BM(HE4-4h) / BM(HE4-8h) / BM(HE10-4h) / BM(HE10-8h) / BM(HE16-8h)

Degree of polymerization	DP	-	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw) HE4-4h / HE4-8h / HE10-4h / HE10-8h / HE16-4h / HE16-8h
			EG-treated raw material	EG30-8 / EG30-24 / EG100-8 / EG100-24 / EG170-8 / EG170-24 / EG240-8 / EG240-24 / EG240 / EG120+Xyn
X-ray diffraction	XRD	Crystallinity index (CrI)	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw) HE4-8h / HE10-8h / HE16-8h
			CNF by US	US(X-4h) / US(X-8h) / US(C+X+S-4h)
			CNF by BM	BM80-30
Thermogravimetric analysis	TGA	Thermal decomposition onset temperature (T _{on}) Maximal weight loss temperature (T _{max})	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw)
			CNF by US	US(C-4h) / US(C-8h) / US(C+X-4h) / US(C+X-8h) / US(X-4h) / US(X-8h) / US(C+X+S-4h) / US(C+X+S-8h)
			CNF by BM	BM80-30
Fiber quality analyzer	FQA	Length-weighted average (Lw) Fines content Average hydrodynamic size	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec) / ET BEKP (Xyn) / ET BEKP (CTec+Xyn) / ET BEKP (CTec+Xyn+Sw)

Dynamic light scattering	DLS	Average hydrodynamic size Polydispersity index (PDI)	CNF by US	US(C-4h) / US(C-8h) / US(C+X-4h) / US(C+X-8h) / US(X-4h) / US(X-8h) / US(C+X+S-4h) / US(C+X+S-8h)
			CNF by BM	BM40-90 / BM40-180 / BM80-90 / BM80-180 / BM120-90 / BM120-180 / BM160-90 / BM160-180 / BM200-90 / BM200-180
				BM(C+X) / BM(EG) / BM(EG+X)
				BM(HE4-4h) / BM(HE4-8h) / BM(HE10-4h) / BM(HE10-8h) / BM(HE16-8h)
Zeta potential	ZP	Zeta potential (ZP) Surface charge	CNF by US	US(C-4h) / US(C-8h) / US(C+X-4h) / US(C+X-8h) / US(X-4h) / US(X-8h) / US(C+X+S-4h) / US(C+X+S-8h)
			CNF by BM	BM40-90 / BM40-180 / BM80-90 / BM80-180 / BM120-90 / BM120-180 / BM160-90 / BM160-180 / BM200-90 / BM200-180
				BM(C+X) / BM(EG) / BM(EG+X)
				BM(HE4-4h) / BM(HE4-8h) / BM(HE10-4h) / BM(HE10-8h) / BM(HE16-8h)
Transmittance	-	Transmittance value	CNF by US	US(C-4h) / US(C-8h) / US(C+X-4h) / US(C+X-8h) / US(X-4h) / US(X-8h) / US(C+X+S-4h) / US(C+X+S-8h)
			CNF by BM	BM40-90 / BM40-180 / BM80-90 / BM80-180 / BM120-90 / BM120-180 / BM160-90 / BM160-180 / BM200-90 / BM200-180
				Ball milled CNF obtained at 20 Hz, BSR of 80-200 g/g for 15-180 min (Fig. 4.3.7a) Ball milled CNF obtained at 10 Hz, BSR of 80-200 g/g for 90 min (Fig. 4.3.7b)
				BM(C+X) / BM(EG) / BM(EG+X)
Sedimentation	-	Sedimentation ratio (Hs/Ho) Aspect ratio (A)	CNF by BM	BM40-90 / BM80-90 / BM120-90 / BM160-90 / BM200-90
			CNF by BM	BM(HE4-4h) / BM(HE4-8h) / BM(HE10-4h) / BM(HE10-8h) / BM(HE16-8h)

Conductometric titration	-	Acidic groups content (AG)	Raw material	BEKP
			Cellulase-treated raw material	HE4-4h / HE4-8h / HE10-4h / HE10-8h / HE16-4h / HE16-8h
Optical microscopy	OM	Fiber length and width distributions	Raw material	BEKP
			Cellulase-treated raw material	ET BEKP (CTec+Xyn)
			EG-treated raw material	EG240 / EG120+Xyn
Scanning electron microscopy	SEM	Morphology	Raw material	BEKP
			CNF by BM	BM(C+X) / BM(EG)
Energy dispersive X-ray spectroscopy	EDS	Elemental composition	CNF by BM	BM80-90
Transmission electron microscopy	TEM	Morphology Width distribution	CNF by BM	BM(HE10-8h)
Atomic force microscopy	AFM	Morphology Width distribution	CNF by US	US(C-8h) / US(C+X-4h) / US(C+X-8h) / US(X-8h)
X-ray diffraction	XRF	Elemental composition	CNF by US	US(X-8h)
			CNF by BM	BM80-90 / BM80-180 / BM(C+X)
Loss on ignition	LOI	Inorganic content	CNF by BM	Ball milled CNF obtained at 20 Hz, BSR of 80-200 g/g for 15-180 min (Fig. 4.3.8a)
				Ball milled CNF obtained at 10 Hz, BSR of 80-200 g/g for 90 min (Fig. 4.3.8b) BM(C+X) / BM(EG) / BM(EG+X)

US: ultrasonication; BM: ball milling.

