UNIVERSIDADE DE SÃO PAULO
ESCOLA DE ENGENHARIA DE LORENA

ISABELLA KAROLINE RIBEIRO DIAS

The study of xylanase effects as an auxiliary enzyme on the production of cellulose nanocrystals through enzymatic hydrolysis with endoglucanase

Lorena

2017

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Dissertation presented to the Escola de Engenharia de Lorena da Universidade de São Paulo to obtain the title of Master of Sciences of the Graduate Program in Industrial Biotechnology in the area of concentration of Biomass Conversion.

Advisor : Prof. Dr. Valdeir Arantes.

Original Version

LORENA

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ISABELLA KAROLINE RIBEIRO DIAS

O estudo dos efeitos da xilanase como uma enzima auxiliar na produção de celulose nanocristalina através da hidrólise enzimática com endoglucanase

Dissertação apresentada à Escola de Engenharia de Lorena da Universidade de São Paulo para obtenção do título de Mestre em Ciências do Programa de Pós-graduação em Biotecnologia Industrial na área de concentração de Conversão de Biomassa.

Orientador: Prof. Dr. Valdeir Arantes.

Versão Original

LORENA

2017

Ficha catalográfica elaborada pelo Sistema Automatizado
da Escola de Engenharia de Lorena,
com os dados fornecidos pelo(a) autor(a)

Dias, Isabella Karoline Ribeiro
O estudo dos efeitos da xilanase como uma enzima auxiliar na produção de celulose nanocristalina através da hidrólise enzimática com endoglucanase / Isabella Karoline Ribeiro Dias; orientador Valdeir Arantes - Versão Original. - Lorena, 2017.
74 p.

Dissertação (Mestrado em Ciências - Programa de Pós Graduação em Biotecnologia Industrial na Área de Conversão de Biomassa) - Escola de Engenharia de Lorena da Universidade de São Paulo. 2017
Orientador: Valdeir Arantes

1. Hidrólise enzimática. 2. Endoglucanase. 3. Xilanase. 4. Celulose nanocristalina. I. Título. II. Arantes, Valdeir , orient.

ACKNOWLEDGEMENTS

First of all, I thank God for all the opportunities and for giving me the strength to face all the challenges that have brought me here.

To my mother Isabel, who always battled hard, doing more than it was possible to her so that I could follow the path I chose.

To my father Airton and my brother Lucas who always protected and supported me.

To my supervisor Prof. Dr. Valdeir Arantes for giving us the opportunity to learn a lot in every meeting and in every conversation. It is a great pride and satisfaction to be with the "Senhor". For all your commitment, patience (lots of patience), dedication and availability, even on holidays. Thank you very much!

To Prof. Adriane for all the support and for making her laboratory available to me for a long time.

To Prof. Dr. Ferraz for the support and also for making his laboratory available.

To Prof. Dr. Segato for his constant teachings and support.

To the teachers of the postgraduate degree in Industrial Biotechnology for their teachings, which contributed to my formation in the master's degree.

To the friends of the Laboratory BBioPro (Barbara, Lisa, Gabriela, Eduarda, Renan and Wilian) and to the friends in the Laboratory of Microbiology and Biochemistry (Daniele, Maiara and Felipe) for the companionship, wisdom, conversations and laughter. Special thanks to Dr. Germano for all the teachings, protection and support, both technical and emotional.

To the laboratory technicians.

To CAPES for financial support.

To EEL-USP for the opportunity to carry out this work.

RESUMO

DIAS, I. K. R. **Estudo dos efeitos da xilanase como uma enzima auxiliar na produção de celulose nanocristalina por hidrólise enzimática com endoglucanase**. 2017. 74p. Dissertação (Mestrado em Ciências) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2017.

Celulose nanocristalina (CNC) é um material de grande ascensão e desenvolvimento no mercado, com um número cada vez maior de aplicações em diversos setores industriais. Contudo, suas aplicações dependem fortemente das propriedades químicas, físicas e ópticas inerentes da CNC, bem como a capacidade de subsequente modificação química. A produção de CNC por via enzimática é um processo controlado e ambientalmente correto que permite obter as CNCs com propriedades desejáveis, porém o processo ainda é pouco estudado. Neste contexto, o objetivo deste trabalho foi aumentar a seletividade da endoglucanase para as regiões amorfas e produzir CNC com alta cristalinidade e alto grau de pureza (baixo teor de hemicelulose). Para isso, foi investigado, pela primeira vez, os efeitos de um preparo enzimático rico em xilanase (Cellic HTec2 ®) para auxiliar na produção de CNC por hidrólise enzimática a partir de polpa kraft de eucalipto branqueada (BEKP). Surpreendentemente, combinações de enzimas com cargas mais elevadas de xilanase em relação a de endoglucanase, mostrou ter um maior potencial para produção de CNC, uma vez que esta foi a única condição que levou ao isolamento de nanopartículas. Essas nanopartículas apresentaram tamanho médio de 420-720nm, índice de cristalinidade entre 65-70%, suas suspensões aquosas permaneceram estáveis por um período maior do que 48h, e apresentaram termoestabilidade muito superior a CNCs obtidas pelo método tradicional de hidrólise com H_2SO_4. A combinação com carga de xilanase 3 e 7 vezes maior do que a de endoglucanase mostrou ser uma combinação ideal para produção de CNCs. Apesar da xilanase utilizada neste trabalho ter solubilizado mais 70% da xilana de BEKP, o teor de xilana encontrado nas CNCs mantiveram alto (13-15%) e não houve correlação com a composição química o resíduo de BEKP após a hidrólise enzimática.

Palavras-chave: Hidrólise enzimática. Endoglucanase. Xilanase. Celulose nanocristalina.

ABSTRACT

DIAS, I. K. R. **The study of xylanase effects as an auxiliary enzyme on the production of cellulose nanocrystals through enzymatic hydrolysis with endoglucanase**. 2017. 74p. Dissertation (Master of Science) - Escola de Engenharia de Lorena, Universidade de São Paulo, Lorena, 2017.

Cellulose nanocrystal (CNC) is a high-value, emerging nanomaterial with an increasing number of applications in various industrial sectors. However, its applications depend heavily on the inherent chemical, physical and optical properties as well as its suitability for subsequent chemical modification. The enzymatic production of CNC is a controlled and ecofriendly process that allows to obtain CNC with improved properties, but the process is still poorly studied. In this context, the objective of this work was to increase the selectivity of an endoglucanase to the amorphous regions of cellulose and to produce CNC with high crystallinity and purity (low hemicellulose content). We investigated, for the first time, the ability of an endoxylanase enriched enzyme preparation (Cellic HTec2 ®) to aid in the production of CNC by enzymatic hydrolysis from a bleached eucalyptus kraft pulp (BEKP). Interestingly, it was found that combinations of enzymes with xylanase load higher than endoglucanase resulted in greater potential for CNC production, since this was the only condition that led to the isolation of nanoparticles. These nanoparticles showed an average particle size of 420-720nm, crystallinity index between 65-70%, and their aqueous suspension could remain stable for a period longer than 48h. The enzymatically produced CNCs showed much higher thermostability than the CNC obtained by the traditional hydrolysis with H_2SO_4. The combination of xylanase loading 3 and 7 times greater than endoglucanase was shown to be an ideal combination for CNC production. Although the xylanase employed in this work solubilized more than 70% of the xylan in BEKP, the content of xylan found in CNC produced remained high (13-15%) and did not correlated with the chemical composition of the enzymatic hydrolysis cellulosic residue.

Keywords: Enzymatic hydrolysis. Endoglucanase. Xylanase. Cellulose nanocrystal.

LIST OF ABBREVIATIONS

AA9 - Auxiliary Activity 9

AFEX - Ammonia fiber expansion

BEKP - Bleached eucalyptus Kraft pulp

CAGR - Compound Annual Growth Rate

CAZy - Carbohydrate-active enzyme

BC - Bacterial cellulose

CBH - Cellobiohydrolases

CBM - Carbohydrate-binding module

CNC - Cellulose nanocrystal

CNF - Cellulose nanofibril

DP - Degree of polymerization

DTG - derivative of thermogravimetric

EC - Enzyme commission

EG - Endoglucanase

EX - Endoxylanase

G - gram

G - Lignin guaiacyl

GH - Glycosyl hydrolases

G-S - Lignin guaiacyl-syringyl

GPa - Gigapascal

HGS - Lignin hydroxypjenyl-guaiacyl-syringyl

kDa - Kilodalton

LPMOs - Lytic polysaccharide monooxigenases

TEM - Transmission electron microscopy

T*max* - Maximum degradation temperature

T*onset* - Onset degradation temperature

SEM - Scanning electron microscopy

mL - Milliliter

nm - Nanometer

OPEC - Organization of the petroleum exporting countries

v/v - volume per volume

w/w - weight per weight

w/v - Weight per volume

µm - Micrometer

µmol - Micromol

IU - International unit

TG - Thermogravimetric

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1 INTRODUCTION

Cellulose nanocrystals (CNC) are rigid, rod-shaped, highly crystalline particles that have at least one of the dimensions within the nanometrical scale. They exhibit excellent physical, chemical, biological and optical properties such as biodegradability, biocompatibility, low density, high aspect ratio, translucency and a highly reactive surface. In addition, CNC has a Yong module (129-250 GPa) comparable to kevlar (60-125GPa) and potential to be stronger than steel (200-220 GPa) (MOON et al., 2011; LIN; DUFRESNE, 2014). These outstanding properties make CNC a unique high-value nanomaterial that can be utilized as building blocks in the development of a range of products for the construction, food, biomedical, pharmaceutical, automotive, aerospace and electronics industries (HABIBI; LUCIA, 2012; LIN; DUFRESNE, 2014; SHATKIN; WEGNER; BILEK, 2014). Examples of promising CNC applications include, but are not limited to, tissue regeneration, drug delivery, LED display and smart windows (HAMAD, 2008; HABIBI; LUCIA; ROJAS, 2010).

Cellulose nanocrystals can be extracted from any cellulosic material such as plants, tunicates, algae and even produced by some microorganisms (such as bacteria of the *Gluconoacetobacter* genus). However, plant-derived biomass (lignocellulosic biomass) is the most promising source as it is a renewable, widely and abundantly available material, allowing for a more sustainable, large-scale CNC production (GEORGE; SABAPATHI, 2015). In fact, bleached kraft pulp, almost 99% holocellulose (cellulose and hemicelluloses), has been the most utilized plant-derived cellulosic material for production of CNC (BECK-CANDANEDO; ROMAN; GRAY, 2005; DE MESQUITA; DONNICI; PEREIRA, 2010; TONOLI et al., 2012; CHEN et al., 2015). This is not surprising since the Kraft process is the world's dominant pulping process, corresponding to 90% of the chemical pulp market (FAOSTAT, 2014). Among the kraft pulps produced worldwide, Bleached Eucalyptus Kraft Pulp (BEKP) is the most abundant and commercially available pulp, particularly in Brazil, the biggest producer and exporter of BEKP (SILVA; BUENO; NEVES, 2016).

The predominant method for CNC production is the hydrolysis of cellulose-rich pulp with strong mineral acid, usually sulfuric acid (64-65% w/w), to preferentially hydrolyze the less organized cellulose domains (amorphous

regions), resulting in the release of the nanocrystals. Alternatively, enzymatic hydrolysis with cellulolytic enzymes has been proposed as an environmentally-friend process to produce CNC (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; SATYAMURTHY et al., 2011; XU et al., 2013; CUI et al., 2016). Compared to the sulfuric-acid hydrolysis, the enzymatic hydrolysis has the advantage of lower energy and water consumption, milder processing conditions, does not generate sugar degradation products and therefore allows for recovery of all hydrolyzed sugars, and preserves the chemical surface of the nanocrystals (KARIM et al., 2017). Additionally, because enzymes are more specific than mineral acids, the use of a suitable enzyme preparation may enable more selective degradation of the amorphous regions without affecting the integrity of the nanocrystals.

Among the cellulolytic enzymes, the glycosyl hydrolyases endoglucanases have the greatest potential in the enzymatic hydrolysis of cellulosic pulp for production of CNC. This is because they catalyze the hydrolysis of β-1,4-glycosid linkages in cellulose molecules, preferentially in the amorphous regions, essential for isolation of the nanocrystals from cellulose fibers. Although most previous work that employed enzymatic hydrolysis to obtain CNC were based on endoglucanase activity (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; XU et al., 2013; TEIXEIRA et al., 2015), many of them have made use of cellulase preparations like Celluclast 1.5L (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; CUI et al., 2016) and Cellusoft L (MEYABADI; DADASHIAN, 2012; FATTAHI MEYABADI et al., 2014), or employed enzymatic hydrolysis with the well-known cellulolytic fungus *Trichoderma reesei* (SATYAMURTHY et al., 2011; ZHANG et al., 2012b). In such cases, the cellulase preparations contain not only endoglucanases, but also other cellulose-hydrolyzing enzymes, like cellobiohydrolases that typically represents more than 30% of enzymes in the cellulase preparations (HU; ARANTES; SADDLER, 2011).

The presence of high amount of cellobiohydrolases in cellulase preparations used for CNC production may be undesirable, since they selectively catalyze the hydrolysis of glycosidic bonds in the crystalline regions of cellulose. This could be the reason why the yield of CNC produced by enzymatic hydrolysis with cellulase preparation is generally only about 10-40% (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; SATYAMURTHY et al., 2011; ANDERSON et al.,

2014; FATTAHI MEYABADI et al., 2014; CUI et al., 2016). Though this CNC yield range is comparable to the typical yield range of CNC produced by mineral acid hydrolysis (BONDESON; MATHEW; OKSMAN, 2006; OLIVEIRA et al., 2016), it has the potential to be higher if hydrolysis could be carried out in the absence, or at lower ratios, of cellobiohydrolases. In general, based on the limited literature available, another drawback of enzyme-mediated production of CNC with cellulase preparations is the need for high enzyme dosage (6.5-13% w/w) (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; FATTAHI MEYABADI et al., 2014) and long reaction time (up to seven days) (ZHANG et al., 2012b).

Thus, although with great potential and some success to date, enzymatic-mediated production of CNC would certainly benefit of a specific cellulase preparation tailored to effectively, and with high selectivity, hydrolyze the amorphous regions of cellulosic pulps. This becomes even more crucial when using BEKP as cellulosic material to produce CNC (PENTTILÄ et al., 2013). This is because in addition to cellulose (typically in the range of 65-80%), BEKP contains about 14-16% of residual, amorphous xylan (SIXTA, 2006). Although a substantial amount (40-65%) of the hemicelluloses are solubilized during kraft pulping, a significant portion of the xylan remains strongly adsorbed to the cellulose in the final BEKP (SIXTA, 2006). It is known that a higher amount of this residual hemicellulose is found on the surface of the cellulose fibers than embedded into the cellulose layers (SHIN; STROMBERG; FALLS, 2007). This is very relevant since residual xylan in cellulosic pulp has been reported to severely restrict cellulose access by cellulases, and therefore, their catalytic performance (ZHANG; TANG; VIIKARI, 2012; PENTTILÄ et al., 2013; GONÇALVES et al., 2015), consequently requiring longer processing time and higher enzyme quantity to hydrolyze the cellulose chains. Moreover, residual hemicellulose (i.e. xylan) can be detrimental to some inherent properties of CNC, such as reactivity, purity and crystallinity (RONCERO, 2005; PENTTILÄ et al., 2013; DUAN et al., 2016).

In this context, the objective of this study was to investigate, for the first time, the beneficial effect of using an endoxylanase as an accessory enzyme in the enzymatic-mediated production of CNC from BEKP with endoglucanase. It was expected that the hydrolysis of the residual xylan by the endoxylanase coul enhance the access and performance da endoglucanase to the amorphous regions

of the cellulose and consequently lead to CNC with relatively high yield, crystallinity and purity.

2 LITERATURE REVIEW

2.1 Lignocellulosic Biomass

2.1.1 Potential use of lignocellulosic biomass as renewable source

Lignocellulosic biomass is a natural resource composed mainly of a complex and intertwined structure of cellulose, hemicellulose and lignin. Over the decades, a large number of studies have been done aiming to use lignocellulose as a potential resource to substitute fossil raw materials, not only energetically (DODD; CANN, 2009; ZHU; SABO; LUO, 2011; HU et al., 2013), but also as feedstock for bioproducts (MATHEW et al., 2011; SHATKIN; WEGNER; BILEK, 2014; ZHONG et al., 2015).

The use of lignocellulosic biomass instead of fossil carbon has many advantages and the use has been encouraged in several countries. The main reasons that led governments to seek strategies for greater production and consumption of fuels and materials that are renewable and sustainable are the imminent shortage of oil reserves, the world's leading energy source, along with society's concerns about environmental preservation.

Lignocellulosic biomass, besides being a source of renewable raw material almost inexhaustible and present in almost all regions of the planet, it is also an environmentally friendly material that enables the reduction of CO_2 emissions, unlike the fossil carbon derivatives used as an energy source that are one of the largest responsible for the emission of CO_2 and is concentrated in only a few regions of the planet. Therefore, lignocellulosic biomass has great potential to help reduce some of the problems faced by today's society, such as the energy crisis, environmental pollution, and holds to potential to guarantee greater economic autonomy and independence from the few big oil producers and exporters.

2.1.2 Composition and structure of lignocellulosic biomass

Lignocellulose is composed basically of three major organic compounds, cellulose, hemicelluloses and lignin. Located in the cell wall of plants, they are responsible for defining the structure of plants, to provide support and protection against physical and chemicals agents, and pathogens (CHEN, 2014).

