Enzymatic-assisted preparation of nanocrystalline cellulose from non-wood fibers

Facundo Beltramino Heffes

Programa de Doctorat d'Enginyeria Tèxtil i Paperera

Enzymatic-assisted preparation of nanocrystalline cellulose from non-wood fibers

PhD Thesis

Facundo Beltramino Heffes

Terrassa, 2016

La Doctora **M. BLANCA RONCERO VIVERO,** Professora Titular i la Doctora **CRISTINA VALLS VIDAL**, del Departament d'Enginyeria Tèxtil i Paperera de la Universitat Politècnica de Catalunya-BarcelonaTech

CERTIFIQUEN:

Que **Facundo Beltramino Heffes**, Llicenciat en Bioquímica i Màster en Enginyeria Tèxtil, Paperera i Gràfica, ha realitzat sota la seva direcció el treball d'investigació titulat **"Enzymatic-assisted nanocrystalline cellulose preparation from non-wood fibers"** que presenta per optar al títol de Doctor.

I perquè així consti s'expedeix el present certificat a Terrassa, 20 de Setembre de 2016.

M. Blanca Roncero Vivero
Professora Titular d'Universitat
Universitat Politècnica de Catalunya

Cristina Valls Vidal
Doctora de la Universitat
Politècnica de Catalunya

A mi familia

A mis amigos

"Our freedom to doubt was born out of a struggle against authority in the early days of science. It was a very deep and strong struggle: permit us to question - to doubt- to not be sure. I think that it is important that we do not forget this struggle and thus perhaps lose what we have gained."

Richard Phillips Feynman - 1955

Agraïments

La realització d'aquesta tesi ha tingut lloc als laboratoris de recerca del grup Celbiotech al departament d'Enginyeria Tèxtil i Paperera de l'Escola Superior d'Enginyeries Industrial, Aeroespacial i Audiovisual de Terrassa (ESEIAAT), de la Universitat Politècnica de Catalunya (UPC BarcelonaTech).
L'execució de la tesi ha estat possible gràcies al finançament d'una beca pre-doctoral FPI MINECO (BES-2011-046674).
La recerca duta a terme ha estat finançada pels següents projectes de recerca nacionals:

- FUNCICEL: Funcionalización de fibras lignocelulósicas mediante sistemas lacasa mediador utilizando compuestos fenólicos de origen natural. MINECO (CTQ 2009-12904).
- BIOFIBRECELL: Nuevos procesos enzimáticos para la obtención de productos celulósicos de alto valor añadido. MINECO (CTQ2010-20238-C03-01).
- BIOSURFACEL: Estudio y desarrollo de procedimientos para la funcionalización superficial de soportes lignocelulósicos mediante sistemas enzimáticos. MINECO (CTQ2012-34109).
- BIOPAPμFLUID: Biomodificación de papeles para la construcción de dispositivos microfluídicos. MINECO (CTQ2013-48995-C2-1-R)

Aquest treball es el resultat d'anys de dedicació durant els quals sempre he estat recolzat, guiat i aconsellat per moltes persones, sense les quals de ben segur avui no em trobaria avui escrivint aquestes línies. Voldria agrair personalment a totes elles:

En primer lloc a les meves directores de tesi, la **Dra. Ma. Blanca Roncero** i la meva co-directora, la **Dra. Cristina Valls**. Voldria agrair-les per la seva passió per la recerca, positivisme davant els moments complicats i sobre tot per transmetre'm els seus coneixements i guiar-me al llarg d'aquest camí. Per ensenyar-me com plantejar i enfocar un problema, i com mirar una mateixa realitat des del cantó oposat, des del qual molts cops es veu un panorama diferent. Per inculcar-me que a vegades el que sembla evident no necessàriament ho és i també per tenir una infinita paciència ensenyant-me a escriure ciència.

Si em trobo avui aquí és gràcies a la **Dra. Teresa Vidal**, que va confiar en mi i em va donar l'oportunitat de treballar en el seu grup de recerca. Voldria donar les gràcies per aquesta confiança, pel seu optimisme i per estar sempre disposada a trasmetre els seus coneixements. Aquesta tesi no hagués estat possible tampoc sense el seu guiatge i les seves bones idees que m'han permès ensortir-me en els moments més complicats.

Al **Dr. Antonio L. Torres**, por su inmensa predisposición a ayudarme en todo lo que estuviese en su mano, por estar siempre disponible para explicarme lo que necesitase, siempre con una visión más "ingenieril". También agradecerle, como no, por el tiempo dedicado al buen funcionamiento de los equipos e instrumentos del laboratorio, permitiéndonos a todos trabajar siempre en excelentes condiciones.

A **Antonio Clemente** y a **Adrián Oliva** por hacer día a día, más fácil el trabajo de laboratorio. Gracias por estar disponibles siempre que lo he necesitado, por procurar que nunca nos faltase material y también por la paciencia y buen humor de todos los días.

Al **Dr. Tzanko Tzanov** y al **Dr. Pere Garriga**, del grup GBMI de la UPC per acollir-me als seus respectius laboratoris i permetre'm utilitzar els seus equips quan els he necessitat.

A la **Dra. Pilar Diaz** i al **Dr. Javier Pastor**, del grup d'enzims microbians de la Facultat de Biologia de la Universitat de Barcelona, gràcies per rebre'm al seu laboratori mentre encara era estudiant i donar-me l'oportunitat de començar aquest camí de la recerca. Gràcies també a la **Dra. Susana Valenzuela** i la **Dra. Silvia Cesarini**, aleshores estudiants de doctorat, per ensenyar-me a treballar al laboratori i per totes les bones estones que vam compartir.

A tots els meus companys paperers amb els que he tingut la sort de compartir aquests anys. A l'**Amanda**, el **Jordi**, l'**Elisabetta**, l'**Oriol**, la **Glòria,** l'**Elisabet,** l'**Alvaro** i la **Mercè**. Gracies per tots els grans moments viscuts al laboratori i també fora d'ell. Gràcies per crear aquest bon ambient, pels esmorzars, dinars i berenars, per les converses i els riures. En resum, estic molt content d'haver compartit aquests anys amb vosaltres. A més a més, voldria agrair especialment als qui he tingut més a prop durant tota la tesi: a l'Ori i en particular a l'Eli. No començaré a enumerar totes les coses perquè no acabaria mai, només diré que he estat afortunat per tenir companys així. Sabem que les tesis tenen una mica de cadascuna de les persones que tens a prop durant el camí. Gràcies per tot!

Als meus amics **Blai** i **Arnau**, per tots els anys que ens portem aguantant i els que encara ens queden. Que podamos seguir compartiendo éxitos toda la vida! Aquí he venido a hablar de mi libro!

A mis amigos de la Uni, a **Isaac**, a **Oscar**, a **Loren**, a **Gerard** a **Marcel** y a **Aran**. Imagino que nuestra realidad común de "doctorandos" nos hacía entendernos de una forma especial. Gracias por todos los viajes, fiestas, reuniones, cafés y un largo etcétera que hicieron de estos años una gran etapa a nivel personal además de profesional. Siempre nos quedará el barro!

A tots els integrants del **Club de Rugby Carboners de Terrassa**. Tot i que cap d'ells en sigui conscient, han aportat l'equilibri necessari al meu dia a dia. En aquest club, a més a més del millor esport del món, he pogut trobar-me amb gent increïble i formar part d'una veritable família. Salut i Rugby!

Finalmente, me gustaría agradecer especialmente a mi **Familia**. Tengo claro que lo que he hecho en la vida lo debo en gran parte a su apoyo, cariño y consejos. A Mamá y Juancho, por todo lo que me han brindado en esta vida, no solo a nivel material (que también) sino por la educación, los valores y el apoyo que he tenido la gran suerte de recibir. Me han demostrado que el (verdadero) deseo es una fuerza muy poderosa! A Joaco, que a medida que crecemos me va mostrando que las diferencias son siempre algo relativo, y que tengo la suerte de contar con una persona brillante como él, que sé que va a estar ahí durante todo el camino. Finalmente a Juanma, que llegó a mi vida como un hermano mayor, alguien de quien aprender, y con el tiempo se convirtió además en un amigo con el que compartir la vida.

The design of the cover of this PhD thesis includes an image extracted from a photogram of the video: “Tappi Nanocellulose video - Rethink Paper”, available at: https://www.youtube.com/watch?v=R3HH4iN8aDM. This image is only used for illustrating this work with a non-commercial, educational purpose.

Index

Abstract

In the current scenario of growing environmental concerns, the search for innovative, renewable, non-polluting materials has never been as intensive as it is today. Cellulose, being the most abundant polymer on earth, offers a wide range of possibilities for fulfilling current and potential future needs for novel materials. In this direction, research in the field of nanocrystalline cellulose (NCC) has attracted a great interest in recent years. However, this great interest has been shadowed by the low yields yet offered by published works concerning this matter. With the aim of addressing this issue from a biotechnological perspective and therefore increasing the industrial feasibility of NCC, this doctoral thesis deals with the introduction of an enzymatic pretreatment into the NCC preparation process in combination with a controlled sulfuric acid hydrolysis.

This work is part of the research conducted by the CELBIOTECH group (UPC_BarcelonaTech) within the framework of Spain's MINECO projects FUNCICEL (CTQ 2009-12904), BIOFIBRECELL (CTQ2010-20238-C03-01), BIOSURFACEL (CTQ2012-34109), and BIOPAPμFLUID (CTQ2013-48995-C2-1-R) projects.

The first part of this thesis involved a comprehensive **characterization of a series of bleached cellulosic raw materials** for the proper selection of the one fitting best each specific study.

In the second part an **enzymatic pretreatment using a cellulase was performed on fibers prior to acid hydrolysis for NCC preparation for the first time.** For this, cotton linters, a pure cellulosic source was used. It was found that this pretreatment was capable of **significantly increasing process yield** while it also influenced other NCC characteristics such as their size and electrical charge. Also, it was observed that the magnitude of the effects of the enzymatic pretreatment on NCC were strongly dependent on the subsequent conditions used for acid hydrolysis.

With the aim of maximizing process yield and the influence of the cellulase pretreatment, the third and fourth parts of the thesis involved the **optimization of the acid hydrolysis and enzymatic pretreatment** steps, respectively. For this, two 2^3 and a complete 2^2 experimental designs were carried out. As a result of these optimizations **a**

total yield of ≈ 82% was reached and the required time for enzymatic pretreatment and acid hydrolysis were reduced in a 90% and a 44%, down to 2h and to 25 minutes, respectively, dramatically increasing the industrial feasibility of the process.

Being high-cellulose content fibers the preferential source for NCC, the fifth part of this thesis involved removal of hemicelluloses for **purification of totally chlorine-free (TCF) sisal fibers throughout treatments with hydrolases** in combination with alkaline extraction. These treatments led to fibers with high- **cellulose content (≈ 96 %).** Then, in the sixth part of this work these **high-cellulose content sisal fibers were used as a cellulose source for NCC using optimal enzymatic and chemical conditions**. This final section demonstrated that the enzymatic pretreatment **was a promising first step for NCC production regardless of the used raw material** and highlighted the benefits of hemicelluloses removal in the efficiency of isolation and NCC quality.

This thesis also sought for a better understanding of reaction mechanisms and chemical and structural modifications of cellulose in each studied step using analytical techniques such as SEM, TEM, DLS, WCA, HPLC, FTIR and UV-visible spectroscopy. Furthermore we understood that the enzymatic pretreatment also allowed a better exploitation of the used natural resources by generating a clean stream of easily-profitable oligosaccharides as a by-product, **better integrating NCC manufacture within the biorrefinery concept**.

Resumen

En el marco actual de creciente preocupación por la conservación del medio ambiente, la búsqueda de materiales innovadores, renovables y no contaminantes es más intensa que nunca. En ésta dirección, la celulosa, el polímero más abundante de la tierra, posee un gran potencial para satisfacer las necesidades actuales y futuras de nuevos materiales. Dentro de éstas posibilidades, la investigación relacionada con la Celulosa Nanocristalina (*NCC, Nanocrystalline cellulose*) ha atraído un gran interés durante los últimos años. Sin embargo, este gran interés se ha visto opacado por los bajos rendimientos generalmente obtenidos en trabajos publicados sobre este tema hasta la actualidad. Con el objetivo general de incrementar la viabilidad industrial de la NCC y abordando este asunto desde una perspectiva biotecnológica, la presente tesis doctoral trata sobre la introducción de un pretratamiento enzimático en el proceso de preparación de NCC en combinación con una hidrólisis controlada usando ácido sulfúrico.

Este trabajo es parte de la investigación llevada a cabo por el grupo CELBIOTECH (UPC_BarcelonaTech) en el marco de los proyectos del Mineco (España): FUNCICEL (CTQ 2009-12904), BIOFIBRECELL (CTQ2010-20238-C03-01), BIOSURFACEL (CTQ2012-34109) y BIOPAPμFLUID (CTQ2013-48995-C2-1-R).

La primera parte de la tesis consistió en la **caracterización detallada de una serie de materias primas celulósicas blanqueadas**, con la finalidad de poder escoger la más adecuada para cada uno de los estudios a realizar.

En la segunda parte de la tesis, **un pretratamiento enzimático sobre fibras con una celulasa como paso previo a la preparación de NCC vía hidrólisis ácida fue realizado por primera vez.** Para ello se utilizaron linters de algodón, una fuente de celulosa pura. Se observó que este pretratamiento era capaz de **incrementar significativamente el rendimiento de la hidrólisis** a la vez que influenciaba otras características de la NCC, como su tamaño y carga eléctrica. Además, advertimos que la magnitud de los efectos del pretratamiento sobre la NCC eran fuertemente dependientes de las condiciones de hidrólisis ácida posteriormente utilizadas.

Con el objetivo de maximizar el rendimiento del proceso y también la influencia del pretratamiento enzimático, la tercera y cuarta parte de la presente tesis consistieron en la **optimización de la hidrólisis ácida y del pretratamiento enzimático**,

respectivamente. Para ello se realizaron dos planes estadísticos del tipo 2^3 y uno 2^2. Como resultado de estas optimizaciones **se alcanzó un rendimiento total de ≈ 82%.** Paralelamente, se consiguió también reducir el tiempo de pretratamiento enzimático y de hidrólisis ácida un 90% y un 44% hasta 2h y a 25 minutos, respectivamente, incrementando así la viabilidad industrial del proceso estudiado.

Siendo las fibras de alto contenido en celulosa la materia prima preferente para la preparación de NCC, la quinta parte de ésta tesis trató sobre **la eliminación de hemicelulosas para la purificación de fibras de sisal TCF** (*Totally chlorine-free*, totalmente libres de cloro) **mediante tratamientos con hidrolasas** en combinación con una extracción alcalina. Por medio de éstos, se **produjeron fibras con un alto contenido en celulosa (≈ 96 %).** Finalmente, en la sexta parte de la tesis **estas fibras con alto contenido en celulosa fueron utilizadas como fuente para preparar NCC usando las condiciones enzimáticas y químicas óptimas**. Esta sección final demostró que el pretratamiento enzimático constituye una prometedora etapa para la preparación de NCC que **funciona independientemente de la materia prima utilizada**. Además, remarcó los beneficios de la eliminación de hemicelulosas en la mejora de la eficiencia de hidrolisis y la calidad de la NCC.

La presente tesis también persiguió un mejor entendimiento de los mecanismos de reacción y las modificaciones químicas y estructurales de la celulosa en cada paso, para lo cual se utilizaron técnicas analíticas como SEM, TEM, DLS, WCA, HPLC, FTIR y espectroscopia UV-visible. Asimismo, se comprendió también que el pretratamiento enzimático implicaba un mejor aprovechamiento de los recursos naturales al generar como sub-producto aguas residuales con un alto contenido en oligosacáridos fácilmente aprovechables. De esta manera se consigue también una **mejor integración de la fabricación de NCC en el concepto de la biorefinería**.

Resum

Dins el marc actual de creixent preocupació per la conservació del medi ambient, la cerca de materials innovadors, renovables, i no contaminants és més intensa que mai. En aquesta direcció, la cel·lulosa, el polímer més abundant a la terra, ofereix un ampli ventall de possibilitats per satisfer les necessitats actuals i futures de nous materials. Dins aquestes possibilitats, la recerca relacionada amb la cel·lulosa nanocristal·lina (*NCC, Nanocrystalline cellulose*) ha atret un gran interès en els darrers anys. No obstant, aquest interès s'ha vist fins ara opacat pels baixos rendiments generalment reportats en els estudis publicats sobre aquest tema. Amb l'objectiu general d'augmentar la viabilitat industrial de la producció d'NCC i abordant aquest tema des d'una perspectiva biotecnològica, la present tesi doctoral estudia la introducció d'un pretractament enzimàtic en el procés de preparació d'NCC tot combinant-lo amb una hidròlisi controlada amb àcid sulfúric.

Aquest treball es part de la recerda duta a terme pel grup CELBIOTECH (UPC_BarcelonaTech) dins el marc dels projectes del Mineco: FUNCICEL (CTQ 2009-12904), BIOFIBRECELL (CTQ2010-20238-C03-01), BIOSURFACEL (CTQ2012-34109) i BIOPAPμFLUID (CTQ2013-48995-C2-1-R).

La primera part de la tesi va consistir en la **caracterització detallada d'una sèrie de matèries primeres cel·lulòsiques blanquejades**, amb l'objectiu de poder escollir la més adequada per cadascun dels estudis a realitzar.

En la segona part de la tesi, **un pretractament enzimàtic sobre fibres amb una cel·lulasa com a pas previ a la preparació d'NCC via hidròlisi àcida va ser realitzat per primera vegada**. Per aquest estudi es van utilitzar línters de cotó, una font de cel·lulosa pura. Es va observar que el pretractament era capaç **d'incrementar significativament el rendiment de la hidròlisi** a la vegada que influenciava altres característiques de l'NCC, com ara la seva mida i càrrega elèctrica. Altrament, es va advertir que la magnitud dels efectes d'aquest pretractament era fortament depenent de les condicions d'hidròlisi àcida posteriorment utilitzades.

Amb l'objectiu de maximitzar el rendiment del procés i també la influència del pretractament enzimàtic, la tercera i quarta part de la tesi va consistir en **l'optimització de la hidròlisi àcida i el pretractament enzimàtic**, respectivament. Per això van ser realitzats dos plans estadístics del tipus 2^3 i un de 2^2. Com a resultat d'aquestes

optimitzacions es **va aconseguir un rendiment total de ≈ 82%.** Paral·lelament, els temps de pretractament enzimàtic i d'hidròlisi àcida van ser reduïts un 90% i un 44%, fins a 2 hores i 25 minuts respectivament, tot augmentant la viabilitat industrial d'aquest procés.

Essent les fibres d'alt contingut en cel·lulosa la matèria primera preferent per a la preparació d'NCC, la cinquena part de la tesi va tractar sobre **l'eliminació d'hemicel·luloses per a la purificació de fibres TCF de sisal** (*Totally chlorine-free*, totalment lliures de clor) **mitjançant tractaments amb hidrolases** en combinació amb una extracció alcalina. Per mitjà d'aquests tractaments **es van produir fibres amb un alt contingut en cel·lulosa (≈ 96 %).** Finalment, en la sisena part de la tesi aquestes fibres d'alt contingut en cel·lulosa van ser utilitzades com a font per la preparació d'NCC fent servir les condicions enzimàtiques i químiques òptimes trobades anteriorment. Aquesta secció final va demostrar que el pretractament enzimàtic constitueix una prometedora etapa per a la preparació d'NCC **que funciona independentment de la matèria primera utilitzada**. A més a més, va remarcar els efectes positius de la eliminació d'hemicel·luloses en la millora de l'eficiència d'hidròlisi i la qualitat de l'NCC.

La present tesi també va perseguir una millor comprensió dels mecanismes de reacció i les modificacions químiques i estructurals de la cel·lulosa a cada pas, per la qual cosa es van utilitzar tècniques analítiques com ara SEM, TEM, DLS, WCA, HPLC, FTIR i espectroscòpia UV-visible. Tanmateix, aquest treball va permetre entendre que el pretractament enzimàtic també implica un millor aprofitament dels recursos naturals al generar com a sub-producte aigües amb un alt contingut en oligosacàrids fàcilment aprofitables. Així, es permet també un **millor encaix de la fabricació d'NCC dins el concepte de la biorrefinería.**

Nomenclature

Abs	Absorbance
AFM	Atomic Force Microscopy
ATR	Attenuated Total Reflectance
BLI	Brightness Loss Index
C, Cx	Fungal Bioproducts Cellulase
C1-6	Glucooligosaccharide from 1 to 6 subunits
CMC	Carboxymethil Cellulose
CSN	Chain Schission Number
D	Chlorine dioxide bleaching stage
DLS	Dynamic Light Scattering
DP	Degree of Polymerization
E (4, 9%)	Alkali extraction with NaOH 4% or 9% (w/v)
E.C.	Enzyme Comission Number
ECF	Elementary Chlorine Free
FAO	Food and Agriculture Organization
FTIR	Fourier-transformed Infrared Spectroscopy
H_2SO_4	Sulfuric acid
HexA	Hexenuronic Acid
HPLC	High-Performance Liquid Chromatography
K	Control treatment
kDa	kiloDaltons
KN	Kappa Number
KN_{HexA}	KN due to HexA

KN_{lig}	KN due to lignin
kV	kiloVolts
kWh	kiloWatt per hour
L(l)	Length-weighted average length
LOI	Lateral Order Index
NaOH-AQ	Sodyum hydroxyde-Antraquinone pulping process
NCC	Nanocrystalline Cellulose
NFC	Nanofribrillar Cellulose
nm	nanometer
O	Oxigen delignification stage
odp	oven-dried pulp
OH-	Hydroxyl group
^{o}SR	Schopper-Riegler degrees
P	Peroxide bleaching stage
Po	Oxigen-pressurized peroxide bleaching stage
Poly-Dadmac	Polydiallyldimethylammonium chloride
Q	Chelating bleaching stage
RH	Relative Humidity
RID	Refractive Index Detector
rpm	revolutions per minute
SEM	Scanning Electron Microscopy
SO_4^{2-}	Sulfate group
TCF	Totally Chlorine Free
TCI	Total Crystallinity Index
TEM	Transmission Electron Microscopy

U	Enzymatic activity Unit
UV	Ultraviolet
w/v	weight/volume concentration
w/w, wt.	weight/weight concentration
WCA	Water Contact Angle
WRV	Water Retention Value
X	Fungal Bioproducts Xylanase
X_{1-5}, Xs_{1-5}	Direct and step-wise treatment with xylansase from 1 to 5h.
X1-4	Xylooligosaccharide from 1 to 4 subunits

List of figures

List of tables

Chapter 1

Introduction

1.1. Nanotechnology

Nanotechnology, a discipline primarily dealing with synthesis, characterization, exploration, and exploitation of nanostructured materials has attracted plenty of interest during the last decades. Nanomaterials are usually characterized by at least one dimension in the nanometer (nm) range. For a tentative picture of these dimensions, one nanometer constitutes approximately the length equivalent to 10 hydrogen or 5 silicon atoms aligned in a line. It is broadly accepted that nanotechnology was introduced for the first time by the Nobel laureate Richard P. Feynman on a historic lecture at the annual meeting of the American Physical Society at the California Institute of Technology (Caltech) in 1959 named "There's plenty of room at the bottom – An invitation to enter a new field of physics" (Feynman, 1960). During it, he suggested the interest of manipulating matter at an extremely small scale, at molecular or even atomic levels, *i.e.*, the Nano-scale, and stated that this interest could be related not only to physics, but also to many other knowledge areas as well. Nowadays, nanotechnology definition at a vague level depends on the field of study it is applied to, commonly being referred to very small structures. However, a broadly accepted definition for nanotechnology is: Understanding, control and restructuring of matter on the order of nanometers (*i.e.* less than 100 nm) to create materials with fundamentally new properties and functions (Executive Office of the President of the United States, 2007).

Two main approaches are usually comprised within this discipline. First, the "top-down" approach consists in reducing larger structures (in size terms) to the nanoscale, while maintaining their original properties and without atomic-level modification. On the other hand, the "bottom-up" approach, also named "molecular nanotechnology" or "molecular manufacturing", as introduced by Drexler et al. (1993) consists in the engineering of materials from atoms or molecules through process of assembly or self-assembly. Up to date, most of the reported investigations in this field are encompassed in the "top-down" approach. However, both approaches account for a great potential in groundbreaking innovations in several fields. Because of this, in recent years, nanotechnology has become a very active and vital area of science and technological research, rapidly developing and spreading and touching across the

whole spectrum of science and engineering. Also, it is gaining interest in public as well as private sectors worldwide. Nanotechnology has contributed to remarkable advances in the field of science and technology in the past two decades, which have led to significant prospective applications in various technological domains; including advanced materials, biotechnology and pharmacy, electronics, scientific tools and techniques, and industrial manufacturing processes. In this direction, nanotechnology has been deemed as a key emerging technology for fulfilling the "Grand challenges of our time", in areas such as health care, energy production, environmental protection (climate change and remediation) and potable water procurement (Swedish EU Presidency, 2009).

1.2. Nanomaterials

1.2.1. Definition

Due to all these reasons, nanotechnology reunites fundamental scientific research with engineering, focusing on the molecular or atomic level, emphasizing the nano size range of the structures and the ability to work at that scale. Among all the possibilities provided by this discipline, the exploitation of properties and functions specific to the nano-scale, compared with previous macro- or micro-scale focus raised a special interest in the forest-based industry, as will be further discussed in the following pages. The emerging number of products ascribed to nanotechnology field has raised the need for an internationally agreed definition of one of its branches: Nanomaterials science. For this purpose, three independent European scientific committees provided aid to the European Comission (EC): the Scientific Committee for Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER), and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR). Of these three, the SCENIHR developed a scientific basis for the definition of nanomaterials by identifying particle characteristics relevant to safety assessment. Following this advice to a considerable extent, in October 2011, the European Comission recommended to define nanomaterials as: "A *natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm*."

(Potocnik, 2011). Alternatively, materials having a specific surface area by volume of the material greater than 60 m^2/cm^3 should be considered as falling into this definition as well. Importantly, the interest in nanomaterials lies in that at nanoscale, some physical and chemical material properties can differ significantly from those of the bulk structured materials of the same composition, allowing them several uses in many industrial, health care and energy sectors (Figure 1-1). For example, melting point of the materials can be as different as 1000ºC between one and the other, as the number of surface atoms or ions becomes a significant fraction of the total, and therefore surface energy plays a significant role in thermal stability. Now, being aware of this evidence, many material properties should be reconsidered, as this increase in surface-to-volume ratio associated to the reduction of the material to the nanoscale has a deep effect on the performance of the material.

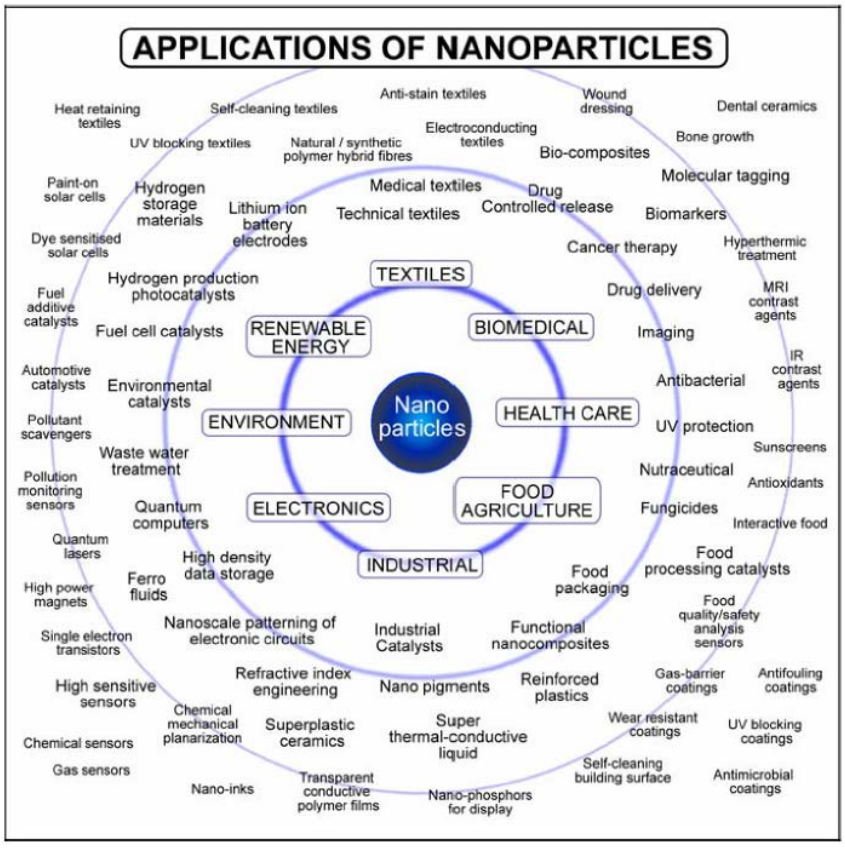

Figure 1-1: Major application fields of nanomaterials. From Tsuzuki, 2009

1.2.2. General classification

Nanomaterials can be classified using different criteria, such as their chemical composition, origin, shape, etc. Due to the innovative nature of nanomaterials science, no definitive classification is yet available considering all aspects of them. However, according to ISO standards TS27687:2008 and TS80004-4:2011, nanomaterials can be classified into two large groups (Figure 1-2). On one hand, Nano-Objects are materials with one, two, and/or three dimensions in the nano-scale, and named nanofiber, nanoplate or nanoparticle, respectively. On the other hand, Nano-structured materials are those having an internal or surface structure in the nano-scale. Among them we can find: nanocomposites, a solid comprising a mixture of two or more phase-separated materials, one or more being nanophase; nanostructured powders, which are powders comprising nanostructure agglomerates or aggregates and fluid nanodispersions, an heterogeneous material in which nano-objects or a nanophase are dispersed in a continuous fluid phase of a different composition.