Appendix A2. Size distribution analysis by LD

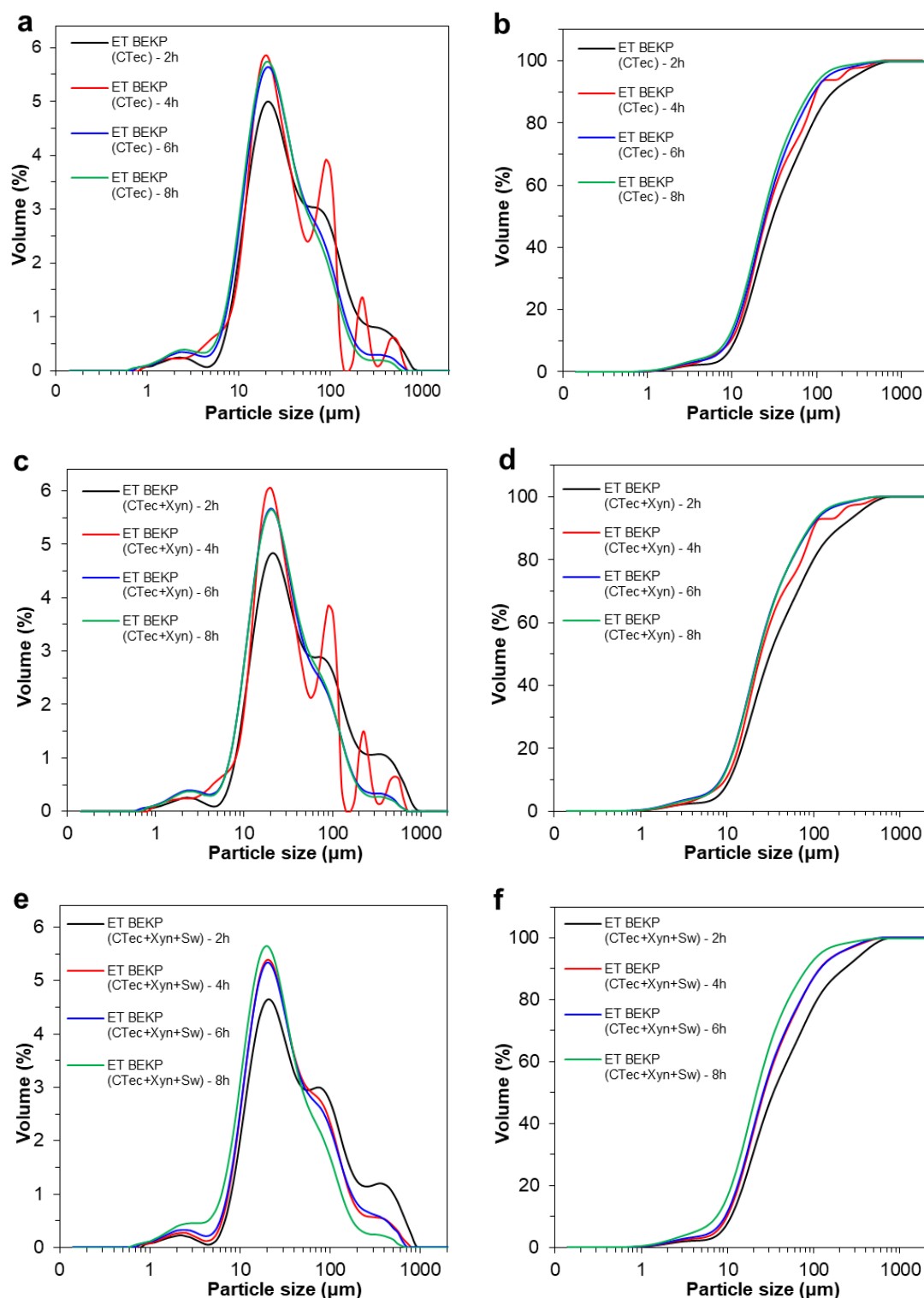


Figure A2.1. Monitoring enzyme-treated (ET) BEKP size distribution during enzyme-mediated pretreatment through laser diffraction (LD) analysis: (a, c, e) probability density functions (b, d, f) cumulative distribution functions.

Appendix A3. Width distribution of enzyme-treated BEKP

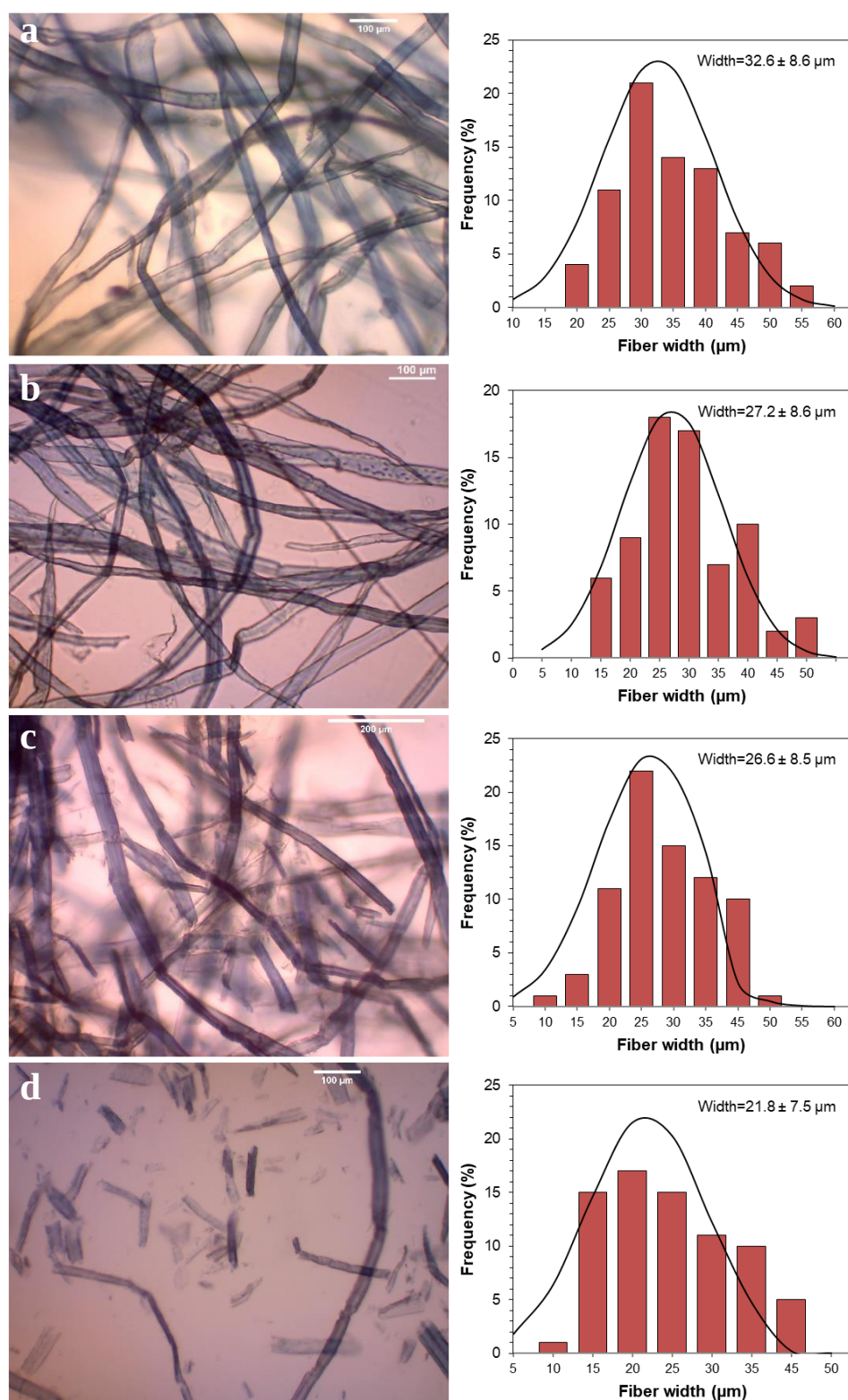


Figure A3.1. Optical microscopy images showing the morphology and width distributions for samples: (a) untreated BEKP, (b) EG-treated at 240 U/g_{BEKP} for 8 h, (c) EG-treated at 120 U/g_{BEKP} for 8 h with xylanase supplementation, and (d) cellulase-treated BEKP at 4.6 FPU/g_{BEKP} for 8 h with xylanase supplementation. The average width and standard deviation are labeled in the figures.