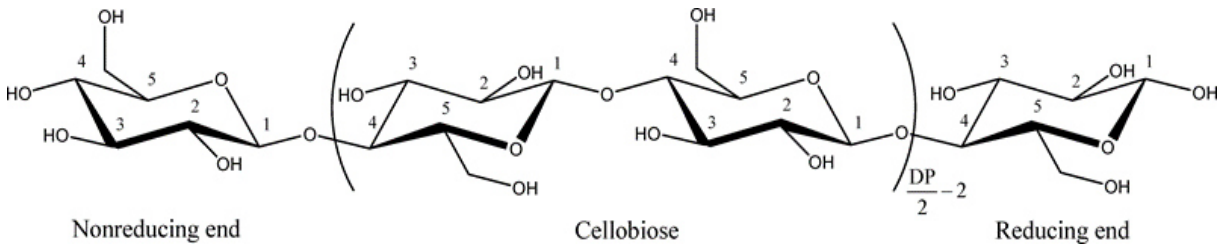
2.1.2.1 Cellulose

Among the elements present in lignocellulosic materials, cellulose is the main component (20-50% dry weight) and also the largest source of renewable polymer available in the biosphere, reaching approximately $7.5x10^{10}$ tons per year (HABIBI; LUCIA, 2012). Cellulose is not only found in plants, but it can also be synthesized by animals (tunicates), bacteria, algae, some fungi and even amoebae. However, access to these non-plant-based sources celluloses is limited (BRINCHI et al., 2013).

Cellulose $(C_6H_{10}O_5)_n$ is a linear, insoluble homopolymer of high molecular mass, composed of β-1,4 glycosidic bonds and β-D-glucopyranose units (FENGEL; WEGNER, 1983). Due to the 180° rotation around the glycosidic bonds, the basic cellulose coupling unit is anhydrocelobiose. Each anhydroglucose monomer in the cellulose chain has three hydroxyl groups, located on carbon C_3 and C_2 (secondary, equatorial) and C_6 (primary) (KLEMM et al., 2005), as shown in Figure 1.

The polymerization of the anhydroglucose form the cellulose chains constituted by a terminal in which C1 is in the form of a hemiacetal and has reducing character, and the other terminal with a pending hydroxyl group, termed non-reducing terminal (Figure 1). The number of glucose present in a cellulose chain or the degree of polymerization (DP) depends very much on the source of the cellulose. It has been shown that the DP for wood pulp is about 10,000 (FENGEL; WEGNER, 1983; WANG et al., 2015).

Figure 1 - Scheme of the molecular structure of cellulose.

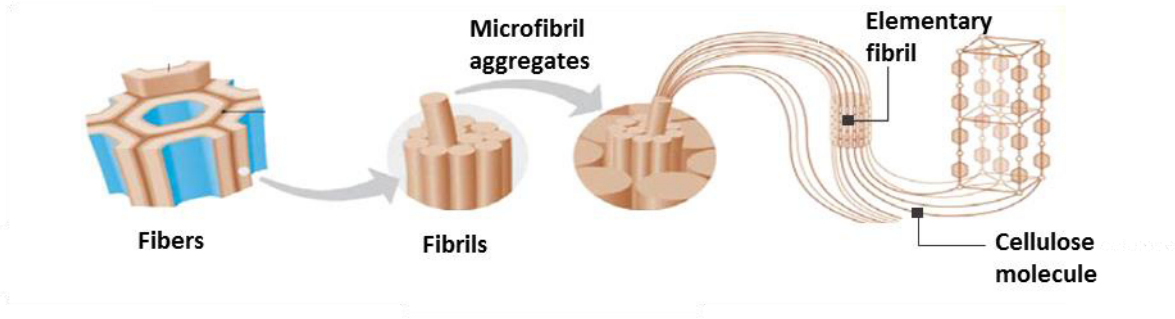


Source: (HABIBI; LUCIA; ROJAS, 2010).

Cellulose has a hierarchical organization (Figure 2). That is, cellulose chains are joint in parallel (in native cellulose) by hydrogen bonds and van der Waals force

to form long agglomerates called elementary fibrils. For a long time, it was considered that each elementary fibril was composed of approximately 36 cellulose chains (FENGEL; WEGNER, 1983). However, with the advances of analytical techniques, recent research has pointed out that 18-24 chains are the most correct (FERNANDES et al., 2011; THOMAS et al., 2013). The set of elementary fibrils form the microfibrils, which are covered by a monolayer of hemicellulose, which in turn, is joined together in a matrix of hemicellulose and lignin (associated with each other by means of physical interactions and covalent bonds) to form the fibrils of cellulose, found in plant cell walls (MOON et al., 2011).

Figure 2 - Representative scheme of the hierarchical organization of cellulose within the cell wall of plants.

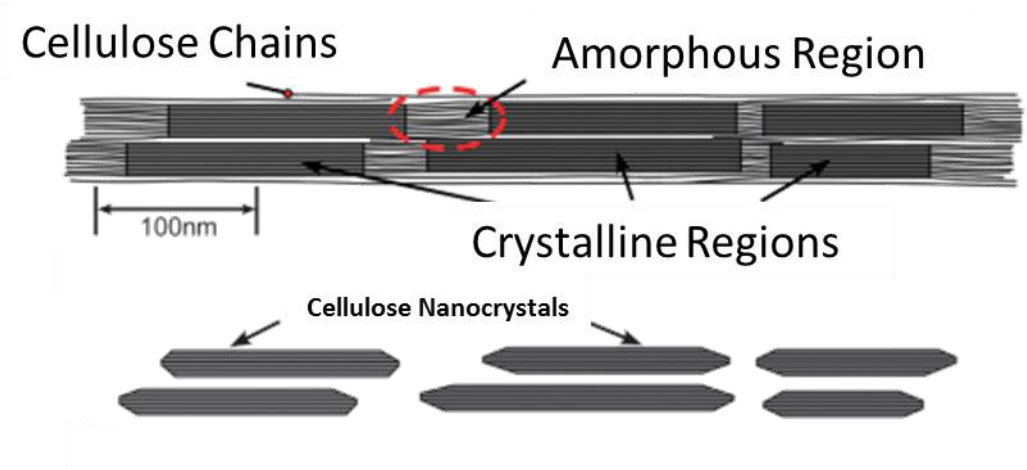


Source: Adapted from (ZIMMERMANN et al.; 2014).

Cellulose elementary fibrils are composed of two different regions. One where the interactions between the molecules are more intense, forming a more organized and crystalline region called the crystalline region. Another, that by perturbations suffered by the internal tension of the fiber during the synthesis of the cellulose, ends up forming a region less organized, denominated, the amorphous region (CHEN, 2014). Due to the lower interaction between the cellulose units of the amorphous region, it becomes more susceptible to chemical or enzymatic catalysts (GAMA; MOTA, 1997; AHOLA et al., 2008). Thus, with selective hydrolysis of such amorphous regions it is possible to obtain cellulose crystals, also called cellulose nanocrystals (CNC) (Figure 3) (BONDESON; MATHEW; OKSMAN, 2006).

Due to its important characteristics, cellulose has been used in the industrial sector for many years, mainly in the paper, textile and chemical industries and more recently as biomass for the production of biofuels. Despite these applications, the search still continues for new applications of cellulose, mainly for composite materials (MOON et al., 2011; HUBER et al., 2012).

Figure 3 - Representation of the amorphous and crystalline regions of the cellulose and the production of cellulose nanocrystals (CNC) by hydrolysis of the amorphous regions.



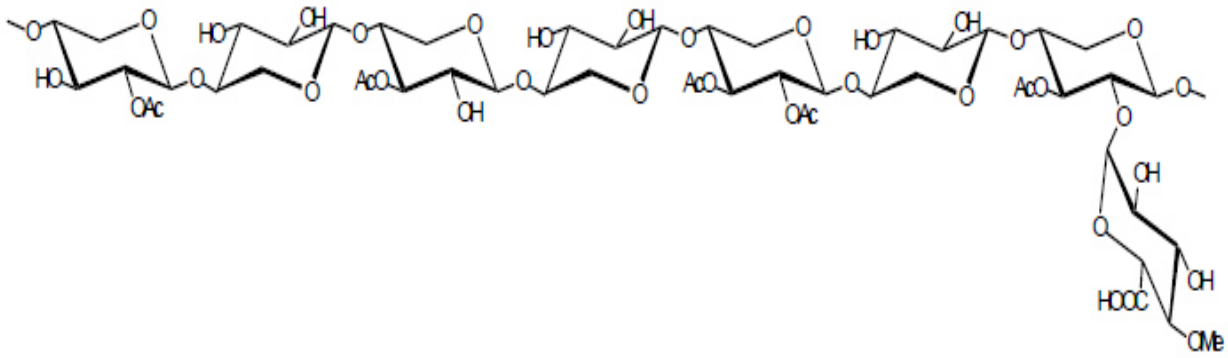
Source: Adapted from (MOON et al., 2011).

2.1.2.2 Hemicellulose

Hemicelluloses, like cellulose, are polymers made up of sugars. However, they are constituted by different types of monosaccharides, have branches and low molecular mass (CHEN, 2014). Generally, their chemical and thermal stability are lower than that of cellulose (GIUDICIANNI; CARDONE; RAGUCCI, 2013). Hemicelluloses are the second most representative constituent (25-35% dry mass) of lignocellulosic materials and act as an interface between cellulose and lignin, facilitating fiber junction and compaction and ensuring the structural reinforcement of the plants. The constituents of the hemicelluloses can be hexoses (D-glucose, D-mannose and D-galactose) and/or pentoses (D-xylose and L-arabinose). Uronic acids (4-O-methylglucuronic acid, D-glucuronic acid, D-galacturonic acid) and acetyl groups are also found in hemicelluloses. In angiosperms (dicotyledons and monocotyledons) the linear chain of hemicellulose is mainly xylan and in gymnosperm is glucomannan. The hemicellulose found in plants such as

eucalyptus and birch is the acetylated glucuronoxylan, more correctly called O-acetyl-4-O-methylglucuron-β-D-xylan (Figure 4). The linear chain of this polymer is formed by β-1,4-linked D-xylose residues. Most xylans isolated from dicotyledonae woods have, on average, one side group of 4-O-methylglucuronic acid (MeGlcA) for every 10 units of D-xylose linked by α-1,2 bonds to xylan. Many of the xylose residues present in the xylan linear chain have an O-acetyl group on the C2 and/or C3 –position (DAHLMAN, 2003).

Figure 4 - Representative scheme of the xylan found in dicotyledoneas.

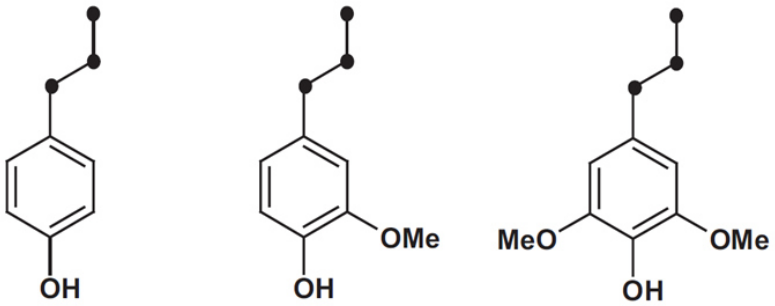


Source: (DAHLMAN, 2003).

2.1.2.3 Lignin

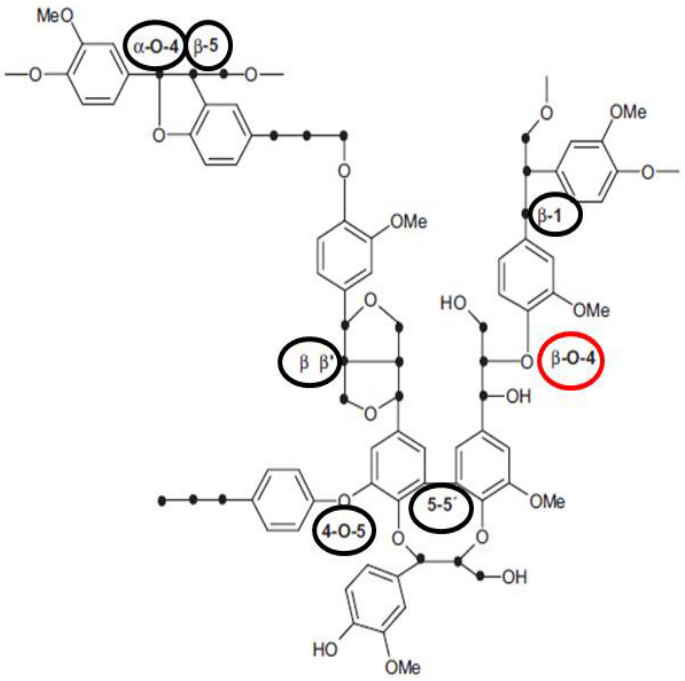
Lignin represents 10-30% of the dry mass of the plant cell wall and has an important function in the structure of plants, giving them greater rigidity, impermeability and resistance. Lignin is an aromatic, amorphous and water insoluble macromolecule, originating from phenylpropanoid precursors. It is the most complex component of a lignocellulosic material, resulting from the polymerization of the heterogeneous mixture of three monomers called monolignols: p-coumaryl, coniferylic and synapylic alcohols, represented in Figure 5. These basic units are interconnected by a variety of linkages, among them the most common are the B-O-4 ether type bonds. Other ether linkages (α-O-4; 5-O-4), as well as carbon-carbon bonds are also present (CHEN, 2014). A hypothetical model of lignin and its bonds are schematized in Figure 6.

Figure 5 - The phenylpropene units precursors of lignin. From left to right, p-coumaryl alcohol, coniferyl alcohol and synapyl alcohol.



Source: (SIXTA, 2006).

Figure 6 - The hypothetical model of lignin with its main linkages.



Source: (SIXTA, 2006).

Due to the different types of monomers, there are three types of lignin, which are classified according to the type and proportion of monolignols. The guaiacyl lignin (G), normally found in gymnosperms, has a basic unit exclusively derived from the coniferylic alcohol. Lignin guaiacyl-syringyl (G-S) is found in dicotyledoneas and has a structural unit derived from coniferylic and synapylic alcohols. Lignin found in monocotiledoneas, called lignin hydroxyphenylguaiacyl-syringyl (HGS), has a mixture derived from the three monolignols (p-coumarilic, coniferilic and synapylic alcohols) (CHEN, 2014).

2.2 Nanocelluloses

With the development of nanotechnology, cellulose, the most important natural polymer on Earth, resurfaced attracting attention on its nanometric scale. It can be used as a building block in the development of products with high technological advancement and added value such as flexible displays for mobile devices, aerogels and bioplastics. (SHATKIN; WEGNER; BILEK, 2014). This allows the advent of a new generation for the use of cellulosic pulp, with the potential to completely change the application of lignocellulosic materials, allowing a possible expansion of the market of the pulping and biorefinery industries. Nanocellulose had an estimated potential market valued at $ 54.9 million for 2014 and it is estimated to reach $ 699.6 million by 2023, expanding at a compound annual growth rate (CAGR) of 33,8% between 2015 and 2023 (TMR, 2016).

Nanocellulose is a nanoscale material with at least one of its dimensions smaller than 100 nm (DUFRESNE, 2012), extracted from native cellulose (plants, animals and bacteria) and with impressive properties which are more relevant than its macromolecular specimen. Its properties, such as biodegradability, biocompatibility, low density, high aspect ratio (length/width), mechanical reinforcement, with a Yong module (129-250 GPa) comparable to kevlar (60-125GPa) and potential to be stronger than steel (200-220 GPa) (MOON et al., 2011; LIN; DUFRESNE, 2014; SIQUEIRA; ARANTES, 2016) allow this material to be applied in various industrial sectors, mainly as composite in the construction, food, biomedical, pharmaceutical, automotive, aerospace, electronics and other industries (HABIBI; LUCIA, 2012; LIN; DUFRESNE, 2014; SHATKIN; WEGNER; BILEK, 2014).

According to its properties, such as degree of crystallinity, purity and dimensions, as well as the source and production method, nanocellulose is basically divided into three main categories: cellulose nanocrystals (CNC), cellulose nanofibrils (CNF) and bacterial cellulose (BC) (DUFRESNE, 2012).

Bacterial cellulose is synthesized by bacteria such as *Acetobacter xylinum* (LIN; DUFRESNE, 2014), has DP of about 2,000-8,000, high purity and crystallinity, and diameter of about 20-100 nm (KLEMM et al., 2005). Figure 7a shows an image of BC. BC requires no treatment for removal of lignin,

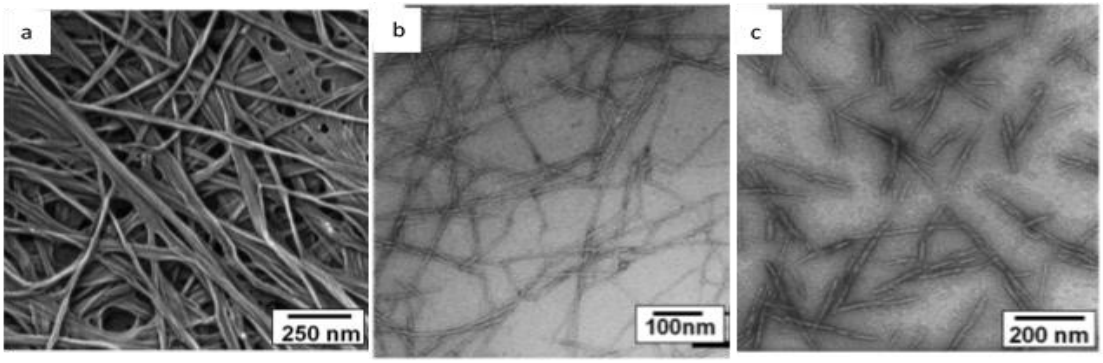
hemicelluloses, pectin and other contaminants. On the other hand, its production yield is very low (the highest BC yield reported is only about 0.38 g/L.h, using an aerosol bioreactor (LEE; BLAKER; BISMARCK, 2009)) and it has a high cost of production (WU; LIU, 2013). The biocompatibility of BC derives primarily from its high purity, making it attractive for a wide variety of applications in various industrial sectors, such as food and, especially, more noble applications such as in the biomedical and pharmaceutical sectors (KLEMM et al., 2005; LIN; DUFRESNE, 2014).