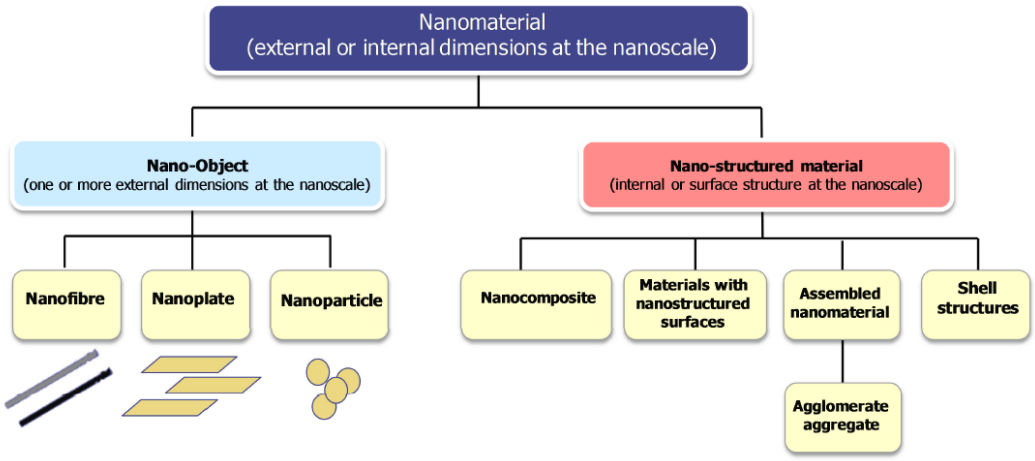

Figure 1-2: Classification of nanomaterials in accordance with ISO TS 27687:2008 and ISO TS 80004-4:2011. From NanosafePACK (Dobón-Lopez, 2013)

1.2.3. Types of nanomaterials according to chemical composition

Besides the classification provided by ISO standards, organizing nanomaterials according to their chemical nature is desirable for a better understanding of the possibilities they could offer. Authors such as Gleiter (2000) and Stone et al. (2010) addressed this issue by identifying common treats among nanometric particles and materials regarding their chemical origin or structure. Based on the work of these authors and particularly on a classification proposed by the latter, the Regulatory Cooperation Council of the USA-Canada nanotechnology initiative proposed in 2013 a general classification (Figure 1-3). This classification comprises 7 categories, including nanometarials composed by carbon (nanotubes or inorganic), metals (oxides or salts), semiconductors, organic nanomaterials and a last category including the ones not falling into any of the former. Falling into the "organics" category we could find nanocellulose, and more precisely, nanocrystalline cellulose.

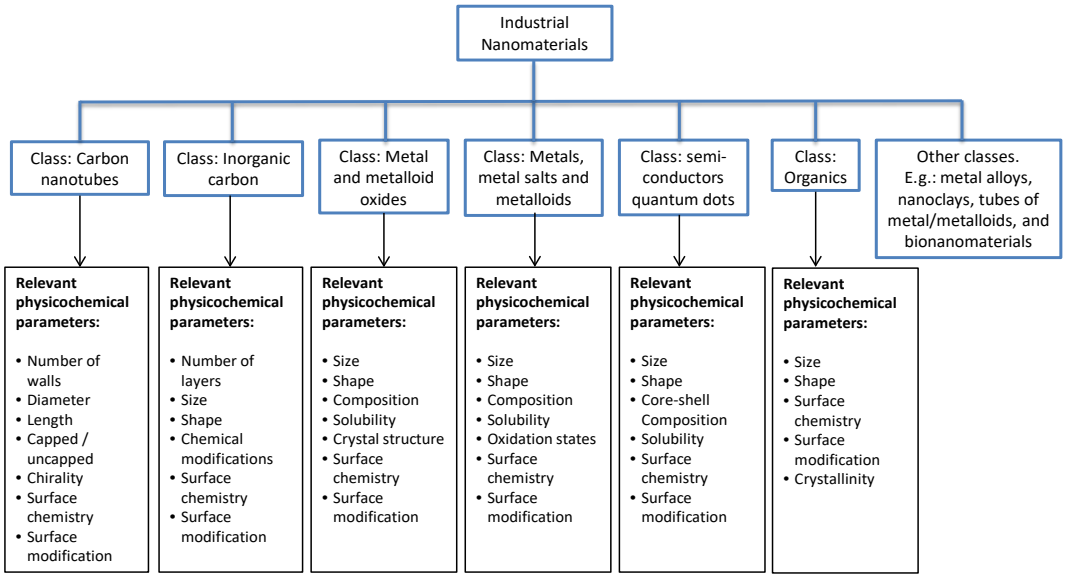

Figure 1-3: Classification of nanomaterials according to their chemical composition. From USA-Canada Regulatory Cooperation Council, 2013

1.3. Nanocellulose

Among all the possibilities nanomaterials have to offer, nanocelluloses, have attracted plenty of interest in recent years (Charreau et al., 2013). This is due to a number of desirable unique physical and chemical characteristics presented by them. Nanocelluloses are nanomaterials with at least one nanoscale dimension and composed by cellulose, the most abundant biopolymer on earth and the main component of plants biomass.

In 1838, the French chemist Anselme Payen described a resistant fibrous solid that remains behind after treatment of various plant tissues with acids and ammonia and after subsequent extraction with water, alcohol and ether (Payen, 1838). Using elemental analysis he determined the molecular formula of this compound to be $C_6H_{10}O_5$ and also observed isomerism with starch. The term "cellulose" for this plant constituent was first used in 1839 in a report of the French academy on the work of Payen (Brogniart et al., 1839). For millennia cellulose, literally "the sugar of the plant cell wall" has been used in the form of wood and plant fibers as an energy source, for building materials and for clothing (Klemm et al., 2005). Since the Egyptian papyri, cellulose products have played a central role in the transmission and recording of human culture. As a chemical raw material, cellulose has been used for about 150 years, during which the gain of knowledge on its structure and reactivity has permitted the step-by-step creation of novel types of materials. Among these we can highlight cellulose esters and cellulose ethers, as well as regenerates and the discovery of polymeric state of molecules (Klemm et al., 2005). Hyatt Manufacturing Company was the responsible, in 1870, of the process for celluloid manufacturing, the first thermoplastic polymeric material, designed from the discovery of the reaction between cellulose and nitric acid to form cellulose nitrate. From this point, chemical modification of cellulose on an industrial scale led to a broad range of products based on wood cellulose. The first example was fabrication of regenerated cellulose filaments by spinning a solution of cellulose in a copper hydroxide and aqueous ammonia, in which tetraamminecopper (II) hydroxide $[Cu(NH_3)_4](OH)_2$ is formed. After this development came a particularly important large-scale process for manufacturing rayon fiber and filament, the viscose process. New materials for coatings, films, membranes, building materials, drilling techniques, pharmaceuticals and foodstuffs

were obtained by large-scale production of other derivatives such as cellulose esters and ethers.

1.3.1. Cellulose structure

Cellulose annual production is estimated to be over 7.5 x 10^{10} tons in our planet (Habibi et al., 2010). It is widely distributed in higher plants, some marine animals (*e.g.* tunicates) and to a lesser degree in algae, fungi, bacteria, invertebrates and even amoeba. In general is a fibrous, tough, water-insoluble substance that plays an essential role in maintaining the structure of plant cell walls. Elucidation of its polymeric structure is responsibility of the pioneering work of Hermann Staudinger, who through acetylation and deacetylation of cellulose recognized that its structure was not merely an aggregation of D-glucose units but molecular chains of covalently linked units (Staudinger, 1920). This evidence, along with Staudinger's research with other molecules marked the discovey of polymeric state of molecules, and the origin of polymer science. In chemical terms, cellulose is a linear polysaccharide consisting of a chain of β-(1,4) linked D-glucopyranose units (Figure 1-4). Glucosydic bonds occur between equatorial OH group of C4 and C1 carbon atoms, through acetal functions. As a result, cellulose is an extense lineal-polymer with a large number of hydroxyl groups (three per anhydroglucose unit). In order to thermodynamically better accommodate the preferred bond angles of acetal oxygen bridges; every second glucose unit is rotated 180º in the plane. In this way, two adjacent subunits define cellobiose, considered the basic repeating unit of cellulose (Habibi et al., 2010). Each chain possesses a directional chemical asymmetry with respect to the end of its molecular axis. One end is a chemically reducing group (*i.e.* a hemiacetal unit), while the other has a pendant hydroxyl group. The degree of polymerization of this polysaccharide (DP), *i.e.* the number of subunits (D-glucose) composing it, varies largely among sources and treatment to which raw material was submitted. Typically, DP comprises values between 300 and 1700, even though higher numbers might be found in non-wood plants (Tanaka et al., 2014). Generally, a β (1-4) linked glucan with 20-30 repeating D-glucose units already offers all properties of cellulose (Kobayashi et al., 2001).

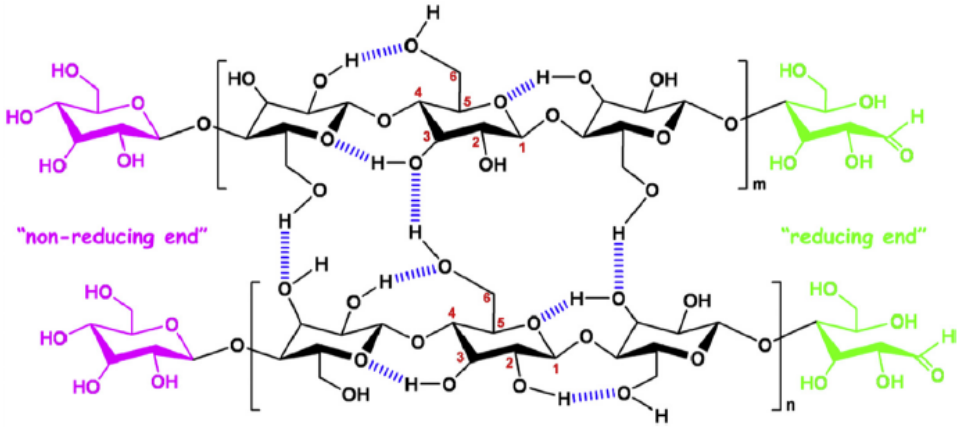

Figure 1-4: Schematic representation of cellulose chains and their intra- and intermolecular bonding structure. From Lin and Dufresne, 2014.

During its biosynthesis, cellulose chains are aggregated in microfibrils that can have dimensions in the range of 2 to 20 nm, depending on the cellulose source (Figure 1-5). Aggregation of these chains occur basically trough van der Waals forces and both intra- and inter-molecular hydrogen bonds (Figure 1-4). Intramolecular hydrogen bonds between an OH (3) and the O (5) ring oxygen and from OH (2) to the OH (6) (Figure 1-4) are responsible for molecules stability (Klemm et al., 2005). Supramolecular structure for cellulose is determined by intermolecular hydrogen bonds, which are so stable that make cellulose insoluble in water, even though it is hydrophilic (Dugan et al., 2013). This complex bonding network stablishes two main microstructures in cellulose microfribils. Amorphous or less ordered regions are consequence of tilts and twists in microfibrils, which hinder the correct packing of cellulose. These "defects" constitute regions were cellulose is not so well packed and are more accessible to chemicals and reactants (Lu and Hsieh, 2010). Between these amorphous zones we find other regions were cellulose is tightly packed, named crystalline regions. On them, intermolecular hydrogen bonds are correctly established and stabilize the structure (Figure 1-4). Hydrogen-bonding network and molecular orientation can vary widely, which gives rise to cellulose polymorphs or allomorphs, depending on the source, method of extraction or treatment (Atalla and VanderHart, 1984). Six interconvertible polymorphs of cellulose, namely I, II, III_I, III_{II}, IV_I, IV_{II} have

been identified (Atalla and VanderHart, 1984). Of these, cellulose I is the structure of native cellulose, which contains two cellulose chains in a parallel orientation with a twofold screw axis. Other forms may occur after chemical modifications of native cellulose. Also, two suballomorphs exist in cellulose I: Iα and Iβ, which have different hydrogen bonding patterns and are present in different proportions depending on the source. Therefore, crystalline structure of cellulose affects physical and mechanical properties of cellulose fibers (Gümüskaya et al., 2003). For example, increasing ratio of crystalline-to-amorphous regions ratio increases fibers stiffness, but decreases flexibility. Finally, crystalline regions are identified as less accessible for chemicals and other elements, such as enzymes (Ahola et al., 2008; Moon et al., 2011).

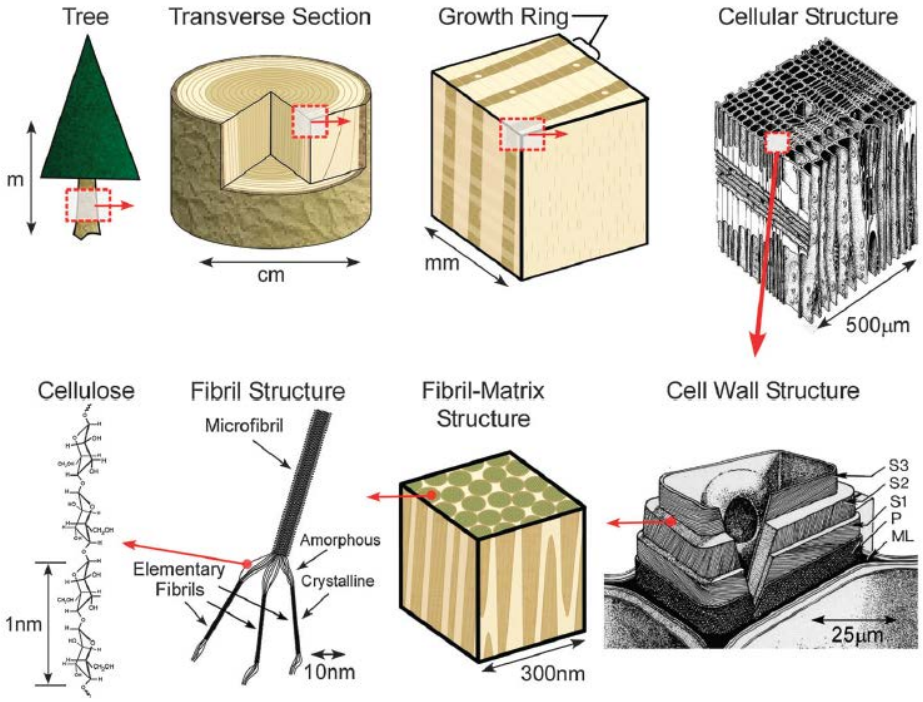

Figure 1-5: Ultrastructure of lignocellulosic fibers. From Postek et al., 2011

1.3.2. Cellulose sources

Plant fibers are by far the main cellulose source for industry nowadays. They are the product of aggregation of cellulose chains into larger structures and found on plant cell walls (Figure 1-5).

1.3.2.1. Wood fibers:

Wood fibers are the most extensively used nowadays by industry for pulp manufacturing. They are obtained from the trees trunks after debarking. From the paper industry perspective, wood fibers are classified into two large groups (García-Hortal, 2007):

- Softwood fibers: They are long and resistant and obtained from conifers. These kinds of trees have a large proportion of fibers in wood, as other elements are basically inexistent. Examples of softwoods would be trees such as pine, spruce, or firs.
- Hardwood fibers: Obtained from homonym plants, which in evolutive terms are newer organisms than conifers. Fibers are shorter than softwoods and are containers of other elements besides fibers which play important roles on plant vital functions, such as vessels. Examples of hardwoods would be trees such as beech, eucalyptus or birch.

1.3.2.2. Non-wood fibers:

Under the non-wood fiber denomination a heterogeneous range of plants with widely differing characteristics is included. The main sources of non-woody raw materials are agricultural residues from monocotyledons, including cereal straw and bagasse, or plants grown specifically for the fiber, such as bamboo, reeds, and some other high-fiber content plants such as flax, hemp, kenaf, jute, sisal, or abaca (Marques et al., 2010). Cellulose fibers obtained from non-wood plants (herbaceous or shrubby) account only for the 9% of the total amount of fibers used worldwide for papermaking (Leponiemi, 2008). However, in some countries with insufficient forest supplies and

non-wood plants abundance such as India and China, these fibers represent the main source for this application (López et al., 2004). Also, other causes such as a growing environmental pressure, restrictions on forest use, the increasing world demand for paper and increases in wood and recycled fibers cost are making manufacturers to have a renewed interest in non-woods (Kissinger et al., 2007).

Interest in non-woods is due to a range of advantages presented by these fibers. Firstly, their possibility of growing in annual cycles in which they develop full fiber potential (*i.e.* renewability) compared to the typical long periods of woody plants. Secondly, they present comparatively smaller amounts of lignin, allowing the use of milder chemical conditions for pulping or bleaching than wood sources (Paavilainen, 1998; Madakadze et al., 2010). Lastly, non-food uses of crops could provide farmers additional incomes derived from food harvests or cattle raising (Kissinger et al., 2007). However, the characteristic high silica content of non-wood fibers appears as a drawback conditioning their applicability (Andrade and Colodette, 2014). Other disadvantages of these fibers include their seasonal availability (rather than all year) and inconvenient handling due to their high volume and low density. In the developed world, non-wood fibers are typically used for the manufacture of specialty papers, which could not be easily obtained from wood fibers.

Among non-wood fibers, sisal and cotton linters attracted a special interest for the studies performed in this thesis, as will be discussed in following chapters.

- **Sisal** provides fibers that are isolated from the leaves of *Agave sisalana* and have traditionally been of the most used natural fibers for applications such as sacking, cordage, carpets backing or twines. Its annual worldwide production was around 241,000 tons in 2011 (FAO, 2012). Regarding its potential as a raw material for pulp and paper industry, sisal pulp presents some positive features including a high tear resistance, alpha cellulose content, porosity, bulk, absorbency and folding endurance, making it excellent for a variety of specialty papers (Aracri and Vidal, 2012).
- **Cotton linters** are an important by-product of the textile industry, being the short fiber that cannot be used in the textile process (Morais et al., 2013). They are obtained from cotton plant (*Gossypium sp.*), an annual shrub harvested for their high industrial interest. When the regular cotton fibers are extracted in the ginning process, the linters remain attached to the seed coat and an

additional process is required for their removal. The amount of linters produced worldwide is around 2.5 million metric tons, considering the 42 million metric tons of cotton produced in 2010 (Morais et al., 2013). Cotton linters consist on high-quality cellulose fibers presenting very high-cellulose content (98-99%) (Sczostak, 2009). They are typically used on special applications such as the production of cellulose derivatives, cellulose regeneration, or the manufacture of high-added value papers (Sczostak, 2009).

1.3.2.3. Other components present in cellulose fibers

a) Hemicelluloses

Hemicelluloses represent the second polysaccharide in plant biomass, in mass terms, after cellulose. Together with lignin they form an amorphous matrix in which cellulose microfibrils are immersed. Hemicelluloses differentiate from celluloses in that they are formed by 5-6 carbon unit monosaccharaides, are branched, are easier to dissolve and their structure is amorphous. Hemicelluloses are generally hydrophilic and play an important role in fibers water uptake during paper fabrication. Therefore, they participate in fiber interaction increasing their flexibility and binding capacity. Also, their degree of polymerization is low compared to that of cellulose, with values between 50 and 300 monosaccharide residues. Their chemical composition varies depending of the analyzed plant type. Among the different fiber sources, softwoods contain a higher value of hexosans (glucomanans, galactoglucomannans and arabinoglucoronoxylans) while hardwoods hemicelluloses are mainly composed by pentosans (glucoronoxylans), but glucomannans could be also present (Sjöström and Westermark, 1999). Regarding non-wood plants, composition of their hemicelluloses varies widely depending on the analyzed species. Plants such as kenaf or sisal have hemicelluloses mainly composed by xylose, while others such as flax or hemp have mannose and galactose as main sugars on their hemicelluloses (Marques et al., 2010).

a.1) Hexenuronic acids (HexA)

HexA are formed during alkaline cooking by a β-elimination of methylglucoronic acid, which is randomly distributed among side-chains of xylans, with an alkaline pH and at temperatures between 110 and 150ºC. HexA presence in pulps is deleterious due to some negative effects it has on further processes and final quality. They contribute to kappa number, as they are also oxidized by potassium permanganate during its determination. This fact difficulties the normal estimation of lignin content of pulps (Costa and Colodette, 2007). Also, they consume reactants during pulp bleaching, increasing process cost. Furthermore, a chelating action on metallic ions was observed, increasing pulp metal content. And above all, they reduce the material durability, as they play an important role in brightness reversion on bleached pulps (Cadena et al., 2010a).

b) Lignin

Lignin is produced by plant cells as they go through a maturing process and it is placed between fibrous walls and mainly, in intercellular regions (middle lamella), producing as a result an stiff and cohesive structure. Chemically, is an aromatic heteropolymer with an irregular 3D structure formed by different combinations of three phenylpropane units: syringyl, guaiacyl, and p-hydroxyphenyl. Lignin presents very high hydrophobicity level and because of this it hinders water uptake and fiber swelling when present. On the other hand, it presents some thermoplastic behavior, very important for mechanical pulping. Importantly, lignin is a chromophore being the main target of bleaching processes through its removal or modification.

1.3.3. Nanocellulose types and preparation procedures

Usually, the denomination “nanocellulose” englobes two different materials: Nanofibrillated cellulose (NFC) and Nanocrystalline cellulose (NCC) (Klemm et al., 2011). Table 1-1 summarizes the sources and basic preparation procedures for nanocelluloses and Figure 1-6 shows images of these two different materials.

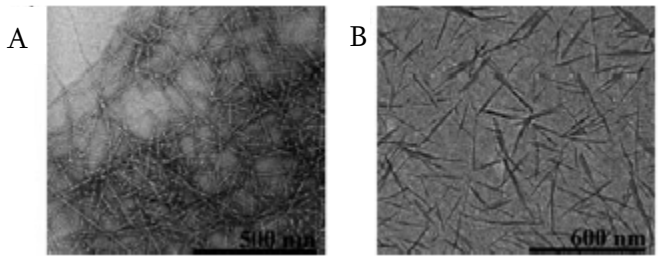

Figure 1-6: Transmission electron microscope images of A)NFC, B)NCC. From Klemm et al., 2011.

Table 1-1: The family of nanocellulose materials. Reproduced from Klemm et al., 2011

Type of nano-cellulose	Selected references and synonyms	Typical sources	Formation and average size
Nanofibrillated cellulose (NFC)	Microfibrillated cellulose, nanofibrils and microfibrils, nanofibrillated cellulose	Wood, sugar beet, potato tuber, hemp, flax	Delamination of wood pulp by mechanical pressure before and/or after chemical or enzymatic treatment Diameter: 5-60 nm Length: Several micrometers
Nanocrystalline cellulose (NCC)	Cellulose nanocrystals, crystallites, whiskers, rodlike cellulose microcrystals	Wood, cotton, hemp, flax, wheat straw, mulberry bark, ramie, Avicel, tunicin, cellulose from algae and bacteria	Acid hydrolysis of cellulose from many sources Diameter: 5-70 nm Length: 100-250nm (plant celluloses); 100 nm to several micrometers (cellulose of tunicates, algae, bacteria)

1.3.3.1. Nanofibrillated cellulose (NFC)

Nanofibrillated cellulose (NFC) was first produced by Turbak et al. at ITT Rayonnier, USA in the late 1970s and early 1980s (Turbak et al., 1983). This material is usually produced by the forcing of suspensions of cellulose fibers through mechanical devices, such as high-pressure homogenizers. This mechanical treatment delaminates the fibers and allows the extraction of microfibrils and microfibril aggregates which

form highly entangled networks (Figure 1-7A). The microfibrils have a high aspect ratio, of around 20 nm wide and up to micrometers long, and exhibit gel-like characteristics in water (Figure 1-7B), with pseudoplastic and thixotropic properties (Klemm et al., 2011). The major impediment for commercial success of NFC has been the very high energy consumption of the production process. This consumption has been estimated to be over 25000 kWh per ton as a result of the required multiple passes through homogenizers. Another problem commonly found during their manufacture is clogging of homogenizers. In the last years, enzymatic and chemical pretreatments have been proposed to reduce the energy input of the process (Henriksson et al., 2007; Pääkko et al., 2007). In reference to NFC uses and potential applications, this material has been mainly used as reinforcing phase in polymer nanocomposites, in the production of papers with increased strength and also in the food and cosmetic industries as thickener, flavor carrier and suspension stabilizer (Charreau et al., 2013).

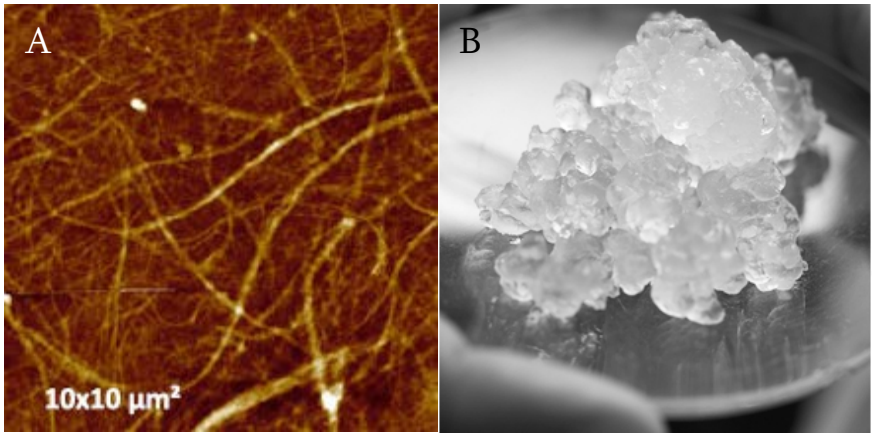

Figure 1-7: AFM image of nanofibrillar cellulose (NFC), from Ferrer et al., 2012) (A) and NFC hydrogel, from Inventia AB (Sweden) (B).

1.4. Nanocrystalline cellulose (NCC)

Exploring efficient ways to extract cellulose crystalline regions from fibers has attracted plenty of attention of authors during the last years, fact that can be observed in the growing number of patents related to this field published since year 2000 (Charreau et al., 2013).

Bengt G. Rånby is thought to be the first author to obtain colloidal suspensions of cellulose crystals by controlled sulfuric acid hydrolysis of cellulose fibers (Rånby, 1951). Previously, Nickerson and Habrle (1947) observed that the degradation induced by boiling cellulose fibers in acidic solution reached a limit after a certain time of treatment. Transmission electron microscopy (TEM) images of dried suspensions revealed for the first time the presence of aggregates of needle-shaped particles, while further analyses of these rods with electron diffraction demonstrated that they had the same crystalline structure as the original fibers (Mukherjee and Woods, 1953). Another milestone in the development of this material was performed by Marchessault et al. in 1959, who observed that colloidal suspensions of nanocrystalline cellulose showed birefringence beyond a critical concentration. Decades later Revol and co-workers demonstrated that this birefringence was produced due to a chiral nematic liquid-crystalline phase (Revol et al., 1992) formed by nanocrystalline cellulose generated by sulfuric acid hydrolysis. This discovery, together with observation that NCC could strongly improve the mechanical properties of nanocomposites and the general growing interest in using renewable resources for our everyday products (Charreau et al., 2013) produced a renaissance of interest among the scientific community towards this material up to our days (Klemm et al., 2011).

1.4.1. Special features and applications

Nanocrystalline cellulose (NCC), also referred as cellulose nanocrystals, microcrystals, whiskers, nanoparticles, microcrystallites, or nanofibers are rod-like cellulose crystals with widths among 5–70 nm and lengths between 100 nm and several micrometers (Klemm et al., 2011) (Figure 1-8). The interest in producing NCC lies on its physicochemical characteristics making it a very promising material for several applications. Firstly, the already explained chiral nematic liquid crystal behavior

confers it special optical properties. Other properties strongly depend on the used raw material used and the preparation procedure. However, generally it could be said that NCC has a high degree of crystal structure (more than 70%) (Fan and Li, 2012), a very high aspect ratio, *i.e.* length-to-diameter ratio (up to 300) (Tanaka et al., 2014), a large surface area (above 150 m^2/g), a very high elastic moduli, (estimated to be over 130-150 GPa) (Sakurada et al., 1962) and a low thermal expansion coefficient (6 ppm/K) (Hori and Wada, 2005).