Appendix A4. Data characterization of ultrasonicated CNF suspensions

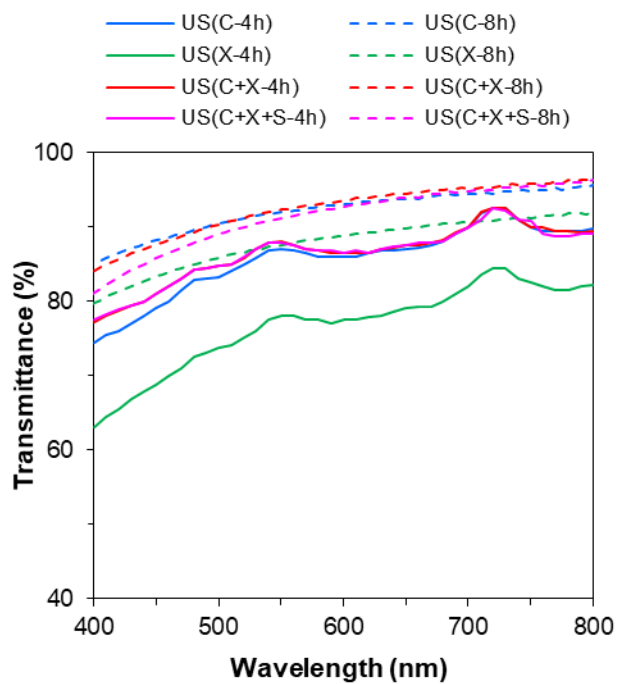
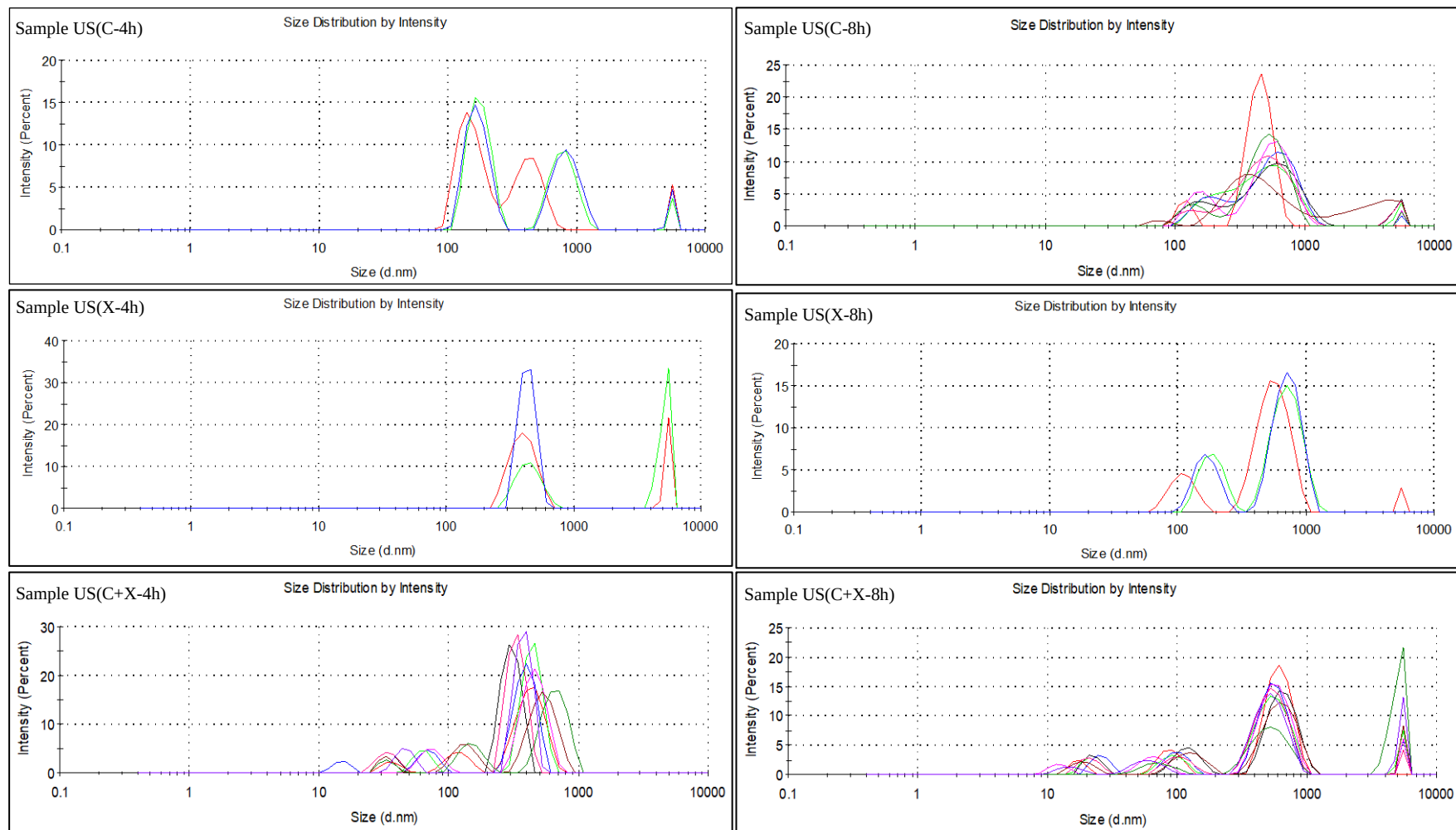
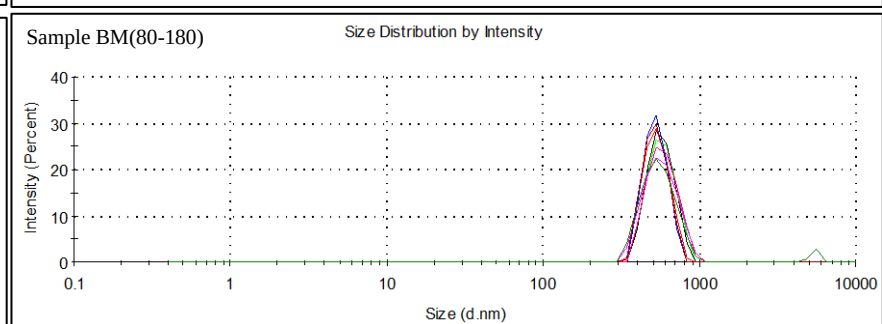
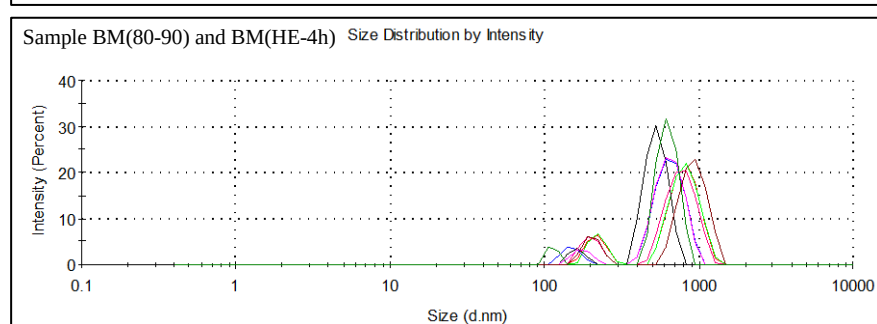
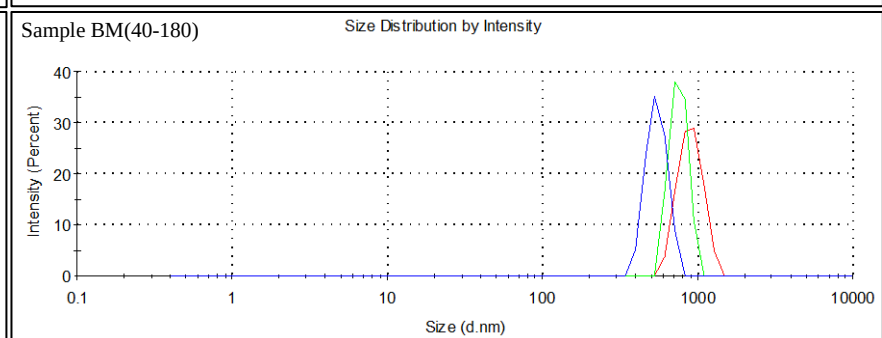
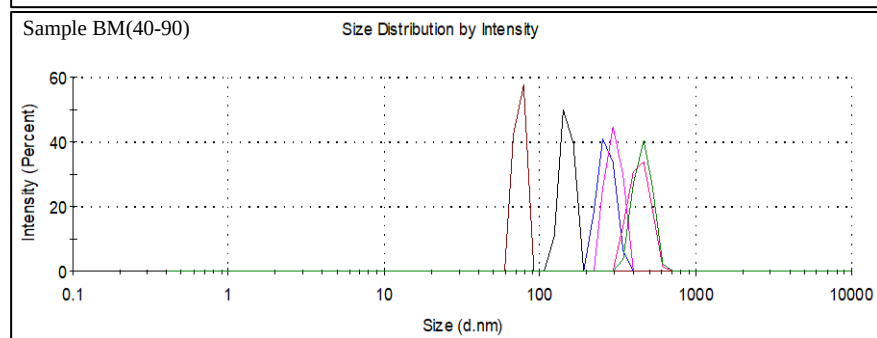