Different from BC that is obtained from a bottom up production process (from glucose to nanocellulose), CNF and CNC are obtained from a top down production process, that is, deconstruction of lignocellulosic biomass to nanocellulose.

Cellulose nanofibrils, the elementary structure of the cellulose, is formed by amorphous and crystalline regions, has a diameter in the range of 10-100 nm and length up to several μm (DUFRESNE, 2012) (Figure 7b). Applications of CNF do not require a high degree of purity, and it typically contains residual hemicellulose and lignin (SIQUEIRA; ARANTES, 2016). CNF is mainly obtained by mechanical defibrillation methods of cellulose fibrils, such as homogenization at high pressure (HENRIKSSON et al., 2007), microfluidization, and nano-grinding by disc refining (IWAMOTO; NAKAGAITO; YANO, 2007; SPOSINA et al., 2015). These defibrillation processes may or may not be accompanied by chemical pretreatment with 2,2,6,6-tetramethylpiperidine N-oxyl mediated oxidation (TEMPO) (GONZÁLEZ et al., 2012), sodium hypochlorite (ZHANG et al., 2012a) or enzymatic with endoglucanase (DE CAMPOS et al., 2013; WANG et al., 2015b) .

Cellulose nanocrystals have more defined geometric shape, diameter between 3 to 15 nm (DUFRESNE, 2012), length from 100 to 280 nm when isolated by acid catalysis and from 250 to 1000 nm when isolated by enzymatic hydrolysis (SIQUEIRA; ARANTES, 2016) (Figure 7c). The isolation of the nanocrystals is carried out by hydrolysis of the amorphous regions of the microfibrils, catalyzed mainly by acid (BONDESON; MATHEW; OKSMAN, 2006). Because it is constituted mainly of crystalline regions (50-90% crystallinity index) (MOON et al., 2011; SIQUEIRA; ARANTES, 2016), it displays greater rigidity and elasticity than CNF and CB, which are more flexible and with a greater plasticity (LIN; DUFRESNE, 2014).

Figure 7 - The three main categories of nanocellulose: (a) BC; (b) CNF; and (c) CNC. A and B are scanning electron microscopy images and C a transition electron microscopy image.



Source: Adapted from (MOON et al., 2011).

2.2.1 Sources of cellulose for production of nanocelluloses

Nanocelluloses can be obtained from different types of cellulosic materials such as cotton (TEIXEIRA et al., 2010), agricultural residues (KAUSHIK; Singh, 2011; CHERIAN et al., 2008), wood (BECK-CANDANEDO; ROMAN; CINZA, 2005; SEHAQUI et al., 2011); bacterial cellulose (SOYKEABKAEW et al., 2009; WOEHLET al., 2010), tunicates (MATHEW; DUFRESNE, 2002) and Kraft pulp.

The Kraft pulp is the most used cellulosic material for production of nanocelluloses (ZHU; SABO; LUO, 2011; TONOLI et al., 2012; WANG et al., 2015b; ZENI; FAVERO; MC, 2015). This is basically because the Kraft process is the dominant pulping process worldwide, accounting for about 90% of the world's chemical pulps (FAOSTAT, 2014). In Brazil, most of the bleached Kraft pulp comes from eucalyptus (BEKP) and it is the most abundant and commercially available cellulosic pulp (TONOLI et al., 2012).

The high availability of BEKP combined with properties of the eucalyptus plant, such as its rapid growth (CAMPINHOS JR., 1999) and the relatively low price (CEPEA, 2015), make BEKP a promising source for nanocellulose in Brazil. The use of BEKP as a source of nanocellulose will benefit not only the nanocellulose industry, but also the pulp and paper industries, allowing a greater diversification of its products with high added value.

2.2.1.1 Bleached eucalyptus kraft pulp (BEKP)

Bleached eucalyptus Kraft pulp is typically composed of approximately 99% of its dry mass of holocellulose (cellulose and hemicellulose). It is produced by an alkaline treatment process with sodium hydroxide (NaOH) and sodium sulfate (Na_2S) at high temperatures (130-160 °C), followed by bleaching of the cellulosic pulp generally with oxidizing agents. Together, these two processes promote the degradation and solubilisation of virtually all lignin through the cleavage of intermolecular bonds (SIXTA, 2006).

During Kraft pulping, cellulose undergoes reduction of the degree of polymerization (<30%), however, the mass loss is small (SIXTA, 2006). Xylan, the major hemicellulose in eucalyptus, is dissolved and modified, but part of it in oligomeric form precipitates on the cellulose fibers. This precipitation phenomenon occurs because under alkaline conditions the acetyl groups, naturally present in hemicelluloses, are cleaved, leaving the xylan deacetylated. The 4-O-methylglucuronic acid side groups are also removed during the alkaline process, and their residues promote the alkaline beta-elimination reaction of methanol, forming hexenoronic acid residues attached to the xylan linear chain. On the other hand, hexenuronic acid under sufficiently strong alkaline conditions can be degraded, making xylan free of side chains (DAHLMAN, 2003). The large loss of xylan's side groups causes xylan to be readsorbed, promoting a strong adsorption on the cellulose surface. Thus, most of the xylan (hemicellulose) in cellulosic Kraft pulp is on the surface of the fibers rather than inside of the layers (SHIN; STROMBERG; FALLS, 2007). The precipitated xylan is a barrier in the bleaching process, reducing the contact of the delignification chemical agents with the residual lignin, therefore, making it difficult to dissolve (RONCERO, 2005).

In order to overcome this barrier caused by the precipitated xylan, the use of xylanase was suggested as a pretreatment in the bleaching process (LIU et al., 2012; SAELEE et al., 2016), increasing the yield of cellulose and reducing the amount of the delignification reagents as well as increasing the overall crystallinity of the pulp (RONCERO, 2005; SAELEE et al., 2016). This precipitated xylan in Kraft pulp is also an obstacle to the access of enzymes to cellulose during enzymatic hydrolysis, reducing the enzyme-substrate contact surface (ZHANG; TANG; VIIKARI, 2012). In spite of the fact that xylanases have not yet been applied in the process of obtaining CNC, they are enzymes with potential to improve the efficiency of CNC production from eucalypt Kraft pulp, as well as to reduce the load

of enzymes used, as discussed in more detail in item 2.3.2. It is important to emphasize that the modifications caused in the lignocellulosic material during the Kraft process can affect the development of the enzymatic activity and can define the type of enzymes necessary for the production of nanocellulose.

2.2.2 Methods to obtain cellulose nanocrystals

2.2.2.1 Acid hydrolysis

CNC was first reported in the late 1940s by Rånby, when a colloidal suspension was observed after hydrolysis of cellulose fibers catalyzed by sulfuric acid (RÅNBY; BANDERET; SILLÉN, 1949; RÅNBY, 1951). As sulfuric and other acids preferentially act on the less organized (amorphous) regions of the cellulose during hydrolysis (ANGELS, 2001; RUIZ, 2000), it is possible to release cellulose in the form of whiskers with crystallinity similar to that of natural cellulose fibers (HABIBI; LUCIA, 2012).

Removal of the amorphous region for release of cellulose nanocrystals can be achieved by various acids, including sulfuric acid, hypochlorite, phosphoric acid, or ammonium persulfate (DUFRESNE, 2012). However, sulfuric acid has been most intensively used under different concentration (typically 55-65% m/m), temperature (45-70 °C) and reaction time usually of two hours, but may vary from a few minutes up to 12 hours (DUFRESNE, 2012; HABIBI; LUCIA, 2012).

After hydrolysis, the resulting suspension is diluted with water (between 5-10 times the volume of the reaction mixture) to quench the reaction and washed several times by centrifugation, and followed by dialysis to remove any residual acid molecules. The obtained material is then subjected to a mechanical sonication treatment in an ultrasonic bath or with the aid of an ultrasonic processor to form a uniform suspension of nanocrystals. Finally, the CNC particles are concentrated by centrifugation and eventually dried, resulting in yields usually below 30% (BONDESON; MATHEW; OKSMAN, 2006; BRINCHI et al., 2013). In this process, the parameters such as time, the source of cellulosic pulp, consistency (solid loading), concentration and type of acid, agitation and temperature are strictly important (HABIBI, 2014; CHEN et al., 2015).

In general, acid hydrolysis leads to a rapid decrease in the DP of cellulose (BATTISTA et al., 1956). The limit of reduction in the DP in some cases has been used to correlate with the longitudinal size of the crystals (HABIBI; LUCIA, 2012), but this limit has been shown to be dependent on the cellulose source. For example, values of about 250, 140-200 and 6.000 were found for cotton, bleached wood pulp (BATTISTA et al., 1956) and *Wallonia sp*. (KAY,1976), respectively.

The use of sulfuric acid to obtain CNC has many disadvantages. This is because this process has been reported to toxic, dangerous, corrosive and an unprofitable treatment (ZHU; SABO; LUO, 2011). In addition, the severe conditions, the large amount of water used in the process and the high cost with anti-corrosive equipment and treatment of residual effluents, make this process unsustainable (SONG et al., 2014). Another disadvantage of obtaining CNCs via hydrolysis with sulfuric acid is the need for a very intense control of the conditions so that there is no uncontrollable loss of the material, leading to the degradation of the crystals to the monomers and their undesirable degradation products such as furfural and hydroxymethylfurfural (SONG et al., 2014) depending on the chemical composition of the cellulosic pulp.

The CNC obtained by hydrolysis with sulfuric acid has a negatively charged surface due to the sulfate groups introduced during hydrolysis (BECK-CANDANEDO; ROMAN; GRAY, 2005). This characteristic allows to obtain a more stable suspension of nanocrystals, however, it is not readily functionalized by covalent bond or incorporation in hydrophobic solvents, polymers and resins (ANDERSON et al., 2014). Thus, sulfate groups alter the reactivity of CNC, one of the most important quality parameters of nanocellulose. This is extremely important as higher reactivity improves homogeneity by increasing the quality of CNC-based final products, reducing the total amount of reagents required for a higher quality composite product (WANG et al., 2015a; DUAN et al., 2016). Another important feature to determine the CNC potential for applications, especially in biological applications, is the ease in which its surface can be readily modified by other groups before the development of practical materials such as the introduction of fluorescent molecules on the CNC surface to be used as a biosensor in the medical area (LIN; DUFRESNE, 2014). Therefore, sulfate groups added on the surface of the CNC after hydrolysis with H_2SO_4 make it difficult to modify the surface, reducing the potential of the CNC application (ANDERSON et al., 2014; LIN; DUFRESNE,

2014). Hydrolysis with sulfuric acid also affects other properties, such as a decrease in the thermal stability of CNCs (LU; HSIEH, 2010). In addition, in spite of the preference for amorphous regions, sulfuric acid is not a totally specific catalyst and interferes with the crystallinity of CNCs (GEORGE et al., 2011). Even if a further step is taken to remove the sulfate groups of CNC, its functionalization and application will still be limited when compared to bacterial cellulose, since acid hydrolysis interferes with thermostability and because it decreases the degree of polymerization of the crystals it reduces the reinforcing properties of nanocomposites (GEORGE et al., 2011).

2.2.2.2 Enzymatic hydrolysis

Research on CNC production by enzymatic hydrolysis is restricted and knowledge is still limited. In the same way as the acid hydrolysis, enzymatic production of CNC is done by preferential hydrolysis of the amorphous regions followed by treatment of the hydrolysis residue by sonication. Filson, Dawson-Andoh and Schwegler-Berry (2009) were the first to use enzymatic hydrolysis to produce CNC, obtaining a maximum yield of 38% of crystals with diameters of 30-80nm and length of 100nm-1.8μm from recycled pulp repeatedly hydrolyzed with the commercial cellulase preparation Celluclast 1.5 L (Novozymes). It is noteworthy that as the surface of the nanocrystals obtained by enzymatic hydrolysis is not negatively charged, the stability of the suspension of the crystals is limited (ANDERSON et al., 2014), unlike CNC obtained with sulfuric acid that possesses very stable colloidal solution, however, its thermostability and mechanical properties are inferior (GEORGE et al., 2011).

To date, studies on enzymatic production of CNC have made use almost exclusively of enzymatic preparation of cellulases (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; ANDERSON et al., 2014; SPOSINA et al., 2015) and microbial hydrolysis (SATYAMURTHY et al., 2011; ZHANG et al., 2012b), which are nonspecific for production of CNC. That is, these cocktails consist of enzymes such as endoglucanases and cellobiohydrolases, that act in the amorphous and crystalline regions, respectively, typically leading to low CNC yield (10-40%) (SIQUEIRA; ARANTES, 2016). The most recent work on CNC

production by enzymatic hydrolysis used a combination of endoglucanase and β-glycosidase, however the yield result was not reported (SPOSINA et al., 2015).

2.3 Enzymatic hydrolysis of cellulosic materials

2.3.1 Cellulases

Cellulases are typically hydrolytic enzymes that catalyze the breakdown of the glycosidic β-1,4 bonds in cellulose chain and are classified basically into three main groups: endo-1,4-β-D-glucanases (EC 3.2.1.4) or endoglucanases (EG), Exo-1,4-β-D-glucanases or cellobiohydrolases (CBH) (EC 3.2.1.91 and EC 3.2.1.176), and 1,4-β-D-glycosidase or cellobiases (EC 3.2.1.21).

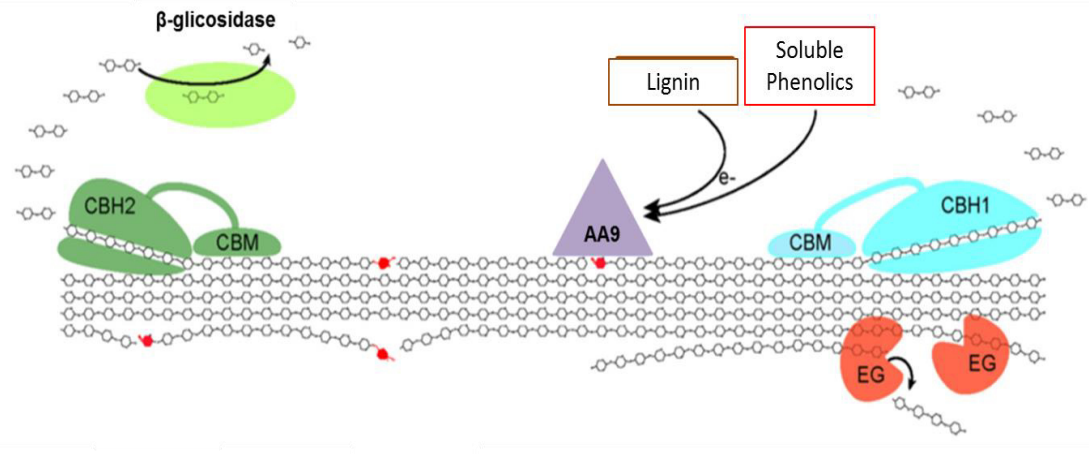
Endoglucanases catalyze the hydrolysis of the β-1,4-glycosidic bonds within the cellulose chain, randomly, creating new terminals in the chain. While the endoglucanase acts preferentially in the less ordered regions of the cellulose (LYND et al., 2002), cellobiohydrolases have a preference for the crystalline regions and act from the reducing process (CBHI) and non-reducing (CBHII) ends, allowing the hydrolysis of the cellulose chains and release of glucose dimers (cellobiose) (ZHANG; LYND, 2006). Endoglucanases and cellobiohydrolases may possess a second domain, whose main function is to approximate the catalytic site of the enzyme to cellulose, called the carbohydrate-binding module (CBM). Finally, cellobiases that although not considered true cellulases are essential for complete hydrolysis of cellulose to glucose. They catalyze the hydrolysis of the β-14 bonds in cellobiose and cellodextrins molecules, which are products released by CBHs and EGs. In addition, they promote a synergistic effect by reducing the amount of cellobiose in the cellulolytic system, an end-product that causes strong inhibition of EGs and especially for CBHs (LYND et al., 2002).

The lytic polysaccharide mono-oxygenase (LPMO), currently classified as Auxiliary Activity 9 (AA9) also acts on cellulose chains (LEVASSEUR et al., 2013). However, unlike EG and CBH, LPMO has oxidative activity (VAAJE-KOLSTAD, 2010) and is electron-donor dependent (QUINLAN et al., 2011). Its catalytic action allows the internal breakdown of the cellulose chain, generating two new ends, one with oxidized sugar (aldonic acid) and the other with non-oxidized sugar (HORN et al., 2012). The great differential of this enzyme is its ability to act in the crystalline

regions of cellulose, creating new terminals for CBHs (HU; ARANTES; PRIBOWO, 2014).

For conversion of cellulose to glucose, the use of the different cellulases is ideal because they act in a collaborative way with one another to reach complete depolymerization of cellulose (Figure 8). In order to obtain CNC, where high selectivity for hydrolysis of the amorphous regions is desire as well as high yield and uniformity of the crystals, the use of cellulases, especially together, should be carried out cautiously. This is because, among the cellulase enzymes that act in the depolymerization of the cellulose chain, EGs are the only ones that can allow the selective hydrolysis of the amorphous regions, leaving the crystalline domains intact.