Nanocrystalline cellulose finds applications in many sectors. One of the most studied applications of this material is its use as filler in nanocomposites, in order to improve their mechanical, thermal and barrier properties. There are several references addressing this topic in bibliography (Hamad, 2006; George et al., 2011; Moon et al., 2011; Brinchi et al., 2013; Mariano et al., 2014). Synthetic polymeric matrixes (such as polypropylene, PP or polyvinyl chloride, PVC) and natural ones (such as starch, or polylactic acid, PLA) have been used for templating with NCC in a vast number of examples existing in bibliography.

Regarding mechanical properties, NCC presence has been found to increase tensile strength, young modulus and elongation break of nanocomposites (Cao et al., 2008). Thermal properties of NCC containing nanocomposites have also been studied. Thermal degradation, or the reduction of mechanical properties at high temperatures are among the major problems limiting NCC application as fillers in nanocomposites (Moon et al., 2011). However, the improvement of glass–rubber transition temperatures (Tg), melting point (Tm), and thermal stability has been investigated by several authors through differential scanning calorimetry (DSC) (Brinchi et al., 2013). In some cases, thermal performances of nanocomposites showed improvements after addition of nanocellulose. Furthermore, barrier properties of materials are very important, for example, for meeting safety regulations in packaging products. Several works have been carried out investigating this property. Saxena and Ragauskas 2009, found that water permeability of xylan/NCC nanocomposites decreased considerably when adding and increasing NCC content on the composite. Morevocer, optical properties of nanocomposites (*e.g.* optical transmittance) are often properties aimed to be preserved, regardless of the gain in mechanical properties provided by the filler. Studies using NCC have demonstrated that the gain in mechanical properties by a nanocomposite did not necessarily imply a loss in light transmittance, due to the

unique optical properties shown by this material (Moon et al., 2011). Finally, the fact that NCC are usually electrically charged on surface allows their use in biomedical applications, due to the possibility of electrostatically absorb enzymes or proteins, or for drug delivery uses (Lin and Dufresne, 2014).

1.4.2. Cellulose sources and dimensions

Cellulose sources for NCC preparation may vary, and their degree of crystallinity strongly conditions the further dimensions of the released crystals. Sources such as wood, cotton or Avicel yield a narrow distribution of highly crystalline (90% crystallinity) nanorods with: 5–10 nm in width and 100–300 nm in length (Dong et al., 1996; Beck-Candanedo et al., 2005; Elazzouzi-Hafraoui et al., 2008). Other sources, such as tunicin (extracted from tunicates, sea animals producing cellulose), bacterial cellulose, and algae, generate NCC with larger polydispersities and dimensions (width 5–60 nm, length: 100 nm up to several micrometers) (Yoshiharu et al., 1997; Elazzouzi-Hafraoui et al., 2008; Hirai et al., 2009). NCC obtained from these sources appears to be similar to NFC in size terms (Turbak et al., 1983). However, in contraposition to NFC, they have very limited flexibility, as they do not contain amorphous regions but instead exhibit elongated crystalline rodlike shapes.

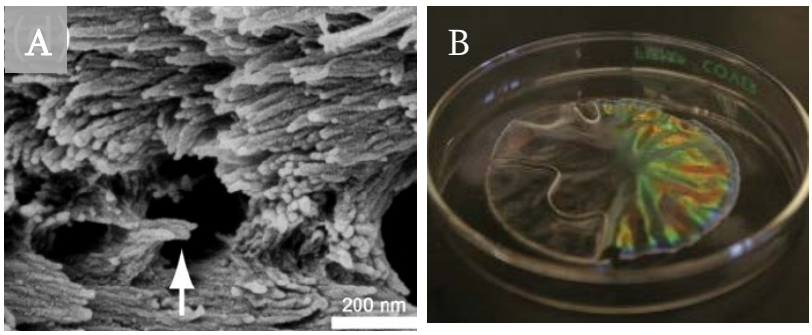

Figure 1-8: SEM micrograph of a NCC film showing chiral nematic organization, from Majoinen et al., 2012, (A). NCC film showing birefringence from Kelly et al., 2013 (B).

1.4.2.1. High-cellulose content fibers

As explained in previous sections, non-wood cellulose sources are a promising source for high-quality fibers with a broad range of environmental advantages. However, their usage as cellulose source for NCC requires the previous elimination of non-cellulosic components. Hemicelluloses are an undesirable impurity in pulps intended for production not only of nanocellulose but also of viscose rayon or other derivatives, as they interfere with chemicals during manufacturing processes, reducing their yield and also quality of final product.

Concerning NCC in particular, high-cellulose content fibers are the preferential source for its production due to a series of reasons. In the first place, other components of lignocellulosic biomass such as hemicelluloses or lignin are known to hinder the acid-cellulose interaction, modifying the kinetics of the acid hydrolysis reaction and thereafter reducing its efficiency (Yoon et al., 2014). Secondly, hemicelluloses or lignin presence in biomass would reduce the NCC yield of the reaction as they are non-cellulosic components. Lastly, hemicelluloses presence in NCC has been related to a decrease in their quality, for example, by increasing their thermal degradability (Jonoobi et al., 2015).

Traditionally, pulps with low hemicelluloses content have been obtained through acid sulphite or pre-hydrolysis Kraft processes (Li et al., 2015). On them, hemicelluloses that are present on fibers suffer a stronger attack than during alkaline processes such as Kraft or NaOH-AQ, reducing their presence on final product. Generally, pulps obtained through acid processes have some drawbacks related to quality of final product or the pollution they generate. Also, these they imply higher costs than alkaline ones in terms of chemical consumption, production rate, inventories and storage space (Barlow and Hillman, 2006). For these reasons, several methods have been studied in order to carry out the selective elimination of hemicelluloses from alkaline pulps (Jackson et al., 1998; Bajpai and Bajpai, 2001; Kopcke et al., 2008). These methods include nitren, cuen and alkaline extraction or enzymatic hydrolysis (Ibarra et al., 2010; Quintana et al., 2013). Also among these alternatives, the use of biotechnology by means of enzymatic hydrolysis of different components of lignocellulose has attracted special attention because of its potential as a "green" process by virtue of its high specificity and environmental friendliness.

1.4.3. NCC Preparation

The most extended method for preparation of nanocrystalline is controlled hydrolysis using mineral acids (Beck-Candanedo et al., 2005; Habibi, 2014). During this hydrolysis polysaccharides bound at the fibril surface are removed first and then more readily accessible amorphous regions are cleaved and destructed in order to liberate rod-like crystalline cellulose sections (Klemm et al., 2011). This differentiated susceptibility to acid attack is thought to be provoked by differences in the kinetics of hydrolysis between amorphous and crystalline domains, where the first ones are more rapidly accessible by acid and thereafter, hydrolyzed first (Habibi et al., 2010). When a desired depolymerization level is reached, hydrolysis reaction is usually quenched by diluting the acid concentration with distilled water and by reducing sample temperature (Klemm et al., 2011). After this, residual acid is eliminated washing samples by centrifugation. Hydrolysis is usually followed by a mechanical process, typically sonication, which disperses the nanocrystals until a uniform stable suspension is formed. Finally, samples are extensively dialyzed against distilled water for complete acid elimination.

Hydrolysis reaction strongly influences structure, properties and phase-separation behavior of nanocrystalline cellulose suspensions. The type of mineral acid used, its concentration; and hydrolysis time and temperature are key parameters determining final properties of NCC (Bondeson et al., 2006; Fan and Li, 2012; Kargarzadeh et al., 2012; Chen et al., 2015). NCC produced with acids such as phosphoric, hydrochloric (HCl) or hydrobromic (HBr) present a low colloidal stability (Araki et al., 2000; Habibi et al., 2010; Beck et al., 2015), whereas the use of sulfuric acid leads to well-stable suspensions produced by strong electrostatic repulsions between negatively charged NCC. This electrical charge, only present in NCC obtained with sulfuric acid is explained by a side reaction occurring during hydrolysis with this acid. In this reaction, sulfate (SO_4^{2-}) moieties are incorporated onto NCC surface throughout an esterification with free OH- groups of cellulose (Abitbol et al., 2013; Beck et al., 2015). Besides the improvement in suspension stability, sulfur content has been related to a higher NCC thermodegradability (Roman and Winter, 2004). Sulfate groups also influence, among other characteristics, the properties NCC could confer to composites if used as fillers. Recently, new NCC preparation methods have been

reported, such as a hydrolysis with phospotungstic acid followed by and extraction and purification (Liu et al., 2014), hydrolysis using a cationic-exchange resins (Tang et al., 2011) and biotechnology, obtaining NCC trough enzymatic hydrolysis (Anderson et al., 2014).

Generally NCC preparation procedures have been characterized by low yields. Studies such as those performed by Bondeson et al. (2006), Chen et al. (2015) or Fan and Li (2012) addressed this issue by studying different reaction conditions in order to increase yield. With this same objective and also with the aim of reducing the environmental impact of NCC manufacture it was considered that the use of biotechnology could be an effective tool to improve the efficiency of NCC isolation.

1.5. Biotechnology in the cellulose-related industry

Cellulose-related industry has traditionally been subjected to a steady and increasing social pressure globally due to environmental concerns and also to changes in market demands. All this has aroused the increasing interest in the use of biotechnology, emerging not only as a very promising approach to developing cleaner processes, but also to obtain novel high added-value products (Valls et al., 2010c; Fillat et al., 2012). Biotechnological methods have the potential to provide substantial improvements in traditional manufacturing processes thanks to their specificity and potential environmental advantages. Among the possibilities of biotechnology, enzymes have centered the focus of research. Enzymatic technology is related to its potential to perform reactions with a larger specificity, decreasing the need for environmentally negative processes, decreasing resources consumption and lately, reducing costs (Kenealy and Jeffries, 2003). Also, enzymes are biodegradable, derived from renewable resources and they can perform reactions in environmentally friendly solvents under mild temperature and pressure conditions (Tąta et al., 2015). Thus, reactions are typically simple to run in conventional manufacturing equipment and are cost-efficient (Tąta et al., 2015). Concerning pulp and paper industry, enzymatic processes have been introduced in most of unitary operations, while some of them have been satisfactorily transferred to industrial scale. Enzymes have been used for fibers bleaching, delignification, biorefining, pitch removal, deinking and surface

modifications (Jeffries, 2008). Two main enzymatic types are used as catalysts in the cellulose-related industry: Xylanases and cellulases.

1.5.1. Xylanases

Xylanases (E.C. 3.2.1.8) are hydrolytic enzymes catalyzing the hydrolysis of xylans, which are complex heteropolysaccharides containing β-1,4 bonds of highly substituted d-xylopyranose units. Due to the heterogeneity and complex chemical nature of plant xylan, its complete breakdown requires the action of several hydrolytic enzymes with diverse specificity and modes of action. The xylanolytic enzyme system degrading has β-1, 4 endoxylanases as the main actor, but other activities are also present for converting xylan into its constituent sugars (Beg et al., 2001) (Figure 1-9).

The major use xylanases have seen in cellulose-related industry was as pre-bleaching agents. In 1986 Viikari et al. observed that xylanases could aid bleaching process by providing savings in bleaching chemicals or raising pulp brightness. Xylanase effects on bleaching processes are explained by the removal of hemicelluloses in cellulose fibers, present between structured cellulose chains and amorphous lignin fraction. The removal of this hemicelluloses implies the elimination of the bonds uniting cellulose and lignin, which increases its susceptibility to the attack of bleaching reactants on further stages (Roncero et al., 2000; Valls and Roncero, 2009; Valls et al., 2014). Also, another usage of xylanases recently identified has been their ability to reduce the content of hexenuronic acids (HexA) in pulps (Aracri and Vidal, 2011; Valls et al., 2014). This effect is usually ascribed to the removal of HexA-containing hemicelluloses on fibers surface.

Xylanases have also been used in literature for the upgrading the quality of paper-grade fibers to high-cellulose content fibers through the removal of hemicelluloses. Usually, xylanases are applied before or after chemical extraction steps using NaOH, which could be used in combination with cellulases (Ibarra et al., 2009; Hakala et al., 2012). These high-cellulose content pulps have then several applications, such as a raw material for viscose process (Ibarra et al., 2009), or nanocellulose production (Fortunati et al., 2013), as previously explained.

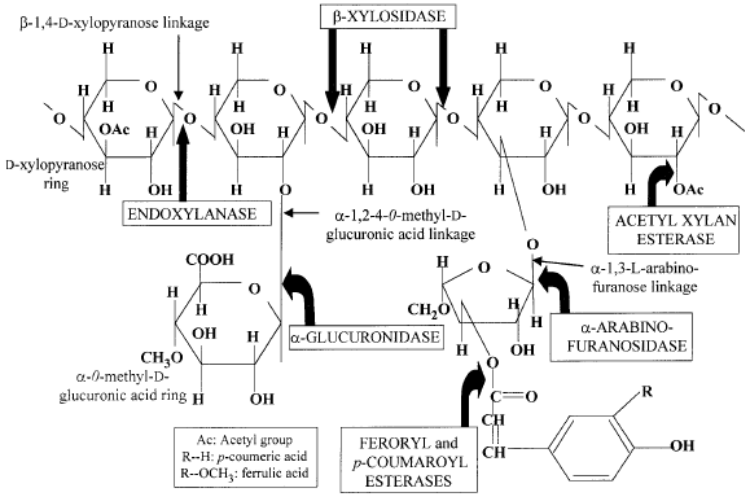

Figure 1-9: Plant xylan showing action sites of the different xylanase activities. From Beg et al. 2001.

1.5.2. Cellulases

Application of cellulases (E.C. 3.2.1.4) in cellulose-related industry is related to their capacity of degrading its chains up to glucose oligosaccharides. Microorganisms degrading cellulose usually have a complex enzymatic system generically named cellulase. Cellulolytic enzymes are conformed by a variety of activities synergistically acting for hydrolyzing this polymer. Three main activities act for this catalysis: endoglucanase, exoglucanases and β-glucosidases (Dashtban et al., 2010) (

Figure **1-10**). Endoglucanases, endo-acting cellulases, are thought to cleave internal β-1,4-glycosidic bonds randomly only, releasing oligosaccharides of different lengths, and appear to have cleft-shaped open active sites (Maki et al., 2009). Cellobiohydrolases (exoglucanases), being exo-acting cellulases, cleave β-1,4-glycosidic bonds from chain ends. Exoglucanases often have a tunnel-shaped closed active site which retains a single glucan chain and prevents it from re-adhering to the cellulose crystal (Maki et al., 2009). The products of exoglucanases and cellobiohydrolases, cellobiose and cellooligosaccharides, respectively, are inhibitory to their activity (Dashtban et al., 2010). Thus, efficient cellulose hydrolysis also requires the presence of

β-glucosidases to cleave the final glycosidic bonds producing glucose (Maki et al., 2009).

Due to the nature of their activity, cellulases find many applications in different industrial sectors, such as pulp and paper, textile, fuels, food, and others (Kuhad et al., 2011). Concerning their application on cellulose fibers, cellulases have been mainly used by authors for energy saving during fibers refining (*i.e.* biorefining) (Cadena et al., 2010b; Garcia-Ubasart et al., 2013), biomass saccharification for biofuels production (Zhang et al., 2012a; Pihlajaniemi et al., 2014) or for upgrading fibers quality through increasing cellulose reactivity (Kopcke et al., 2008; Miao et al., 2014; Quintana et al., 2015a, 2015b).

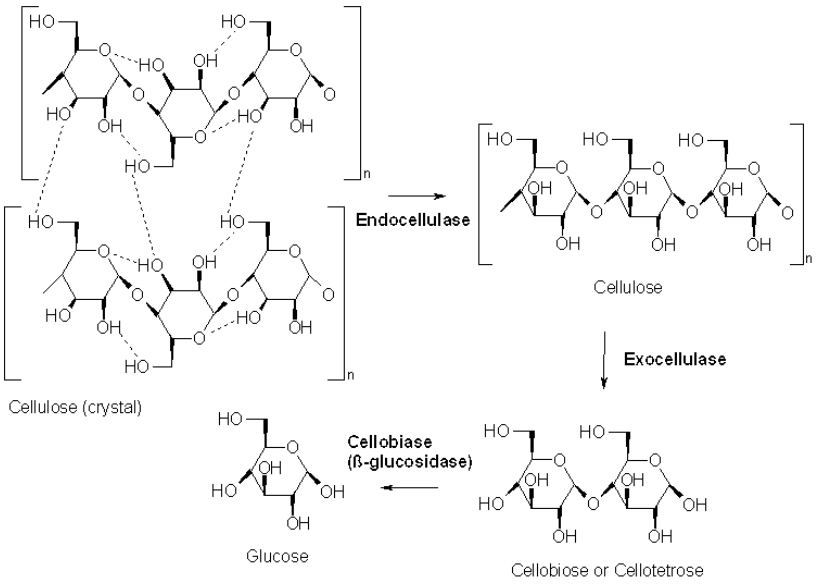

Figure 1-10: Schematic representation of the action of the three main cellulolytic activities. From Wikipedia commons.

1.6. Objectives

The process of innovation is defined by Oxford dictionary as "Make changes in something established, especially by introducing new methods, ideas, or products". In the business world, it is broadly accepted that innovation is crucial for the survival of organizations through time. Social concerns about the environment have considerably grown in the past years. Because of this, it is well known that cellulose-related industry has been submitted to a high social and legal pressure in order to increase the sustainability and efficiency of its processes. The research activity held by Celbiotech Paper Engineering research group at Universitat Politècnica de Catalunya deals with this issue using innovative procedures meanwhile it also seeks for new innovative products.

The present thesis studies an innovative material such as nanocrystalline cellulose (NCC), up to date typically obtained throughout chemical procedures, in combination of biotechnological methods. This was made in an attempt to provide a new manufacturing perspective for this innovative material. Figure 1-11 presents a general scheme of the different parts in which this work is divided. The first part involved the selection of the raw materials to use after their characterization. In the second part an enzymatic pretreatment was introduced prior to NCC isolation with sulfuric acid hydrolysis. Third and fourth parts involved the optimization of chemical and enzymatic conditions for hydrolysis and pretreatment, respectively. Fifth part involved the production of high-cellulose content fibers through enzymatic and chemical treatments. In the last part, NCC was obtained from these high-cellulose content fibers using optimal enzymatic and chemical conditions.

Therefore, the main objective of the present thesis was:

To develop a new nanocrystalline cellulose preparation process using biotechnology in combination with traditional chemical methods for maximizing its yield while reducing its environmental impact.

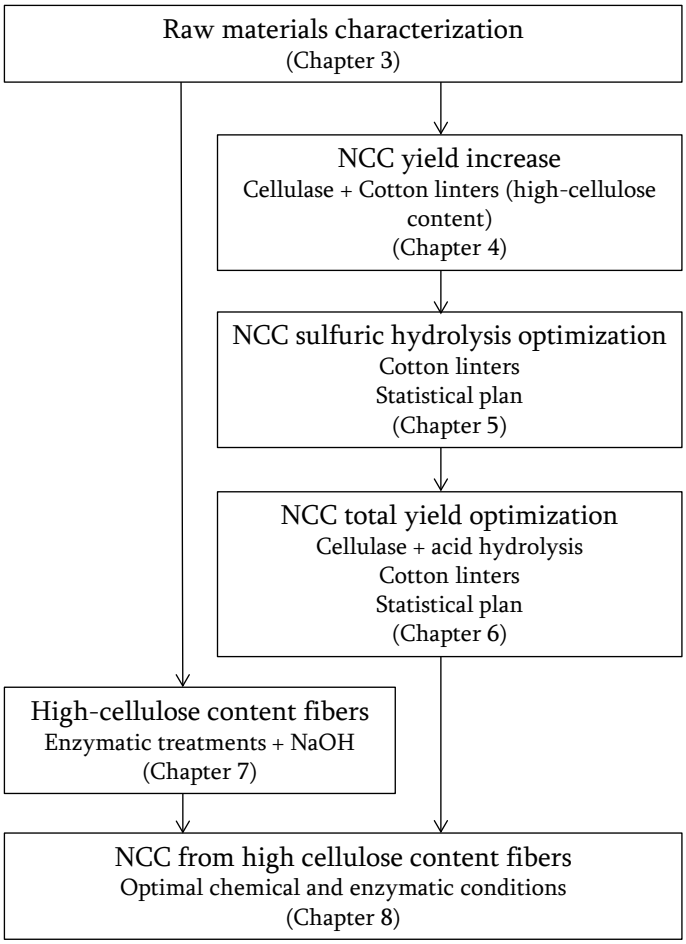

Figure 1-11: General overview of the present thesis

Specific objectives were:

- To study the effects of a cellulase pretreatment prior to hydrolysis using sulfuric acid on the process of NCC isolation and also on final product.
- To find the acid hydrolysis conditions maximizing yield and also cellulase effects, from enzymatically-pretreated fibers.

- To find the compromise solution between the loss of cellulose mass and the gain in yield due to cellulase action optimizing enzymatic pretreatment in order to maximize total yield.
- To assess the best combination of treatments with xylanase and cellulase for the removal of hemicelluloses from fibers.
- To obtain high-cellulose content fibers from a non-wood source such as sisal using enzymatic and chemical treatments.
- To obtain NCC from enzymatically obtained high-cellulose content fibers, making any raw material a suitable source for high-quality NCC.

The main novelties presented in this thesis were:

- The use of unconventional raw materials such as sisal or cotton linters to obtain high added-value cellulose products.
- The use of an enzyme (cellulase) as a pretreatment step in NCC isolation through sulfuric acid hydrolysis.
- The optimization of chemical conditions for NCC isolation from enzymatically pretreated fibers.
- The search for an enzymatic-assisted approach for NCC preparation with a novel two-step high-yield enzymatic and chemical method, combining advantages of both procedures.
- The application of enzymatic xylanolytic treatments in bleached fibers.
- The use of a single enzymatic preparation in combination with NaOH for the preparation of high-cellulose content fibers and the adjustment of their viscosity and reactivity.
- The possibility of using any non-wood source for high-quality NCC production through the enzymatic removal of hemicelluloses and enzymatic cellulose degradation prior to sulfuric acid hydrolysis.

1.7. Thesis format

The experimental results of this thesis are presented as chapters based on papers published as journal articles. These chapters are based on 6 publications, detailed as follows:

Publication 1:

Beltramino, F.; Valls, C.; Vidal, T.; Roncero, M.B.; "Exploring the effects of treatments with carbohydrases to obtain a high-cellulose-content pulp from a non-wood alkaline pulp". *Carbohydrate Polymers vol. 133, 302-312 (2015).*

Publication 2:

Beltramino, F.; Roncero, M. B.; Vidal, T.; Torres, A.L.; Valls, C.; "Increasing yield of nanocrystalline cellulose preparation process by a cellulase pretreatment". *Bioresource Technology vol. 192, 574-581 (2015).*

Publication 3:

Beltramino, F.; Roncero, M. B.; Torres, A.L.; Vidal, T.; Valls, C.; "Optimization of sulfuric acid hydrolysis conditions for preparation of nanocrystalline cellulose from enzymatically pretreated fibers". *Cellulose vol. 23 (3), 1777-1789 (2016).*

Publication 4:

"Facilitating the selection of raw materials: Evaluation of the effects of TCF and ECF bleaching sequences on different wood and non-wood pulps". Currently in writing process.

Publication 5:

"A novel enzymatic approach to nanocrystalline cellulose preparation". Currently in writing process.

Publication 6:

Currently in writing process.

1.8. References

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. Enzymatic Hydrolysis of Native Cellulose Nanofibrils and Other Cellulose Model Films: Effect of Surface Structure. Langmuir 2008, 24 (20), 11592–11599.

Anderson, S. R.; Esposito, D.; Gillette, W.; Zhu, J. Y.; Baxa, U.; McNeil, S. E. Enzymatic Preparation of Nanocrystalline and Microcrystalline Cellulose. Tappi J. 2014, 13 (5), 35–42.

Andrade, M. F.; Colodette, J. L. Dissolving Pulp Production from Sugar Cane Bagasse. Ind. Crops Prod. 2014, 52, 58–64.

Aracri, E.; Vidal, T. Xylanase- and Laccase-Aided Hexenuronic Acids and Lignin Removal from Specialty Sisal Fibres. Carbohydr. Polym. 2011, 83 (3), 1355–1362.

Aracri, E.; Vidal, T. Enhancing the Effectiveness of a Laccase-TEMPO Treatment Has a Biorefining Effect on Sisal Cellulose Fibres. Cellulose 2012, 19 (3), 867–877.

Araki, J.; Wada, M.; Kuga, S.; Okano, T. Birefringent Glassy Phase of a Cellulose Microcrystal Suspension. Langmuir 2000, 16 (6), 2413–2415.

Atalla, R. H.; VanderHart, D. L. Native Cellulose: A Composite of Two Distinct Crystalline Forms. Science (80). 1984, 223, 283–285.

Bajpai, P.; Bajpai, P. K. Development of a Process for the Production of Dissolving Kraft Pulp Using Xylanase Enzyme. Appita J. 2001, 54 (4), 381–384.

Barlow, F.; Hillman, D. Do Dissolving Pulps Really Dissolve? Pap. Asia 2006, 22 (7), 12–18.

Beck, S.; Me, M.; Bouchard, J. General Procedure for Determining Cellulose Nanocrystal Sulfate Half-Ester Content by Conductometric Titration. 2015, 101–116.

Beck-Candanedo, S.; Roman, M.; Gray, D. G. Effect of Reaction Conditions on the Properties and Behavior of Wood Cellulose Nanocrystal Suspensions. Biomacromolecules 2005, 6 (2), 1048–1054.

Beg, Q. K.; Kapoor, M.; Mahajan, L.; Hoondal, G. S. Microbial Xylanases and Their Industrial Applications: A Review. Appl. Microbiol. Biotechnol. 2001, 56 (3-4), 326–338.

Bondeson, D.; Mathew, A.; Oksman, K. Optimization of the Isolation of Nanocrystals from Microcrystalline Cellulose by Acid Hydrolysis. Cellulose 2006, 13 (2), 171–180.

Brinchi, L.; Cotana, F.; Fortunati, E.; Kenny, J. M. Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications. Carbohydr. Polym. 2013, 94 (1), 154–169.

Brogniart, A.; Pelonze, A. B.; Dumas, R. Rapport Sur Un Mémoire de M. Payen, Relatif À La Composition de La Matière Ligneuse. Comptes Rendus 1839, 8, 51–53.

Cadena, E. M.; Vidal, T.; Torres, A. L. Influence of the Hexenuronic Acid Content on Refining and Ageing in Eucalyptus TCF Pulp. Bioresour. Technol. 2010a, 101 (10), 3554–3560.

Cadena, E. M.; Chriac, a I.; Pastor, F. I. J.; Diaz, P.; Vidal, T.; Torres, A. L. Use of Cellulases and Recombinant Cellulose Binding Domains for Refining TCF Kraft Pulp. Biotechnol. Prog. 2010b, 26 (4), 960–967.

Cao, X.; Chen, Y.; Chang, P. R.; Muir, a. D.; Falk, G. Starch-Based Nanocomposites Reinforced with Flax Cellulose Nanocrystals. Express Polym. Lett. 2008, 2 (7), 502–510.

Charreau, H.; Foresti, M. L.; Vázquez, A.; Vazquez, A.; Vázquez, A. Nanocellulose Patents Trends: A Comprehensive Review on Patents on Cellulose Nanocrystals, Microfibrillated and Bacterial Cellulose. Recent Pat. Nanotechnol. 2013, 7 (1), 56–80.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Costa, M. M.; Colodette, J. L. The Impact of Kappa Number Composition on Eucalyptus Kraft Pulp Bleachability. Brazilian J. Chem. Eng. 2007, 24 (1), 61–71.

Regulatory Cooperation Council: USA-Canada Nanotechnology Initiative. Work Element 2, Priority Setting : Development of a Joint Nanomaterials Classification Scheme; 2013.

Dashtban, M.; Maki, M.; Leung, K. T.; Mao, C.; Qin, W. Cellulase Activities in Biomass Conversion: Measurement Methods and Comparison. Crit. Rev. Biotechnol. 2010, 30 (4), 302–309.

Dobón-Lopez, A. NanosafePACK. Development of a Best Practices Guide for the Safe Handling and Use of Nanoparticles in Packaging Industries D4 . 1 Analysis of the Consideration of Physic-Chemical and Toxicological Endpoints in LCA Studies; 2013.

Dong, X. M.; Kimura, T.; Gray, D. G. Effects of Ionic Strength on the Isotropic - Chiral Nematic Phase Transition of Suspensions of Cellulose Crystallites. Langmuir 1996, 12 (8), 2076–2082.

Drexler, E.; Peterson, C.; Pergamit, G.; Brand, S. Unbounding the Future: The Nanotechnology Revolution; 1993; Vol. 15.

Dugan, J. M.; Gough, J. E.; Eichhorn, S. J. Bacterial Cellulose Scaffolds and Cellulose Nanowhiskers for Tissue Engineering. Nanomedicine 2013, 8 (2), 287–298.

Elazzouzi-Hafraoui, S.; Nishiyama, Y.; Putaux, J. L.; Heux, L.; Dubreuil, F.; Rochas, C. The Shape and Size Distribution of Crystalline Nanoparticles Prepared by Acid Hydrolysis of Native Cellulose. Biomacromolecules 2008, 9 (1), 57–65.

Executive Office of the President of the United States. NSTC, The National Nanotechnology Initiative – Strategic Plan, December 2007.