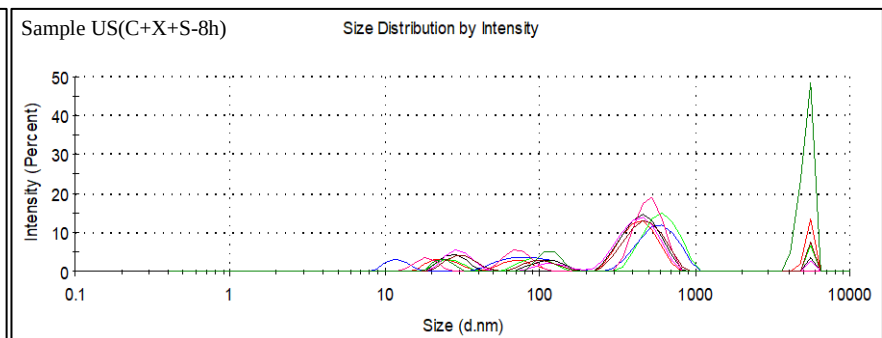
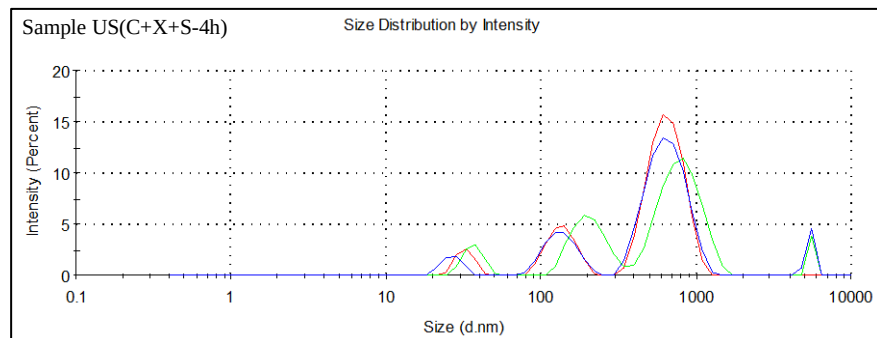
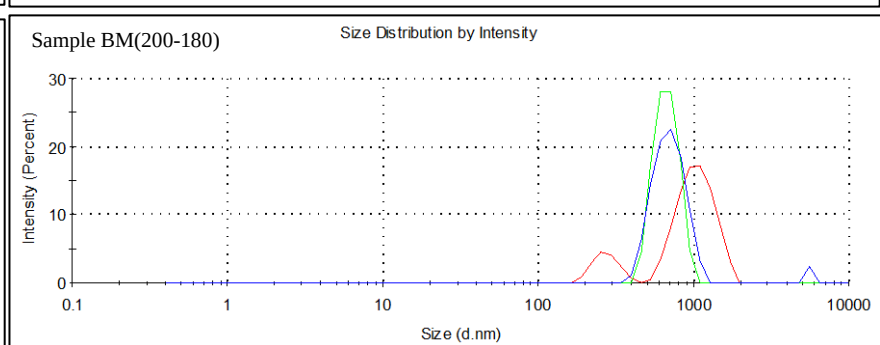
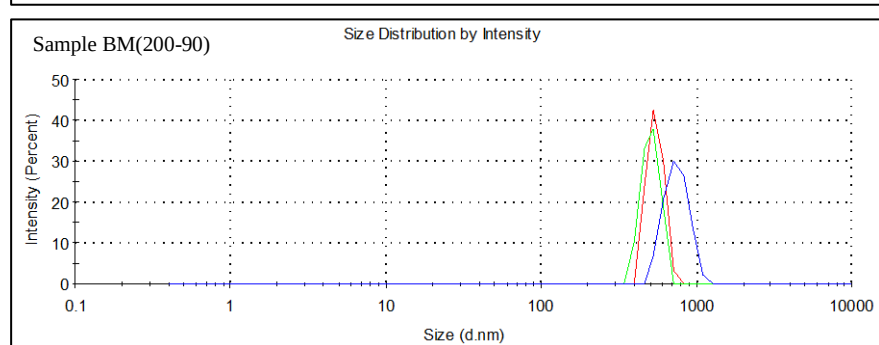
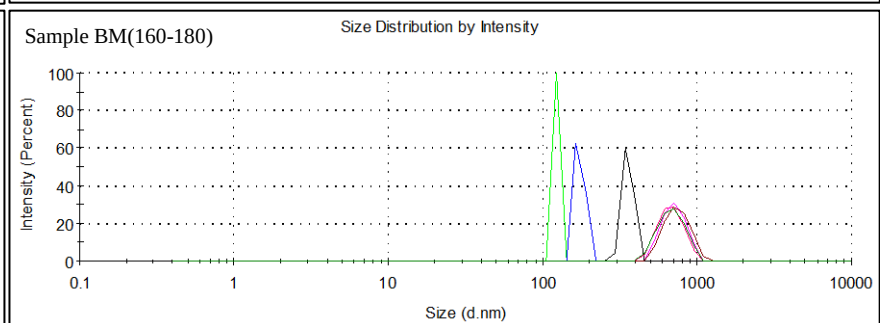
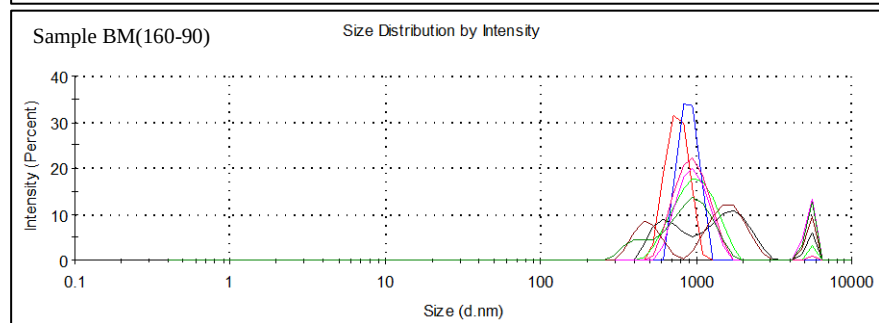
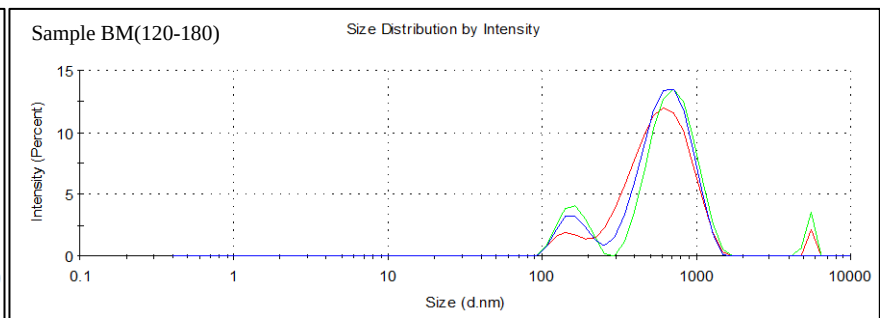
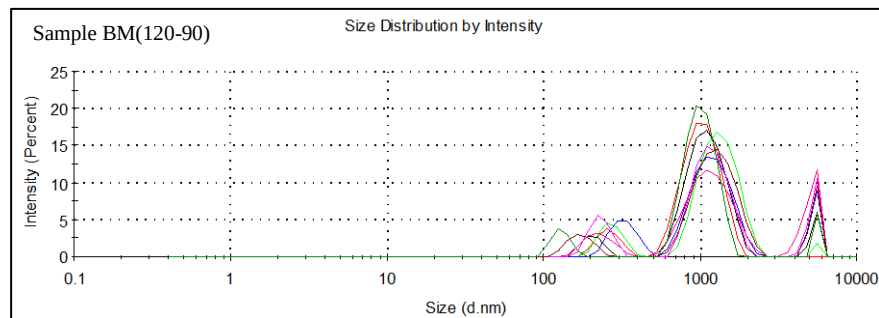