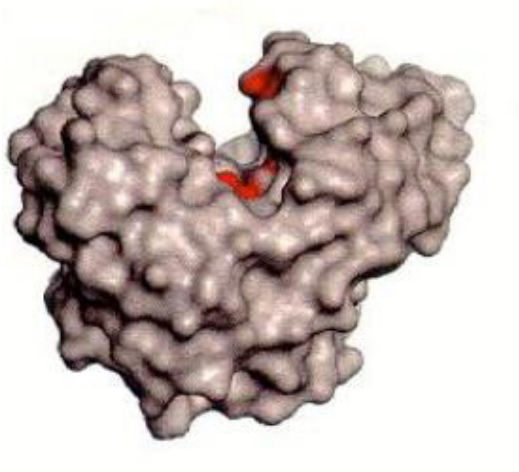
Figure 8 - Representative scheme for the deconstruction of cellulose by the cooperative action of hydrolytic and oxidative enzymes. Abbreviations: CBH - cellobiohydrolase; CBM - carbohydrate binding modules; EG-endoglucanase; AA9 - Auxiliary Activity 9.



Source: Adapted from (HORN et al., 2012).

Endo-1,4-β-D-glucanases (EC 3.2.1.4) are grouped according to their similarity in the genetic sequence and three-dimensional structure. To date, they are grouped in approximately 14 glycosyl hydrolase (GH) families, including GH 5, 6, 7, 8, 9 , 10, 12, 26, 44, 45, 48, 51, 74 and 124 according to the Carbohydrate-Active Enzymes (CAZy) database. Endoglucanases have a catalytic site with an open cleft active site allowing the catalysis of the glycosidic binding to occur at any portion of the chain in the absence of steric hindrance (DAVIES; HENRISSAT, 1995).

Figure 9 - Three-dimensional structure of the Thermononospora fusca endoglucanase II showing its cleft active site.



Source: (DAVIES; HENRISSAT, 1995).

Among the endoglucanase GH families, those belonging to the GH45 family are the most commonly used in the production of nanocellulose fibrils (HENRIKSSON et al., 2007; ZHU; SABO; LUO, 2011; ANDERSON et al., 2020), since it is a commercially available enzyme. GH45 enzymes are generally small (~ 20 kDa) and versatile enzymes, which may degrade other polysaccharides in plant cell wall (PAYNE et al., 2015). An interesting characteristic of GH45s is that they are structurally and evolutionarily related to expansins and swolenins (PAYNE et al., 2015). EG GH45, such as the commercial preparation FiberCare® R, is also commonly used in the textile industry (biopolymerization and stonewashing) and also added in soap powder as a tissue color restorer by removing loose fibrils from the surface of cotton fibers (PAYNE et al., 2015) and pulping industry. Generally, EGs used in the pulping industry promote fiber swelling, increase tensile strength, decrease viscosity and increase fiber reactivity (IBARRA et al., 2010).

2.3.2 Xylanases

Xylanases are glycosyl hydrolases (GH) that act in the decomposition of xylan, the main hemicellulose in dicotyledonous and monocotyledonous angiosperms. These enzymes are also widely used in the textile, pulp, paper and food industries (WILSON, 2008).

Endoxylanases (endo-β-1,4-xylanases EC 3.2.1.8) catalysis the cleavage of β-1,4 bonds typically in the inner part of the linear chain of xylan. Most of them are active in xylo-oligomers, but with higher affinity for larger oligomers (POLAINA; MACCABE, 2007). These enzymes have gained prominence among the enzymes in biomass conversion as they allow the residual hemicellulose that acts as a barrier to cellulolytic enzymes to be removed, thus increasing the access of the cellulases to their substrate, consequently improving hydrolysis yield and reducing the total enzyme dosage (HU; ARANTES; SADDLER, 2011; GONÇALVES et al., 2015). Enzymes with xylanolytic activity have been reported and classified as GH 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 52, 62 and 98 (CAZy). However, GH10 and GH11 are the most abundant and commonly found in microorganisms, therefore, the best known and studied xylanases (COLLINS; GERDAY; FELLER, 2005).

The endoxylanases of the GH 11 family have activity only on xylan and are therefore said to be true xylanases (BIELY et al., 1997). They are more specific than any other xylanase (COLLINS; GERDAY; FELLER, 2005), small in size (approximately 20 kDa) (BIELY et al., 1997), and also allow better adsorption to the substrate, increasing the efficiency of the hydrolysis. On the other hand, the endoxylanase of the GH10 family is a highly versatile enzyme that attacks glycosidic bonds near the regions of side chain insertion and near the non-reducing ends (SUBRAMANIYAN; PREMA, 2002), have a molar mass greater than 30 KDa, and low isoelectric point (BIELY et al., 1997). The differences between these two main families of endoxylanases cause them to have different catalytic activities and results (BIELY et al., 1997; GONÇALVES et al., 2015).

One of the main interferences in the catalytic efficiency of the xylanase enzymes is related to the characteristics of the substrate and the pretreatment conditions to which the material was submitted to (HU; ARANTES; SADDLER, 2011; HU et al., 2013). Endoxylanase belonging to GH11 has been shown to be more efficient in substrates that have been subjected to alkaline pre-treatments (CLARKE et al., 1997; GEORIS et al., 2000), whereas GH10 enxoxylansae has been shown to be more efficient in substrates submitted to acid-based pretreatment (ZHANG et al., 2011; HU et al., 2013).

It is important to emphasize that the pretreatment severity and the source of the cellulosic material are factors that may alter this assertion, as described by Gao

et al (2011), where a xylanase from the GH10 family performed better than the GH11 family when the pretreatment used was the ammonia fiber expansion (AFEX), which despite being a pretreatment conducted in alkaline medium, it is a mild treatment, which maintains most of the hemicellulose's side groups, making GH10 more suitable for better results.

Although endoxylanases aid in the hydrolysis efficiency lignocellulosic materials by cellulases, it is necessary to consider that the product resulting from xylan hydrolysis (xylobiose) by endoxylanase can act as inhibitors of cellulase enzymes (QING; YANG; WYMAN, 2010; QING; WYMAN, 2011). Therefore, the use of endoxylanases together with β-xylosidase (β-1,4-xylosidases EC 3.2.1.37), an enzyme that act at the reducing and non-reducing ends of xylan and especially in the catalysis of xylobiose hydrolysis (POLAINA; MACCABE, 2007) may be indispensable.

In the context of CNC production from eucalyptus Kraft pulp, which typically has a residual xylan content of 15% (SIXTA, 2008), it is expected that xylanases (endoxylanases and β-xylosidases) can improve endoglucanase access to the amorphous regions of cellulose. This could lead to an increase in CNC yield and higher crystallinity of the material.

2.4 Considerations on the methods for production of cellulose nanocrystals

Cellulose nanocrystals have potential to be applied in biocomposite for bone and dental implants, pharmaceuticals products and drug delivery, cosmetic and food additives, improvement of paper and construction materials, coatings additives, adhesives, reinforcing polymers, bioplastics, among others. The infinite applicability of CNC in various sectors is due to its outstand properties such as: uniformity, biodegradability, biocompatibility, no toxicity, reactivity, viscosity, and thermostability such as the reinforcement and tensile index. These properties can be modified or lost according to the type of treatment and its severity to which the cellulose is submitted for the release of the nanocrystals. The presence of impurities such as hemicellulose and lignin also reduces the potentiality of CNC

properties and limit the access of the catalysts to the amorphous regions of cellulose (RONCERO, 2005; PENTTILÄ et al., 2013).

Sulfuric acid is currently the only acid that has been used industrially for CNC production(CelluForce in Canada that produces NCC^{TM}), even though it is an abrasive acid resulting in low yield, unsustainable process and affects some of the CNC properties (ANDERSON et al., 2020). An alternative to the sulfuric acid hydrolysis method is the enzymatic hydrolysis. However, this method has been poorly studied.

The use of enzymes for extraction of cellulose nanocrystals is an environmentally friendly, promising and very attractive alternative. The enzymatic hydrolyses can overcome the hydrolyses acid bottleneck, making the process of isolation of CNCs more economically and environmentally sustainable. Enzymatic hydrolysis reduces energy consumption because it requires milder conditions, decreases water consumption, does not generate co-products or toxic waste and does not compromise the chemical surface of the crystals. The use of a suitable enzyme preparation may enable more selective degradation of the amorphous regions without affecting the integrity of the crystals, resulting in CNCs with more uniform dimensions and maintaining their chemical properties.

Production of CNC from lignocellulosic materials using a combination of enzymes that allow the production of intact CNCs with purity similar to bacterial cellulose would unite the advantages and unique properties of using lignocellulosic material, enzymatic hydrolysis and the properties of bacterial cellulose.

The high degree of purity found in BC and its highly reactive chemical surface due to the conversation of the hydroxyl groups that can be readily modified by incorporation of other chemical groups (LIN; DUFRESNE, 2014), allied with the almost inexhaustible lignocellulosic raw material (GELLERSTEDT; EK; HENRIKSSON, 2009), make this material unique and required for a wide range of CNC applications, attractive characteristics that can lead to an increase its applicability in various industrial sectors.

The properties of CNC obtained by the enzymatic hydrolysis method associated with the high availability of the lignocellulosic material can allow a greater diversification in the applications of CNCs, covering the market possibilities and guaranteeing the supply of CNC. However, due to lack of specific enzymes studied so far in the production of CNC, the yield of CNC from the enzymatic

hydrolysis is still low, even if comparable with the mineral acid hydrolysis method (SIQUEIRA; ARANTES, 2016).

3 OBJECTIVE

3.1 General objective

To determine the influence of xylanase as an auxiliary activity on the production of cellulose nanocrystals from bleached eucalyptus kraft pulp by enzymatic hydrolysis with endoglucanase.

3.2 Specific objectives

- To characterize the cooperative action of the endoxylanase auxiliary enzyme with endoglucanase during hydrolysis of BEKP;
- To identify proportions of the enzymes (endoxylanase and endoglucanase) more suitable for production of CNC;
- To characterize the properties of CNC produced by enzymatic hydrolysis and compare with CNC produced by acid hydrolysis.

4 MATERIAL AND METHODS

4.1 Cellulose pulp and enzymes

The never dried bleached eucalyptus kraft pulp (BEKP), provided by Fibria Celulose S.A. (Jacareí, Brazil), was used as received for CNC production. The chemical composition (% w/w) of the pulp (78.6% cellulose, 14.6% xylan and 2.7% lignin) was determined by a two-stage acid hydrolysis according to Sluiter et al. (2012).

The two commercial enzyme preparations, provided by Novozymes (Araucária, Brazil), FiberCare® R (Batch CGK20074) and Cellic® HTec2 (Batch VHN00003) were used as received. FiberCare® R, hereafter referred to as EG, is a monocomponent endoglucanase, with an optimum temperature and pH between 20-65 °C and 6.0-8.0, respectively. Cellic® HTec2, hereafter referred to as EX, is a hemicellulase preparation enriched in endoxylanase, with an optimum temperature and pH of 50 °C and 5.0, respectively.

4.2 Enzyme activities

Endoglucanase (Endo-1,4-β-glucanase), exoglucanase (exo-1,4-β-glucanase) and endoxylanase (endo-1,4-β-xylanase) activities were determined according to methods described by Tanaka et al. (1981), Wood and Bhat (1988) and Bayley et al. (1981), followed by quantification of reducing sugars released from 3,5-carboxymethylcellulose (CMC, Sigma), Avicel (Sigma) and birch wood xylan (Sigma), respectively, with the 3,5-dinitrosalicylic acid-DNS method (Miller, 1959). β-D-glycosidase and β-D-xylosidase activities were determined according to Tan et al. (1987) using *p*-nitrophenyl-β-D- glucopyranoside (Sigma-Aldrich) and *p*-nitrophenyl-β-D-xylopyranoside (Sigma-Aldrich) as substrates, respectively. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of product per minute.

4.3 Enzymatic hydrolysis

Enzymatic hydrolyses of BEKP were performed in a 100-mL erlenmeyer flask containing 2% (w/w) pulp, citrate-phosphate buffer (0.05M pH 6.0), 0.01%

w/w sodium azide (to avoid microbial contamination), with a total reaction weight of 40g, under agitation in an orbital shaker incubator (Thermo Scientifc) at 200 rpm and 50 °C for 72 h. Two sets of hydrolysis experiments were carried out. The first set was with the enzymes individually at loadings of 100, 200 and 400 U per gram of pulp based on the endoglucanase and xylanase activities of EG (FiberCare® R) and EX (Cellic® HTec2), respectively.

Table 1 - Enzyme loadings of EG (FiberCare® R) and EX (Cellic® HTec2) during hydrolysis of BEKP at 2% (w/w) pulp.

Total enzyme loading (U/g pulp)	Abbreviation	EG [1] (U/g pulp)	EX [2] (U/g pulp)
100	100EG	100	0
	100EX	0	100
200	200EG	200	0
	175EG25EX	175	25
	150EG50EX	150	50
	100EG100EX	100	100
	200EX	0	200
	25EG175EX	25	175
	50EG150EX	50	150
400	400EG	400	0
	400EX	0	400

[1] based on the endoglucanase activity

[2] based on the endoxylanase activity

Source: Author source

The second set of hydrolysis was conducted with supplementation of endoglucanase (EG) with the xylanase (EX) at a total enzyme loadings of 200 U/g pulp, but at different rations of the two enzymes as shown in Table 1. All enzymatic hydrolyses were performed in duplicates. At the end of 72h, each hydrolysis was terminated by boiling the mixture at 100 °C for 10 min to inactivate the enzymes. Then, the reaction mixture was centrifuged at 10,618 rpm (15,000 g) at 10 °C for 10 minutes, and the supernatant was decanted and collected for sugar analysis by

high performance liquid chromatography (HPLC). The cellulosic solid residues (CSR) collected after centrifugation was washed to collect the CNC particles produced.

4.4 Cellulose and xylan hydrolysis yield, selectivity, and chemical composition of CSR

The supernatants collected after centrifugation of the 72-h hydrolysis mixture were used to determine the amount of cellulose and xylan solubilized. They were first subjected to a mild acid hydrolysis with H_2SO_4 at a final concentration of 4% w/w and autoclaved at 121 °C, 1 atm for 1 h to hydrolyze soluble oligosaccharides released from to monosaccharides. The amount of glucose and xylose in the acid-hydrolyzed supernatant fractions were quantified by HPLC (Waters), equipped with a HPX87H column (Bio-Rad Laboratories) at 45°C, eluted at a rate of 0.6 ml/min with 5 mM H_2SO_4. Sugars were detected using a temperature controlled refractive index detector at 35°C. Cellulose and xylose hydrolysis yields were calculated according the following equations 1 and 2:

$$\%\ Cellulose\ hydrolysis = \frac{C_{glu} x 0.90}{C_{pulp} x C_{\%}} x 100 \quad (1)$$

where C_{glu} is the concentration of glucose (g/L) in the acid-hydrolyzed supernatant fractions, 0.90 is the glucose to cellulose conversion factor, C_{pulp} is the initial concentration of BEKP (g/L), and $C_{\%}$ is the cellulose content in percentage in BEKP.

$$\%\ Xylan\ hydrolysis = \frac{C_{xyl} x 0.88}{C_{pulp} x X_{\%}} x 100 \quad (2)$$

Where C_{xyl} is the concentration of xylose (g/L) in the acid-hydrolyzed supernatant fractions, 0.88 is the xylose to xylan conversion factor, C_{pulp} is the initial concentration of BEKP (g/L), and $X_{\%}$ is the xylan content in percentage in BEKP.

The selectivity of the enzymatic hydrolysis of BEKP for xylan solubilisation over cellulose solubilization was calculated by diving the % xylan hydrolysis per % cellulose hydrolysis.

The cellulose and xylan content in the CRSs were determined by subtracting the amount of cellulose and xylan hydrolysed (equation 1 and 2) from their contents in the original BEKP (100%) multiplied for 0.786 or 0.146, respectively.

4.5 Washing of the csr to release cnc particles

Ten milliliters of deionized water were added to each CSR obtained after 72h-enzymatic hydrolysis, manually agitated, centrifuged at 500 rpm (54 g) for 10 min, and allowed to decant at room temperature. Then, the supernatant was removed and the washing step was repeated several times until there was no more turbidity in the supernatant (turbidity is a possible signal of presence of nanocellulose particles). The turbid fractions were collected and dispersed by sonication using an ultrasonic liquid processor (Vibra-Cell ™ VCX-750, Sonics) coupled with a 13 mm-diameter titanium probe, for 10 or 30 minutes at 60% power. To prevent overheating, the sample was placed in an ice batch and then sonicated. After sonication, the turbid solution was allowed to decant for 30 min, and the supernatant containing the CNC nanoparticles were collected and used for subsequent analyzes.

4.6 Stability of CNC suspensions

The CNC suspensions were allowed to stand at room temperature for 3 days. During this period, 200 µL of each of the CNC suspensions were collected periodically, and the absorbance at 600 nm of the samples was immediately determined using a spectrophotometer (Epoch/2 Biotek). To determine the stability of the CNC suspension, the absorbance readings were converted to transmittance using equation 3:

$$T = 100x10^{-A} \quad (3)$$

Where T is transmittence and A is absorbance.

4.7 Particle size and distribution of the CNC suspensions

Mean particle size (PS_M) and particle size distribution (PS_D) of the CNC suspensions were determined by Low Angle Laser Light Scattering (LALLS), also called laser diffraction technique, using a laser diffraction particle size analyzer (Mastersizer 3000, Malvern Instruments) with two sources of light, red (632.8 nm) and blue (470 nm), allowing for a measurement range of 10nm-3,500µm. The equipment was coupled with a medium volume automated sample dispersion unit (Hydro MV) with a built-in centrifugal pulp to circulate the suspended sample within the analysis cell, an in-line ultrasonic probe and a stirrer to prevent sedimentation and aggregation of the particles in the sample unit tank. Analyses were conducted at low obscuration (0,5-4%) – a measure of the concentration of the suspension during analysis –, with stir rotation speed at 3500 rpm, without sonication. The Mie-scattering theory is used by the Malvern Mastersizer 3000 software to convert the light scattering data to particle size distributing. Therefore, for nanocellulose particles, the input parameters were manually set to assume a non-spherical particle model and the refractive index (RI) to 1,4683, the RI for cellulose. The final particle-size distributions were reported as particle size density number. Size analyses reported in this paper are the average of three runs, each with five successive laser diffraction runs, a total of 5 readings per sample. Before each analysis, the dispersion unit was automatically cleaned 3 times with ultrapure water.