Fan, J. S.; Li, Y. H. Maximizing the Yield of Nanocrystalline Cellulose from Cotton Pulp Fiber. Carbohydr. Polym. 2012, 88 (4), 1184–1188.

FAO. FAOstat Reports: Jute, Kenaf, Sisal, Abaca, Coir and Allied Fibers.; 2012.

Ferrer, A.; Quintana, E.; Filpponen, I.; Solala, I.; Vidal, T.; Rodríguez, A.; Laine, J.; Rojas, O. J. Effect of Residual Lignin and Heteropolysaccharides in Nanofibrillar Cellulose and Nanopaper from Wood Fibers. Cellulose 2012, 19 (6), 2179–2193.

Feynman, R. P. There's Plenty of Room at the Bottom. Eng. Sci. 1960, 23 (5), 22–36.

Fillat, A.; Roncero, M. B.; Vidal, T. Elucidating the Effects of Laccase-Modifying Compounds Treatments on Bast and Core Fibers in Flax Pulp. Biotechnol. Bioeng. 2012, 109 (1), 225–233.

Fortunati, E.; Puglia, D.; Monti, M.; Peponi, L.; Santulli, C.; Kenny, J. M.; Torre, L. Extraction of Cellulose Nanocrystals from Phormium Tenax Fibres. J. Polym. Environ. 2013, 21, 319–328.

García-Hortal, J. A. Fibras Papeleras; UPC, E., Ed.; 2007.

Garcia-Ubasart, J.; Torres, A. L.; Vila, C.; Pastor, F. I. J.; Vidal, T. Biomodification of Cellulose Flax Fibers by a New Cellulase. Ind. Crops Prod. 2013, 44, 71–76.

George, J.; Ramana, K. V.; Bawa, a. S.; Siddaramaiah. Bacterial Cellulose Nanocrystals Exhibiting High Thermal Stability and Their Polymer Nanocomposites. Int. J. Biol. Macromol. 2011, 48 (1), 50–57.

Gleiter, H. Nanostructured Materials: Basic Concepts and Microstructure. Acta Mater. 2000, 48 (1), 1–29.

Gümüskaya, E.; Usta, M.; Kirci, H. The Effects of Various Pulping Conditions on Crystalline Structure of Cellulose in Cotton Linters. Polym. Degrad. Stab. 2003, 81 (3), 559–564.

Habibi, Y. Key Advances in the Chemical Modification of Nanocelluloses. Chem. Soc. Rev. 2014, 43 (5), 1519–1542.

Habibi, Y.; Lucia, L. A.; Rojas, O. J. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Hakala, T. K.; Liitiä, T.; Suurnäkki, A. Enzyme-Aided Alkaline Extraction of Oligosaccharides and Polymeric Xylan from Hardwood Kraft Pulp. Carbohydr. Polym. 2012, 93 (1), 102–108.

Hamad, W. On the Development and Applications of Cellulosic Nanofibrillar and Nanocrystalline Materials. Can. J. Chem. Eng. 2006, 84 (5), 513–519.

Henriksson, M.; Henriksson, G.; Berglund, L. A.; Lindström, T. An Environmentally Friendly Method for Enzyme-Assisted Preparation of Microfibrillated Cellulose (MFC) Nanofibers. Eur. Polym. J. 2007, 43 (8), 3434–3441.

Hirai, A.; Inui, O.; Horii, F.; Tsuji, M. Phase Separation Behavior in Aqueous Suspensions of Bacterial Cellulose Nanocrystals Prepared by Sulfuric Acid Treatment. Langmuir 2009, 25 (1), 497–502.

Hori, R.; Wada, M. The Thermal Expansion of Wood Cellulose Crystals. Cellulose 2005, 12 (5), 479–484.

Ibarra, D.; Kopcke, V.; Ek, M. Exploring Enzymatic Treatments for the Production of Dissolving Grade Pulp from Different Wood and Non-Wood Paper Grade Pulps. Holzforschung 2009, 63 (6), 721–730.

Ibarra, D.; Kopcke, V.; Larsson, P. T.; Jaaskelainen, A.-S.; Ek, M. Combination of Alkaline and Enzymatic Treatments as a Process for Upgrading Sisal Paper-Grade Pulp to Dissolving-Grade Pulp. Bioresour. Technol. 2010, 101 (19), 7416–7423.

Jackson, L. S.; Heitmann Jr., J. A.; Joyce, T. W. Production of Dissolving Pulp from Recovered Paper Using Enzymes. Tappi J. 1998, 81 (3), 171–178.

Jeffries, T. W. Introduction of a Special Issue on Biotechnology for the Pulp and Paper Industry. Enzyme Microb. Technol. 2008, 43 (2), 77-77.

Jonoobi, M.; Oladi, R.; Davoudpour, Y.; Oksman, K.; Dufresne, A.; Hamzeh, Y.; Davoodi, R. Different Preparation Methods and Properties of Nanostructured Cellulose from Various Natural Resources and Residues: A Review. Cellulose 2015, No. 22, 935–969.

Kargarzadeh, H.; Ahmad, I.; Abdullah, I.; Dufresne, A.; Zainudin, S. Y.; Sheltami, R. M. Effects of Hydrolysis Conditions on the Morphology, Crystallinity, and Thermal Stability of Cellulose Nanocrystals Extracted from Kenaf Bast Fibers. Cellulose 2012, 19 (3), 855–866.

Kelly, J. a.; Yu, M.; Hamad, W. Y.; Maclachlan, M. J. Large, Crack-Free Freestanding Films with Chiral Nematic Structures. Adv. Opt. Mater. 2013, 1 (4), 295–299.

Kenealy, W. R.; Jeffries, T. W. Enzyme Processes for Pulp and Paper : A Review of Recent Developments. Society 2003, 845, 210–241.

Kissinger, M.; Fix, J.; Rees, W. E. Wood and Non-Wood Pulp Production: Comparative Ecological Footprinting on the Canadian Prairies. Ecol. Econ. 2007, 62 (3-4), 552–558.

Klemm, D.; Heublein, B.; Fink, H. P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie - Int. Ed. 2005, 44 (22), 3358–3393.

Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. Angew. Chemie - Int. Ed. 2011, 50 (24), 5438–5466.

Kobayashi, S.; Sakamoto, J.; Kimura, S. In Vitro Synthesis of Cellulose and Related Polysaccharides. Prog. Polym. Sci. 2001, 26 (9), 1525–1560.

Kopcke, V.; Ibarra, D.; Ek, M. Increasing Accessibility and Reactivity of Paper Grade Pulp by Enzymatic Treatment for Use as Dissolving Pulp. Nord. Pulp Pap. Res. J. 2008, 23 (4), 363–368.

Kuhad, R. C.; Gupta, R.; Singh, A. Microbial Cellulases and Their Industrial Applications. Enzyme Res. 2011, 2011, 1-10.

Leponiemi, A. Non-Wood Pulping Possibilities - A Challenge for the Chemical Pulping Industry. Appita J. 2008, 61 (3), 234–243.

Li, J.; Zhang, H.; Duan, C.; Liu, Y.; Ni, Y. Enhancing Hemicelluloses Removal from a Softwood Sulfite Pulp. Bioresour. Technol. 2015, 192, 11–16.

Lin, N.; Dufresne, A. Nanocellulose in Biomedicine: Current Status and Future Prospect. Eur. Polym. J. 2014, 59, 302–325.

Liu, Y.; Wang, H.; Yu, G.; Yu, Q.; Li, B.; Mu, X. A Novel Approach for the Preparation of Nanocrystalline Cellulose by Using Phosphotungstic Acid. Carbohydr. Polym. 2014, 110, 415–422.

López, F.; Alaejos, J.; Díaz, M. J.; García, M. M.; Alfaro, A. Vegetales No Madereros Para La Fabricación de Pasta Celulósica. Revisión de Su Utilización Actual. Ing. Química 2004, 36, 141–144.

Lu, P.; Hsieh, Y.-L. Preparation and Properties of Cellulose Nanocrystals: Rods, Spheres, and Network. Carbohydr. Polym. 2010, 82 (2), 329–336.

Madakadze, I. C.; Masamvu, T. M.; Radiotis, T.; Li, J.; Smith, D. L. Evaluation of Pulp and Paper Making Characteristics of Elephant Grass (Pennisetum Purpureum Schum) and Switchgrass (Panicum Virgatum L.). African J. Environ. Sci. Technol. 2010, 4 (July), 465–470.

Majoinen, J.; Kontturi, E.; Ikkala, O.; Gray, D. G. SEM Imaging of Chiral Nematic Films Cast from Cellulose Nanocrystal Suspensions. Cellulose 2012, 19 (5), 1599–1605.

Maki, M.; Leung, K. T.; Qin, W. The Prospects of Cellulase-Producing Bacteria for the Bioconversion of Lignocellulosic Biomass. Int. J. Biol. Sci. 2009, 5 (5), 500–516.

Marchessault, R.; Morehead, F.; Walter, N. Liquid Crystal Systems from Fibrillar Polysaccharides. Nature 1959, 184, 632–633.

Mariano, M.; El Kissi, N.; Dufresne, A. Cellulose Nanocrystals and Related Nanocomposites: Review of Some Properties and Challenges. J. Polym. Sci. Part B Polym. Phys. 2014, 52 (12), 791–806.

Marques, G.; Rencoret, J.; Gutierrez, A.; del Jose C., R. Evaluation of the Chemical Composition of Different Non-Woody Plant Fibers Used for Pulp and Paper Manufacturing. Open Agric. J. 2010, 4, 93–101.

Miao, Q.; Chen, L.; Huang, L.; Tian, C.; Zheng, L.; Ni, Y. A Process for Enhancing the Accessibility and Reactivity of Hardwood Kraft-Based Dissolving Pulp for Viscose Rayon Production by Cellulase Treatment. Bioresour. Technol. 2014, 154, 109–113.

Moon, R. J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose Nanomaterials Review: Structure, Properties and Nanocomposites. Chem. Soc. Rev. 2011, 40 (7), 3941–3994.

Morais, J. P. S.; Rosa, M. D. F.; de Souza Filho, M. D. S. M.; Nascimento, L. D.; do Nascimento, D. M.; Cassales, A. R. Extraction and Characterization of Nanocellulose Structures from Raw Cotton Linter. Carbohydr. Polym. 2013, 91 (1), 229–235.

Mukherjee, S. M.; Woods, H. J. X-Ray and Electron Microscope Studies of the Degradation of Cellulose by Sulphuric Acid. Biochim. Biophys. Acta 1953, 10, 499–511.

Nickerson, R.; Habrle, J. Cellulose Intercrystalline Structure. Ind. Eng. Chem. 1947, 1507–1512.

Pääkko, M.; Ankerfors, M.; Kosonen, H.; Nykänen, a.; Ahola, S.; Österberg, M.; Ruokolainen, J.; Laine, J.; Larsson, P. T.; Ikkala, O.; et al. Enzymatic Hydrolysis Combined with Mechanical Shearing and High-Pressure Homogenization for Nanoscale Cellulose Fibrils and Strong Gels. Biomacromolecules 2007, 8 (6), 1934–1941.

Paavilainen, L. European Prospects for Using Nonwood Fibers. Pulp Pap. Int. 1998, No. 40, 61–66.

Payen, A. Mémoire Sur La Composition Du Tissu Propre Des Plantes et Du Ligneux. Comptes Rendus 1838, 7, 1052–1056.

Pihlajaniemi, V.; Sipponen, S.; Sipponen, M. H.; Pastinen, O.; Laakso, S. Enzymatic Saccharification of Pretreated Wheat Straw: Comparison of Solids-Recycling, Sequential Hydrolysis and Batch Hydrolysis. Bioresour. Technol. 2014, 153, 15–22.

Postek, M. T.; Vladár, A.; Dagata, J.; Farkas, N.; Ming, B.; Wagner, R.; Raman, A.; Moon, R. J.; Sabo, R.; Wegner, T. H.; et al. Development of the Metrology and Imaging of Cellulose Nanocrystals. Meas. Sci. Technol. 2011, 22 (2), 1-10.

Potocnik, J. Commission Recommendation of October 2011 on the Definition of Nanomaterial. Of. J. Eur. Union 2011, L275, 38–40.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. An Enzyme-Catalysed Bleaching Treatment to Meet Dissolving Pulp Characteristics for Cellulose Derivatives Applications. Bioresour. Technol. 2013, 148, 1–8.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part I: On a Fully Bleached, Commercial Acid Sulfite Dissolving Pulp. Cellulose 2015a, 2067–2079.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part II: On a Biobleached Acid Sulphite Pulp. Cellulose 2015b, 22 (3), 2081–2093.

Rånby, B. G. III. Fibrous Macromolecular Systems. Cellulose and Muscle. The Colloidal Properties of Cellulose Micelles. Discuss. Faraday Soc. 1951, 11, 158–164.

Revol, J. F.; Bradford, H.; Giasson, J.; Marchessault, R. H.; Gray, D. G. Helicoidal Self-Ordering of Cellulose Microfibrils in Aqueous Suspension. Int. J. Biol. Macromol. 1992, 14 (3), 170–172.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Roncero, M. B.; Torres, A. L.; Colom, J. F.; Vidal, T. Effects of Xylanase Treatment on Fibre Morphology in Totally Chlorine Free Bleaching (TCF) of Eucalyptus Pulp. Process Biochem. 2000, 36 (1-2), 45–50.

Sakurada, I.; Nubushina, Y.; Nukushina, Y.; Ito, T. Experimental Determination of the Elastic Modulus of Crystalline Regions in Oriented Polymers. J. Polym. Sci. 1962, 57, 651–660.

Saxena, A.; Ragauskas, A. J. Water Transmission Barrier Properties of Biodegradable Films Based on Cellulosic Whiskers and Xylan. Carbohydr. Polym. 2009, 78 (2), 357–360.

Sczostak, A. Cotton Linters: An Alternative Cellulosic Raw Material. Macromol. Symp. 2009, 280 (1), 45–53.

Sjöström, E.; Westermark, U. Chemical Composition of Wood and Pulps: Basic Constituents and Their Distribution. In Analytical Methods in Wood Chemistry, Pulping, and Papermaking; Springer Berlin Heidelberg, 1999; 68–82.

Staudinger, H. Über Polymerisation. Berichte der Dtsch. Chem. Gesellschaft 1920, 53 (6), 1073–1085.

Stone, V.; Nowack, B.; Baun, A.; van den Brink, N.; von der Kammer, F.; Dusinska, M.; Handy, R.; Hankin, S.; Hassellov, M.; Joner, E.; et al. Nanomaterials for Environmental Studies: Classification, Reference Material Issues, and Strategies for Physico-Chemical Characterisation. Sci. Total Environ. 2010, 408 (7), 1745–1754.

Swedish EU Presidency. Lund Declaration, "Europe Must Focus on the Grand Challenges of Our Time"; 2009.

Tanaka, R.; Saito, T.; Ishii, D.; Isogai, A. Determination of Nanocellulose Fibril Length by Shear Viscosity Measurement. Cellulose 2014, 21, 1581–1589.

Tang, L. R.; Huang, B.; Ou, W.; Chen, X. R.; Chen, Y. D. Manufacture of Cellulose Nanocrystals by Cation Exchange Resin-Catalyzed Hydrolysis of Cellulose. Bioresour. Technol. 2011, 102 (23), 10973–10977.

Tąta, A.; Sokołowska, K.; Świder, J.; Konieczna-Molenda, A.; Proniewicz, E.; Witek, E. Study of Cellulolytic Enzyme Immobilization on Copolymers of N-Vinylformamide. Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 2015, 149, 494–504.

Tsuzuki, T. Commercial Scale Production of Inorganic Nanoparticles. Int. J. Nanotechnol. 2009, 6 (5), 567-578.

Turbak, A. F.; Snyder, F. W.; Sandberg, K. R. Microfibrillated Cellulose, a New Cellulose Product: Properties, Uses, and Commercial Potential. J. Appl. Polym. Sci. Symp. 1983, 37, 815–837.

Valls, C.; Roncero, M. B. Using Both Xylanase and Laccase Enzymes for Pulp Bleaching. Bioresour. Technol. 2009, 100 (6), 2032–2039.

Valls, C.; Gallardo, Ó.; Vidal, T.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B. Performance of New and Commercial Xylanases for ECF and TCF Bleaching of Eucalyptus Kraft Pulp. Wood Sci. Technol. 2010, 45 (3), 433–448.

Valls, C.; Vidal, T.; Roncero, M. B. Enzymatic Strategies to Improve Removal of Hexenuronic Acids and Lignin from Cellulosic Fibers. Holzforschung 2014, 68 (2), 229–237.

Viikari, L.; Ranua, M.; Kantelinen, A.; Sundquist, J.; Linko, M. Bleaching with Enzymes. In 3rd International conference on Biotechnology in the pulp and paper industry. A challenge for change. Proceedings; 1986; 67–69.

Yoon, S. Y.; Han, S. H.; Shin, S. J. The Effect of Hemicelluloses and Lignin on Acid Hydrolysis of Cellulose. Energy 2014, 77, 1–6.

Yoshiharu, N.; Shigenori, K.; Masahisa, W.; Takeshi, O. Cellulose Microcrystal Film of High Uniaxial Orientation. Macromolecules 1997, 30 (20), 6395–6397.

Zhang, J.; Tang, M.; Viikari, L. Xylans Inhibit Enzymatic Hydrolysis of Lignocellulosic Materials by Cellulases. Bioresour. Technol. 2012, 121, 8–12.

Chapter 2

Materials and methods

2.1. Raw material

In the present PhD thesis, several raw materials were analyzed; being them in all cases bleached cellulose fibers from different plant sources. Bleached fibers were used with the aim of evaluating the possibility of obtaining NCC from different sources. In order to find the best option fitting each of the following studies, a total of eight different raw materials were employed along the different sections:

- **TCF** and **ECF** bleached fibers from **Eucalyptus**, provided by Ence (Spain).
- **TCF** and **ECF** bleached fibers from **Flax**, provided by Celesa (Spain).
- **TCF** and **ECF** bleached fibers from **Sisal**, provided by Celesa (Spain). Used in chapters 7 and 8 for the preparation of high-cellulose content fibers and NCC from them, respectively.
- Bleached **Cotton linters**, provided by Celsur (Spain). Used in chapters 4, 5 and 6 for the studies concerning NCC preparation.
- **Dissolving-grade** fibers from Norway spruce and Scots pine, provided by Domsjö Fabriker (Sweden). Used in chapter 8 for NCC preparation.

A comprehensive study of chemical and morphological characteristics of fibers from Eucalyptus, Flax, Sisal and cotton liters is exposed in Chapter 3.

2.1.1. Cotton linters pre-beating

In order to reduce the average length of cotton linters and to allow their proper manipulation, fibers were pre-beated in a valley mill during 90 minutes using a 2200 g weight. This operation provided the initial cotton linters used in all indicated chapters.

2.2. Enzymatic treatments

2.2.1. Enzymes

Three enzymes were used in the present thesis: a **xylanase** (named "**X**"), a **cellulase** ("**Cx**" or "**C**") and another cellulase named "Celluclast". The first two enzymes were provided by Fungal Bioproducts (Spain) and obtained from *Cerrena sp* fungus, whilst "Celluclast" enzyme was provided by Novozymes (Denmark). Xyalanse (X) only had xylanolytic activity and cellulase (Cx) consisted on a cellulase + xylanase preparation, having carboxymethylcellulase (CMCase) and xylanase activities. Activities as U/g dried enzyme powder were: 11000 U/g for the xylanase (X) and 1700 U/g and 680 U/g for the cellulase and xylanase activity on the mixture (Cx), respectively. Concerning "Celluclast", CMCase activity was 640 U/mL of product.

An activity unit (U) is defined as the amount of enzyme capable of converting 1 μmol of substrate per minute. Enzymatic activity was determined using Spiro method to quantify released reducing sugars (Spiro, 1966), which will be further explained in section 2.2.2. Prior to treatments, reducing sugars presence on enzymatic preparations was analyzed using HPLC (see section 2.7.3), discarding any sugar presence on these preparations.

2.2.2. Enzymatic activity assay

Hydrolytic activity of xylanase and cellulase was measured using the method proposed by Spiro in 1966 for quantifying reducing sugars in a solution. For this, a microscale enzymatic reaction was performed in Eppendorf tubes with a total volume of 100 μL. Enzymatic reactions were held for 15 minutes and they were immediately stopped cooling samples in an ice bath when finished.

Temperature and pH of microscale reaction were those used for enzymatic treatments on fibers and were different for X and Cx enzymes. Xylanase activity was measured using 50 mM Tris-HCl buffer at pH 7. Temperature was set at 50ºC and 0.5% w/v birchwood xylan was used as substrate for reaction. Cellulase activity was measured using 50 mM acetate buffer at pH 5, 55ºC and 1.5% w/v carboxymethil cellulose (CMC) was used as a substrate. Microscale reaction was conducted in

triplicate. Enzymatic preparation was added diluted to each tube, with the dilution factor being different in each case depending on the activity of the enzyme. A blank measurement was also conducted for each measurement for quantification of previously present sugars, skipping the enzyme addition step. Enzyme was added to blank samples only after reaction, in order to detect any sugar present on preparations, if available.

Sugar quantification as a result of the enzymatic reaction was done using the formerly stated method, based on the reaction between reducing ends of sugars with an alkaline copper reactant to form copper oxide. Later, this copper oxide reacts with an arsenomolybtade reactant to form a blue-colored compound having absorbance at 520 nm (Figure 2-1). The absorbance difference between enzyme containing samples and blank (representing previous sugar content on samples) was interpolated into calibration curves run from standard solutions of xylose (for xylanase activity) and glucose (for cellulase activity) for knowing the amount of sugars released by enzyme. Activity was then expressed as (Equation 2-1):

$$Enzymatic\ Units\ (U) = \frac{\mu mol\ of\ sugar\ released}{min}$$

Equation 2-1

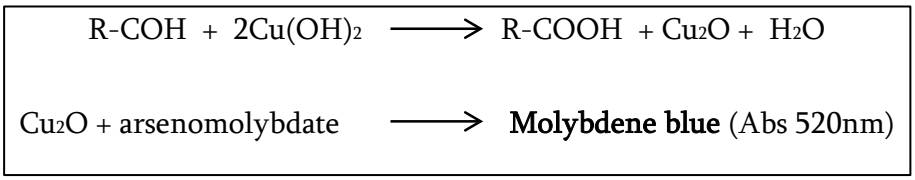

Figure 2-1: Chemical principle of the Spiro method for quantification of reducing sugars in a solution.

2.2.3. Xylanase treatment

In chapter 7 treatments with xylanase (X) were applied in polyurethane plastic bags acting as reactors on a thermostatic water bath with a 10 U/g oven-dried pulp

(odp) dose at 50 ºC, 10 % consistency, pH 7 (adjusted with 50 mM Tris-HCl buffer) and manual agitation every 10 min. In all cases, control fibers were prepared using the same conditions as for treatments but skipping enzyme addition. After treatments fibers were filtered and washed with decalcified water three times and one with distilled water in a fritted glass funnel (porosity 40-100 μm). Two different procedures were used for X application:

2.2.3.1. Direct

Total enzymatic dose (10U/g odp) was added and reaction was carried out up to 5h. Samples were collected after each hour for characterization (X_1, X_2, X_3, X_4 and X_5).

2.2.3.2. Stepwise addition

2U/g odp xylanase were added in 1h periods which were immediately followed by washing with deionized water (Xs_1, Xs_2, Xs_3, Xs_4 and Xs_5). At the end of treatment (5h) a final dose of 10 U/g odp was applied, equivalent to that used on "direct" treatment.

2.2.4. Cellulase treatment

Cellulase treatments were performed in different chapters of this thesis (4, 5, 6, 7 and 8). In all cases, they were performed at 55ºC and pH 5. Control treatments were prepared using the same conditions as of enzymatic treatments, but with no enzyme addition. Different experimental setups were used for treatments:

2.2.4.1. Treatments in plastic bag

Polyurethane plastic bags acting as reactors were placed in a thermostatic water bath for treatments in chapter 7. Treatments were held at 10% consistency, with a dose of 10 U/g odp and using 50 mM pH 5 acetate buffer during 2h. Agitation was done manually every 10 minutes. After treatments fibers were filtered and washed with

decalcified water three times and one with distilled water in a fritted glass funnel (porosity 40-100 μm).

2.2.4.2. Treatments in cylindrical blade-stirred reactor

A thermostatic 4 liter cylindrical blade-stirred reactor (Figure 2-2) was used for cellulase treatments exposed in chapters 4 and 5. In this case a 10 U/g odp enzymatic dose was applied at 5% consistency and using 50 mM pH 5 acetate buffer. Agitation was performed by rotating blades and set at 30 rpm and treatment was performed during 24 hours. After treatments enzyme was deactivated by increasing reactor temperature to 105 ºC for 15 minutes and fibers were then filtered in a fritted glass funnel (porosity 40-100 μm).

Figure 2-2: Cylindrical blade-stirred reactor.

2.2.4.3. Treatments in a Datacolor AHIBA easydye

In chapters 4, 6, 7 and 8 enzymatic treatments were performed in an Ahiba easydye apparatus (Figure 2-3) equipped with closed 250 mL vessels which were loaded with 10-12 g odp of fibers. In these independent vessels treatments were performed at

5% consistency. As this reactor was used for different studies, other paremeters depended on the specific study: treatment time varied from 2 h to 24 h and enzyme dose from 2 U/g odp to 20 U/g odp. Finally, 50 mM acetate buffer was used in most treatments for pH 5 maintenance, although in some treatments performed in chapter 4, H_2SO_4 was used to set reaction liquor pH to 5 before treatment. After treatments enzyme was deactivated by increasing reactor temperature to 105 ºC for 15 minutes and fibers were then filtered in a fritted glass funnel (porosity 40-100 µm).

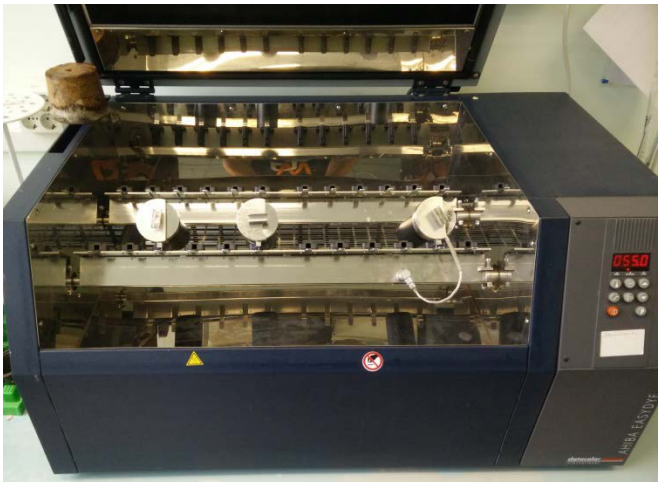

Figure 2-3: AHIBA Easydye apparatus with its 250 mL vessels.

2.3. Alkaline extraction (E stage)

After enzymatic treatments (X, Cx and Cx+X treatments) in chapter 7, an alkaline extraction with NaOH was applied on fibers for hemicelluloses removal. E stage was performed at 25ºC and 5% consistency, using two different NaOH concentrations and reaction times: 4 % (w/v) NaOH for 2 h; and 9 % (w/v) NaOH for 1 hour. After E stage, fibers were filtered and extensively washed with decalcified water and then with distilled water one time, in a fritted glass funnel (porosity 40-100 µm).

2.4. NCC preparation

Nanocrystalline cellulose was obtained by a controlled hydrolysis via sulfuric acid, using a protocol proposed by Dong et al. (1998) (Figure 2-5). Previous to acid hydrolysis, dried fibers were fluffed in a non-cutting mill and oven-dried at 105 ºC in a laboratory oven for 2 h. Fibers were cooled in a desiccator after drying.

For hydrolysis, typically 1.5 g of fluffed fibers weighted immediately from desiccator were treated with sulfuric acid. Different acid concentrations were used for this: 62%, 64% and 65% wt. Hydrolysis was performed using an acid-to-pulp ratio of 10:1 (10 mL/1g cellulose) with magnetic stirring. Temperature was maintained using a thermostatic water bath (Figure 2-4), and set to 45ºC, 47ºC or 60ºC depending on the study. Reaction time also depended on the specific study with samples hydrolyzed for 25, 45 and 50 minutes. Studies concerning NCC preparation are exposed in chapters 4, 5, 6 and 8.

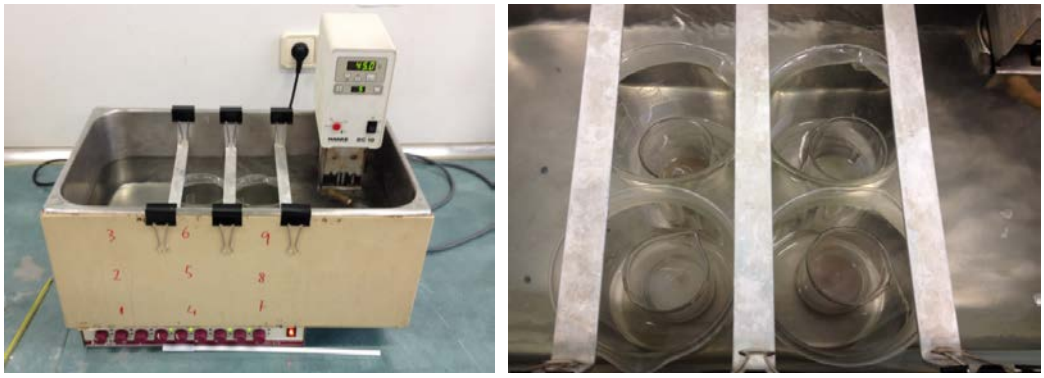

Figure 2-4: Thermostatic bath with magnetic stirring (left), samples during hydrolysis (right).