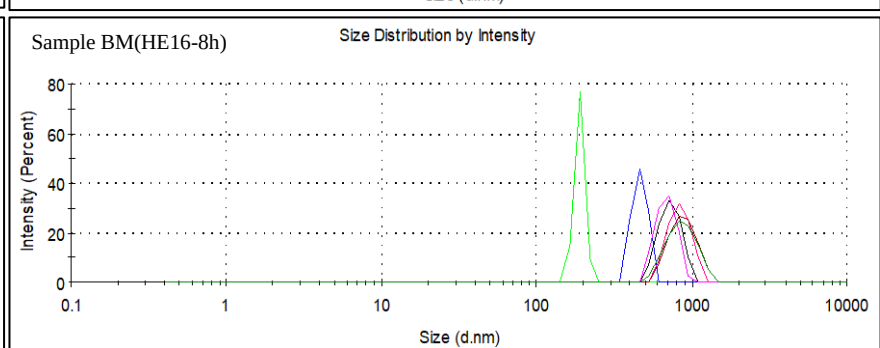
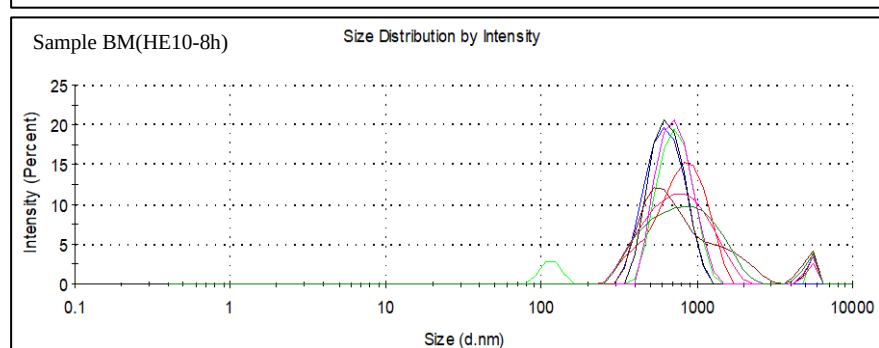
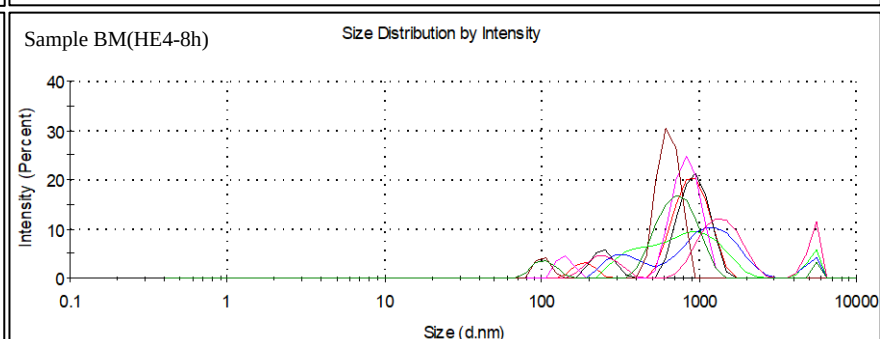
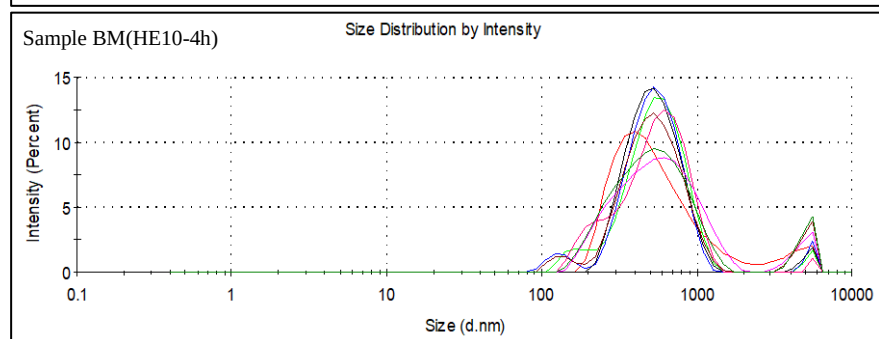
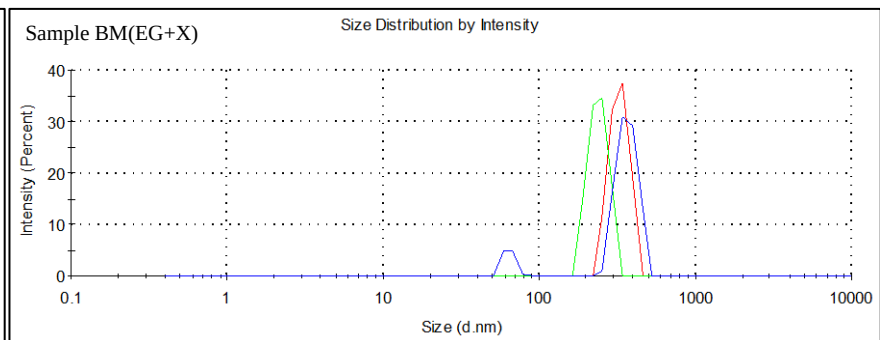
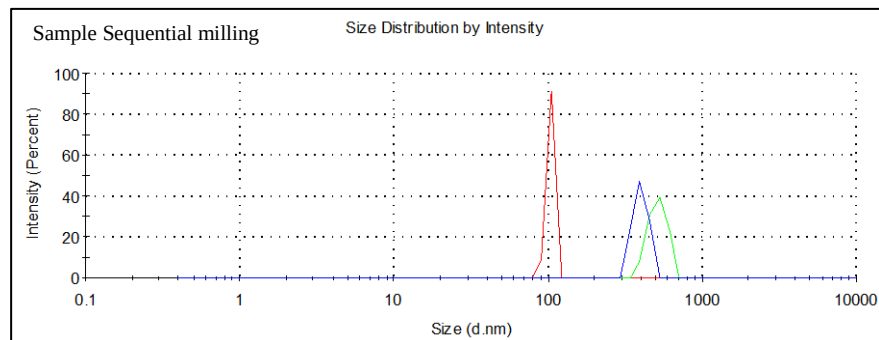
Figure A4.1. UV-Vis transmittance spectra of CNF suspensions.