4.8 Crystallinity index and crystal size of CNC

X-ray diffractograms were collected using a diffractometer (XRD – 6000, Shimadzu) at room temperature, with CuKα radiation and a graphite monochromator (reflection mode analysis). Before analysis, CNC suspensions were frozen at -60 °C and freeze dried (Edwards Super Modulyo Freeze Dryer). The dried CNC samples were placed in a glass support and the measurement conditions were $10 < 2\theta < 40$; 2θ step: 0.02°, 30 s per step. Measurements in the XRD were done in triplicates. The XRD peaks were separated by deconvolution (curve fitting) using a Gaussian function in the Origin software (version 2017, OriginLab). The crystallinity index (CrI) of the CNC suspensions was determined

according to Segal et al. (1959), but using the deconvoluated peak height method (DPHM), which makes useof the fitted XRD data to calculate CrI (CrI) also according to equation 4, where I_{002} is the peak apex around 2θ = 22.5° and I_{am} is the minimum around 2θ = 18°.

$$\mathrm{CrI} = \frac{(\mathrm{I}_{002} - \mathrm{I}_{\mathrm{am}}) \times 100}{\mathrm{I}_{002}} \quad (4)$$

The X-ray diffractograms were also used to calculate the cellulose crystallite lateral dimension in respect to the (200) plane (L_{200}) (nm), according to the Scherrer equation (equation 5), where K is the correction factor (0.91), λ is the radiation wavelength (for CuKα, λ=1.54060 Á), θ is the diffraction angle (around 11.25° for the (200) plane), and $\beta_{1/2}$ is the full width (in radians) at half maximum (FWHM) intensity of the same peak (200) (Kumar et al. 2014).

$$L_{200} = \frac{\mathrm{K}\lambda}{\beta_{1/2} cos\theta} \quad (5)$$

4.9 Chemical composition of CNC

For quantitative determination of the chemical composition, the CNC suspensions were centrifuged at 10,618 rpm (15,000 g) and the precipitated nanoparticles were collected and dried at 105 °C until constant weight. Subsequently, dried CNC samples were subjected to a two-step acid hydrolysis to depolymerize the carbohydrates into monosaccharides according to Sluiter et al. (2012), expected that the analysis was scaled down. Briefly, about 30 mg of dried CNC were mixed with 0,3 mL of 72% (w/w) H_2SO_4 in a 10 mL-borosilicate test tube, and placed in a water bath at 30 °C for 60 min. During this period, each reaction mixture was stirred carefully with a glass rod every 10 min. After 60 min, the acid-sample mixture was diluted with 8.4 g of distilled water to reach a final concentration of 4% (w/w) H_2SO_4. The test tube was sealed and autoclaved for 60 min at 121°C and 1 atm along with a series of glucose and xylose standards in the range of 0.1-4.0 mg/mL in 4% (w/w) H_2SO_4. About 2 mL of the sample or standard

in centrifuge tube was vortexed, filtered using a 0.45µm membrane and analyzed for glucose and xylose by the previously described HPLC procedure. The cellulose and xylan contents in CNC samples were determined from the glucose and xylose contents multiplied by the conversion factors 0.90 and 0.88, respectively, to account for the water molecules released during hydrolysis.

4.10 Thermogavymetry analysis

Thermal analysis was performed on a simultaneous TA Instruments - SCT TGA-DSC Q600 system. Analytical nitrogen (N_2) with volumetric flow rate of 100 mL/min, heating rate of 10 °C/min, and data acquisition from 30 °C to 600 °C was used for analysis. The sample mass used in the thermal analysis assays was between 0.3-0.5 mg. Alumina crucible was used because of its low reactivity.

Thermogravimetric (TG) curves and derivative TG (DTG) curves were analyzed with the aid of the Origin Software (Version 2017) in an effort to determine the peak onset temperature (T*onset*) and peak maximum temperature (T*max*).

Onset degradation temperature (T*onset*) was calculated from the TG curve by extrapolation. It denotes the temperature at which the dominant mass loss begins. Maximum degradation temperature (T*max*) was calculated from the DTG curve by extrapolation. It denotes the temperature at which the mass loss is maximum.

5 RESULTS AND DISCUSSION

To produce CNC by enzymatic hydrolysis of PKBE that contains about 15% xylan, two types of enzymes were chosen: an endoglucanase and an endoxylanase. The cellulose-depolymerizing endoglucanase was chosen for selective hydrolysis of the amorphous regions of the cellulose whereas the xylan-depolymerizing xylanase was for hydrolysis of the residual xylan.

Xylanases are enzymes that catalyze the hydrolysis of xylan, the major hemicellulose found in dicotyledonous angiosperms like eucalyptus. Industrially, xylanases are already widely used in the textile, pulp, paper and food industries (JUTURU; WU, 2012). Among the xylanases, the endoxylanases (endo-β-1,4-xylanases EC 3.2.1.8) are those that typically act within the linear chain of xylan through hydrolysis of β-1,4 linkages (BIELY et al., 1997; BIELY; SINGH; PUCHART, 2016). These enzymes have also gained great prominence among the enzymes in conversion of lignocellulosic biomass to sugars, since they remove the residual hemicellulose that acts as a barrier to cellulolytic enzymes, thus increasing the access of cellulases to cellulose. Consequently, they lead to improvements in the overall hydrolysis and reduction of the total enzyme dosage (HU; ARANTES; SADDLER, 2011; GONÇALVES et al., 2015).

5.1 Enzymatic activity profile of EG and EX

In this study, FiberCare® (EG) a single-component endoglucanase was used as a source of endoglucanase. EG is commercialized for application in kraft, sulphite, and recycled pulps to reduce energy consumption (15-30%) during mechanical refining, and increase tensile strength of the pulp (Novozymes). Additionally, its cellulose fragmentation capability has been widely exploited in the pretreatment of cellulose for production of CNF and dissolving pulp (IBARRA et al., 2010; WANG et al., 2015b). In all cases, cellulose hydrolysis is aimed at the lowest level as possible. For the endoxylanase, we used Cellic® HTec2 (EX), an enzyme preparation enriched in xylanase activity. EX was developed to be used as a hemicellulase supplement for the cellulase cocktail Cellic® CTec2, intended for the conversion of lignocellulosic biomass into fermentable sugars to produce cellulosic ethanol. EX has been reported to reduce the residual xylan content left in the

cellulosic pulp after a hydrothermal or thermochemical pretreatment, thereby improving the conversion of xylan to xylose and cellulose to glucose (Novozymes).

The enzymatic activity profile of EG and EX are shown in Table 2. Both enzyme preparations have little or no activity in crystalline cellulose (Avicel), typical of cellobiohydrolases, enzyme found in cellulase preparations like Celluclast 1.5L (HU; ARANTES; SADDLER, 2011). As expected, EG is constituted primarily of endoglucanase activity as can be seen for its high activity (840 U/mL) towards CMC, an amorphous cellulosic substrate, and the absence or very low activities of other cellulases and hemicellulases. On the other hand, EX is a more complex enzyme preparation as it is constituted of several cellulolytic and xylanonolytic activities. However, the most expressive enzymatic activity in EX is the endoxylanase activity (10733 U/mL), which is several folds higher than all other detected activities (Table 2). The level of endoglucanase activity in EX (850 U/mL) is similar to that found in EX. β-xylosidase, that was found only in EX, is an enzyme that hydrolyzes the products (short and soluble xylo-oligosaccharides) released by endoxylanases, known to be strong inhibitors of cellulase enzymes like endoglucanases and cellobiohydrolases (QING; YANG; WYMAN, 2010). β-glycosidase, that was also found in EX, catalyzes the hydrolysis of the products (short and soluble cellodextrins) of endoglucanases and cellobiohydrolases, therefore, decreasing inhibition of these enzymes by their hydrolysis products (JØRGENSEN; KRISTENSEN; FELBY, 2007; HU et al., 2013).

Table 2 - Enzyme activity profile of EG and EX, and their relative activities at 100, 200, and 400 U/g pulp based on endoglucanase activity.

		Endoglucanase (CMC)	**Cellobiohydrolase (Avicel)**	**β-glucosidase (pNPG)**	**Endoxylanase (Birch wood Xylan)**	**β-xylosidase (pNPX)**
EG	U/mL	840	0.7	0.2	2.4	0,0
	U/g pulp	**100**	0.1	0.0	0.3	0,0
		200	0.2	0.0	0.6	0,0
		400	0.3	0.1	1.1	0,0
EX	U/mL	850	28	7.1	10733	157
	U /g pulp	7.9	0.3	0.1	**100**	1.5
		15.8	0.5	0.1	**200**	2.9
		31.7	1.0	0.3	**400**	5.8

Source: Author source

The main activities present in the two enzyme preparations at the activity level employed in this study for EG and EX, are shown also in Table 2. For example, when EX was used at 100, 200 and 400U EX, the level of all other activities in EX were extremely low, since the endoxylanase activity is comparatively several folds higher. It is also noteworthy that the endoglucanase activity of EX (850 U/ml), that is similar to that of EG (840 U/ml), is dramatically reduced to 7.9, 15.8, and 31.7 U when the endoxylanase activity of EX is set at 100, 200 or 400 U loading. These endoglucanase activity levels at the levels of EX used in this study are about 12-fold lower when compared to the fixed endoglucanase loads (100, 200 and 400 U) for EG. These observations indicate that at the enzyme dosages used in the hydrolysis experiments (Table 1), the endoxylanase activity was the only at a significant level for EX. Similarly, when the endoglucanase activity of EG was used at 100, 200, and 400 U, the level of the other activities detected in EG, that was already very low, became even lower and practically at a negligible level during the enzymatic hydrolysis runs (Table 2).

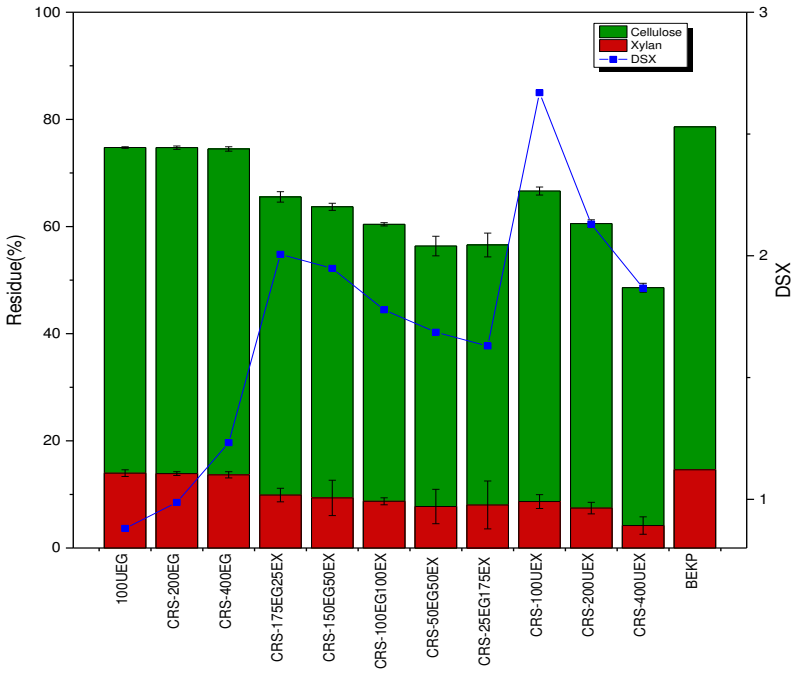
According to the activities found in the two preparations and the relative enzymatic activities employed in the hydrolysis treatments, it was hoped that they could cooperate with each other in the production of CNC from BEKP. That is, the endoxylanase activity would allow greater accessibility of the endoglucanases to the amorphous regions of cellulose facilitated by the hydrolysis of xylan by the endoxylanase, without affecting the integrity of the crystals, since the two preparations showed little or no cellobiohydrolase activity.

5.2 Enzymatic hydrolysis of bekp with EG and EX and their combinations

The cellulose and xylan contents in BEKP after enzymatic hydrolysis with only EG and only EX or combinations thereof in different proportions together with the degree of selectivity towards xylan solubilization (DSX) are shown in Figure 10 Treatments with EG alone at endoglucanase activity loading of 100, 200 and 400 U per gram of pulp did not hydrolyze significantly cellulose or xylan, since their CSR content remained practically unchanged compared to the control (non-hydrolyzed) pulp. For example, at the highest enzyme dose (400 U), the solubilization of cellulose and xylan was only about 5 and 7%, respectively. As

discussed later, when EG was used alone, it also did not appear to result in a turbid suspension - indication of CNC production (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; CUI et al., 2016) - during the CNC isolation step. This may be associated with the low efficiency of EG for cellulose hydrolysis, although a cellulose hydrolyzing enzyme. Recently, Wang et al. (2015) also reported a low hydrolytic performance for similar enzymatic hydrolysis and did not detect solubilization of more than 6% cellulose after 48 h of hydrolysis using and endoglucanase activity loading of 0.1, 10 and 100 mg per gram of pulp. One possible reason for the low hydrolysis efficiency of BEKP by EG could also be the relatively high residual xylan content, which is known to be mostly absorbed on the surface of the cellulose (SIXTA, 2006), and therefore could be hindering the access of EG to most of the cellulose, as it has been observed for hydrolysis of other xylan containing cellulosic substrates by cellulases (HU; ARANTES; SADDLER, 2011; ZHANG; TANG; VIIKARI, 2012; PENTTILÄ et al., 2013; GONÇALVES et al., 2015; PRZYBYSZ BUZAŁA et al., 2016). In such cases, xylanase has been used as an auxiliary enzyme, acting synergistically with cellulases to achieve near complete cellulose hydrolysis.

Figure 10 - Percentage of cellulose and xylan in the cellulosic solid residue (CSR) obtained after hydrolysis of BEKP with different enzymatic combinations, and the degree of selectivity towards xylan during (DSX) hydrolysis.



Source: Author source

To try to improve the hydrolysis efficiency of BEKP by the EG, EX, with very high endoxylanase activity and very low cellulase side activity (Table 2), was used as an auxiliary enzyme, by supplementing it to EG at ratios EG:EX of 175EG25EX, 150EG50EX, 100EG100EX, 50EG150EX, 25EG175EX, with 200 U as the final total enzyme loading. When compared to EG alone, all EG:EX mixtures showed improvement in hydrolysis yield of both cellulose and xylan, even when EG:EX loading was lower than that of EG alone (400 U, Figure 10). Contrary to the cellulose and xylan contents in the cellulosic residue that remained almost unchanged when BEKP was treated only with EG, cellulose content reduced about 17-29% (from 78.6% to 65-56%) and xylan approximately 32-35% (from 14.6% to 10-8%) when BEKP was treated with EG supplemented with EX (Figure 10). It was also possible to observe that as EG loading decreased (and EX loading increased), the hydrolysis of the cellulose and xylan increased. Thus, greater hydrolysis of cellulose and xylan was obtained when lower loading of endoglucanase and higher loading of endoxylanase (50EG150EX, 25EG175EX) was used (Figure 10). Very similar results were obtained by Hu, Arantes and Saddler (2011), when an enzymatic preparation containing only a small amount of cellulase (Celluclast 1.5L, Novozymes) and a significantly higher amount of xylanase (Multifect® Xylanase, Genencor International) led to higher hydrolysis yield of both the xylan and cellulose in a lignocellulosic material pretreated by steam explosion than by any other combination of xylanase and cellulase or when both were used individually. Interestingly, both the results presented here and those of Hu, Arantes and Saddler (2011) demonstrate the importance of using a higher amount of endoxylanase in relation to the amount of cellulase in order to achieve better efficiency in the hydrolysis of cellulose and xylan.

According to Penttilä et al. (2013), the greater the removal of the xylan loosely bound to cellulose, the lower the selectivity and the greater the loss of crystallinity of the cellulose. Therefore, another parameter to monitor is the degree of selectivity for xylan (DSX), since crystallinity is an important parameter for the quality of CNC. Although the hydrolysis of cellulose and xylan were higher as the EX supplementation increased in EG:EX mixtures, DSX decreased progressively (Figure 10). For example, DSX decreased from 2 to 1.9, 1.8, 1.7 and 1.6 according to the supplementation level of EX in the combinations 175EG25EX, 150EG50EX, 100EG100EX, 50EG150EX, 25EG175EG, respectively. That is, with low EX

supplementation, the EG:EX mixture preferentially hydrolyzed xylan, whereas the amount of EX supplementation increased, the hydrolysis of the cellulose showed proportionally higher increase, thereby leading to a reduction of the selectivity for xylan. This may have occurred because the greater the proportion of EX supplemented, the greater the amount of xylan hydrolyzed. However, the access of the most accessible xylan (poorly bound to cellulose) in the material is limited and further decreases as the content of xylan is reduced in the residue. Consequently, with a greater removal of xylan, an increase in the accessible surface area of cellulose is expected, allowing for greater adsorption of EG present in the binary mixture to cellulose, and higher hydrolysis of the cellulose and thus reduced DSX.