Once reaction time ended, hydrolysis reaction was stopped using chilled (4ºC) distilled water to dilute samples on a 10-fold basis, while samples were also cooled in a water ice bath. After this, suspensions were washed by centrifugation into a Hettich EBA 21 centrifuge at 6000 rpm for 15 min. Centrifuge supernatant was discarded and samples re-suspended in distilled water. Centrifugation step was repeated until supernatant became turbid and not able to be discarded. Turbidity in supernatant indicated presence of NCC. Hence, turbid supernatants were never discarded. After

centrifugation, samples were sonicated for NCC dispersion using a UP100H ultrasonic processor (Hielscher, Germany) at 100% amplitude and 0.75 cycles. Sonication was held for 25-40 min on an ice bath to prevent heating which is known to be capable of causing desulfation (Dong et al., 1998), until complete dispersion. Dispersed samples were then dialyzed against distilled water using a 10 kDa Thermo Fischer dialysis membrane for three days. Outer distilled water was changed 3 times during dialysis. After dialysis NCC suspension pH was controlled to be around 3 ± 0.1, extending the process if a lower pH was observed. Once dialyzed, sonication step was repeated for 20 minutes. Finally, suspensions were filtered through Whatman ashless paper membranes Nº 40 or 41 and the total volume of suspension obtained was determined using a measuring cylinder. At least two independent hydrolysis were performed for each fiber sample.

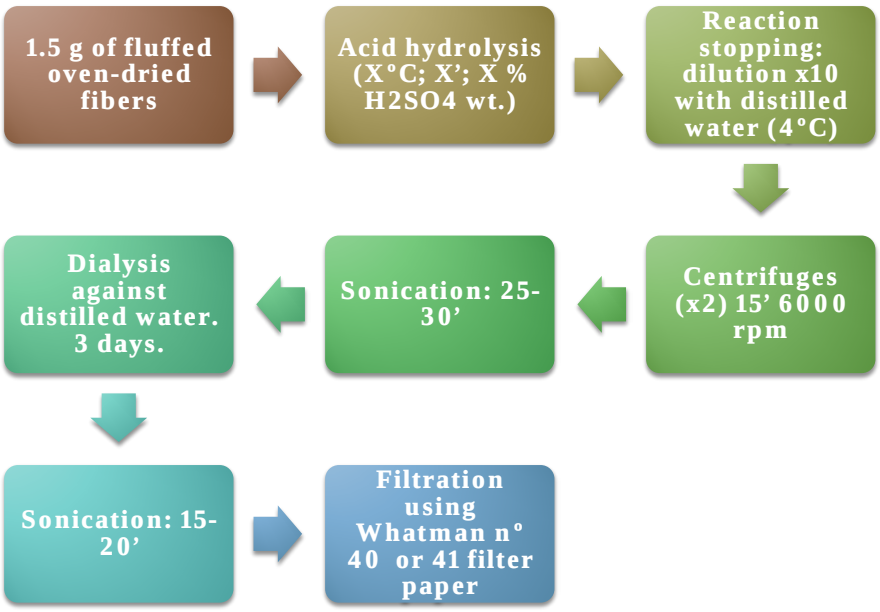

Figure 2-5: General overview of NCC preparation protocol.

2.5. Experimental design

2.5.1. Complete

A 2^2 experimental design was used in chapter 6 for investigating the effects of different enzymatic doses and reaction times of a cellulase pretreatment on nanocrystalline cellulose. Experimental results were analyzed using the Microsoft Excel analytical tool "regression" and the "stepwise backwards regression" method.

The statistical analysis of fiber and NCC properties was based on the results of a planned sequence of tests (Figure 2-6). For this purpose a stepwise-conducted experimental design was performed *i.e.,* the information obtained in the previous step allowed to decide whether the next step could be done or not. Firstly, a factorial design must be stablished in order to find a linear model accurately describing the principal variables affecting the response and their mutual interactions (step 1). To this end, a homoscedasticity test is performed by calculating Snedecor's *F*-value using the variance value at the central point and the residual mean square of the factorial design. The model is called homoscedastic if the variance result homogeneous (*i.e.* the variance is constant throughout the examined experimental region) and then, the study can be extended to the detection of potential curvature in the fitted data (Step 2). For the potential curvature detection, after the estimation of the linear model from the terms deemed significant in the previous factorial design, three replications at the central point have to be performed in order to check whether any quadratic terms are significant (Step 3). If significance is found for quadratic terms, then the experimental plan is expanded with new experiments in order to deconvolute quadratic terms (Step 4). Finally, new samples are prepared and characterized in order to verify obtained models (Step 5).

2.5.2. Linear saturated models

In chapter 5 the effects of the presence of a cellulase pretreatment, different hydrolysis temperatures and reaction times were studied. For this, two 2^3 factorial designs were used. In this case, only the linear saturated model was studied in order to acknowledge the variables influencing each parameter and their interactions, *i.e.* steps

2, 3 and 4 (see section 2.5.1) were not performed. The intention of this first statistical study was to acknowledge whether the enzymatic pretreatment was (or not) influential in NCC preparation. Hence, this sort of study did not allow the analysis of central points.

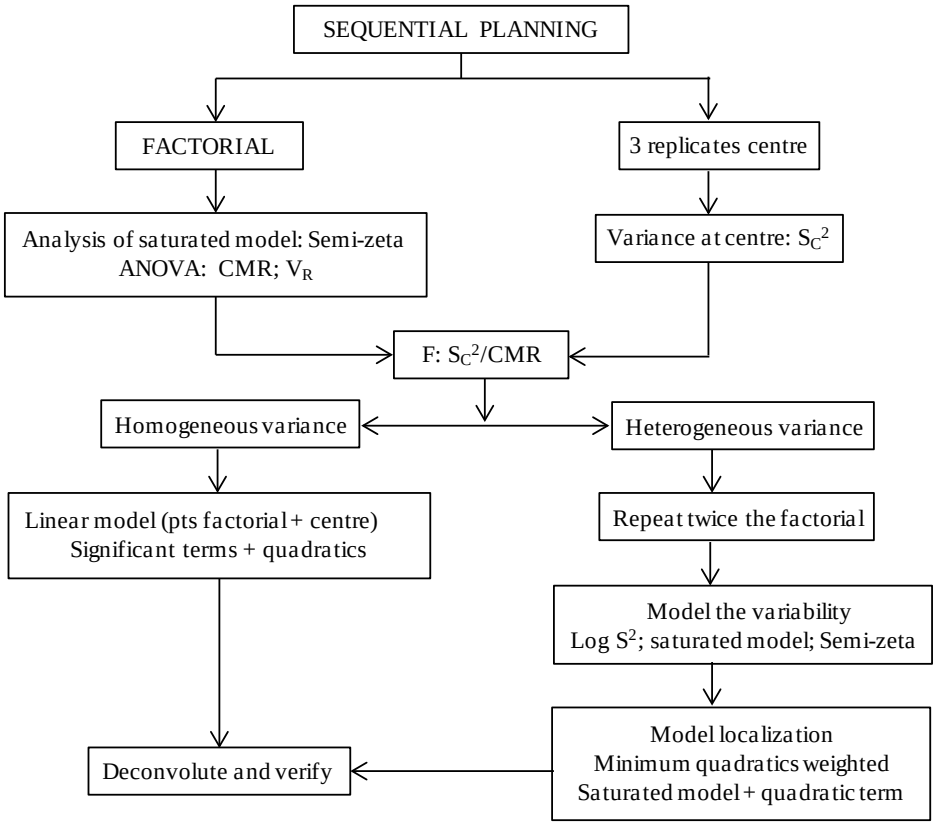

Figure 2-6: Scheme of the sequential testing plan, from Pepió and Polo, 2000

2.6. Cellulosic fibers characterization

2.6.1. Fibers chemical properties

2.6.1.1. Kappa number

Kappa number (KN) was determined in accordance with ISO 302:2004. This provides a measure of the lignin content of pulp. However, it is widely described that other compounds, present in pulp such as hexenuronic acids could affect kappa number determination (Li and Gellerstedt, 1997). Because of this, KN was also determined after the removal of hexenuronic acid groups using mercury acetate, according to the method described by Gellerstedt and Li, 1996. Each sample was analysed in duplicate.

2.6.1.2. Hexenuronic acids (HexA)

Hexenuronic acid content was analyzed in order to assess the improvement in fibers quality produced by the removal of hemicelluloses.

The method used to quantify hexenuronic acids (HexA) in chapter 3 in pulps was based on a procedure proposed by Li and co-workers (Gellerstedt and Li, 1996; Chai et al., 2001; Valls et al., 2010b). We used a mercuric acetate–sodium acetate solution to ensure selective hydrolysis of HexA from pulp. HexA were determined with a sensitive colour test involving periodate oxidation to β-formyl pyruvic acid and coupling of the latter with thiobarbituric acid to form a chromogen with a light-absorption maximum at 549 nm (Gellerstedt and Li, 1996). Two samples per pulp were hydrolysed and the filtrate from each reaction was analysed in quadruplicate.

Later, due to the apparition of a new protocol, HexA content in fibers was determined according to the procedure described in Tappi T 282 om-13 method (Zhu et al., 2014) for samples analyzed in chapter 7. All samples were analyzed in duplicate. This method could replace the procedure proposed by Gellerstedt and Li as it provides more repetitive results, produces less highly-polluting waste and strongly reduces the time needed for analysis.

2.6.1.3. Viscosity

Viscosity measurements were used to assess changes in the degree of polymerization of cellulose after enzymatic treatments. They were also used to determine the degree of polymerization of NCC in chapter 6. Fibers and NCC viscosity (as intrinsic viscosity of a sample of cellulose dissolved in a diluted solution of cupriethylenediamine) was determined in accordance with ISO 5351:2001. Viscosity measurements were made in duplicate. Degree of polymerization was calculated from the intrinsic viscosity [η] using the equation of (SCAN-CM 15:88) (Equation 2-2):

$$DP^{0.085}=1.1\cdot[\eta]$$

Equation 2-2

Also, the number of scissions in the cellulose strain by a certain process are indicated by Chain scission number (CSN) which is defined mathematically as (Equation 2-3) (Bouchard et al., 2006):

$$CSN = \left[\frac{1}{DP} - \frac{1}{DPo}\right] DPo$$

Equation 2-3

Where DP_O is the degree of polymerization of initial fibers and DP that after the studied treatment.

2.6.1.4. Carbohydrate composition

Carbohydrate composition of fiber samples was determined by high performance liquid chromatography (HPLC) after Soxhlet extraction with acetone and fibers fluffing. Two replicates of the resulting samples were hydrolyzed using a modified version of the TAPPI 249 cm-09 test method. Hydrolysis was performed in a two-step process. The first step, a pre-hydrolysis with concentrated sulfuric acid, involved placing approximately 50 mg of fibers sample in a test tube and soaking it with 5 ml of 72% w/w sulfuric acid, with the tube placed in a water bath at 30 ± 0.5 °C

for 1 h with occasional stirring. The second step, a final hydrolysis with dilute sulfuric acid consisted on placing the content of the former tubes in 250-ml flask in order to obtain a final solution of 4% in sulfuric acid adding distilled water. The flask was then placed in an autoclave at 103 ± 7 kPa for 1 h. Once the reaction was complete, the specimen solution was cooled at room temperature and passed through a gooch filter No. 3 to remove lignin insoluble in sulfuric acid, which was taken to represent Klason lignin. Resulting solution was filtered through a Whatman membrane of 0.45 μm pore size before HPLC analysis. The chromatographic determination was performed with an Agilent 1100 HPLC instrument furnished with column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 6 mM sulfuric acid; flow rate, 0.7 ml/min; column temperature, 60 °C. Data was collected by a refractive index detector (RID) in HPLC equipment. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from Sigma–Aldrich). Because the column failed to resolve xylose, mannose and galactose, their combined content was expressed as xylose (Garrote et al., 2001).

2.6.1.5. Free hydroxyl content

Free hydroxyl content of cellulose was determined according to the protocol proposed by Genung (1950). The method is based in the titration of an acetylated derivative of cellulose obtained via an acetylation reaction with a reagent composed by acetic anhydride and pyridine (Verley & Bölsing reagent). For the acetylation reaction, 1 g of oven-dried cellulose was soaked with 40 mL of a reagent containing pyridine and acetic anhydride in a 95:5 proportion. After this, erlenmeyers containing samples were set to reflux at 80ºC in a thermostatic bath for 12 hours (Figure 2-7). A blank with no cellulosic sample was also refluxed. Reaction was stopped by the addition of 5 mL of distilled water, which decomposes acetate anhydride. With this reaction, cellulose acetate was obtained, which was then titrated using NaOH 0.5 N monitoring pH, until a pH value of 9 was reached. OH- group content is directly proportional to the difference in the consumed value of NaOH between the blank and the analyzed sample. Samples were acetylated in duplicate. Results were expressed as % of free OH (w/w) and are calculated according to the following formula (Equation 2-4):

$$\%\ free\ OH{-}{=}\ \frac{(mL\ NaOH\ blank - mL\ NaOH\ sample) * Normality\ NaOH * 1.7}{sample\ dried\ weight\ (g)}$$

Equation 2-4

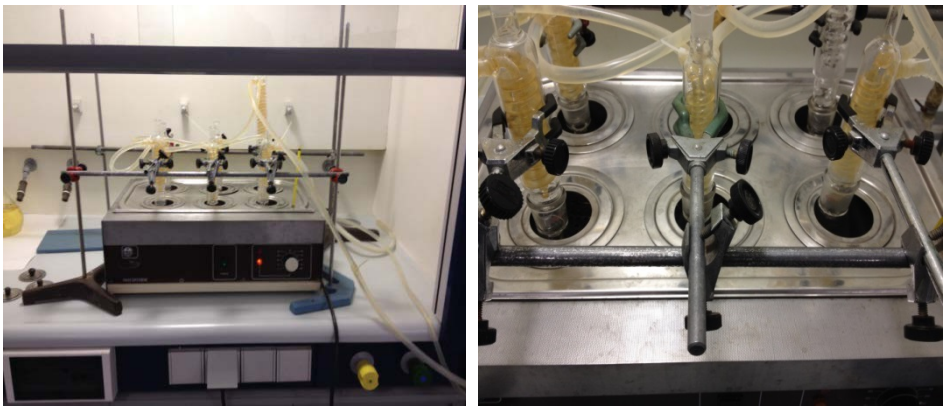

Figure 2-7: Experimental setup for acetylation reaction in a thermostatic bath with reflux refrigerated by water.

2.6.1.6. Cellulose reactivity

Reactivity values of samples were determined according to slightly modified version of Fock's method (Fock, 1959; Ibarra et al., 2010). The Fock method is a micro-scale process simulating the industrial viscose process for the manufacture of regenerated cellulose. Prior to analysis, samples were dried overnight in a controlled atmosphere (25 ºC and 50% RH). The first step involved the production of viscose from cellulose sample, adding to 50 mg of cellulose 50 mL of 9% (w/v) NaOH and 1.3 mL of CS_2, which was stirred for 4h at room temperature. 10 mL of diluted cellulose were neutralized using 29% H_2SO_4 and left overnight. The next day samples were mixed with 20 mL of 68% H2SO4 and stirred for 1h. Then samples were diluted to 50 mL and 10 mL of 1 N $K_2Cr_2O_7$ were added. Solution was then refluxed at boiling temperature for 1h to fully oxidize regenerated cellulose. When cooled, all samples were taken to 100 mL. 40 mL of these samples were mixed with 0.5 g of KI and titrated with 0.1N

$Na_2S_2O_3$. Reactivity measurements were conducted in duplicate and expressed as the regenerated cellulose yield (Ibarra et al., 2010) (Equation 2-5):

$$X = 100 * 10(a)\ \frac{M(V_1C_1 - (V_2C_2 * \frac{100}{40\ (b)})/6)}{4Y}$$

Equation 2-5

Where: X is the reacted cellulose (%), Y is the weight of sample (g), M is the molecular mass of glucopyranosyl residue, $C_6H_{10}O_5$ (162 g/mol), V_1 is the volume of added $K_2Cr_2O_7$ (L), V_2 is the volume of titrated $Na_2S_2O_3$ (L), C_1 is the concentration of $K_2Cr_2O_7$ (mol/L), C_2 is the concentration of $Na_2S_2O_3$ (mol/L), *a* is the first dilution to 100 g and outtake of 10 mL (10.4 g) = 100/10.4 = 9.62, and *b* is the second dilution of the sample to 100 mL and outtake of 40 mL = 100/40.

2.6.1.7. Cationic demand

Cationic demand of fibrous suspensions (1.2 % consistency using distilled water) was determined using a particle charge detector (PCD03PH, Mütek, Germany). 10 mL sample of filtrate obtained from each fibrous suspension (then, the aqueous fraction only) was placed in the measuring cell (Figure 2-8). As the piston moved, it produced an electrical current that was measured by two electrodes. In order to neutralize negative charge, the filtrate was directly titrated with a cationic polyelectrolyte (0.001 N Poly-Dadmac at a rate of 0.2 mL/min) to an output signal of 0 mV (charge neutrality). Titrations were conducted in duplicate and cationic demand was calculated according to the following formula (Equation 2-6):

$$Cationic\ demand\ (\frac{meq}{g\ fibers}) = \frac{VxC}{Vs * Cn}$$

Equation 2-6

Where V and C are the volume and the concentration of the titration agent (poly-dadmac), respectively, and Vs is the volume to be titrated (10 mL) and Cn the fibrous suspension consistency.

Figure 2-8: Mütek particle charge detector.

2.6.1.8. Zeta potential

Zeta potential of fibers was determined using a streaming potential detector (SZP-06, Mütek, Germany). Fibers were sucked into the suction tube by applying vacuum which induced the formation of a pad of fibers in the measuring cell. The flow passing the fibers pad moved mobile charges from the shear plane (the boundary between the adsorbed counter-ion layer and diffuse layer), thus generating a streaming current and hence a potential difference that was measured with two electrodes. Motion of the liquid was a result of the pressure difference between the two sides of the fiber pad. Zeta potential measuremanets were conducted in duplicate for each fiber sample and was calculated from the Smoluchowski equation (Cadena et al., 2009) (Equation 2-7):

$$Zeta\ potential\ (mV) = \frac{\Delta U}{\Delta p} * \frac{n\ L}{\varepsilon\ \varepsilon 0\ Q\ R}$$

Equation 2-7

Where ΔU is the streaming potential, Δp the pressure difference, n is the viscosity, L/Q he cell constant, R the electric resistance and ε and $\varepsilon 0$ the dielectric constant and electric permittivity of vacuum, respectively.

2.6.1.9. FTIR spectroscopy

Modifications in cellulose crystalline structure by cellulase treatments were assessed via FTIR analysis in chapters 5 and 6. Cellulose fibers FTIR spectra were recorded in duplicate at room temperature using an ATR-FTIR spectrophotometer (Spectrum 100, Perkin Elmer, USA). FTIR spectral analysis was conducted within the wavenumber range of 600-4000 cm^{-1}. A total of 64 scans were run to collect each spectrum at a 1 cm^{-1} resolution. Total crystallinity index (TCI), proposed by Nelson and O`Connor (Nelson and O'Connor, 1964), was estimated from the ratio between the absorption peaks at 1370 cm^{-1} and 2900 cm^{-1} bands.

2.6.2. Fibers physical and morphological properties

2.6.2.1. ISO brightness

Fibers brightness was tested in accordance with ISO 3688:1999. Tests were performed by quadruplicate in each sample using a Technidyne Color Touch reflectance measurement apparatus equipped with the standard illuminant/observer combination C/2º. Samples preparation involved the filtration of a fibrous suspension on a Büchner funnel furnished with filter paper for the preparation of a handsheet of grammage superior to 200 g/m^2. ISO brightness was determined by the intrinsic reflectance factor of samples at 457 nm. Reflectance factor is defined as the ratio of radiation reflected by a body compared to that of a perfect reflecting diffuser.

2.6.2.2. Moist heat ageing

In order to assess the changes in brightness durability of samples after treatments, they were aged in an ageing vessel at 80 ºC and 65 % of relative humidity (RH) for 72 h according to ISO 5630-3:1996. ISO brightness of samples was tested at the beginning and final of the ageing process. After ageing essays, brightness loss index (BLI) was calculated using the following formula (Equation 2-8):

$$Brightness\ loss\ index\ (\%) = \frac{B_0 - B_{72}}{B_0} * 100$$

Equation 2-8

Where B_0 is the initial ISO brightness value for pulp and B_{72} is the brightness value after 72 h of moist heat exposure.

2.6.2.3. Fiber analysis

Morphological properties of fibers such as their length, width and fines content were determined using the method described in TAPPI T 271 om-02 method in a Kajaani fiber analyzer (FS300, Metso automation, Finland). Measurements technique is based on fibers capability of modifying the direction of a polarized light beam. Pre-soaked fiber samples were disintegrated and resulting suspension diluted to for an average particle count rate of around 100 pieces per second. The instrument provides three different calculations for fiber length (numerical, L(n); length-weighted, L(l); and weight-weighted, L(w); Equation 2-9). In this thesis, fiber length was expressed as length-weighted fiber length, L(l):

$$L(l) = \frac{\sum(n.l\hat{}2)}{\sum(n.l)}$$

Equation 2-9

Where n is the particle number count and l the length in millimeters of each particle.

2.6.2.4. Drainage resistance

Fibers drainage resistance was measured using Schopper-Riegler, and expressed using the homonym degrees (ºSR) in accordance to ISO 5267-1:1999. Measurements were conducted in duplicate.

2.6.2.5. Water retention value

Fibers water uptake was determined by measuring their water retention value (WRV) in accordance to ISO 23714:2014. Measurements were conducted in duplicate.

2.6.2.6. Fiber fractionation

A laboratory Bauer-MacNett classifier with screen sizes of 30 and 200 mesh was used in chapter 3 for separating flax long and short fibers in accordance to TAPPI T233-cm-06. For separation, 10 g of dried fibers were disintegrated in 3L of water and poured into the first chamber. A continuous water flow was passed through chambers for 20 minutes. At the end, small fraction was recovered into the chamber with the 200 mesh screen, while the smaller particles passing this screen were considered fines and lost during classification.

2.6.2.7. Wet Zero-span tensile strength

Wet zero-span tensile strength measures the maximum tensile load of wet fibers in a paper sample clamped between two jaws in mutual contact (Zero-span). This value is used to refer to the average axial resistance of fibers in independence of the fibrous bonding network. Measurements were performed in accordance of ISO 15361:2000 in a Zero-span 1000 Pulmac tester. For analysis, strips obtained from paper sheets prepared in a laboratory Rapid-Köthen lab former (ISO 5269-2:2004) were previously soaked in distilled water for 5 seconds. At least 10 measurements were made for each sample. Values were expressed as wet zero-span Index, *i.e.* correcting the instrument reading by the paper sample grammage.

2.6.2.8. Water contact angle (WCA)

WCA measurements provide information about the affinity of liquids to solid surfaces. The use of water allows the determination of the hydrophobic or hydrophilic condition of a surface. Generally, it is assumed that hydrophilic surfaces will have WCA values smaller than 90º, while hydrophobic surfaces WCA will be comprised

between 90º and 180º (Cusola et al., 2013). Contact angle was determined using the sessile drop method, which consists in dispensing a water drop of a few microliters from the needle of a precision syringe and slowly approaching the solid material until contact. Contact angle is the one formed between the tangent to drop's profile and tangent to surface at intersection of vapor, liquid and solid.

Measurements were made using a Dataphysics OCA15EC contact angle goniophotometer using an image capture ratio of 25 frames/s. A 4 µL water drop was delivered to sample surface (paper strips) and at least 8 measurements were made for each sample.

2.6.2.9. Scanning electron microscopy (SEM)

Handsheets formed by the different fiber samples were analyzed through scanning electron microscopy. Small pieces of paper of each sample were used for SEM analysis with a JEOL JSM-6400 microscope operating at 10 kV. Samples were first coated with a very thin layer (14 nm thick) of gold–palladium (Au/Pd) in a sputter coater SCD005 in order to obtain a conductive surface.

2.6.2.10. Optical microscopy

Individual fibers were analyzed by light microscopy according to ISO 9184-3:1990. Images were recorded using a DeltaPix digital camera model Infinity X placed on an Olympus BH-2 microscope. The imaging process involved a small amount of fibers to be stained with 1-2 drops of Herzberg stain (zinc-chloro-iodide) and individualized mechanically. Examination was performed at x40 and x100 magnifications.

2.7. Effluents characterization

2.7.1. Residual activity

Enzymatic residual activity on effluents was determined using the same method as for enzymatic stocks (see section 2.2.2).

2.7.2. Protein content

Protein content on effluents was assessed via Bradford's micromethod (Bradford, 1976). Bradford method is a spectroscopic colorimetric procedure for the determination of the protein content in a solution. It is based on an absorbance shift of the dye Coomassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being quantified. This bound form of the dye has an absorption spectrum maximum at 595 nm. The procedure involves the realization of a calibration curve with different concentrations of bovin seric albumin (BSA) acting as protein standard. Protein content in enzymatic reaction effluents was determined after reaction with Bradford reagent and was calculated by interpolation into calibration curve. Analyses were conducted in triplicate.

2.7.3. Released reducing sugars

Reducing sugars released from fibers as a consequence of enzymatic treatments were identified and quantified using HPLC analysis. Prior to analysis 12.5 mL of effluents were taken to pH 7 using NaOH or HCl. Then all samples were flushed to 25 mL using distilled water and filtered using a Whatman 0.45 μm filter. HPLC analysis was performed using an 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-42A ion-exchange column. Two analyses were made for each effluent sample. Operating conditions were: 0.35 ml/min flow, column temperature 65 ºC and the mobile phase was MQ water. Identification and quantification of compounds was done by interpolation into calibration curves run from glucose-oligosaccharides (DP 1 to 6) and xylose oligosaccharides (DP 1 to 4).

2.8. NCC characterization

2.8.1. Yield

Process yield of NCC preparation was determined by calculating the total obtained NCC mass compared to the initially hydrolyzed mass of fiber. First, 25 mL

glass beakers were oven dried at 105ºC for 2 hours and cooled in a desiccator. The tare weight of each beaker was determined. Then 25 mL of each suspension measured with pipette were poured into tared beakers by triplicate (Figure 2-9). NCC suspensions were evaporated at 60ºC until constant weight. After evaporation beakers were cooled again in a desiccator and total NCC mass was determined.

NCC yield and solids content in suspensions were determined using the following equations (Eq. 2-10 and 2-11):

$$Yield\ (\%) = \left(\frac{(a-b) * total\ volume\ (mL)}{dried\ volume\ (mL) * initial\ fibers\ mass\ (g)}\right) * 100$$

Equation 2-10

$$Solids\ content\ \left(\frac{g}{mL}\right) = \left(\frac{(a-b)}{dried\ volume\ (mL)}\right)$$

Equation 2-11

Where: a is the weight of beaker + dried NCC (g) and b is the beaker tare weight (g).

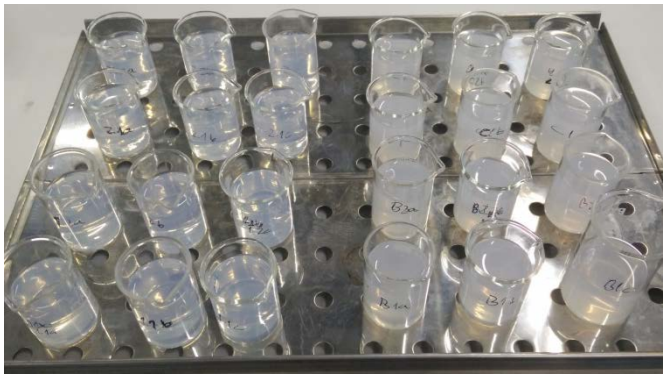

Figure 2-9: Tared 25 mL glass beakers with NCC suspension samples.

2.8.2. Sulfur content

Sulfur content provided by SO_4^{2-} groups incorporated onto NCC during sulfuric hydrolysis was determined according to the protocol proposed by Abitbol et al. (2013). This method consisted on a potentiometric titration using NaOH. First, 10 mL of NCC suspensions of known solids content were added to a beaker containing 120 g of a 1 mM NaCl solution for the complete protonation of sulfate groups. Samples were then titrated using 1.25 mM NaOH using magnetic stirring and monitoring solution conductivity (Figure 2-10). Three phases could be distinguished in this titration (Figure 2-10). The first phase involved a decrease in solution conductivity due to the consumption of protons by the added alkali (NaOH). The second phase, neutralization, consisted on the equivalence point in which all sulfate groups were neutralized by NaOH. Finally, the addition in excess of NaOH leaded to an increase in conductivity due to the excess in sodium ions present in sample. Inflexion point, determined by the intersection of the two linear regions provided the volume of NaOH at a determined concentration necessary for neutralization. This allowed then to calculate NCC sulfur content through the following equation (Equation 2-12):

$$\% S = \frac{V_{NaOH}\, C_{NaOH}\, M_W(S)}{V_{susp}\, C_{susp}} X100$$

Equation 2-12

Where V_{NaOH} is the volume of NaOH used for titration (mL), C_{NaOH} is the concentration of titrant solution (mol) and V_{susp} y C_{susp} is the volume (in mL) and concentration (in g/mL) of NCC in the suspension and Mw(S) is sulfur atomic mass (32 g/mol). Three independent determinations were made for each sample.