Appendix A5. DLS spectra of CNF samples









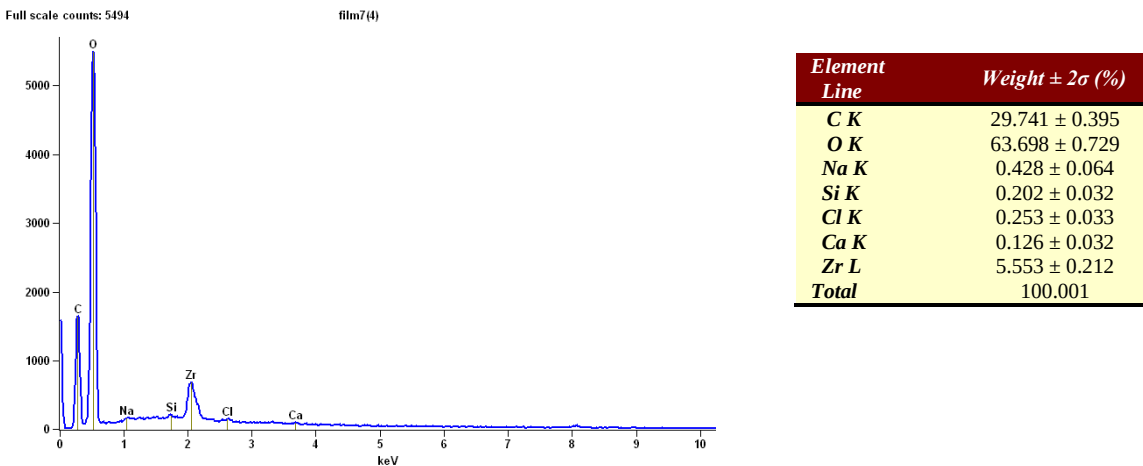
Appendix A6. Pictures of sedimentation tests for ball milled CNF samples

BSR (g/g)	-	40	40	80	80	120	120	160	160	200	200
Milling time (min)	-	90	180	90	180	90	180	90	180	90	180
0 h											
4 h											
24 h											
48 h											
72 h											

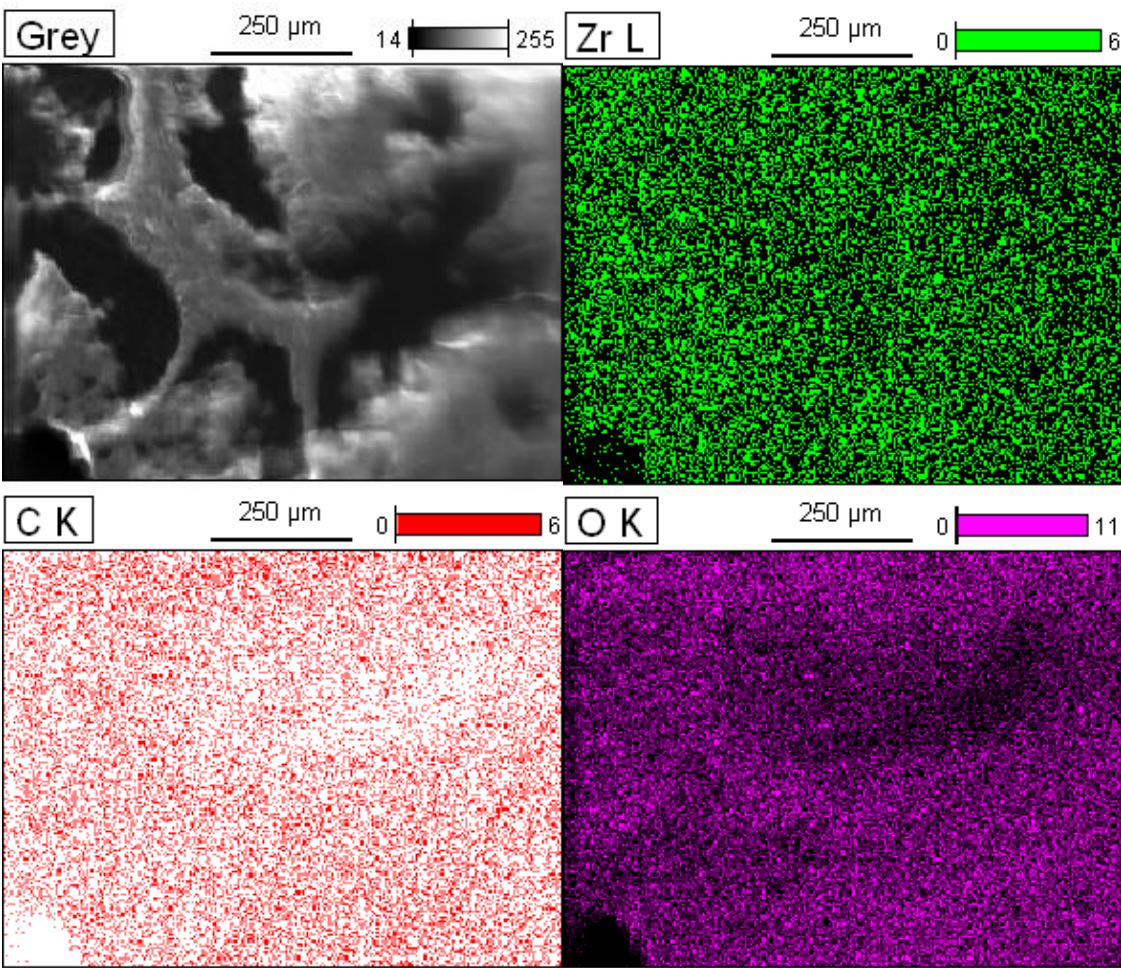
Appendix A7. EDS in SEM analysis of ball milled CNF sample

EDS collected data in SEM for sample CNF BM80-90:

- Quantitative results of elemental concentrations from area:



- Qualitative results of elemental (C, O, Zr) concentrations from maps:



Appendix A8. XRD spectra for ball milled CNF

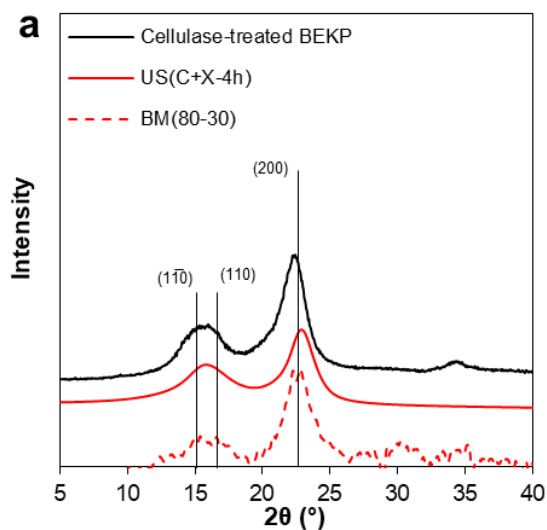


Figure A8.1. XRD pattern of ball milled CNF (sample BM80-30, solid red line) and its comparison to cellulase-treated BEKP (black line) and ultrasonicated CNF (sample US(C+X-8h), dashed red line).