Based on the above results, increased EX supplementation and consequent EG decrease led to high levels of hydrolysis. Thus, in order to determine whether the higher yields were only due to the presence of higher loading of EX or actually due to synergism between EG and EX, hydrolysis of BEKP was also carried out with only EX at 100, 200, and 400 U and are shown in Figure 10 as 100EX, 200EX and 400EX, respectively. Surprisingly, EX effectively solubilized both cellulose and xylan, and maintained a high degree of selectivity for xylan. For example, EX was more efficient in cellulose hydrolysis (15-40%) than EG (5%) and some of the combinations between EG and EX. This result was unexpected, since EX is an enzyme preparation developed to be used as a complement to cellulases for hydrolysis of lignocellulosic materials pretreated in acid medium, which typically results in a low but representative content of residual xylan. It is important to emphasize that the ratio between the endoxylanase and endoglucanase activity in EX is approximately 13: 1 (Table 2), i.e. for the endoxylanase activity loadings of 100, 200 and 400U used, the endoglucanase activity corresponded to only 9, 16, and 32 U, respectively, and even much lower amounts of other cellulases (i.e. cellobiohydrolase and β-glycosidase) (Table 2). The hydrolysis of BEKP with EX at an endoxylanase activity of 200U per gram (200EX) of pulp was greater than or equal to the binary mixtures EG:EX 175EG25EX, 150EG50EX, 100EG100EX, 50EG150EX, 25EG175EG) at the same total enzyme loading (200U) (Figure 10). The only combinations of EG with EX that obtained better hydrolysis of cellulose than 200EX were the 50EG150EX and 25EG175EX mixtures, combinations having approximately 75% and 87% EX, respectively. These proportions of xylanase and

cellulase were very similar to those found by Hu, Arantes and Saddler (2011), who obtained the best hydrolysis values using 75% and 86% xylanase in the enzymatic preparation.

In the hydrolysis of the xylan in BEKP, 200EX was superior to all combinations of EG:EX at a total enzyme loading of 200U tested. With a higher loading of EX (400EX), more than 70% xylan hydrolysis was achieved, resulting in a high purity CSR (48% cellulose) with only 4% xylan (Figure 10).

EX alone showed a good selectivity for the hydrolysis of xylan, solubilizing approximately 2-3 times more xylan than cellulose, likely due to its very high xylanase activity compared to cellulases (Table 2). The increase in EX loading (from 100 to 200 and 400 U) decreased the selectivity towards xylan (Figure 10), as was observed with the proportional increase of EX in the EG:EX combinations. This result also shows that besides the type of enzymes, their proportions are important and should be controlled.

5.3 Isolation of CNC and their particle size analysis

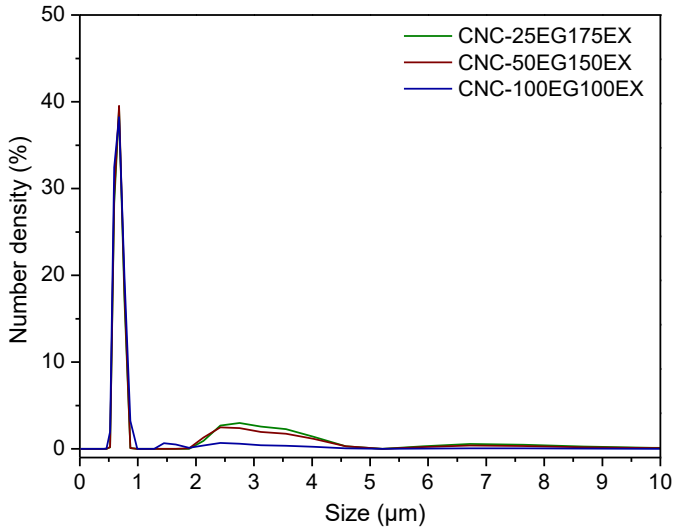
The CSRs collected after enzymatic hydrolysis with different dosages and combinations of EG and EX were further processed for isolation of CNC particles and analysed for particle size and distribution. The dimensions of nanocellulose particles depend greatly on the raw material as well as on the cellulose fractionation processes and the nanocrystals isolation procedure employed (SACUI et al., 2014; SIQUEIRA; ARANTES, 2016), and so on the technique used to determine their sizes. In addition, the sizes of nanocellulose particles and their distribution (uniformity) is one of the most important characteristics for CNC. In general, the dimensions for CNCs obtained by enzymatic treatment reported in previous work have been around 4-80nm and 100-1000nm in diameter and length, respectively (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY, 2009; XU et al., 2013; CUI et al., 2016).

Though the formation of turbidity may not be the best qualitative indicator of CNC production, many studies have associated the formation of a turbid suspension during the washing steps following cellulose hydrolysis to the release of CNC particles in solution (FILSON; DAWSON-ANDOH; SCHWEGLER-BERRY,

2009; XU et al. 2013; CUI et al., 2016;). In this study, when the supernatant of at least one of the 10 washing steps performed did not become turbid, it was assumed that the hydrolysis condition did not lead to the isolation of CNC or that the amount of CNC particles released was very low.

The hydrolyses using only EG (100, 200, 400U) were not efficient to produce a visible turbid suspension during the washing steps and likely did not produce CNC or only very low amounts of it. This may be due to the low EG activity towards BEKP (Figure 10), as a consequence of the likely limited access of the enzyme to the cellulose chains. When EG was supplemented with an auxiliary enzyme (EX), the formation of nanoparticles (<1 μm) was observed for three (25EG175EX, 50EG150EX and 100EG100EX) of the five combinations used, evidencing the importance of auxiliary enzyme and its proportion in the enzyme-mediated production of CNC. The treatments that resulted in CNC formation were the ones conducted with EX supplemented to EX at equivalent or higher loading. The LALLS results for CNC-25EG175EX, CNC-50EG150EX and CNC-100EG100EX suspensions showed PS_M of 675nm, indicating that 50% of the nanoparticles generated in the three different enzyme combinations were smaller than 675nm (Figure 11).

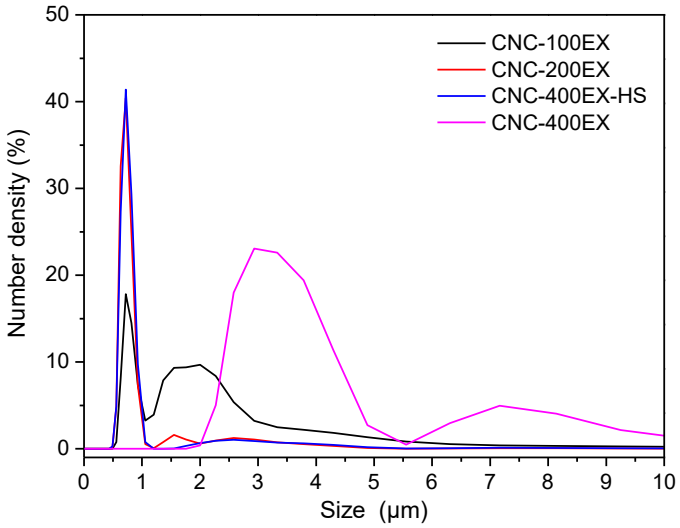
Figure 11 - Particle size distribution of CNC suspensions produced by different enzymatic loadings of endoglucanase (EG) e endoxylanase (EX).



Source: Author source.

Interestingly, BEKP hydrolysis with only EX, condition with very low endoglucanase activity (7.9, 15.8, and 31.7U) compared to endoxylanase activity (100, 200 and 400U), also resulted in formation of nanoparticles. A PS_M of 720 nm was observed for CNC-100EX, CNC-200EX and CNC-400EX suspensions. It is noteworthy that in the initial LALLS analysis of CNC-400EX suspension, no particles in the nanometer scale were detected, only particles larger than 2.3 µm (Figure 12). were detected These larger particles observed under more severe hydrolysis condition (higher enzyme dose) may be due to the aggregation of smaller particles, since the enzymes used in this study are not expected to alter the surface chemistry of the nanocrystals, thus preserving their hydroxyl groups, which have been reported as responsible for the interaction of the suspended nanocellulose particles, and consequently, their aggregation (HABIBI; LUCIA; ROJAS, 2010). To test this hypothesis, the CNC-400EX suspension was (again) sonicated, but this time, for a longer period (30 min instead of 10 min). It was expected that with this more severe sonication condition, possible aggregates of nanocellulose particles in the CNC-400EX suspension would be broken down, releasing the nanoparticles into suspension. In fact, sonication for 30 min promoted fragmentation of the large particles in CNC-400EX, resulting in CNC suspension with PS_M of 720 nm and a PS_D similar in size to CNC-200EX (Figure 12). Based on this result, all other CNC suspensions were also sonicated for 30 minutes. However, their sizes (PS_M and PS_D) remained unchanged.

Figure 12 - Particle size distribution of CNC suspensions produced by different combinations of endoglucanase (EG) with endoxylanase (EX). CNC-400EX was also submitted to a high instensity sonication (HS).



Source: Author source

The results shown here demonstrate that both the presence of EX and a high load of it have great influence on the production of CNC from BEKP, and that the beneficial effect of adding the auxiliary enzyme EX seems to be more than only xylan hydrolysis. These observations are evident when the hydrolysis treatment with combinations containing higher proportions of EG than EX (150EG50EX and 175EG25EX), that did not produce CNC, are compared with combinations of 100EX and 100EG100EX, which produced CNC. Although 175EG25EX and 150EG50EX hydrolyzed a slightly higher amount of cellulose than 100EX, and hydrolyzed a smaller amount of cellulose than 100EG100EX, the xylan hydrolyzed was similar (Figure 10). These results suggest that there should be a minimal loading of EX for CNC production, because even with a similar hydrolysis of xylan, the form of action of the enzymes present in EX has great importance in the release of CNCs.

The higher purity (cellulose content) of the CNC-400EX particles due to the lower xylan content as a result of the higher enzyme loading used in the treatment may have influenced the interaction and agglomeration of the nanoparticles in the CNC-400EX suspension. This is because it would be expected that CNC with lower xylan content and therefore higher purity, would have more free hydroxyl groups available for interactions with free hydroxyl groups available on the surface of other

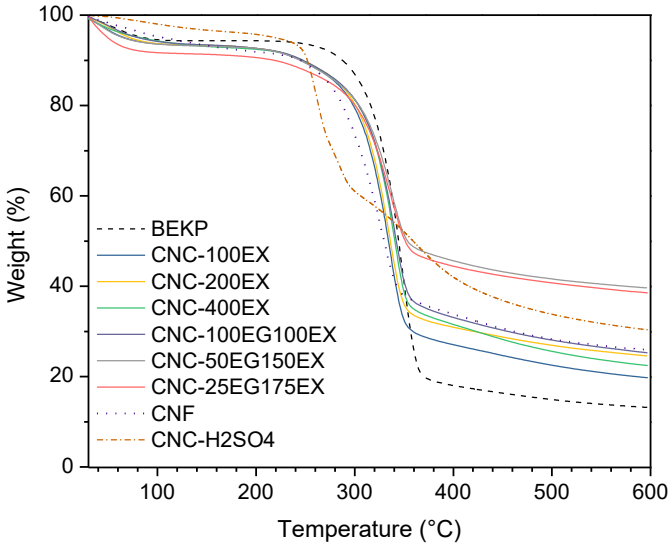
nanocrystals, leading to agglomeration. This CNC's agglomeration effect may be one of the reasons for the typical low yield of CNC obtained by enzymatic route and reported in previous work. However, one should bear in mind that enzymatic hydrolysis followed by sonication, unlike hydrolysis with sulfuric acid, keeps the hydroxyls unmodified, and the CNC surface more reactive, an important feature for various CNC applications (ANDERSON et al., 2014).

5.4 Thermostability of CNC

The thermal behavior of the nanocrystals is an important parameter for many applications (i.e. reinforcement material, polymer nanocomposites) (LIN; HUANG; DUFRESNE, 2012; CSISZAR et al., 2016). Therefore, one of the advantages of the CNCs produced by enzymatic hydrolysis is the possibility to maintain some of the characteristics of native cellulose, such as thermal stability.

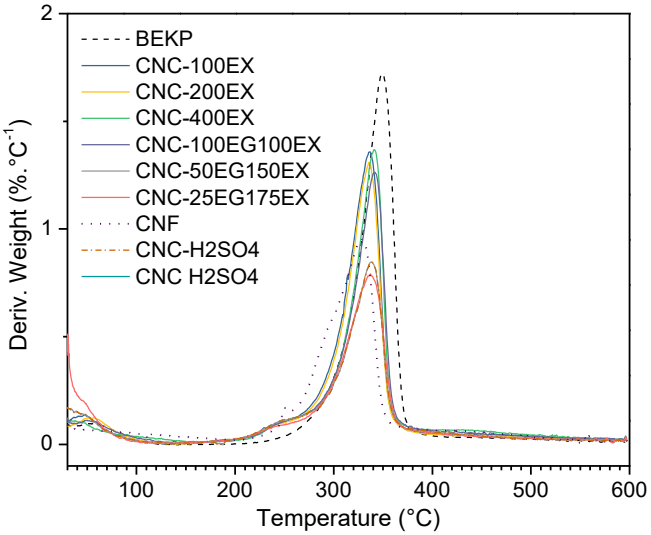
In order to observe the thermostability of the CNCs obtained in this work, thermogravimetric (TG) analyzes were performed on the 6 CNCs (CNC-100EX, CNC-200EX, CNC-100EX, CNC-100EG100EX, CNC-50EG150EX, CNC-25EG175EX) and compared with TG results of the starting material (BEKP) and to CNCH_2SO_4 obtained via the traditional acid hydrolysis method (H_2SO_4 64% w/w) according to Bundeson et al. 2006 (Figure 13).

Figure 13 - Thermogravimetric analyzes (TG) of CNC produced using different conditions.



Source: Author source.

Figure 14 – DTG curves of CNC produced using different conditions.



Source: Author source.

Unlike CNCH_2SO_4, which showed a unique pyrolysis behavior, all other CNC samples (CNC-100EX, CNC-200EX, CNC-100EX, CNC-100EG100EX, CNC-50EG150EX, CNC-25EG175EX, and BEKP) displayed three distinct stages of material loss. That is, an initial stage between 30 °C to 100 °C corresponding to evaporation of the water absorbed in the material (ROMAN; WINTER, 2004). A second stage with a more expressive mass loss referring to the loss of mass of non-cellulosic and cellulosic materials between the range of 200 °C to 400 °C. Finally, the third phase that generally corresponds to a slower mass loss due to the pyrolysis of the degraded material in the second stage ranged from 400 °C to 600 °C.

Except for CNC-25EG175EX which displayed a mass loss of about 8 % at low temperatures (<100 °C), all enzymatic produced CNC samples displayed a smaller mass loss (approximately 5%), similar to that of BEKP (starting material). CNC-H_2SO_4 showed a higher thermostability at this stage (30 °C-100 °C). However, as expected, it was observed that the start of the thermal degradation of the cellulosic material (T*onset*) of CNC-H_2SO_4 began at 242 °C and the maximum degradation temperature of the cellulosic material (T*max*) was 261 °C, showing the low thermostability of CNC obtained by sulfuric acid hydrolysis. This low thermostability of CNC-H_2SO_4 compared to BEKP and the CNCs obtained by enzymatic hydrolysis is more clearly shown on the DTG plot (Figure 14). This low

thermostability is because thermal degradation of nanoscritals containing sulfate groups (inserted into the cellulose chains during hydrolysis) occurs at lower temperatures, as demonstrated by other authors (ROMAN; WINTER, 2004; WANG; DING; CHENG, 2007; LU; HSIEH, 2010).

The CNCs CNC-100EX, CNC-100EX, CNC-100EG100EX, CNC-50EG150EX, CNC-25EG175EX showed a very similar second stage-thermostability profile with a high T*onset* (250 °C) and T*max* (340 °C), which were only slightly lower than that of the starting material (BHKP, T*onset* at 286 °C and T*max* at 350 °C) (Figure 13 and 14). This small loss in the thermal stability of the CNCs may be related to the smaller size of the nanocrystals that allows a larger surface area of exposure to the heat (WANG; DING; CHENG, 2007). Meyabadi et al., (2014) also reported a small decrease of the T*onset* temperature for enzymatically produced CNC (250 °C) when compared to cotton, the initial material (300 °C). Nevertheless, the CNCs obtained by enzymatic hydrolysis have high thermal stability and higher than the CNC obtained by sulfuric acid (FATTAHI MEYABADI et al., 2014; CUI et al., 2016). Although not observed in the TG analysis, it is important to note that analysis of the DTG detected that the enzymatic CNCs displayed a differentiated peak (started at around 220 °C) compared to BEKP (Figure 14). This peak corresponds to the degradation of a non-cellulosic material, such as the residual hemicellulose or the residual proteins contained in the CNC. This material may be responsible for the lower stability of CNCs observed in TG (Figure 13). According to Cui et al., (2016) the residual enzymes likely contained in the CNCs may be responsible for the decrease of the thermal stability. The low molecular weight of the hemicellulose (residual xylan) fragments on the outer surface of the crystals could also have contributed to this decrease in thermal stability since hemicellulose has a lower thermal stability (220°C-315°C) than cellulose (300°C-400°C) (YANG et al., 2007). One evidence for this is the fact that the same peak was observed in DTG analysis of a CNF sample (Figure 14), which was produced purely by a mechanical disc refining process and its composition is similar to the starting material (BEKP) and contains about 15% xylan.

At the last stage (350 °C-600 °C), a slower degradation of the material occurred for all samples, indicating a char yield at 600 °C. The amount of the char residue from the CNCs, ranging from 20% to 40%, was noticeably greater than the initial pulp (BEKP, 13%). The higher char yields may be related to higher

crystallinity of the materials, as materials with higher crystallinity have a higher energy requirement for degradation (Wang et al., 2007). The smaller particle sizes of the CNCs may also interfere with the higher amount of char. Roman and Winter (2004) reported that smaller particle sizes leaves a larger number of free chains ends, and that they begin to decompose at a higher temperature, which in this study would lead to the higher yield of CNC char.