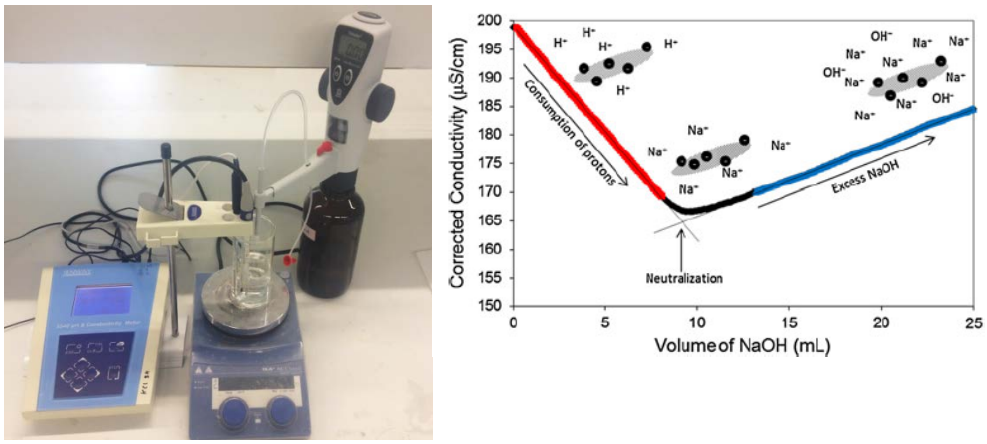

Figure 2-10: Experimental setup for potentiometric titration (left) and chemical principle of the titration from Abitbol et al., 2013 (right).

2.8.3. Particle size analysis

Dynamic light scattering (DLS) is a technique usually employed for the determination of particle size in the nanometric region. It is also called photon correlation spectroscopy (PCS) or quasi-elastic Light Scattering (QELS). DLS measures the random movement of particles (Brownian motion) suspended within a liquid medium. A monochromatic light beam (*e.g.* a laser) shining on a solution with suspended particles in Brownian motion is known to produce a Doppler Shift when the light hits the particle. This interference can change the wavelength of the incoming light, and the magnitude of this change is related to particle size. Size of the particles is inversely related to their Brownian motion speed. The velocity of a particle under Brownian motion is defined by its translational diffusion coefficient (D_t), which in turn can be used to determine the hydrodynamic radius (r_h) of the particles as derived from the Stokes-Einstein equation (Equation 2-13):

$$r_h = \frac{(k_b\, T)}{6\pi\eta D_t}$$

Equation 2-13

Where r_h is the hydrodynamic radius of the particle (in m), k_b is the Boltzmann constant (in $J.K^{-1}$), T is the absolute temperature (in K), η is the medium viscosity (in $kg.m^{-1}.s^{-1}$), Dt is the translational diffusion coeficient (in $m^2.s^{-1}$). It is noteworthy that experimental parameters like temperature and viscosity values should be stable and accurately known.

The hydrodynamic radius r_h, also called Stokes radius, or Stokes-Einstein radius, represents the radius of a hypothetical hard sphere having the same diffusion coefficient or viscosity as the particle under examination. In the case of nanocrystalline cellulose (NCC), non-spherical and solvated particles, hypothetical hard spheres have no real existence and the hydrodynamic radius is only indicative of the 'apparent' size of the dynamic hydrated/solvated NCC particle (Fraschini et al., 2014). For rod-like particles like NCCs, a slight change in their length will directly affect the measured DLS size, while changes in the cross-section will hardly affect the diffusion velocity. Tough useful and easily determined, r_h represents only the hydrodynamic size which can significantly differ from the true physical size. Therefore, it is only used for size comparisons among samples. Also, the coefficient D_t depends not only on the physical size and size-related behavior (*e.g.* diffusion or viscosity), but also on any surface structure and texture (topography, roughness, charges), as well as concentration and size of ions present in the medium. If the particle shape changes in a way that affects the diffusion velocity, then r_h will also change.

In the present thesis DLS measurements were used for apparent size determination of NCC suspensions in order to assess the size variations produced by the different preparation conditions. It was determined using a particle size analyzer (DL 135, Cordouan Technologies, France). Size distribution was determined with dynamic light scattering (DLS) at room temperature (25ºC). Aqueous suspensions were placed directly in the measuring cell and laser power was adjusted for counting around 2000 particles per minute. All samples were analyzed in triplicate.

2.8.4. Surface charge

Surface charge of NCC was determined using the same method as described in section 2.6.1.7. 10 mL of suspensions were titrated using 0,001 N Poly-Dadmac

(cationic poly-electrolyte). Surface charge density was calculated according to the following formula (Equation 2-14):

$$Surface\ charge\ (\frac{meq}{g}) = \frac{VxC}{wt}$$

Equation 2-14

Where V and C are the volume (L) and the concentration (N) of the titration agent (poly-dadmac), respectively, and wt is the solid weight (g) of the NCC sample.

2.8.5. Electrophoretic mobility

Stability of the NCC suspensions was assessed via zeta potential measurements, which is determined studying the electrophoretic mobility of colloidal particles. Measurements were performed in a Malvern Zetamaster model ZEM apparatus equipped with a laser Doppler velocimeter and data was averaged from 12 measurements per sample.

Electrophoresis is an electrokinetic phenomenon during which charged particles (colloids) move with respect to a stationary liquid phase under the effect of an applied electric field (Shaw, 1993). Charged particles are attracted towards the electrode of opposite charge and viscous forces acting on the particles tend to oppose to this movement. When equilibrium is reached between these two opposed forces particles arrive to a constant movement speed. Velocity of the particle is dependent on several factors such as the strength of the electric field, voltage gradient, dielectric constant of the medium and its viscosity. This velocity is the factor commonly referred to as electrophoretic mobility and is calculated through the Henry equation (Equation 2-15):

$$U_E = 2\varepsilon z f(ka)/3\eta$$

Equation 2-15

Where z stands for zeta potential, U_E for the electrophoretic mobility, ε for the dielectric constant, η for the viscosity of the medium and f (ka) is the Henry's function.

Two different values are usually assigned to f (ka): 1.5 or 1.0. For aqueous solutions of moderate electrolyte concentration and particles larger than 0.2 μm the value of 1.5 is usually used, the Smoluchowski approximation. Smaller particles in low dielectric constant media f (ka) is assignes as 1.0, the Huckel approximation.

2.8.6. Viscosity

Intrinsic viscosity of NCC was determined using a modified version of ISO 5351:2010, using 0.2 - 0.3 g of dried NCC suspensions as cellulose samples, a similar procedure as described elsewhere (Satyamurthy et al., 2011). This viscosity values were then used to estimate the degree of polymerization of cellulose in NCC (see section 2.6.1.3).

2.8.7. Water contact angle (WCA)

WCA of dried NCC films was determined using the same procedure as for cellulose fibers (see section 2.6.2.8).

2.8.8. FTIR spectroscopy

Possible modifications in chemical composition and in crystalline structure of NCC after isolation were assessed via FTIR spectra of dried NCC films. Measurements were conducted using the same procedure as for fibers, exposed in section 2.6.1.9.

2.8.9. SEM imaging

NCC dried films were observed by scanning electron microscopy according to Majoinen et al., 2012, who stated that individual rod-like NCC could be observed at the breaking points of the film. Observation procedure was the same as for cellulosic fibers described in section 2.6.2.9, obtaining in this case images of the cross-sections of NCC films.

2.8.10. TEM imaging

Transmission electron microscopy (TEM) was used to examine NCC morphology using a similar protocol to that described elsewhere (Chen et al., 2015). Carbon-coated Cu-grids were firstly glow-discharged for 30 seconds and then floated on 5 μL drops of NCC suspensions (0.1-0.5 % w/v) for 5 minutes. After that, NCC was negatively stained by floating grids consecutively into two 50 μL drops of 2% aqueous uranyl acetate for 30 seconds. Excess stain was removed by capillary action and gentle blotting. Samples were analyzed using a JEOL JEM-1010 transmission electron microscope operating at 80 kV.

2.9. References

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Bouchard, J.; Méthot, M.; Jordan, B. The Effects of Ionizing Radiation on the Cellulose of Woodfree Paper. Cellulose 2006, 13 (5), 601–610.

Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 1976, 72, 248–254.

Cadena, E. M.; Garcia, J.; Vidal, T.; Torres, A. L. Determination of Zeta Potential and Cationic Demand in ECF and TCF Bleached Pulp from Eucalyptus and Flax. Influence of Measuring Conditions. Cellulose 2009, 16 (3), 491–500.

Chai, X.-S.; Zhu, J. Y.; Li, J. A Simple and Rapid Method to Determine Hexeneuronic Acid Groups in Chemical Pulps. J. Pulp Pap. Sci. 2001, 27 (5), 165–170.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Cusola, O.; Valls, C.; Vidal, T.; Roncero, M. B. Application of Surface Enzyme Treatments Using Laccase and a Hydrophobic Compound to Paper-Based Media. Bioresour. Technol. 2013, 131, 521–526.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Fock, W. Eine Modifizierte Methode Zur Bestimmung Der Reaktivität von Zellstoffen Für Die Viskoseherstellung. Das Pap. 1959, 13 (3), 92–95.

Fraschini, C.; Chauve, G.; Berre, J.-F. Le; Ellis, S.; Méthot, M.; Connor, B. O. Critical Discussion of Light Scattering and Microscopy Techniques for CNC Particle Sizing. Nord. Pulp Pap. Res. J. 2014, 29 (1), 31–40.

Garrote, G.; Domínguez, H.; Parajó, J. C. Kinetic Modelling of Corncob Autohydrolysis. Process Biochem. 2001, 36 (6), 571–578.

Gellerstedt, G.; Li, J. An HPLC Method for the Quantitative Determination of Hexeneuronic Acid Groups in Chemical Pulps. Carbohydr. Res. 1996, 294, 41–51.

Genung, L. Analysis of Cellulose Derivatives. Anal. Chem. 1950, 14 (12), 935–940.

Ibarra, D.; Kopcke, V.; Larsson, P. T.; Jaaskelainen, A.-S.; Ek, M. Combination of Alkaline and Enzymatic Treatments as a Process for Upgrading Sisal Paper-Grade Pulp to Dissolving-Grade Pulp. Bioresour. Technol. 2010, 101 (19), 7416–7423.

Li, J.; Gellerstedt, G. The Contribution to Kappa Number from Hexeneuronic Acid Groups in Pulp Xylan. Carbohydr. Res. 1997, 302 (3-4), 213–218.

Majoinen, J.; Kontturi, E.; Ikkala, O.; Gray, D. G. SEM Imaging of Chiral Nematic Films Cast from Cellulose Nanocrystal Suspensions. Cellulose 2012, 19 (5), 1599–1605.

Nelson, M. L.; O'Connor, R. T. Relation of Certain Infrared Bands to Cellulose Crystallinity and Crystal Lattice Type. Part II. A New Infrared Ratio for Estimation of Crystallinity in Celluloses I and II. J. Appl. Polym. Sci. 1964, 8 (3), 1325–1341.

Pepió, M.; Polo, C. Apunts de L'assignatura de Tercer Cicle: "Disseny D'experiments I Optimització de Processos." Dep. d'estadistica i Investig. Oper. Univ. Politècnica Catalunya 2000.

Satyamurthy, P.; Jain, P.; Rudrapatna, H. B.; Vigneshwaran, N. Preparation and Characterization of Cellulose Nanowhiskers from Cotton Fibres by Controlled Microbial Hydrolysis. Carbohydr. Polym. 2011, 83 (1), 122–129.

Shaw, D. J. Introduction to Colloid and Surface Chemistry (4th Edition). 1993, 26 (3), 7506.

Spiro, R. G. R. Analysis of Sugars Found in Glycoproteins. Methods Enzymol. 1966, 566 (C), 3–26.

Valls, C.; Gallardo, O.; Vidal, T.; Ignacio, F.; Pastor, J.; Díaz, P.; Roncero, M. B.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B.; et al. New Xylanases to Obtain Modified Eucalypt Fibres with High-Cellulose Content. Bioresour. Technol. 2010, 101 (19), 7439–7445.

Zhu, J. Y.; Zhou, H. F.; Chai, X. S.; Johannes, D.; Pope, R.; Valls, C.; Roncero, M. B. Inter-Laboratory Comparisons of Hexenuronic Acid Measurements in Kraft Eucalyptus Pulps Using a UV-Vis Spectroscopic Method. Tappi J. 2014, 13 (1), 57–61.

Chapter 3

Raw materials characterization

Abstract

Properties of cellulosic raw materials are known to vary widely depending on their sources. The interest in the usage of non-conventional fibers makes necessary a better knowledge of the peculiarities of each source and their behavior under different bleaching processes. ECF and TCF bleached pulps (ISO brightness ≥ 82%) from eucalyptus, flax, sisal and cotton linters were analyzed. Eucalyptus showed the highest zero-span tensile strength (1.2-1.1 N.m/g), higher than that of sisal (0.95-0.85 N.m/g) and flax (0.8-0.7 N.m/g) which were also found to be linearly correlated to their viscosity, independently of the cellulose source. Sisal and eucalyptus showed the largest hemicelluloses content (≈13-16%) while cotton linters appeared as a high-cellulose content (97.7%) source for high-quality fibers. ECF and TCF bleaching processes produced different effects on fibers, as the latter showed a slightly lower quality than the former, difference that may not be significant if the great environmental benefit of TCF bleaching is considered.

3.1. Introduction

Nowadays global cellulose market offers a wide variety of fibers obtained from diverse sources bleached throughout different sequences. Each fiber source has unique characteristics, hampering the decision of which raw material suits best the different applications always seeking for optimal processability and product quality. Wood fibers are by far the main cellulose source for worldwide pulp and paper industry (Klemm et al., 2005). However, non-wood fibers sources are gaining attention due to the fact that they are usually derived from annual crops, offer high-quality fibers and in many cases they are obtained as by-product, improving biomass utilization (Ververis et al., 2004).

Among woody fibers, hardwoods such as eucalyptus, birch or poplar provide short fibers of thick walls and a narrow lumen compared to softwoods, such as pine, spruce or fir. Generally, hardwoods yield less-resistant papers but offer a better formation, smoothness, bulk and opacity (Ververis et al., 2004). Eucalyptus has been increasingly used for papermaking since 1960's and is currently becoming one of the

most important fiber sources worldwide (Valls et al., 2009). It was predicted that by 2015, market pulp production would reach 70 million tons, with about 35 million coming from hardwoods and more than 50% of this coming from eucalyptus (Hart and Santos, 2015).

On the other hand, non-wood plants are a heterogeneous group of organisms providing fibers with a great morphological and chemical diversity. Flax fibers, for example, are derived from plants usually harvested for both the production of fibers and seeds (raw material of the linseed oil) (Barneto et al., 2010). Flax fibers have been used since ancient time for the manufacture of linen, being nowadays the greatest flax producers Canada, China, India, and United States (Barneto et al., 2010). Flax pulp usually consists of two different types of fibers, namely: "bast fibers", obtained from the bark, which are long and strong (10–55 mm long and 12–30 μm thick), and "core fibers," obtained from the log or xylem, which are shorter and stiffer (0.05 to 0.5 mm long and 10-30 μm in thick). The usage of both fractions responds basically to an economical reason, as bast fibers are up to four times more expensive than core fibers (Fillat et al., 2012).

Another example of non-wood fibers could be found in sisal, which provides fibers with a great potential for several applications. Traditional uses for sisal included manufacture of natural ropes, cordage and sacking, although the apparition of synthetic materials and the lack of technological development has diminished the typical sisal markets (Hurter, 1997). In papermaking terms, sisal presents a high tear resistance, alpha cellulose content, porosity, bulk, absorbency and folding endurance, making it an interesting choice for specialty papers (Aracri and Vidal, 2012). Also, sisal has been reported to have better physical properties than softwood kraft fibers (Maddern and French, 1995).

Cotton linters are another sort of non-wood fiber presenting the peculiarity of not undergoing a cooking process. They constitute a byproduct of the textile industry, being the short fiber that cannot be used in the textile process and presenting a very high cellulose content (Sczostak, 2009). The ginning process used for cotton fibers extraction leaves linters attached to seed coat and another mechanical process is then necessary for their removal. As extracted from seeds, cotton linters also contain other compounds such as pectin, proteinaceous matter, waxes, ashes and minor soluble polysaccharides. These compounds are usually removed by boiling into a diluted

caustic solution under nitrogen in order to obtain pure cellulose fibers (Gümüskaya et al., 2003). In 2010, 42 million metric tons of cotton was produced, which lead to the production of 2.5 million metric tons of cotton linters (Morais et al., 2013). Traditional products made from linter are: absorbent cotton, special papers or derivatives such as cellulose nitrate or acetate (Sczostak, 2009).

Simultaneously, environmental concerns affecting the pulp and paper industry, increased interest in removing chlorine from bleaching processes, compounds known to be highly polluting. Firstly, ECF (elemental chlorine free) sequences and subsequently TCF (totally chlorine free) sequences were developed decades ago in order to reduce this environmental impact. In this work, pulps obtained from eucalyptus, sisal and flax bleached through ECF or TCF bleaching sequences linters were analyzed. For comparative purposes, cotton linters were also characterized as an example of fibers not produced by a pulping process. The aim of this analysis was to expose the characteristics of fibers from different sources while comparing the effects of ECF and TCF sequences on them.

3.2. Materials and methods

For details concerning all materials and methodologies used in the present study, please refer to **chapter 2**.

3.2.1. Raw materials

Commercial TCF and ECF bleached pulps from sisal (*Agave sisalana*), flax (*Linum usitatissimum*), eucalyptus (*Eucalyptus globulus*) and cotton linters (*Gossypium sp.*) were analyzed.

Sisal and flax fibers were obtained through a NaOH-AQ cooking process, bleached through DPo (ECF) or QPo (TCF) sequences and provided by Celesa (Spain). Eucalyptus fibers were obtained through a kraft process, bleached through ODEoD (ECF) or OOQ(PoP) (TCF) sequences and provided by Ence (Spain). Cotton linters were provided by Celsur (Spain) and prior to analysis, they were pre-beated in a valley mill for 90 minutes in order to reduce their average length (see chapter 2).

3.2.2. Pulps characterization

ISO brightness, Kappa number (KN) and viscosity of samples was determined in accordance to ISO 3688:1999, ISO 302:2004 and ISO 5351:2010, respectively.

HexA content of samples was determined through UV detection following the procedure described by Chai et al., 2001. Two hydrolysis for sample and four oxidations of each hydrolysis were performed giving rise to eight measures of HexA content.

Carbohydrate composition of samples was determined using high performance liquid chromatography (HPLC) following a modified version of TAPPI T 249 cm-09 method (Aracri and Vidal, 2011). Chromatographic analysis was performed using a 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-87H ion-exchange column. Data was collected by the refractive index detector (RID). Operating conditions were as follows: 0.6 mL/min, mobile phase H_2SO_4 6 mM and temperature 60 ºC.

Fibers drainage resistance (Schopper-Riegler degree, ºSR) and Water retention value (WRV) were measured in accordance to ISO 5267-1:1999 and ISO 23714:2014, respectively.

Fiber length was determined in accordance to TAPPI T 271 om-02 method in a Kajaani fiber analyzer (FS300, Metso automation, Finland).

Fibers zero-span tensile index was determined according to ISO 15361:2000 in a Zero-span 1000 Pulmac tester. For analysis, strips obtained from paper sheets prepared in a laboratory Rapid-Köthen lab former (ISO 5269-2:2004) were previously soaked in distilled water for 5 seconds.

TCF flax fibers core and bast fractions were separated for further analysis using a laboratory Bauer-MacNett classifier with screen sizes of 30 and 200 mesh in accordance to TAPPI T233-cm-06.

Scanning electron microscope (SEM) images of fibers were obtained using a JEOL JSM-6400 microscope operating at 10 kV. Samples were first coated with a very thin layer (14 nm thick) of gold–palladium in a sputter coater SCD005 in order to obtain a conductive surface.

3.3. Results and discussion

3.3.1. Chemical characterization

Raw materials characterized in the present study had undergone a bleaching process, and because of this, all observed brightness values were greater than 80% ISO (Figure 3-A). Also, it was observed that ECF sequences led to higher brightness values than TCF sequences, as illustrated in the chart. Concerning kappa number (Figure 3-B), TCF bleached samples presented higher values than ECF ones, which could be explained by their content in HexA (also indicated in figure). Although lignin content of these pulps was very low, it is well known that HexA could interfere into KN determination increasing its value (Li and Gellerstedt, 1997). HexA, formed during alkaline cooking, result highly attacked by chlorine-derived reagents during ECF bleaching (Costa and Colodette, 2007), which does not happen during TCF processes. Among samples, TCF bleached eucalyptus and sisal showed a high content in hexenuronic acids, while flax content was lower and cotton linters lacked of them.

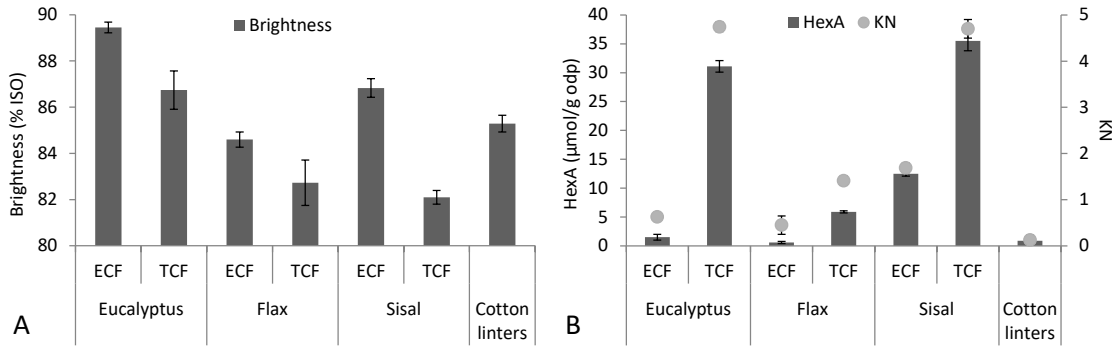

Figure 3-1: ISO brightness (A) and Kappa number (KN) and hexenuronic acid content (HexA) (B) of samples.

Carbohydrate composition of pulps is a key factor determining their behavior in further papermaking steps, but also in other processes such as the manufacture of cellulose derivatives. As could be predicted by HexA (which are contained into hemicelluloses), sisal and eucalyptus accounted for the highest hemicelluloses content,

while flax showed a significantly lower value (Table 3-1). Surprisingly, cotton linters showed a small content in xylans (2%). These hemicelluloses are not naturally contained in cotton fibers (pure cellulose fibers) and are assumed to proceed from seed cloak rests dragged during linters extraction. Finally, carbohydrate composition was also found to depend on the bleaching process. ECF bleached samples showed a content in hemicelluloses ≈ 3 points lower compared to TCF, indicating that hemicelluloses suffered a greater chemical attack during ECF bleaching.

Table 3-1: Carbohydrate composition of pulps.

% of sugar (w/w)		Glucans	Xylans	Ramnans	Glucuronic	Acetyls
Eucalyptus	ECF	84.8 ± 1.3	14.8 ± 1.1	0.1 ± 0.1	0.3 ± 0.1	-
	TCF	83.6 ± 0.4	15.9 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	-
Flax	ECF	96 ± 0.7	3.2 ± 0.3	-	0.8 ± 0.1	-
	TCF	95.3 ± 0.9	3.7 ± 0.8	-	1 ± 0.1	-
Sisal	ECF	86.6 ± 0.5	12.8 ± 0.2	-	0.6 ± 0.1	-
	TCF	82.7 ± 0.6	16.1 ± 0.3	0.2 ± 0.2	0.7 ± 0.1	0.3 ± 0.2
Cotton linters		97.7 ± 0.3	2 ± 0.2	0.1 ± 0.1	0.1 ± 0.05	0.1 ± 0.1

3.3.2. Physical characterization

Fibers viscosity is a characteristic frequently used as an indicator of cellulose integrity, as it is directly correlated to its degree of polymerization (DP). Figure 3-2A shows viscosity of samples, where it can be observed that cotton linters showed the largest viscosity whereas flax accounted for the smallest. It is widely described that TCF sequences produce a slightly larger cellulose degradation than ECF (Valls et al., 2010c), fact that is well illustrated by data. Conservation of cellulose integrity is important due to its implication on fibers mechanical performance. In this direction, wet zero-span tensile strength is an indicator of the maximum tensile load assumable by a single fiber. Observation of zero-span strength values of fibers (Figure 3-2A) revealed a tendency to correlate to cellulose viscosity, as shown in Figure 3-2B, highlighting the relation between cellulose DP and fibers mechanical performance. The only sample skipping this correlation was cotton linters (black dot in chart), which

was not obtained through a pulping process, and thereafter should be considered separately. Data also indicated that non-wood fibers such as sisal could have equivalent mechanical resistance as that of eucalyptus fibers and that TCF process slightly diminished this resistance compared to ECF bleaching.

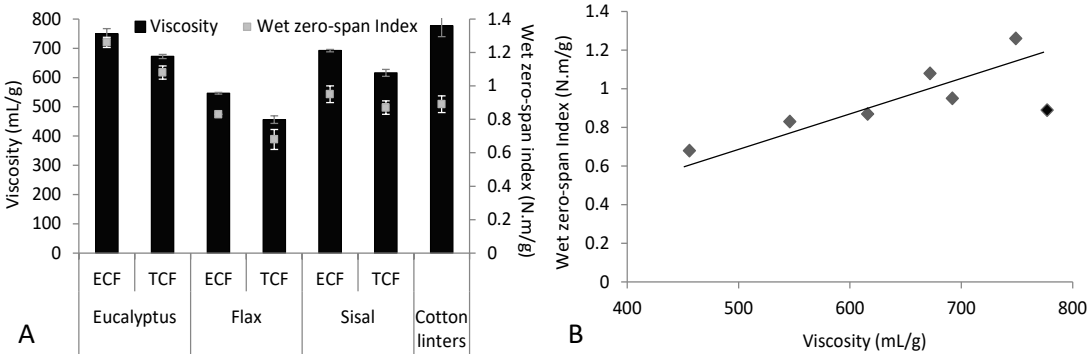

Figure 3-2: Viscosity (bars, left axis) and wet zero-span tensile index (points, right axis) (A) and correlation between both variables (B).

Concerning other physical properties, fibers water-binding capacity showed some differences depending on fibers origin (Table 3-2). Also, ECF fibers presented a smaller water retention value than when bleached without chlorine, possibly due to the smaller content in hemicelluloses, compounds with a well-known tendency for water intake. Moreover, fibers drainage resistance, as ºSR degrees is usually taken as an indicator of fibers beating degree. Table 3-2 indicates ºSR degrees of pulps, highlighting the high value showed by flax fibers, and by cotton linters, due to the pre-refining process to which linters were submitted prior to characterization, the same happening with WRV.

Lastly, fiber length (Table 3-2) showed significant differences between sources, while it did not show any affectation caused by different bleaching sequences. Differences between two calculations (nominal and length-weighted), especially notorious in flax pulp but also in sisal and cotton linters, were caused by the morphological heterogeneity among fibers. Nominal calculation favors the shorter fibers as they usually outnumber the longer ones. Because of this, length-weighted

length (L(l)) usually provides a more realistic value of average fiber longitude. As can be observed, eucalyptus provided the shortest fibers, while all non-woods were longer, with the biggest values showed by sisal and cotton linters.

Table 3-2: Water retention value (WRV), Schopper-Riegler drainage resistance (ºSR), nominal fiber length (L(n)) and length-weighted fiber length (L(l)).

		WRV	ºSR	L(n) (mm)	L(l) (mm)
Eucalyptus	ECF	1.12 ± 0.03	20 ± 1	0.65 ± 0.1	0.82 ± 0.03
	TCF	1.36 ± 0.02	16 ± 1	0.6 ± 0.05	0.79 ± 0.04
Flax	ECF	0.95 ± 0.05	27 ± 1	0.45 ± 0.04	1.45 ± 0.13
	TCF	1.08 ± 0.01	33 ± 2	0.42 ± 0.05	1.31 ± 0.15
Sisal	ECF	0.91 ± 0.01	15 ± 1	1.4 ± 0.15	1.91 ± 0.1
	TCF	0.99 ± 0.01	16 ± 1	1.48 ± 0.12	2.16 ± 0.16
Cotton linters		1.38 ± 0.02	47 ± 2	1.08 ± 0.09	1.95 ± 0.1

As stated on introduction, flax fibers are composed by two well-differentiated fractions. For a better characterization of this raw material, we proceeded to their separation and characterization in two separate groups. Core fibers accounted only for ≈30% of total fiber mass, while bast fibers represented the largest amount (≈70%). Table 3-3 shows carbohydrate composition and HexA content of both fractions, where remarkable differences in composition between them can be observed. Core fibers showed a higher hemicelluloses and HexA content, highlighting the quality difference between fractions explained in introduction. Surprisingly, we observed that hemicelluloses and HexA content in unfractionated pulp was different than the content of each fraction after fractionation (proportionally weighted). The reason for this difference was thought to be caused by separation procedure in the Bauer McNett equipment, process in which fines fraction is lost and thereafter pulp composition modified.