Appendix A9. Conductometric titration curves

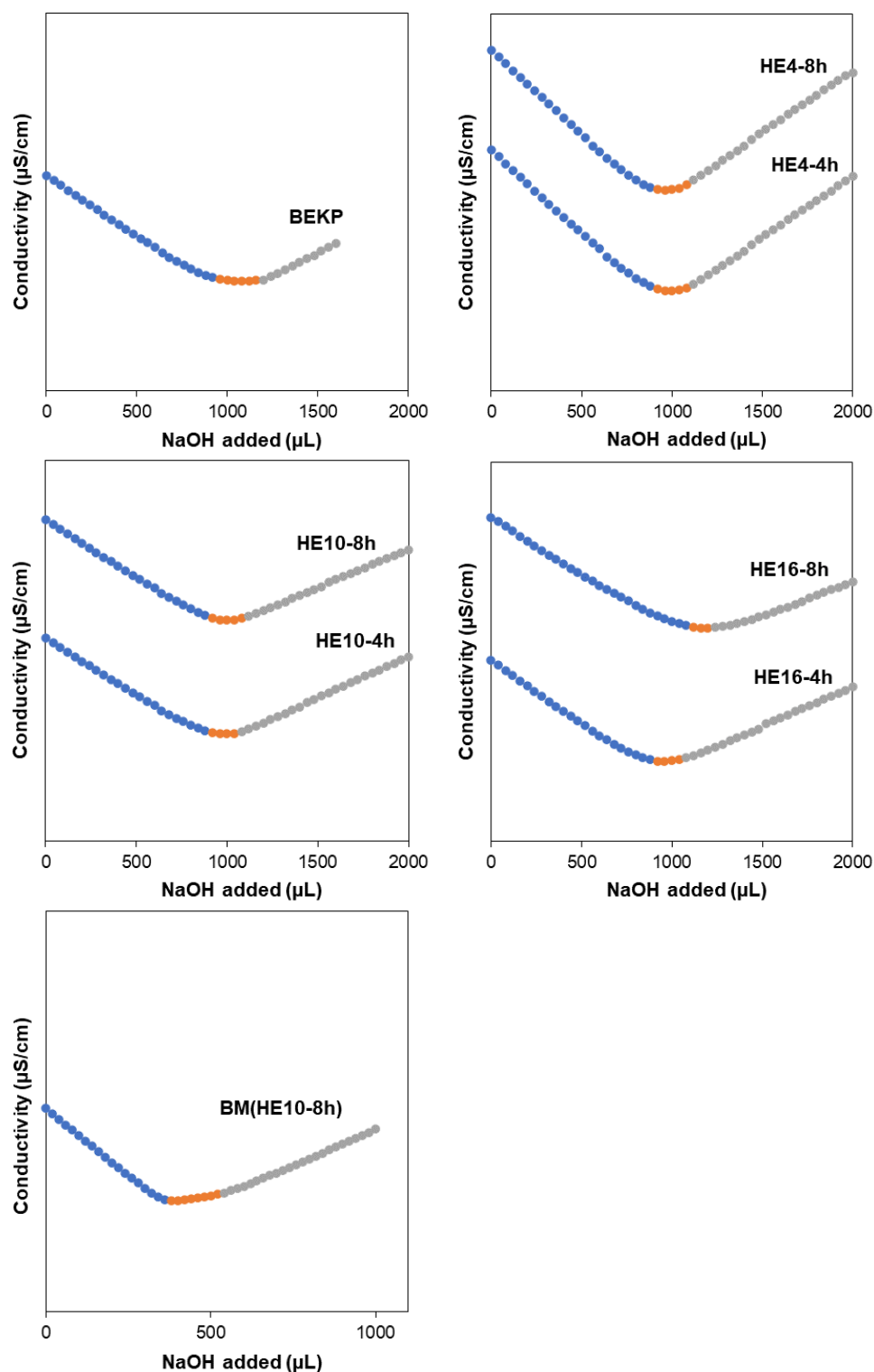


Figure A9.1. Conductometric titration curves of untreated (BEKP), enzyme-treated (HE4-4h, HE4-8h, HE10-4h, HE10-8h, HE16-8h) and CNF (BM(HE10-8h)) samples.