5.5 Crystallinity index and crystal size of CNCs

The crystallinity indexof nanoparticles is one of the most important parameters determining their thermal and mechanical properties (ANDERSON et al., 2014; LIN; DUFRESNE, 2014; CUI et al., 2016).

Table 3 - Crystallinity Index (CrI) and Crystal width (CrW) of CNCs and BEKP.

Treatments	CrI (%)	CrW (nm)
CNC-200EX	67 ± 2,9	4,03 ± 0,17
CNC-100EX100EG	70 ± 0,6	4,07 ± 0,17
CNC-400EX	65 ± 2,1	3,89 ± 0,07
BEKP	71 ± 0,5	3,95± 0,005

Source: Author source

The size of the crystal was recently proposed to be of great importance to describe the crystalline structure of the cellulose (NISHIYAMA; JOHNSON; FRENCH, 2012). Therefore, it is important to make use of a more selective CNC treatment for the amorphous regions in order to maintain the integrity and size of the CNC crystals.

In comparison with the initial material (BEKP) there was no increase in the crystallinity of the analyzed CNCs (CNC-200EX, CNC-100EX100EG, CNC-400EX). However, CNC-400EX showed a decrease in the crystallinity index (Table X). This decrease in the CrI may have occurred due to the degradation of the crystalline region due to the higher enzyme loading used in the treatment of CNC-400EX, which also reflects the smaller size of the CNC-400EX crystal (Table 3). The CNC-100EX100EG was the one that showed a higher CrI, maintaining the CrI

of BEKP. These results indicate the importance of the combination of endoglucanase and xylanase for a better balance of hydrolysis and preservation of the crystallinity. The range of CrI (65-70%) for the CNCs produced in this work is in agreement with the CrI that has been reported in other works. For example, Texeira et al., (2014) reported that their enzymatically produced CNC after 72h hydrolysis of eucalyptus hallocellulose had a CrI of 65.9%.

5.6 Chemical composition of CNCs

The cellulose and xylan content in the CSRs was calculated based on the conversion yields obtained after enzymatic hydrolysis of BEKP and compared with the chemical composition of the CNCs (Table 4). It was assumed that the chemical composition in the CSRs could reflect the chemical composition in the CNCs. However, this was not the case. For example, the CSR with less xylan did not result in CNC with less xylan. This was the case for CSR-400EX that was obtained after enzymatic hydrolysis which solubilized more than 70% of the xylan, enriching the cellulose content from 78.6% in the initial pulp to 92.2% in the CSR (Table 4), but the CNC obtained with this residue had a chemical composition similar to the other CNCs (Table 4). The CNC-50EG-175EX and CNC-25EG175EX showed a composition similar to the CNC-400EX with xylan content of 14.4%, 12.9%, 13.3%, respectively (Table 4).

Table 4 - Cellulose and xylan content in the cellulosic solid residues (CSRs) from hydrolysis of BEKP and their corresponding CNC particles produced.

Treatments	CSR[2]		CNC	
	Cellulose (%)	Xylan (%)	Cellulose (%)	Xylan (%)
100EX	88,5	11,5	78,2 ± 0,3	15,3 ± 0,1
200EX	89,0	11,0	78,9 ± 0,5	14,7 ± 0,1
100EX100EG	87,4	12,6	78,9 ± 1,2	14,7 ± 0,2
50EG150EX	87,9	12,1	79,6 ± 2,8	14,4 ± 0,5
25EG175EX	87,6	12,4	80,3 ± 0,5	12,9 ± 0,2
400EX	92,1	7,9	82,1 ± 1,2	13,3 ± 0,2
64% H_2SO_4, 2h, 45 °C[1]	---	---	91,8 ± 0,4	3,2 ± 0,1

[1] CNC produced by acid hydrolysis of BEKP according to Bondeson, Mathew and Oksman (2006)

[2] Relative percentage of the CSR

Source: Author source

In general, the residual xylan (12.9% to 15.3%) in the CNCs were relatively high and similar among all CNCs (Table 4), indicating that even with high xylanase loading it was not possible to completely remove the residual xylan from BEKP and produce CNCs with very low xylan content. This result suggests that this remaining xylan was hardly accessible/hydrolysable with the xylanase treatment employed. Enzymes are very specific to their substrate and require the substrate to be configured so that it is possible to activate its catalytic site allowing the enzyme-substrate binding to occur. As for GH10s, the xylanase present in EX (HU et al., 2013), are enzymes which have been shown to perform better on branched xylan. This is because xylanase belonging to the GH10 family attacks glycosidic bonds near the regions of insertion of the side chains and near the non-reducing end. With the exception of the reducing end, xylanase GH10 requires two consecutive unsubstituted D-xylopyranosyl residue to attack the xylan backbone. The two glycosidic bonds following the 4-O-methyl-D-glucuronic acid (MeGlcA) branch are not attacked by GH 10.

The chemical characterization of the CNC produced by the traditional acid hydrolysis with sulfuric acid (CNC-H_2SO_4) was also determined (Table 4) and

compared with the CNCs obtained by enzymatic hydrolysis. CNC-H_2SO_4 showed a lower content of xylan (about 3.2%) compared to the CNCs obtained by enzymatic hydrolysis (Table 4). The low amount of xylan found in CNC-H_2SO_4 is due to the low specificity of the acid that attacks all glycosidic bonds, making possible a greater removal of xylan.

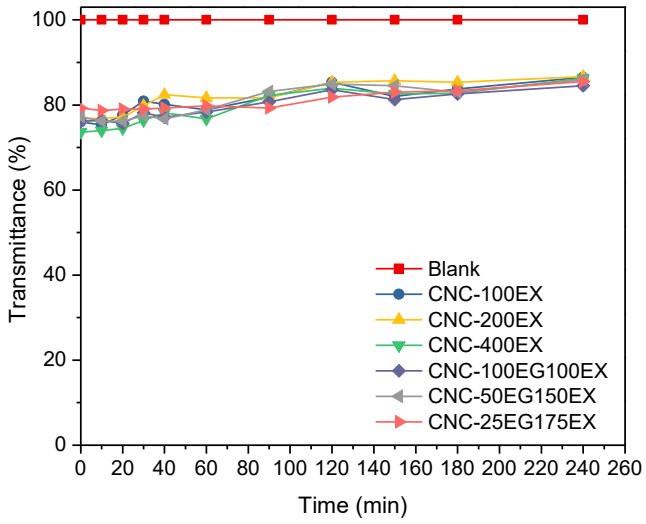
It has become clear that in order to decrease the xylan content in CNC obtained by enzymatic hydrolysis, it is necessary to further study the combination or use other xylanases such as xylanase from GH10 with CBM, GH11, GH11 with CBM and xylobiohydrolase which may be capable of acting or allowing better access of the endoxylanase to the residual xylan found in BEKP.

5.7 Stability of CNC suspensions

Another important characteristis of CNCs is the formation of a stable colloidal suspension. However, due to the preservation of the hydroxyl groups in the CNCs obtained by enzymatic treatment, the stability of the CNC suspensions is quite critical. Actually, this is one of the disadvantages of obtaining CNC by enzymatic hydrolysis.

By monitoring the transmittance of a CNC suspensions over time, it was possible to observe that the stability of the suspensions of the different CNCs are very similar (Figure 15).

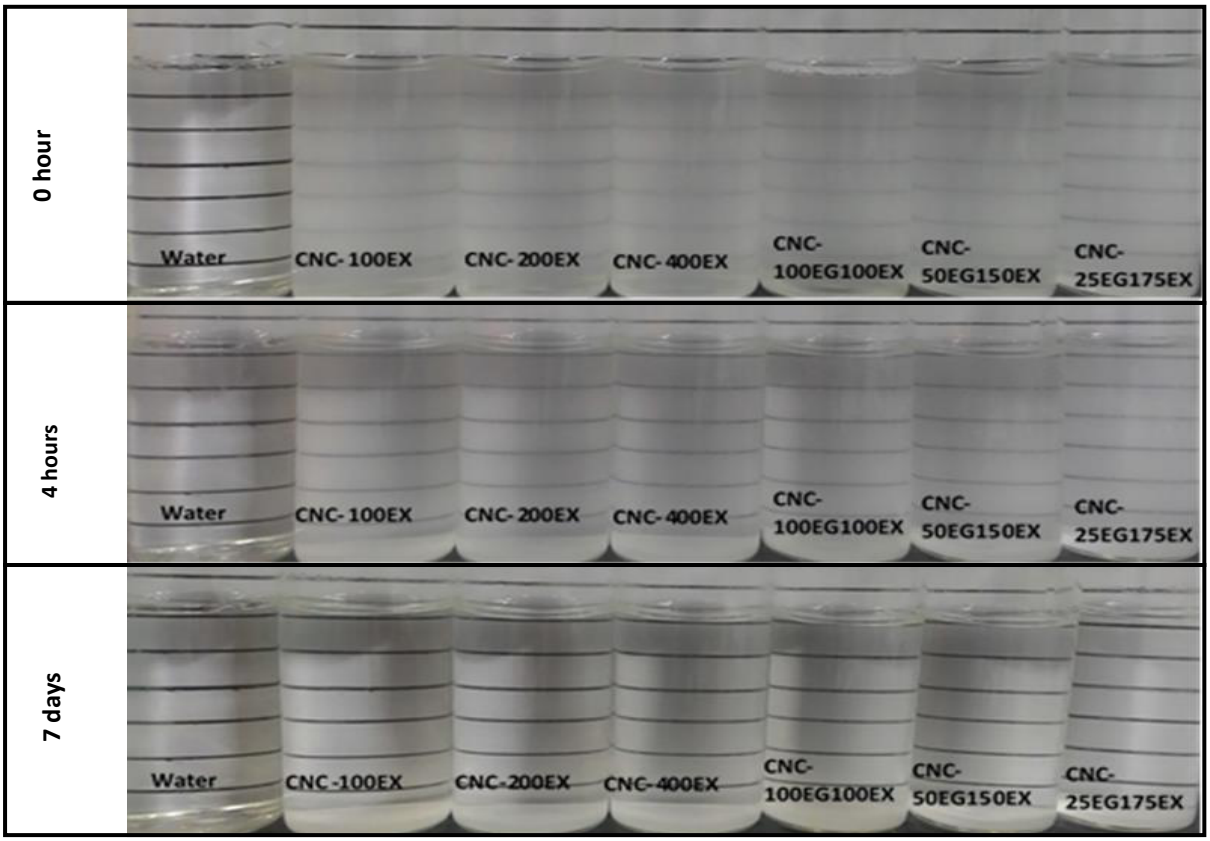
Figure 15 – Transmittance of the CNCs-suspension monitored-over time



Source: Author source.

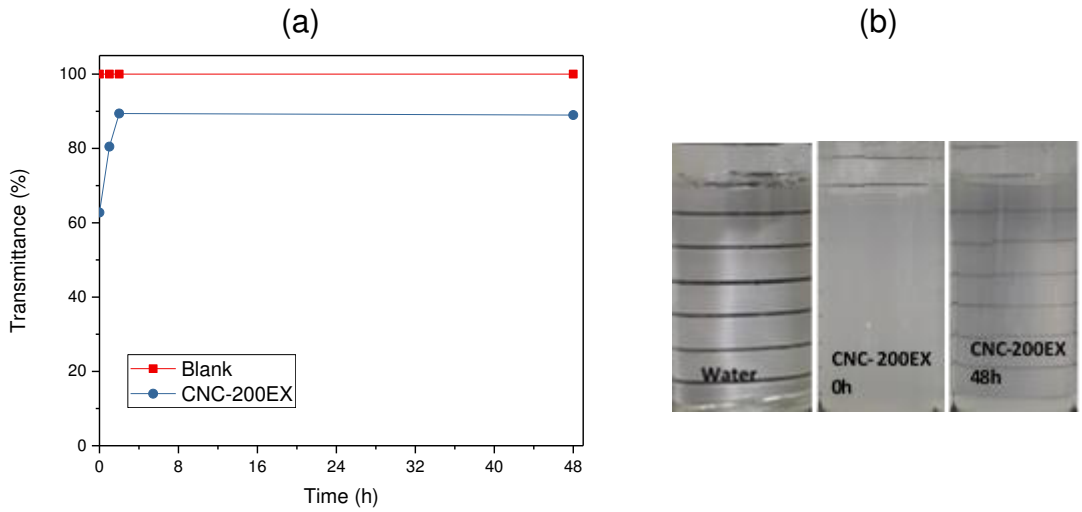
With four hours, it was possible to observe that CNC particles still formed a colloidal suspension. However, after 7 days the nanocrystals were fully aggregated (Figure 15). It appears that the maximum time of suspension stability is about 48 hours (Figure 16), indicating a relatively good stability of the CNC produced by enzymatic hydrolysis, which in fact is in agreement with Filson et al 2009 that also found enzymatically produced nanocrystals in suspension after 48h.

Figure 16 – Stability of the CNCs suspensions monitored over time: Water, CNC-100EX; CNC-200EX; CNC-400EX; CNC-100EG100EX; CNC-50EG150EX; CNC-25EG175EX



Source: Author source

Figure 17 - (a) Transmitance-of-CNC-20EX-suspension over 48h; (b) Stability of CNC-200EX-suspension over 48h



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6 CONCLUSION

The use of endoxylanase (Cellic® HTec2) as the main accessory enzyme, as evaluated here, was shown to be an approach of great importance in CNC isolation from BEKP. This is because only endoglucanase (FiberCare®) was unable to promote the release of nanocrystals, whereas all hydrolyses carried out using xylanase as the main accessory enzyme could produce CNC. The nanoparticles obtained, except for the CNC-100EX that displayed two expressive peaks of particle size, had similar size (PS_M) and uniformity (PS_D), with mean particle size between 500 and 1000nm. The enzymatic hydrolyses using only EX or in combination with EG produced CNCs with high thermostability (T*max* 340°C), similar to BEKP (T*max* 350°C) and more stable than CNCs produced by sulfuric acid hydrolysis (T*max* 261°C). The small difference between the CNCs produced by enzymes and the BEKP can be related to the xylan residual content. CNCs produced by enzymatic hydrolysis showed a xylan content of 13-15%, even when a high dosage of EX (400EX) was used. The xylan content in the cellulosic solid residue recoverd after the hydrolyses (10-17%) was not similar to the CNCs' xylan content (13-15%). CNCs with higher crystallinity index (70%) were produced using a combination of EX and EG. When only EX was used the CNCs showed a lower crystallinity index (65-67%). Regarding stability, all CNC suspensions obtained by enzymatic hydrolysis were similar, remaining stable for about 48h.

REFERENCES

AHOLA, S. et al. Enzymatic Hydrolysis of native cellulose nanofibrils and other cellulose model films: effect of surface structure. **Langmuir**, v. 24, n. 20, p. 11592–11599, 21 out. 2008.

ANDERSON, S. R. et al. Enzymatic prepapartion of nanocrystalline and microcrystalline cellulose. **TAPPI Journal**, v. 13, n. 5, p. 35–42, 2014.

BATTISTA, O. A. et al. Level-off degree of polymerization. **Industrial & Engineering Chemistry**, v. 48, n. 2, p. 333–335, fev. 1956.

BECK-CANDANEDO, S.; ROMAN, M.; GRAY, D. G. Effect of reaction conditions on the properties and behavior of wood cellulose nanocrystal suspensions. **Biomacromolecules**, v. 6, n. 2, p. 1048–1054, mar. 2005.

BIELY, P.; SINGH, S.; PUCHART, V. Towards enzymatic breakdown of complex plant xylan structures: state of the art. **Biotechnology Advances**, v. 34, n. 7, p. 1260–1274, nov. 2016.

BIELY, P. et al. Endo-P - 1,4-xylanase families: differences in catalytic properties. **Journal of Biotechnology**, v. 57, p. 151–166, 1997.

BONDESON, D.; MATHEW, A.; OKSMAN, K. Optimization of the isolation of nanocrystals from microcrystalline cellulose by acid hydrolysis. **Cellulose**, v. 13, n. 2, p. 171–180, 5 abr. 2006.

BRINCHI, L. et al. Production of nanocrystalline cellulose from lignocellulosic biomass: technology and applications. **Carbohydrate Polymers**, v. 94, n. 1, p. 154–169, abr. 2013.

CAMPINHOS, JR., E. No Title. **New Forests**, v. 17, n. 1/3, p. 129–143, 1999.

CEPEA. **Centro de Estudos Avançados em Economia Aplicada**São Paulo, 2015.

CHEN, H. **Biotechnology of Lignocellulose**. Dordrecht: Springer Netherlands, 2014.

CHEN, L. et al. Tailoring the yield and characteristics of wood cellulose nanocrystals (CNC) using concentrated acid hydrolysis. **Cellulose**, v. 22, n. 3, p. 1753–1762, 1 jun. 2015.

CLARKE, J. H. et al. Family-10 and Family-11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. **Applied Microbiology and Biotechnology**, v. 48, n. 2, p. 177–183, 25 ago. 1997.

COLLINS, T.; GERDAY, C.; FELLER, G. Xylanases, xylanase families and extremophilic xylanases. **FEMS Microbiology Reviews**, v. 29, n. 1, p. 3–23, 2005.

CSISZAR, E. et al. Ultrasonics sonochemistry the effect of low frequency ultrasound on the production and properties of nanocrystalline cellulose suspensions and films. v. 31, p. 473–480, 2016.

CUI, S. et al. Green preparation and characterization of size-controlled nanocrystalline cellulose via ultrasonic-assisted enzymatic hydrolysis. **Industrial Crops and Products**, v. 83, p. 346–352, 2016.