Table 3-3: Carbohydrate composition and HexA content of flax fiber fractions.

	Flax TCF	
% of sugar (w/w)	Core fibers	Bast fibers
Glucans (%)	92.2 ± 0.9	95.6 ± 0.5
Xylans (%)	7.1 ± 0.4	3.8 ± 0.2
Arabinans (%)	-	-
Ramnans (%)	0.05 ± 0.01	0.05 ± 0.01
Glucoronic (%)	0.6 ± 0.3	0.3 ± 0.1
Acetyls (%)	-	0.2 ± 0.2
HexA (μmol/g odp)	3.6 ± 0.3	2.4 ± 0.4

3.3.3. SEM microscopy

Images of fibers (Figure 3-3) suggested the presence of cleaner fiber surfaces in ECF fibers (left pictures) compared to TCF (right), *i.e.* TCF fibers presented a larger amount of small fibrils on their surface. Possibly, the greater removal of hemicelluloses produced by ECF bleaching (previously commented) enhanced the cleaning of fibers surface. Among fiber sources, flax pulp showed a higher amount of thin elements forming the network observable in Figure 3-3C and D and not present in other fibers. Considering the higher ºSR value presented by these pulps (Table 3-2), these smaller elements could be consequence of a beating process to which fibers are occasionally submitted before commercialization. This evidence is also shown by Figure 3-3G, which represents cotton linters, beated before analysis.

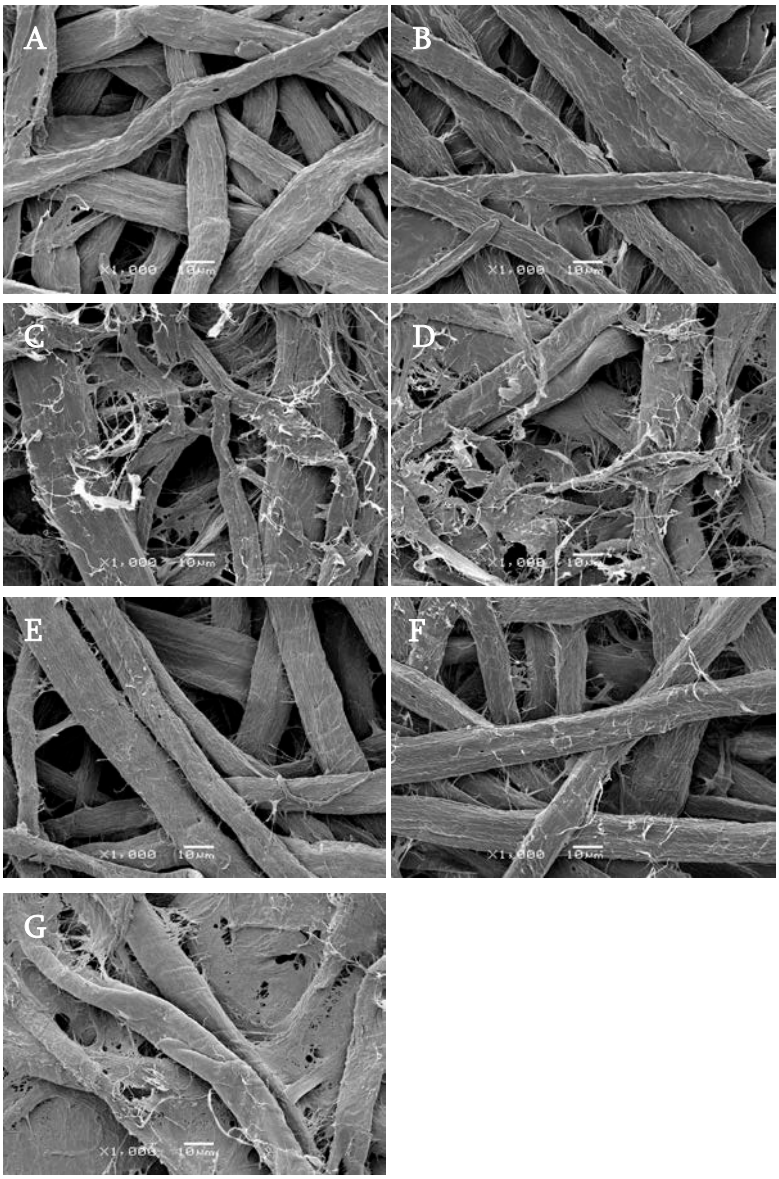

Figure 3-3: SEM micrographs of cellulose fibers. Images correspond to: Eucalyptus ECF (A), TCF (B); flax ECF (C), TCF (D); sisal ECF (E), TCF (F) and cotton linters (G).

3.4. Conclusions

Characterization of raw materials for the present thesis provided a general overview of their characteristics and allowed choosing the best option fitting each application. Generally, fiber properties showed significant differences between sources, including a correlation found between their viscosity and mechanical performance. Also, we observed that TCF fibers showed a slightly lower quality compared to their ECF counterparts for each fiber source. However, the enormous environmental benefits provided by completely eliminating chlorine from bleaching processes could compensate this quality loss, making TCF fibers a good choice for several applications. In the light of the results obtained, cotton linters was chosen as the raw material for the optimization of NCC preparation process aided with biotechnological methods due to its non-wood origin and naturally high-cellulose content. On the other hand, TCF sisal pulp was chosen for the studies concerning hemicelluloses removal via enzymatic treatments, in order to obtain high-cellulose content fibers for NCC preparation. The reason of the selection of sisal must be found also in its non-wood origin and in the fact that in presented the highest hemicelluloses content, being the best target for the study of hemicelluloses-removing treatments.

3.5. References

Aracri, E.; Vidal, T. Xylanase- and Laccase-Aided Hexenuronic Acids and Lignin Removal from Specialty Sisal Fibres. Carbohydr. Polym. 2011, 83 (3), 1355–1362.

Aracri, E.; Vidal, T. Enhancing the Effectiveness of a Laccase-TEMPO Treatment Has a Biorefining Effect on Sisal Cellulose Fibres. Cellulose 2012, 19 (3), 867–877.

Barneto, A. G.; Vila, C.; Ariza, J.; Vidal, T. Thermogravimetric Measurement of Amorphous Cellulose Content in Flax Fibre and Flax Pulp. Cellulose 2010, 18 (1), 17–31.

Chai, X.-S.; Zhu, J. Y.; Li, J. A Simple and Rapid Method to Determine Hexeneuronic Acid Groups in Chemical Pulps. J. Pulp Pap. Sci. 2001, 27 (5), 165–170.

Costa, M. M.; Colodette, J. L. The Impact of Kappa Number Composition on Eucalyptus Kraft Pulp Bleachability. Brazilian J. Chem. Eng. 2007, 24 (1), 61–71.

Fillat, A.; Roncero, M. B.; Vidal, T. Elucidating the Effects of Laccase-Modifying Compounds Treatments on Bast and Core Fibers in Flax Pulp. Biotechnol. Bioeng. 2012, 109 (1), 225–233.

Gümüskaya, E.; Usta, M.; Kirci, H. The Effects of Various Pulping Conditions on Crystalline Structure of Cellulose in Cotton Linters. Polym. Degrad. Stab. 2003, 81 (3), 559–564.

Hart, P. W.; Santos, R. B. Changing the Face of Short Fiber - A Review of the Eucalyptus Revolution. Tappi J. 2015, 14 (6), 353–359.

Hurter, R. W. TCF Bleached Sisal Market Pulp: Potential Reinforcing Fibre for Commodity Papers .1. Tappi Proc. 1997, 501–512.

Klemm, D.; Heublein, B.; Fink, H. P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie - Int. Ed. 2005, 44 (22), 3358–3393.

Li, J.; Gellerstedt, G. The Contribution to Kappa Number from Hexeneuronic Acid Groups in Pulp Xylan. Carbohydr. Res. 1997, 302 (3-4), 213–218.

Maddern, K. N.; French, J. The Potential Application of Nonwood Fibers in Papermaking - an Australian Perspective. Appita J. 1995, 48 (3), 191–196.

Morais, J. P. S.; Rosa, M. D. F.; de Souza Filho, M. D. S. M.; Nascimento, L. D.; do Nascimento, D. M.; Cassales, A. R. Extraction and Characterization of Nanocellulose Structures from Raw Cotton Linter. Carbohydr. Polym. 2013, 91 (1), 229–235.

Sczostak, A. Cotton Linters: An Alternative Cellulosic Raw Material. Macromol. Symp. 2009, 280 (1), 45–53.

Valls, C.; Molina, S.; Vidal, T.; del Río, J. C.; Colom, J. F.; Martínez, Á. T.; Gutiérrez, A.; Roncero, M. B. Influence of Operation Conditions on Laccase-Mediator Removal of Sterols from Eucalypt Pulp. Process Biochem. 2009, 44 (9), 1032–1038.

Valls, C.; Gallardo, Ó.; Vidal, T.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B. Performance of New and Commercial Xylanases for ECF and TCF Bleaching of Eucalyptus Kraft Pulp. Wood Sci. Technol. 2010, 45 (3), 433–448.

Ververis, C.; Georghiou, K.; Christodoulakis, N.; Santas, P.; Santas, R. Fiber Dimensions, Lignin and Cellulose Content of Various Plant Materials and Their Suitability for Paper Production. Ind. Crops Prod. 2004, 19, 245–254.

Chapter 4

Introducing an enzymatic pretreatment into NCC isolation process

Abstract

In this chapter the introduction of a cellulase pretreatment prior to NCC isolation was assessed. For this purpose and according to chapter 3, a naturally pure cellulosic source such as cotton linters was used. NCC was produced using sulfuric acid at two different concentrations (62 and 64 % wt.). The effect of pore size for filtration step was also assessed. The smaller acid dose leaded to yields up to 65-70 % and average size up to 160 nm. It also produced crystals with reduced sulfur content (0.6-1%). Cellulase pretreatment influenced NCC characteristics, as it increased overall yield in 12 points, increased average particle size in around 35 nm and reduced NCC sulfur content up to a 0.8 points. We found that different conditions of enzymatic treatments led to quantitative differences on their effects on NCC. The evidence presented in this chapter suggested that pretreating fibers with this cellulase represents a very interesting option to partially replace chemicals on NCC isolation process.

4.1. Introduction

Nowadays, with the world facing an alarming situation of shortage of non-renewable resources such as coal, petroleum and natural gas, there is a growing interest in the use of renewable resources to fulfill the necessities of our society (Brito et al., 2012; Xu et al., 2013). In this scenario, cellulose, which is considered to be one of the most important renewable polymers on earth, offers a wide range of possibilities (Brinchi et al., 2013). Cellulose annual production is estimated to be over 7.5 x 10^{10} tons in our planet (Habibi et al., 2010). Due to this availability, it has been used for centuries in the form of wood or plant fibers as an energy source, building materials, paper or clothing. Many of these uses continue nowadays, fact that is verified by the huge number of cellulose-based industries existing in the present day (paper, textiles, etc.). Although these long-known applications are still benefiting our society in the present, during the last decade cellulose has been receiving a new and growing interest due to the understanding that fibers are built by smaller entities that could be extracted from them under proper conditions (Charreau et al., 2013). Exploring efficient ways to extract these smaller entities (crystalline regions) from fibers has attracted plenty of

attention of authors during the last years, fact that can be observed in the growing number of patents related to this field published since year 2000 (Charreau et al., 2013).

The more extensively used method to obtain these crystalline regions consists of a controlled hydrolysis with sulfuric acid, basically due to the stability of the resulting suspensions (Abitbol et al., 2013; Brinchi et al., 2013). During this reaction, amorphous domains are attacked preferentially, while crystalline regions present higher resistance to acid attack (Klemm et al., 2011). Microfibrils are then destructed at their "defects", leading to the release of rod-like particles, the former crystalline regions, now nanocrystals. This differentiated susceptibility to acid attack is thought to be provoked by differences in the kinetics of hydrolysis between amorphous and crystalline domains, where the first ones are more rapidly accessible by acid and thereafter, hydrolyzed first (Habibi et al., 2010).

During the last decades, authors have untiringly studied ways to introduce biotechnology in the cellulose-related industry. Within these studies, among all the available options, enzymes have been preferentially chosen due to special features they present (high specificity, environmental friendliness, etc.). According to Brinchi et al., 2013, limited literature has yet been published for ways to introduce enzymes in the preparation process of nanocrystalline cellulose (NCC). From the wide range of options of enzymatic activities existing on nature, cellulases (E.C. 3.2.1.4) have a special interest for our objective of breaking down the hierarchical structure of cellulose (Garcia-Ubasart et al., 2013). Examples of cellulase application for NCC (nanocrystalline cellulose) or MFC (microfibrilar cellulose) isolation available in literature generally include a first chemical step in which cellulose is treated in order to weaken its structure and then a second step in which it is enzymatically treated to finally isolate NCC or MFC (Filson et al., 2009; Chen et al., 2012; Anderson et al., 2014). Furthermore, the use of enzymes could be a good strategy to address one of the most important obstacles to the industrial production of NCC, which are the low yields typically offered by isolation processes (Chen et al., 2015).

In chapter 3 it was exposed that cotton linters constitute a raw material naturally presenting a high-content in cellulose, the preferential source for NCC preparation. In this chapter, the objective was to facilitate the acid hydrolysis step by pre-weakening cellulose structure in cotton linters with a cellulase pretreatment. To our best knowledge, this was the first time that a cellulase pretreatment was reported

to be used before sulfuric acid hydrolysis for NCC preparation. The objective was then to evaluate the consequences of this pretreatment on the characteristics presented by NCC, in order to partially replace the use of harsh chemicals in this process and possibly to increase process yield.

4.2. Materials and Methods

For details concerning all materials and methodologies used in the present study, please refer to **chapter 2**.

4.2.1. Raw material and enzymatic treatment

Cotton linters (cellulose content 97.7 ± 0.3 %) were used as cellulose source. They were provided by Celsur (Spain) and were refined for 90 minutes on a valley mill in order to reduce their average length. Obtained fibers were named as initial.

A cellulase preparation (named "C"), provided by Fungal Bioproducts® (Spain) and obtained from *Cerrena sp.* fungus was used for treatments. Activity as U/g from enzyme stock was 1700. Activity was expressed as CMCase units, that is to say, the amount of enzyme degrading 1 μmol of CMC (carboxymethilcellulose) per minute. Treatments with C were carried out according to three different conditions (Table 4-1). Two different reactors were used for treatments; treatment 1 was carried out in a cylindrical 4 L reactor with agitation produced by rotating blades at 30 rpm. Treatments 2 and 3 were performed in an Ahiba Easydye (Datacolor, USA) apparatus having 250 mL independent units with agitation consisting on upside-down inversions at 20 oscillations per minute. When corresponding (Table 4-1), 50 mM acetate buffer was used to set the pH to 5 during enzymatic treatment. In all cases enzyme was deactivated after reaction by increasing temperature to 105 ºC for 15 min. Fibers were filtered using a Nº2 filter and reaction liquor was passed through fibers 3 times in order to recover fines. No washing was performed after enzymatic treatments to avoid sample loss. Control fibers, as indicated on Table 4-1, were treated in all cases with the same conditions as enzyme-treated fibers but without enzyme addition and were referred as "KC".

Table 4-1: Enzymatic treatment conditions.

		Treatment 1 (C1)	Treatment 2 (C2)	Treatment 3 (C3)
Enzyme dose	10 U/g odp			
Treatment time	24 h	✓	✓	✓
Temperature	55 ºC			
pH	5 (50 mM acetate buffer)	✓	✓	
	5 (adjusted with H_2SO_4)			✓
Reactor	4L blade stirred reactor	✓		
	250mL Ahiba Easydye®		✓	✓
Agitation	Rotating blades - 30 rpm	✓		
	Oscillating - 20 rpm		✓	✓
Control fibers	No enzyme addition	KC1	KC2	KC3

4.2.2. Nanocrystalline cellulose preparation

Nanocrystalline cellulose (NCC) was obtained by a controlled sulfuric acid hydrolysis, adapting to our conditions the protocol proposed by Dong et al., 1998. Fibers were fluffed prior to hydrolysis, then oven dried and cooled in a desiccator. Typically, 1.5 g of sample weighted immediately from desiccator were hydrolyzed with 62 % or 64 % (w/w) sulfuric acid for 45 min at 45 ºC with magnetic stirring and an acid-to-fibers ratio of 10:1 (*i.e.* 10 mL/1 g cellulose). Final samples were filtered through Whatman ashless paper filters, Nº 40 (pore size 8 µm) or 41 (pore size 20-25 µm). The whole process of preparation was repeated three times for each sample in order to ensure repeatability. In text, NCC samples will be noted as X_NCC (*e.g.* Initial or C1 indicate fiber samples, while C1_NCC or initial_NCC refering to the NCC isolated from these fibers).

4.2.3. Fibers and NCC characterization

Initial, enzymatically treated and control fibers were characterized in terms of fiber length, viscosity, zeta potential, cationic demand, free hydroxyl content, crystallinity and water contact angle.

NCC samples were characterized in terms of yield, sulfur content, particle size, surface charge, zeta potential, water contact angle and viscosity. NCC films were prepared by evaporation of aqueous phase of NCC suspensions (water) in an air circulating oven at 60ºC.

Fibers length, width and % of fines were measured using Kajaani fiber analyzer (FS300, Metso automation, Finland) according to TAPPI T 271 om-02 and viscosity of fibers and NCC suspensions was determined according to ISO 5351:2010.

Zeta potential of fibers was determined according to Cadena et al., 2009 using a Mütek zeta potential equipment (SZP-06, Mütek, Germany), while electrophoretic mobility of aqueous NCC suspensions was determined using Malvern instrument Zetamaster (model ZEM, Malvern instruments, UK).

Free hydroxyl content of fibers was determined according to the protocol described by Genung, 1950.

Water contact angle of fibers and NCC film samples was measured using a Dataphysics OCA15EC contact angle goniophotometer (Dataphysics, USA).

Surface charge of fiber and NCC suspensions was determined using Mütek particle charge detector (PCD03PH, Mütek, Germany).

Yield of NCC isolation process was determined by quantifying total solids in suspension and expressed as % of NCC mass compared to initial fiber mass.

Sulfur content of NCC was determined according to a procedure proposed by Abitbol et al., 2013.

Particle size of NCC samples was determined using a particle size analyzer (DL135, Cordouan Technologies, France).

Cellulose fibers FTIR spectra were recorded at room temperature using an ATR-FTIR spectrophotometer (Spectrum 100, Perkin Elmer, USA). FTIR spectral analysis was conducted within the wavenumber range of 600-4000 cm^{-1}. Total crystallinity index (TCI), proposed by Nelson and O'Connor, 1964, was estimated from the ratio between the absorption peaks at 1370cm^{-1} and 2900cm^{-1} bands.

4.2.4. Optical and SEM microscopy

Images of fibers were obtained using an Olympus BH-2 optical microscope connected to a digital camera. Images of NCC films were obtained using a scanning electron microscope (SEM) (JSM-6400, JEOL, Japan).

4.2.5. Released oligosaccharides

Reducing sugars were identified and quantified in supernatant of centrifuges of NCC preparation process and liquid phase of final suspensions using a HPLC instrument (1100, Agilent technologies, USA) furnished with a BIO RAD Aminex HPX-42A ion-exchange column.

4.3. Results and discussion

As stated on introduction, the objective of the present chapter was to evaluate the effects of an enzymatic pretreatment with a cellulase on the NCC isolation process itself and on its final characteristics. For this purpose, enzymatic treatments were held and resulting fibers characterized. After this characterization, NCC were prepared from the different fiber samples.

4.3.1. Cellulase treatments

In literature, studied cellulases could produce a fibrillation effect on fiber surface, which does not necessary imply a reduction in average length or viscosity, as seen by other authors (Garcia-Ubasart et al., 2013). However, previously reported results treating dissolving-grade fibers (Quintana et al., 2015a, 2015b) , indicated that the cellulase used in this study ("C") produced a shortening effect in fiber length, different to the fibrillation effect produced by another cellulase used on the same study. Being the action of isolating cellulose crystalline regions a deconstruction process which takes advantage of the hierarchical structure of this material (Habibi et al., 2010; Brinchi et al., 2013), it was likely that a cellulase could take part on it. Treatments with this enzyme were performed under three different conditions for a long period (24 h) in order to ensure the noticeability of its effects (Table 4-1).

Firstly, fiber width (Table 4-2) showed the effects of C, as it was reduced a 9 % after treatments in the three cases, compared to control fibers. Regarding treatment 1, viscosity was highly reduced, suffering a reduction of about 500 mL/g (Table 4-2). This reduction was of course related to enzyme's cleavage effect of cellulose microfibrils, which leaded to a reduction of their average degree of polymerization. Also, this

cutting effect of C modified fibers macroscopically, reducing average fiber length in ≈0.3 mm and increasing fines amount in about 2 points compared to KC1. Treatment 2, which was conducted at the same pH, temperature and enzyme dose as in treatment 1 but in a different reactor, produced different effects on fibers. These differences can then only be attributable to the reactor, where the smaller volume and the stronger mixing led to a different interaction between fibers and enzyme. This statement was supported by fiber length data as C2 fibers showed a lower value (≈0.5 mm) compared to C1 fibers (≈1.5 mm). Fines amount, also experienced a higher increase in this case compared to control pulps (≈11 points). One plausible explanation for these two contrasting evidences relays on the stronger mixing performed by Ahiba Easydye® reactor, which could have enhanced the physical separation of enzymatically pre-hydrolyzed fibers, leading to a higher fines amount and also to shorter fibers. Treatment 3 was carried out with the objective of studying the influence of the absence of buffer during enzymatic treatments for NCC preparation. Comparing to treatment 2, it produced the same effect on fibers in terms of fiber length and viscosity. Enzymatic effects on shortening fibers can also be evidenced in optical microscope images (Figure 4-1).

Finally, FTIR analysis provided evidence that C treatment increased average crystallinity of fibers. Obtained TCI values for KC and C fibers were 0.69 and 0.92, respectively, indicating an increase in fibers crystallinity. Generally, TCI is claimed to be proportional to cellulose crystallinity index (Široký et al., 2010), and therefore this increase suggested that enzyme attacked preferentially the amorphous regions of cellulose.

Table 4-2: Fiber characteristics of initial, enzymatically treated (C) and control (KC) samples after treatments 1, 2 and 3.

	Fiber length (mm)	Fiber width (μm)	Fines (%)	Viscosity (mL/g)
Initial	1.95 ± 0.10	22 ± 0.2	39.7 ± 0.7	777 ± 37
C1	1.51 ± 0.02	20.2 ± 0.2	43.5 ± 0.4	256 ± 17
KC1	1.82 ± 0.04	22.1 ± 0.1	41.2 ± 0.1	737 ± 20
C2	0.52 ± 0.10	20.1 ± 0.2	52.9 ± 0.6	346 ± 13
KC2	1.64 ± 0.07	22 ± 0.1	42.1 ± 0.4	790 ± 24
C3	0.55 ± 0.20	20 ± 0.4	54.6 ± 1.2	369 ± 24
KC3	1.67 ± 0.03	22 ± 0.2	44.2 ± 0.1	775 ± 15

Regarding surface characteristics (Table 4-3), fibers resulted chemically modified after cellulase treatment. It can be seen how the amount of free hydroxyl groups on cellulose fibers increased a 9 % after cellulase treatment. Probably, after cutting cellulose chains, OH- groups that were hidden resulted then exposed, increasing the total number of free (accessible) hydroxyl groups in relation to cellulose mass. It is important to remark as well that these newly accessible OH- could be in more than one carbon position of each glucose residue, being not all of them equally reactable (Gu et al., 2013). Concerning cationic demand, values of fibers were very low and no differences were observable between samples. Zeta potential values showed the effect of buffer, as both C and KC treatments had smaller values than initial fibers. This difference in zeta potential was thought to be caused by a modification of ionic distribution around cellulose fibers caused by ions provided by buffer. Contact angle of fibers was not affected by enzymatic treatments, and the observed value (≈23º) indicated that fibers were hydrophilic. A reported contact angle value for initial cotton linters by another author (Morais et al., 2013) indicated values around 70º, very different from those reported in Table 4-3. This difference was possibly caused by the presence of non-cellulosic compounds on the raw cotton linters used in that study, different from the washed white linters used in this case. Finally, yield (as % of recovered dried fibers) was calculated after treatment 1, obtaining a value of 90.2 % after C1 and 97.8 % after KC1.

Table 4-3: Surface characteristics of initial, control and treated fibers after treatment 1.

	Free OH- (%)	Surface charge (μeq/g)	Zeta potential (mV)	Contact angle (º)
Initial	5.58 ± 0.02	1.8 ± 0.64	-123.9 ± 15.3	23 ± 1
C1	6.22 ± 0.13	1.3 ± 0.32	-74.1 ± 12.2	24 ± 1
KC1	5.71 ± 0.03	1.34 ± 0.1	-53.2 ± 10.6	23 ± 2

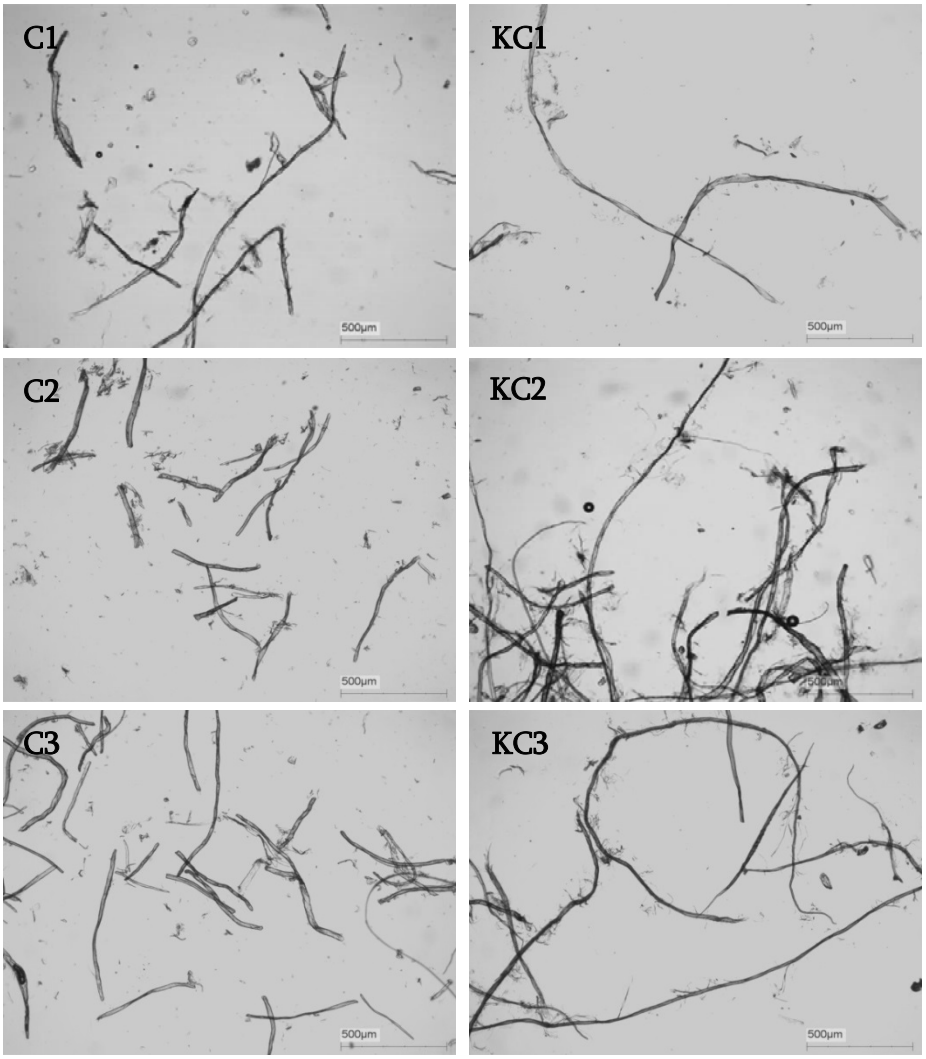

Figure 4-1: Optical microscope images of enzymatically treated fibers (left) and control fibers (right).

4.3.2. Effect of conditions of cellulase treatment on NCC preparation

In order to further understand the effects of this enzyme on NCC preparation and final characteristics, NCC were prepared after fibers obtained in treatments with C (1, 2 and 3). Two different acid doses were used for trials. One dose (64 % wt.) which

was chosen based on evidence obtained from previous work of our group and considered a standard concentration for NCC preparation, and another dose thought to be weaker (62 % wt.). Results are indicated in Table 4-4 and all samples were filtered through the №41 membrane.