Appendix A10. Data characterization of ball milled CNF suspensions

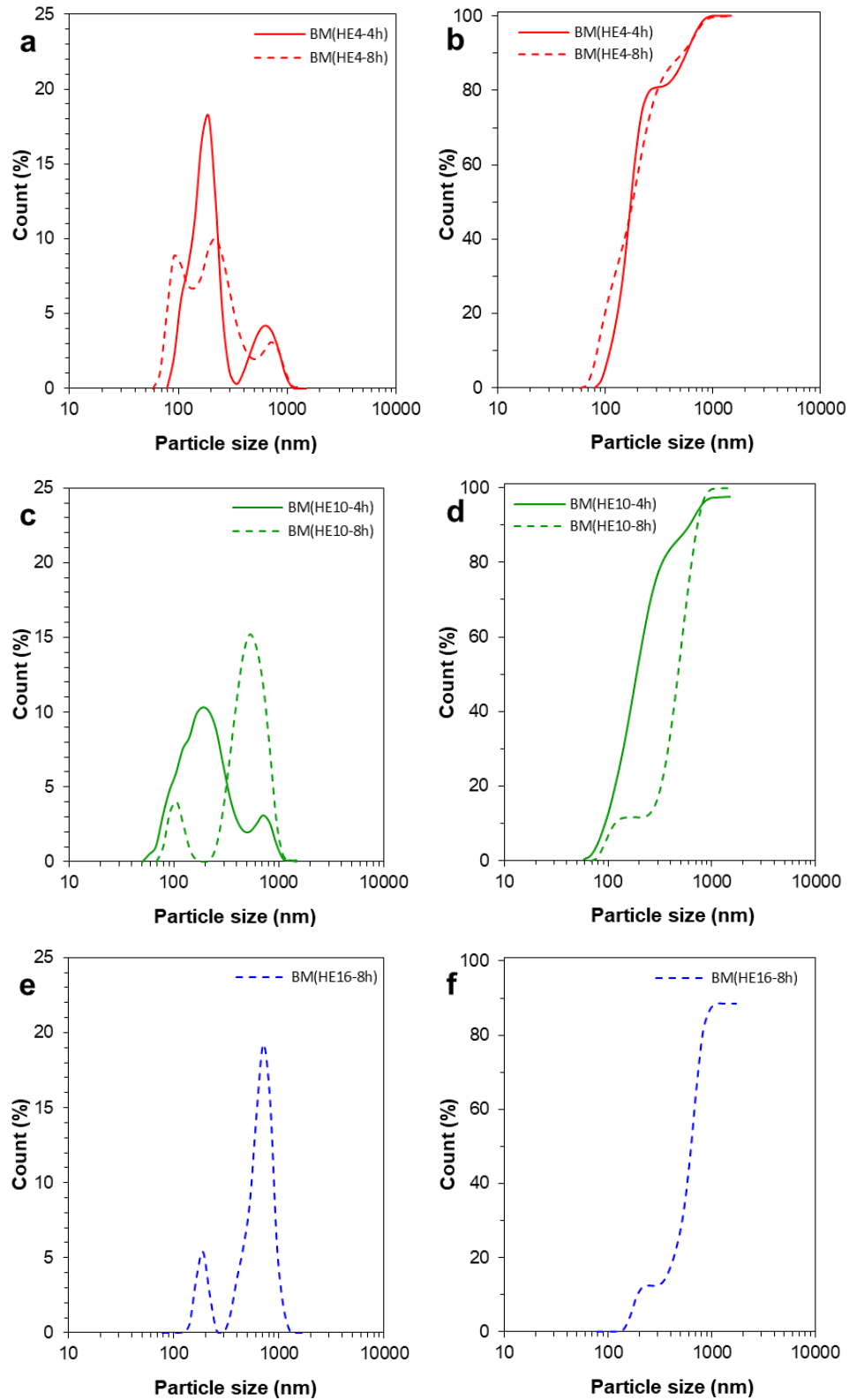


Figure A10.1. (a, c, e) Probability density, and (b, d, f) cumulative distribution (right) functions for CNF samples.

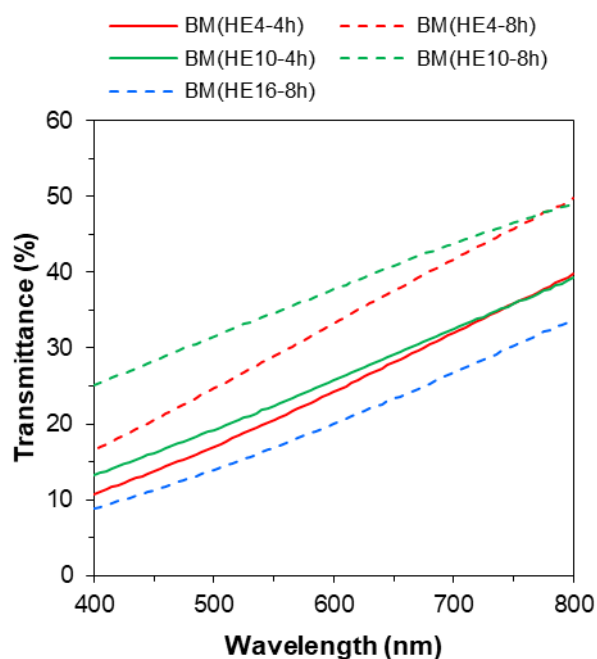
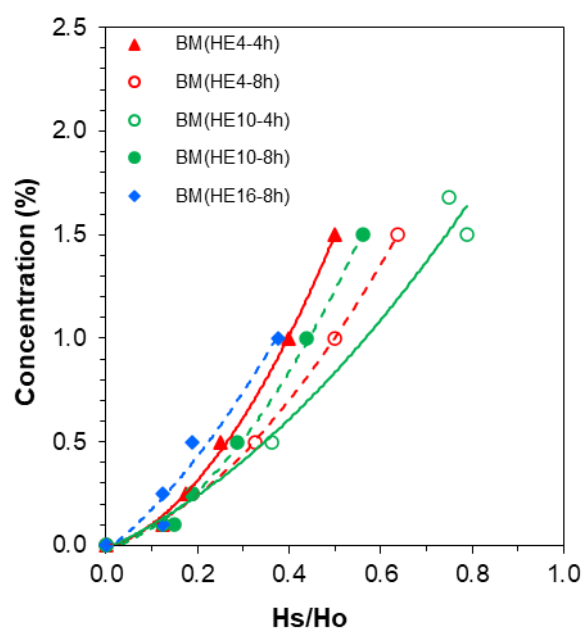


Figure A10.2. UV-Vis transmittance spectra of CNF suspensions.



Samples	Line in graph	Fitting equation	Gel point solids concentration (kg/m ³)
BM(HE4-4h)	—	$y=4.60x^2 + 0.73x - 0.01$, $R^2=0.99$	0.73
BM(HE4-8h)	- - -	$y=2.53x^2 + 0.74x$, $R^2=0.99$	0.74
BM(HE10-4h)	—	$y=1.39x^2 + 1.00x - 0.02$, $R^2=0.99$	1.00
BM(HE10-8h)	- - -	$y=3.62x^2 + 0.70x - 0.02$, $R^2=0.99$	0.70
BM(HE16-8h)	- - -	$y=2.78x^2 + 1.73x - 0.03$, $R^2=0.99$	1.73

Figure A10.3. Gel point solids concentrations from sedimentation method of CNF suspensions.

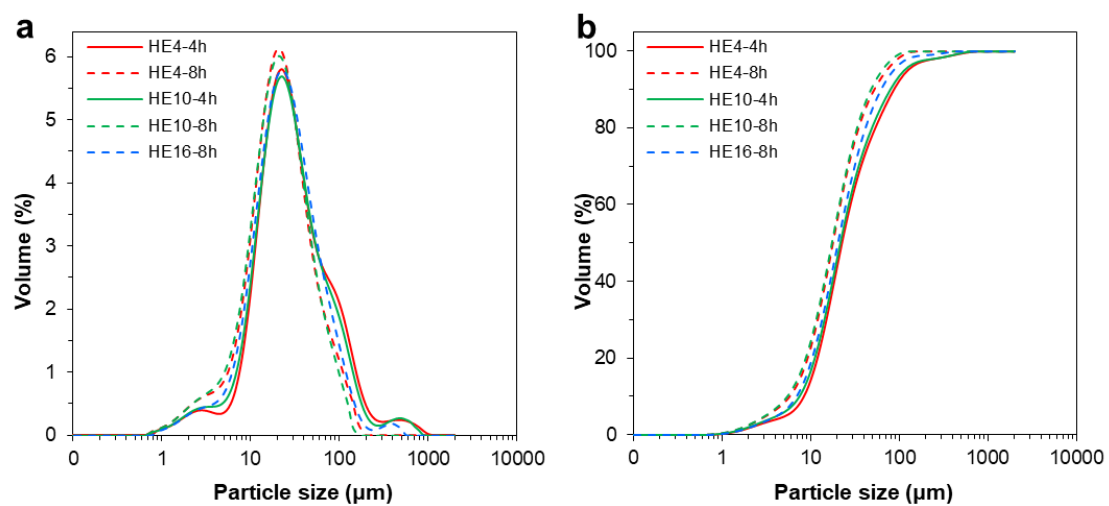


Figure A10.4. Size distribution of enzyme-treated BEKP samples through laser diffraction analysis: (a) probability density function and (b) cumulative distribution function.