DAHLMAN, O. et al. Effects of hardwood xylan dissolutinon/sorpition on fibre charge and pul yield. v.1. p. 59-64. 2003.

DAVIES, G.; HENRISSAT, B. Structures and mechanisms of glycosyl hydrolases. **Structure**, v. 3, n. 9, p. 853–859, set. 1995a.

DAVIES, G. J.; HENRISSAT, B. Structures and mech of glycosyl hydrolases. **Structure**, v. 3, n. 9, p. 853–859, 1995b.

CAMPOS, A. et al. Obtaining nanofibers from curau?? and sugarcane bagasse fibers using enzymatic hydrolysis followed by sonication. **Cellulose**, v. 20, n. 3, p. 1491–1500, 2013.

MESQUITA, J. P. et al. Biobased nanocomposites from layer-by-layer assembly of cellulose nanowhiskers with chitosan. **Biomacromolecules**, v. 11, n. 2, p. 473–480, 2010.

DODD, D.; CANN, I. K. O. Enzymatic deconstruction of xylan for biofuel production. **Global change biology. Bioenergy**, v. 1, n. 1, p. 2–17, 2009.

DUAN, C. et al. Combination of mechanical, alkaline and enzymatic treatments to upgrade paper-grade pulp to dissolving pulp with high reactivity. **Bioresource Technology**, v. 200, p. 458–463, jan. 2016.

DUFRESNE, A. **Nanocellulose : From Nature to High Performance Tailored Materials.** Munchen, DEU: Walter de Gruyter, 2012.

FAOSTAT. **FOOD AND AGRICULTURE ORGANIZATION THE UNITED NATION**, 2014. .

FATTAHI MEYABADI, T. et al. Spherical cellulose nanoparticles preparation from waste cotton using a green method. **Powder Technology**, v. 261, p. 232–240, 2014.

FENGEL, D.; WEGNER, G. **Wood: chemistry, ultrastructure, reactions.** Berlin: Walter de Gruyter. 1983.

FERNANDES, N. et al. Nanostructure of cellulose microfibrils in spruce wood. **Proceedings of the National Academy of Sciences**, v. 108, n. 47, p. E1195–E1203., 2011.

FILSON, P. B.; DAWSON-ANDOH, B. E.; SCHWEGLER-BERRY, D. Enzymatic-mediated production of cellulose nanocrystals from recycled pulp. **Green Chemistry**, v. 11, n. 11, p. 1808, 2009.

GAMA, F. M.; MOTA, M. Enzymatic hydrolysis of cellulose (i): relationship between kinetics and physico-chemical parameters. **Biocatalysis and Biotranformation**, v. 15, p. 221–236, 1997.

EK, M.; GELLERSTEDT, G.; HENRIKSSON, G. **Wood chemistry and biotechnology**. Walter de Gruyer, Berlin. 2009

GEORGE, J. et al. Bacterial cellulose nanocrystals exhibiting high thermal stability and their polymer nanocomposites. **International Journal of Biological Macromolecules**, v. 48, n. 1, p. 50–57, jan. 2011.

GEORGE, J.; SABAPATHI, S. Cellulose nanocrystals: synthesis, functional properties, and applications. **Nanotechnology, Science and Applications**, v. 8, p. 45, nov. 2015.

GEORIS, J. et al. Purification and properties of three endo- beta -1,4-xylanases produced by Streptomyces sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulps. **Enzyme and Microbial Technology**, v. 26, n. 2, p. 178–186, 2000.

GIUDICIANNI, P.; CARDONE, G.; RAGUCCI, R. Cellulose, hemicellulose and lignin slow steam pyrolysis: Thermal decomposition of biomass components mixtures. **Journal of Analytical and Applied Pyrolysis**, v. 100, p. 213–222, mar. 2013.

GONÇALVES, G. A. L. et al. Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. **Enzyme and Microbial Technology**, v. 72, p. 16–24, maio 2015.

GONZÁLEZ, I. et al. Nanofibrillated cellulose as paper additive in eucalyptus pulps. **BioResources**, v. 7, n. 4, p. 5167–5180, 2012.

HABIBI, Y. Key advances in the chemical modification of nanocelluloses. **Chemical Society**

reviews, v. 43, n. 5, p. 1519–42, 2014.

HABIBI, Y.; LUCIA, L. A. **Polysaccharide building blocks: a sustainable approach to the developmet of renewblw biomaterials**. New York: John Wiley & Sons, 2012.

HABIBI, Y.; LUCIA, L. A.; ROJAS, O. J. Cellulose nanocrystals: chemistry, self-assembly, and applications. **Chemical Reviews**, v. 110, n. 6, p. 3479–3500, 9 jun. 2010.

HAMAD, W. On the development and applications of cellulosic nanofibrillar and nanocrystalline materials. **The Canadian Journal of Chemical Engineering**, v. 84, n. 5, p. 513–519, 19 maio 2008.

HENRIKSSON, M. et al. An environmentally friendly method for enzyme-assisted preparation of microfibrillated cellulose (MFC) nanofibers. **European Polymer Journal**, v. 43, n. 8, p. 3434–3441, 2007.

HORN, S. et al. Novel enzymes for the degradation of cellulose. **Biotechnology for Biofuels**, v. 5, n. 1, p. 45, 2012.

HU, J.; ARANTES, V.; PRIBOWO, A. Substrate factors that influence the synergistic interaction of AA9 and cellulases during the enzymatic hydrolysis of biomass. **Energy & Environmental Science**, v. 7, p. 2308–2315, 2014.

HU, J. et al. The synergistic action of accessory enzymes enhances the hydrolytic potential of a “cellulase mixture” but is highly substrate specific. **Biotechnology for biofuels**, v. 6, n. 1, p. 112, 2013.

HU, J.; ARANTES, V.; SADDLER, J. N. The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? **Biotechnology for Biofuels**, v. 4, n. 1, p. 36, 2011.

HUBER, T. et al. A critical review of all-cellulose composites. **Journal of Materials Science**, v. 47, n. 3, p. 1171–1186, 2012.

IBARRA, D. et al. Combination of alkaline and enzymatic treatments as a process for upgrading sisal paper-grade pulp to dissolving-grade pulp. **Bioresource Technology**, v. 101, n. 19, p. 7416–7423, out. 2010.

IWAMOTO, S.; NAKAGAITO, A. N.; YANO, H. Nano-fibrillation of pulp fibers for the processing of transparent nanocomposites. **Applied Physics A: Materials Science and Processing**, v. 89, n. 2, p. 461–466, 2007.

JØRGENSEN, H.; KRISTENSEN, J. B.; FELBY, C. Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. **Biofuels, Bioproducts and Biorefining**, v. 1, n. 2, p. 119–134, out. 2007.

JUTURU, V.; WU, J. C. Microbial xylanases: Engineering, production and industrial applications. **Biotechnology Advances**, v. 30, n. 6, p. 1219–1227, 2012.

KARIM, Z. et al. Necessity of enzymatic hydrolysis for production and functionalization of nanocelluloses. **Critical Reviews in Biotechnology**, v. 37, n. 3, p. 355–370, 3 abr. 2017.

KLEMM, D. et al. Polymer Science Cellulose : Fascinating Biopolymer and Sustainable Raw Material Angewandte. **Polymer Science**, v. 44, p. 3358–3393, 2005.

LEE, K. Y.; BLAKER, J. J.; BISMARCK, A. Surface functionalisation of bacterial cellulose as the route
to produce green polylactide nanocomposites with improved properties. **Composites Science and Technology**, v. 69, n. 15–16, p. 2724–2733, 2009.

LEVASSEUR, A. et al. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. **Biotechnology for biofuels**, v. 41, p. 1–14, 2013.

LIN, N.; DUFRESNE, A. Nanocellulose in biomedicine: Current status and future prospect. **European Polymer Journal**, v. 59, p. 302–325, out. 2014.

LIN, N.; HUANG, J.; DUFRESNE, A. Preparation, properties and applications of polysaccharide nanocrystals in advanced functional nanomaterials: a review. **Nanoscale**, v. 4, n. 11, p. 3274, 2012.

LIU, N. et al. Pulp properties and fiber characteristics of xylanase-treated aspen apmp. **BioResources**, v. 7, n. 3, p. 3367–3377, 2012.

LU, P.; HSIEH, Y. Preparation and properties of cellulose nanocrystals: rods, spheres, and network. **Carbohydrate Polymers**, v. 82, n. 2, p. 329–336, set. 2010.

LYND, L. R. et al. Microbial cellulose utilization. **Fundamentals and Biotechnology**. v. 66, n. 3, p. 506–577, 2002.

MATHEW, B. et al. Cellulose nanocomposites with nanofibres isolated from pineapple leaf fibers for medical applications. **Carbohydrate Polymers**, v. 86, n. 4, p. 1790–1798, 2011.

MEYABADI, T. F.; DADASHIAN, F. Optimization of enzymatic hydrolysis of waste cotton fibers for nanoparticles production using response surface methodology. **Fibers and Polymers**, v. 13, n. 3, p. 313–321, 3 mar. 2012.

MOON, R. J. et al. Cellulose nanomaterials review: structure, properties and nanocomposites. **Chemical society reviews**. v. 40. p. 32-34, may/jun. 2012

NISHIYAMA, Y.; JOHNSON, G. P.; FRENCH, A. D. Diffraction from nonperiodic models of cellulose crystals. **Cellulose**, v. 19, n. 2, p. 319–336, 20 abr. 2012.

OLIVEIRA, F. B. de. et al. Production of cellulose nanocrystals from sugarcane bagasse fibers and pith. **Industrial Crops and Products**, v. 93, p. 48–57, dez. 2016.

PAYNE, C. M. et al. Fungal cellulases. **Chemical Reviews**, v. 115, n. 3, p. 1308–1448, 2015.

PENTTILÄ, P. A. et al. Xylan as limiting factor in enzymatic hydrolysis of nanocellulose. **Bioresource Technology**, v. 129, p. 135–141, 2013.

POLAINA, J.; MACCABE, A. P. Industrial enzymes - structure, function and applications. Amsterdan: Springer Netherlands. 2007. p. 66-75.

PRZYBYSZ BUZAŁA, K. et al. Effect of cellulases and xylanases on refining process and kraft pulp properties. **PLOS ONE**, v. 11, n. 8, p. e0161575, 24 ago. 2016.

QING, Q.; WYMAN, C. E. Supplementation with xylanase and β-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover. **Biotechnology for biofuels**, v. 4, n. 1, p. 18, 2011.

QING, Q.; YANG, B.; WYMAN, C. E. Xylooligomers are strong inhibitors of cellulose hydrolysis by enzymes. **Bioresource Technology**, v. 101, n. 24, p. 9624–9630, 2010.

QUINLAN, R. J. et al. Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 37, p. 15079–84, 2011.

RÅNBY, B. G. Fibrous macromolecular systems. Cellulose and muscle. The colloidal properties of cellulose micelles. **Discussions of the Faraday Society**, v. 11, n. 111, p. 158, 1951.

RÅNBY, B. G.; BANDERET, A.; SILLÉN, L. G. Aqueous colloidal solutions of cellulose micelles.

Acta Chemica Scandinavica, v. 3, p. 649–650, 1949.

ROMAN, M.; WINTER, W. T. Effect of sulfate groups from sulfuric acid hydrolysis on the thermal degradation behavior of bacterial cellulose. **Biomacromolecules**, v. 5, n. 5, p. 1671–1677, 2004.

RONCERO, M. The effect of xylanase on lignocellulosic components during the bleaching of wood pulps. **Bioresource Technology**, v. 96, n. 1, p. 21–30, jan. 2005.

SACUI, I. A. et al. Comparison of the properties of cellulose nanocrystals and cellulose nanofibrils isolated from bacteria, tunicate, and wood processed using acid, enzymatic, mechanical, and oxidative methods. **ACS Applied Materials and Interfaces**, v. 6, n. 9, p. 6127–6138, 2014.

SAELEE, K. et al. An environmentally friendly xylanase-assisted pretreatment for cellulose nanofibrils isolation from sugarcane bagasse by high-pressure homogenization. **Industrial Crops and Products**, v. 82, p. 149–160, 2016.

SATYAMURTHY, P. et al. Preparation and characterization of cellulose nanowhiskers from cotton fibres by controlled microbial hydrolysis. **Carbohydrate Polymers**, v. 83, n. 1, p. 122–129, jan. 2011.

SHATKIN, J. O. A.; WEGNER, T. H.; BILEK, E. M. T. E. D. Market projections of cellulose nanomaterial-enabled products − Part 1: Applications. **TAPPI Jounal**, v. 13, n. 5, p. 9–16, 2014.

SHIN, N. H.; STROMBERG, B.; FALLS, G. Xylan ' s impact on eucalyptus pulp yield and strength: myth or reality ? In: INTERNATIONAL COLLOQUIUM ON EUCALYPTUS PULP., 2007.

SILVA, C. A. F. e; BUENO, J. M.; NEVES, M. R. The pulp and paper industry in brazilguia abtcp-fornecedores&fabricantes. **Celulose e Papel**, 2016.

SIQUEIRA, G.; ARANTES, V. Nanocelluloses from Lignocellulosic Biomass. In. Valorization of Lignocellulosic Biomass in a Biorefinery: From Logistics to Environmental and Performance. Nova Scien ed. 2016.

SIXTA, H. Pulp Properties and Applications. In: HANDBOOK OF PULP. 2006 1009–1067.

SONG, Q. et al. Nanofibrillated cellulose (NFC): A high-value co-product that improves the economics of cellulosic ethanol production. **Energies**, v. 7, n. 2, p. 607–618, 2014.

SPOSINA, R. et al. Combining biomass wet disk milling and endoglucanase/glucosidase hydrolysis for the production of cellulose nanocrystals. **Carbohydrate Polymers**, v. 128, p. 75–81, 2015.

SUBRAMANIYAN, S.; PREMA, P. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. **Crit Rev Biotechnol**, v. 22, n. 1, p. 33–64, 2002.

TEIXEIRA, R. S. S. et al. Combining biomass wet disk milling and endoglucanase/B-glucosidase hydrolysis for the production of cellulose nanocrystals. **Carbohydrate Polymers**, v. 128, p. 75–81, 2015.

THOMAS, L. H. et al. Structure of cellulose microfibrils in primary cell walls from collenchyma. **Plant Physiology,** v. 161, n. 1, 2013.

TMR. **Transparency Market Research In depth Analysis Accurate Results**.

TONOLI, G. H. D. et al. Cellulose micro/nanofibres from Eucalyptus kraft pulp: Preparation and properties. **Carbohydrate Polymers**, v. 89, n. 1, p. 80–88, 2012.

VAAJE-KOLSTAD, G. An oxidative enzyme boosting the. **Science**, v. 330, p. 219–222, Oct. 2010.

WANG, N. et al. Thermal degradation behaviors of spherical cellulose nanocrystals with sulfate groups. **Polymer**. v. 48, p. 3486–3493, 2007.

WANG, Q.; LIU, S.; YANG, G.; CHEN, J.; NI, Y. Cationic polyacrylamide enhancing cellulase treatment efficiency of hardwood kraft-based dissolving pulp. **Bioresource Technology**, v. 183, p. 42–46, 2015a.

WANG, W. et al. Production of cellulose nanofibrils from bleached eucalyptus fibers by hyperthermostable endoglucanase treatment and subsequent microfluidization. **Cellulose**, v. 22, n. 1, p. 351–361, 2015b.

WU, J.-M.; LIU, R.-H. Cost-effective production of bacterial cellulose in static cultures using distillery wastewater. **Journal of Bioscience and Bioengineering**, v. 115, n. 3, p. 284–290, mar. 2013.

XU, Y. et al. Feasibility of nanocrystalline cellulose production by endoglucanase treatment of natural bast fibers. **Industrial Crops and Products**, v. 51, p. 381–384, nov. 2013.

YANG, H. et al. Characteristics of hemicellulose, cellulose and lignin pyrolysis. **Fuel**, v. 86, n. 12–13, p. 1781–1788, ago. 2007.

ZENI, M.; FAVERO, D.; MC, A. G. Preparation of microcellulose (mcc) and nanocellulose (ncc) from eucalyptus kraft ssp pulp. **Polymer Sceiences**, v. 1, p. 1–7, 2015.

ZHANG, J. et al. Microfibrillated cellulose from bamboo pulp and its properties. **Biomass and Bioenergy**, v. 39, p. 78–83, abr. 2012a.

ZHANG, J.; TANG, M.; VIIKARI, L. Xylans inhibit enzymatic hydrolysis of lignocellulosic materials by cellulases. **Bioresource Technology**, v. 121, p. 8–12, 2012.

ZHANG, J. et al. Comparison of the synergistic action of two thermostable xylanases from GH families 10 and 11 with thermostable cellulases in lignocellulose hydrolysis. **Bioresource Technology**, v. 102, n. 19, p. 9090–9095, 2011.

ZHANG, Y. et al . Preparation and characterization of nano crystalline cellulose from bamboo fibers by controlled cellulase hydrolysis. **Journal of Fiber Bioengineering and Informatics**, v. 5, n. 3, p. 263–271, 5 set. 2012b.

ZHANG, Y. P.; LYND, L. R. A functionally based model for hydrolysis of cellulose by fungal cellulase. **Biotechnology and Bioenergy**, v. 5, n. 94, p. 888-98, 2006.

ZHONG, J. et al. Self-Powered Human Interactive Transparent Nanopaper . **ACSNano**, n. 7, p. 7399–7406, 2015.

ZHU, J. Y.; SABO, R.; LUO, X. Integrated production of nano-fibrillated cellulose and cellulosic biofuel (ethanol) by enzymatic fractionation of wood fibers. **Green Chemistry**, v. 13, n. 5, p. 1339, 2011.