Yield of NCC preparation process is a key aspect to be analyzed due to its evident impact on the economic cost of the whole process. Acid dose had a major impact on yield, as NCC yields about a 30-35 points bigger were obtained for hydrolysis carried out with 62 % H_2SO_4 compared to 64 % (Figure 4-2). This evidence accorded with the results reported by several authors, where smaller acid concentrations resulted in higher yields due to weaker hydrolysis (Bondeson et al., 2006; Lu et al., 2013). Regarding enzymatic treatments, C1 and C2 fibers led to higher yields with both acid concentrations compared to NCC obtained after C3 fibers.

Table 4-4: Yield, average size and sulfur content of NCC samples obtained from the different enzymatic treatments.

	62 % wt. H_2SO_4			64 % wt. H_2SO_4	
Cellulase treatment	C1	C2	C3	C1	C3
Yield (%)	71.3 ± 4.4	74.2 ± 0.1	61.7 ± 2.8	28.4 ± 2.6	26.4 ± 2.6
Z average (nm)	158 ± 5	163 ± 2	123 ± 4	92 ± 3	98 ± 1
Sulfur content (% S)	0.69 ± 0.01	0.75 ± 0.03	0.87 ± 0.06	1.17 ± 0.09	1.89 ± 0.5

Size of NCC, by its side, as measured by DLS did not provide a real value of their dimensions due to the rod-like structure they present (Fraschini et al., 2014). DLS uses laser scattering to measure radio (diameters) of particles and with this data calculates particles hydrodynamic diameters considering all elements to be spheres (Fraschini et al., 2014). Still, authors have used DLS data to establish comparisons between NCC samples, being aware that this value was not necessary representing their true physical size (Habibi et al., 2010; Brito et al., 2012). Hydrodynamic diameters in Figure 4-2A show that acid dose also had great influence on size. Bigger NCC seemed to be obtained with the weaker acid treatment (62 %) with values around 30-60 nm bigger compared to those obtained with 64 % acid. Regarding cellulase

treatments, C1_NCC and C2_NCC again showed very similar values, yielding ≈35 nm bigger crystals with 62 % sulfuric acid compared to C3_NCC. With 64 % acid, differences were small. On Figure 4-2A it can be observed that both particle size and yield seem to be related, fact that will be further discussed in the following section.

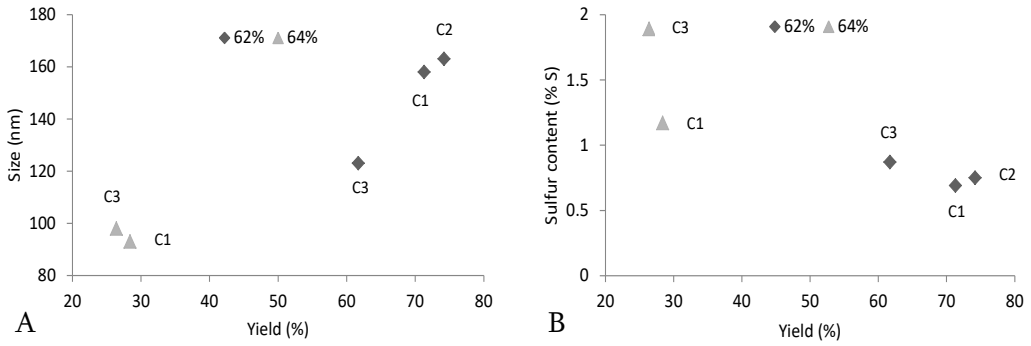

Figure 4-2: Size (A) and sulfur content (B) of NCC samples expressed as a function of yield for 62% (diamonds) and 64% (triangles) H_2SO_4.

Sulfur content of NCC, in the form of sulfate groups attached to their surface determines their behavior in several aspects. Among other, their presence is crucial for the stabilization of crystal suspensions in water, (Abitbol et al., 2013; Habibi et al., 2010). In spite of this benefit, they compromise NCC thermostability (Roman and Winter, 2004; Gu et al., 2013). Thus, an optimal sulfur content value considering advantages and inconveniences should be found. Regarding acid dose, sulfur content (Figure 4-2B) showed bigger values for NCC obtained with sulfuric acid at the higher concentration (64 % wt.) compared to the lower (62 % wt.), with differences among 0.5-0.8% sulfur. This evidence has already been reported and is explained by the fact that SO_4^{2-} groups are incorporated to crystals by an esterification reaction (Habibi 2010; Abitbol et al. 2013) (Figure 4-3B). As water is a reaction product in esterifications, having it in smaller amounts (as happens for the acid at 64 %) enhances its occurring compared to smaller acid concentrations, where water content is higher (Roman and Winter, 2004). Among samples, C3_NCC presented the highest sulfur content for both acid concentrations studied, with higher differences between C1_NCC and C3_NCC at

64 % sulfuric acid. In this case, sulfur content 0.7 points higher was measured in C3_NCC. Data on Figure 4-2 suggested that acetate buffer affected NCC isolation process, fact that arises from the comparison of C3_NCC with C2_NCC, as the lack on buffer on the former treatment is the only difference between these samples. In this direction, buffer presence seemed to increase yield and particle size and reduce sulfur content.

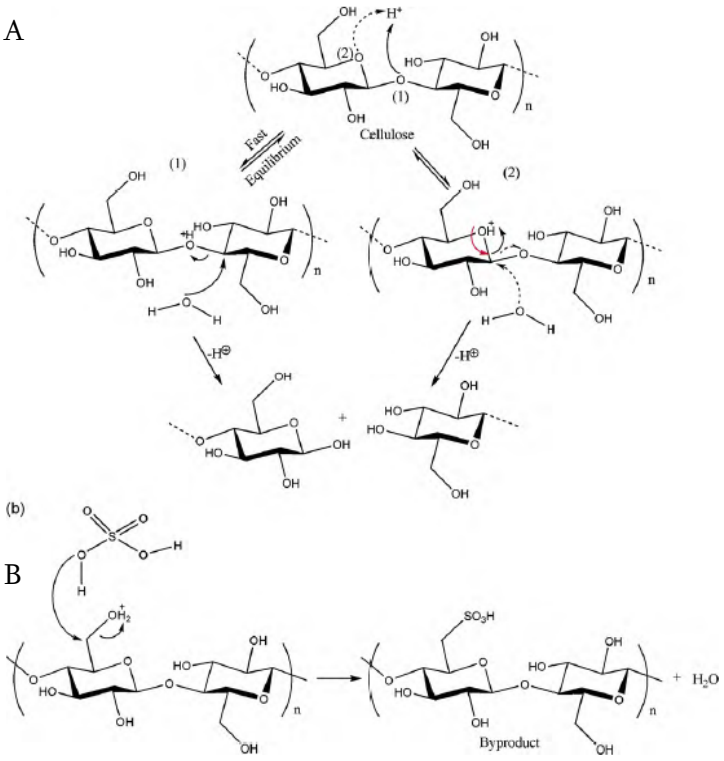

Figure 4-3: Acid hydrolysis mechanism (A) and sulfate esterification on NCC surface (B). From Lu and Hsieh, (2010).

Table 4-5 indicates schematically the different effects of cellulase treatments on NCC properties related to initial_ NCC. At both acid doses, data reflected an: Increase in yield, decrease in sulfur content and increase in average size as a consequence of cellulase treatments. A further study will be discussed in the following section for properly studying this evidence. Data remarked the fact that C effects were in all cases

more visible at the lower acid concentration (62 % wt.). Also, it can be observed how cellulase treatments in buffer presence provided better results than in its absence (C3). Additionally, with 62 % acid, C3 fibers performed worse yielding NCC than initial fibers did, providing evidence that treatment 3 was not useful for NCC production. Finally, treatments 1 and 2 provided very similar results on NCC. However, the reactor used for treatment 1 offered better conditions for a treatment intended for its industrial application. Therefore, the influence of C1 treatment on NCC will be further thoroughly analyzed.

Table 4-5: Effects of enzymatic treatments as differences in NCC characteristics between each indicated NCC sample and NCC from initial fibers. Samples were filtered using Whatman 41 membrane.

	H_2SO_4 62%			H_2SO_4 64%	
	C1_NCC	C2_NCC	C3_NCC	C1_NCC	C3_NCC
Increase in yield (% yield)	5.8	8.7	-3.8	5.1	3.1
Decrease in sulfur content (% S)	0.43	0.37	0.25	1.14	0.42
Increase in size (nm)	31	36	-4	14	19

4.3.3. Effects of enzymatic treatment and acid dose on NCC

As no agreement seems to exist in bibliography regarding pore size for the filtration step in nanowhisker preparation process, we used two different filter papers with two different pore sizes to study their influence. Yield was found to be affected by the used filter, with yields between 3 and 8 % bigger obtained with Whatman 41 filter compared to Whatman 40 (Figure 4-4). Particle size was also affected in the same direction. The rest of properties did not show any affectation.

4.3.3.1. NCC properties

For a proper understanding of C enzyme influence on NCC, characterization results (Table 4-6) were compared with KC1_NCC, attributing further differences from initial pulp to buffer effect.

Table 4-6: Yield, sulfur content and surface charge values of NCC samples from C1, KC1 and initial fibers.

Sulfuric acid (% wt.)	Filter nº (Whatman®)	Enzymatic treatment	Yield (%)	Sulfurs (% S)	Surf. charge (μeq/g)
62	40	C1	68.4 ± 3.1	0.65 ± 0.03	0.208 ± 0.007
		KC1	59.7 ± 1.4	0.74 ± 0.03	0.215 ± 0.012
		Initial	57.2 ± 1.6	1.16 ± 0.05	0.238 ± 0.006
	41	C1	71.3 ± 4.4	0.69 ± 0.01	0.216 ± 0.002
		KC1	64.3 ± 3	0.75 ± 0.03	0.217 ± 0.003
		Initial	65.5 ± 1.1	1.12 ± 0.08	0.241 ± 0.003
64	40	C1	27.6 ± 2.8	1.18 ± 0.03	0.240 ± 0.021
		KC1	26.3 ± 1.2	1.27 ± 0.07	0.263 ± 0.013
		Initial	23.0 ± 0.7	2.30 ± 0.10	0.261 ± 0.009
	41	C1	28.4 ± 2.6	1.17 ± 0.09	0.252 ± 0.005
		KC1	26.4 ± 0.3	1.26 ± 0.12	0.245 ± 0.007
		Initial	23.3 ± 0.8	2.31 ± 0.06	0.265 ± 0.011

Again, enzymatic pre-treatment on fibers increased NCC yield, particularly with 62 % acid, with yields 7-8 points higher (Figure 4-4). Our hypothesis for explaining this behavior was related to a different interaction between acid and cellulose fibrils after being cleaved by C action. Reduction of cellulose chains length by enzyme (indicated by viscosity values) and reduction in fibers dimensions (Table 4-2) might have modified fiber-acid interaction. Crystalline regions are more difficult to be degraded by cellulases (Ahola et al., 2008), and according to previously mentioned crystallinity measurements, amorphous regions seem to have been selectively degraded by C1 treatment, improving accessibility of acid to crystalline regions. We understand that this improvement in accessibility reduced the existence of obstacles, reducing the need of degrading cellulose mass to reach crystalline regions. This evidence was also

supported by % of free OH-, which indicated that C fibers had a bigger number of OH-groups exposed compared to KC and initial fibers (Table 4-2). This meant that a larger fraction of surface of fibrils was exposed, increasing accessibility to acid on fibers internal structure. Finally, in order to have a more realistic idea of the gain in yield provided by C treatment, the yield of the enzymatic treatment on fibers (90.2 %) must be considered, as cellulolytic activities imply some cellulose mass conversion to oligosaccharides. After taking this into consideration, only treatments with 62 % sulfuric acid provided higher yields comparing C and KC treatments, as the gain in yield produced by cellulase was smaller with 64 % sulfuric acid.

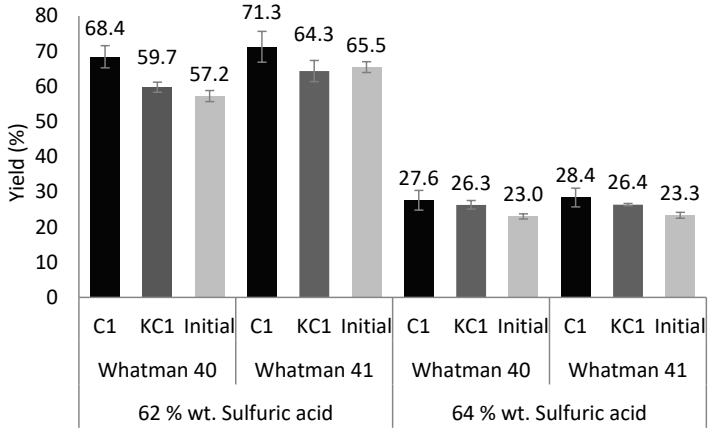

Figure 4-4: Yield of NCC preparation process from C1, KC1 and initial fibers at the different studied conditions.

Regarding crystals size, differences produced by enzyme were only significant for samples at 62% acid, as C1_NCC crystals were 25-30 nm larger than KC1_NCC (Table 4-7). Then, if yield is plotted against sample size (Figure 4-5) a linear correlation seemed to be found linking both parameters. This linear relation suggested that the increase in yield, meaning an increase in obtained NCC mass, was related to an increase in average particle size, which ultimately implied a higher mass per crystal unit. This finding supports our hypothesis for the mechanism behind the increase in yield after C treatment, which consisted in a facilitated interaction between cellulose and acid, reducing unnecessary cellulose mass loss.

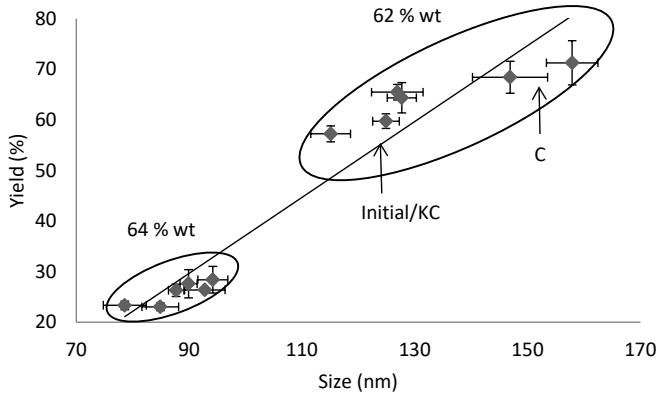

Figure 4-5: NCC average size vs yield. Circles indicate data with 62% and 64% wt. H_2SO_4. All samples were obtained from initial, C1 and KC1 fibers.

Table 4-7: Average size, electrophoretic mobility and water contact angle of NCC samples.

Sulfuric acid (% wt)	Filter nº (Whatman®)	Enzymatic treatment	Z average (nm)	Zeta potential (mV)	WCA (º)
62%	40	C1	147 ± 7	-53.7 ± 1.1	41 ± 5
		KC1	125 ± 2	-53.6 ± 1.1	50 ± 5
		Initial	115 ± 4	-55.5 ± 0.6	47 ± 3
	41	C1	158 ± 5	-53.5 ± 1.3	39 ± 4
		KC1	128 ± 3	-54.8 ± 1	50 ± 2
		Initial	127 ± 5	-55.9 ± 0.8	48 ± 4
64%	40	C1	90 ± 2	-50.1 ± 1	44 ± 4
		KC1	88 ± 1	-52.7 ± 1.2	51 ± 7
		Initial	85 ± 3	-54.5 ± 1.6	54 ± 3
	41	C1	93 ± 3	-49.6 ± 1.3	43 ± 3
		KC1	92 ± 4	-54.6 ± 1.2	48 ± 2
		Initial	79 ± 4	-56.5 ± 1.1	52 ± 3

Comparison between C1_NCC and KC1_NCC also confirmed that C treatment affected the incorporation of sulfate groups onto NCC (Figure 4-6). C1_NCC sulfur content was smaller (0.1 % sulfur) than KC1_NCC. If compared to initial_NCC,

enzymatic effects were bigger. This might be related to the difference in free OH- groups available in fibers after C treatment (Table 4-2). It is well known that sulfate groups incorporation occurs by an esterification with free OH- present in cellulose (Habibi et al., 2010). However, this reaction is not equally allowed to occur in all free OH- positions from glucose residues, as OH- reactivity depends on its specific carbon position (Gu et al., 2013). A modification in OH- distribution on cellulose surface may have been the cause for this reduction in SO_4^{2-} incorporation onto NCC. Sulfur content was also influenced by acetate (Figure 4-6). Generally, sulfur contents 1 point higher were obtained on initial_NCC hydrolyzed with 64 % acid and 0.4 points higher for 62 % acid compared to KC1_NCC. Supporting the observed in the previous section, acetate presence also affected the incorporation of sulfate moieties to NCC.

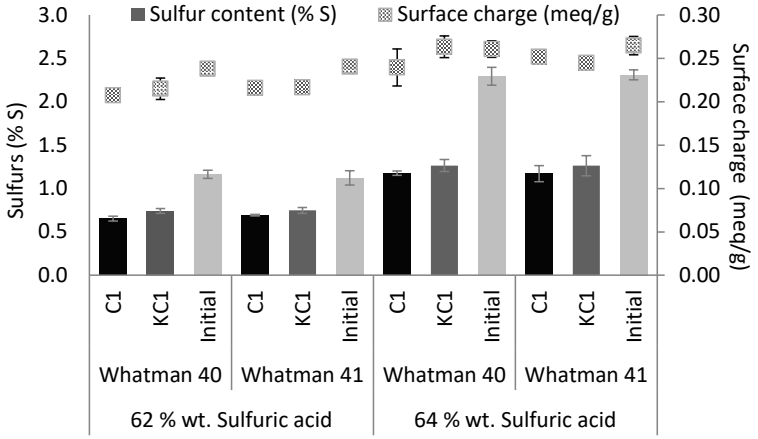

Figure 4-6: Sulfur content of NCC, as % of elemental sulfur (bars, left axis). Surface charge of NCC as cationic demand of suspensions (dots, right axis).

For a deeper understanding of cellulase effects, NCC was characterized in additional terms. Surface charge (cationic demand) of NCC is important as electric charge of NCC affects their applicability (Lin and Dufresne, 2014). Firstly, big differences between NCC and original fibers were found. Charges about 100 times bigger were observed for NCC compared to fibers, responsibility of the negatively charged sulfate groups attached to surface during hydrolysis (Beck et al., 2011). Cationic demand values of NCC suspensions, as indicated in Figure 4-6 follow a similar

tendency as that observed for sulfur content, providing evidence that both parameters were related. As a regard of acid dose, NCC obtained from 64 % acid showed greater surface charges than those observed for 62 % acid, a similar behavior as that observed for sulfate groups. This parameter failed to show differences between C_NCC and KC_NCC samples, though, suggesting this methodology was less sensitive than conductimetric titration used for sulfur content determination.

Electrophoretic mobility of particles, expressed as zeta potential is a good indicator of colloidal stability of suspensions (Teixeira et al., 2010; Boluk et al., 2011). It has been reported that NCC colloidal stability provided by sulfate groups facilitates their further applicability, as well as it permits a broader range of uses (Lin and Dufresne, 2014). Among samples, in all cases zeta potential values were around -50 mV (Table 4-7), which is considered to be a value indicating high stability (Hornig and Heinze, 2008). Values were very similar to reported by other authors for similar samples (Teixeira et al., 2010). Furthermore, they indicated higher stabilities on final suspensions compared to those reported by Filson et al., 2009, obtaining NCC only by means of enzymatic treatments and who reported values around -31 mV. This difference remarked the utility of using both enzymatic treatment and also sulfuric hydrolysis. In this way, benefits of enzymatic treatment and also the benefit in suspension stability provided by sulfate groups attached to NCC surface were obtained. Acid dose and enzymatic treatment produced small differences on zeta potential. 1-3 mV less-negative values were observed for NCC obtained with 62 % acid compared to 64 %. Also, 3-5 mV smaller values were observed for C_NCC samples compared to KC_NCC and initial_NCC, fact that was observable only for 64 % acid. Considering zeta potential is both influenced by colloidal particle size and electrical charge (Hornig and Heinze, 2008), these small differences could also be caused by the already stated existing difference in size between samples.

Surfaces hydrophobicity as WCA (water contact angle) was determined. WCA values for NCC dried films were given in Table 4-7. All samples had WCA values around 50 º, indicating that surfaces were hydrophilic. This hydrophilicity was caused by sulfate groups esterified on crystals surface (Morais et al., 2013; Anderson et al., 2014). In comparison with WCA of original fibers, NCC films showed to be more hydrophobic, taking WCA from ≈23 º to ≈50 º. The explanation for this could be found in the fact that crystalline regions are less accessible for water than amorphous regions,

and NCC are lacking on the former. Also, the chiral ordering of NCC on films could have helped to difficult interaction with water. These results differ from those reported by Morais et al., 2013 who observed a reduction in hydrophobicity after isolating crystalline regions from cotton linter. Regarding enzyme effect, a certain tendency of an increased NCC hydrophilicity was observed for C1_NCC compared to KC_NCC and initial_NCC (Table 4-7).

Finally, viscosity was measured for NCC suspensions obtained with 62% acid and filter 41 from KC and C, obtaining values of 45 and 40 mL/g, respectively. These values provided evidence of the high scission cellulose chains were submitted to during NCC preparation, as they were considerably smaller to those observed on fibers.

4.3.3.2. Assessing the effects of cellulase and reaction conditions: HPLC and SEM

For a better understanding of enzyme and acid action over cotton linters, content in oligosaccharides was determined in centrifuges supernatants (washing waters during NCC preparation) and also in liquid phase of final NCC suspensions. The intention was to elucidate the effects of the used hydrolase (C) and also the effects of acid hydrolysis in relation to the generation of short oligosaccharides. These short oligosaccharides could then be used as a feedstock for the production of other compounds, such as bioethanol (Filson and Dawson-Andoh, 2009). Results are given on Table 4-8.

First, sugar concentration of samples decreased with centrifuge washings (comparing C/KC 64 1st vs 2nd washing). Between the different oligosaccharides, it seemed that the shorter species were produced in a greater quantity than the longer forms (C1≥C6), with concentrations about 10 times higher of glucose compared to the amount of cellopentaose and cellohexose produced (Table 4-8). A similar work reported by other authors (Filson and Dawson-Andoh, 2009) found only glucose and cellobiose as hydrolysis products, being glucose the main released sugar, as in our case. Among samples, fewer sugars were released with lower acid concentrations (initial 62 % vs. initial 64 %, Table 4-8). Lower sugar concentrations were found on washing waters of the hydrolysis of C treated fibers compared to initial. This was consistent with the higher NCC yields obtained from C treated fibers compared to initial_NCC and indirectly supported the statement of C enzymatic treatment modifying sulfuric

acid effect over cellulose fibers during NCC isolation. Finally, it was evidenced that remaining dissolved sugars on samples were removed from suspensions during dialysis, as can be noticed comparing 2º washing samples with the two columns on the right.

Table 4-8: Oligosaccharide concentration after centrifuges (washings) and in liquid phase of final suspensions. Cellulose source and acid concentration is indicated for each sample. C1, C2, C3, C4, C5 and C6 stand for glucose, cellobiose, cellotriose, cellotetraose, cellopentaose and cellohexose, respectively.

	Oligosaccharide concentration (mg/mL)							
	Washing waters						Final suspensions	
Sample	C1 64 %		KC1 64 %		Initial 62 %	Initial 64 %	C1 64 %	KC1 64 %
Washing	1st	2nd	1st	2nd	1st	1st	-	-
C6	0.055	0.015	0.048	0	0.044	0.073	0	0
C5	0.074	0.018	0.065	0.009	0.056	0.098	0	0
C4	0.126	0.024	0.108	0.009	0.080	0.163	0	0
C3	0.189	0.035	0.157	0.013	0.091	0.224	0	0
C2	0.301	0.077	0.246	0.028	0.118	0.302	Traces	0
C1	0.722	0.170	0.570	0.060	0.268	0.665	Traces	0

Finally, SEM images from NCC films (Figure 4-7) showed the typical chiral nematic organization, in which crystals are deposited by layers during drying of suspensions. In each of these layers there is one axis determining the main orientation for these rod-like nanostructures. This organizing property is determined by shape and electric properties of CNC and has already been reported elsewhere (Revol et al., 1992). The obtained SEM images for our films were very similar to those reported by Majoinen et al., 2012 using a similar procedure for observation. On images the rods (NCC) can be spotted individually, confirming their nano-scale diameter, while their length could not be observed with this technique.

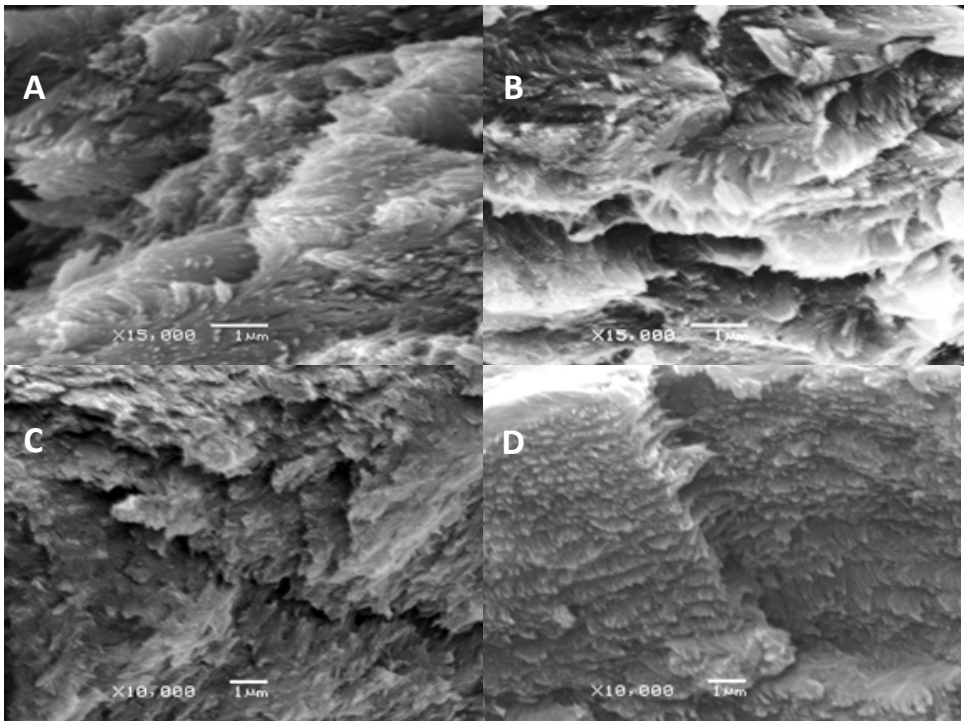

Figure 4-7: SEM images of dried NCC films. A: C1_NCC 62 % wt.; B: KC1_NCC 62 % wt.; C: C1_NCC 64 % wt.; D: KC1_NCC 64 % wt. All samples on images were filtered through Whatman 41 filter.

4.4. Conclusions

Data exposed in this chapter showed that a cellulase pretreatment could be a promising first step for partially replacing the use of harsh chemicals and to save energy during the isolation of nanocrystalline cellulose. Concerning enzymatic pretreatment, we observed that the used buffer affected NCC isolation by increasing its yield and size and reducing sulfur content. Also, the type of reactor used influenced the effects produced on fibers by the enzyme, while it did not seem to affect NCC isolation. Also, it was exposed that as a result of cellulase action, NCC yields up to 12 points greater compared to NCC from initial fibers and 7-8 points bigger compared to NCC from control fibers were obtained. Cellulase also affected NCC dimensions, with values up to 35 nm higher as a result of its action. Enzyme reduced the incorporation of

sulfate moieties onto crystals surface, yielding NCC with contents up to 0.8 % sulfur lower but maintaining suspensions stability (zeta potential). Evidence presented in this chapter highlighted the utility of the studied pretreatment, also suggesting the necessity of optimizing conditions of both sulfuric acid hydrolysis and enzymatic pretreatment for maximizing yield and the benefits of pretreatment.

4.5. References

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. Enzymatic Hydrolysis of Native Cellulose Nanofibrils and Other Cellulose Model Films: Effect of Surface Structure. Langmuir 2008, 24 (20), 11592–11599.

Anderson, S. R.; Esposito, D.; Gillette, W.; Zhu, J. Y.; Baxa, U.; McNeil, S. E. Enzymatic Preparation of Nanocrystalline and Microcrystalline Cellulose. Tappi J. 2014, 13 (5), 35–42.

Beck, S.; Bouchard, J.; Berry, R. Controlling the Reflection Wavelength of Iridescent Solid Films of Nanocrystalline Cellulose. Biomacromolecules 2011, 12 (1), 167–172.

Boluk, Y.; Lahiji, R.; Zhao, L.; McDermott, M. T. Suspension Viscosities and Shape Parameter of Cellulose Nanocrystals (CNC). Colloids Surfaces A Physicochem. Eng. Asp. 2011, 377 (1-3), 297–303.

Bondeson, D.; Mathew, A.; Oksman, K. Optimization of the Isolation of Nanocrystals from Microcrystalline Cellulose by Acid Hydrolysis. Cellulose 2006, 13 (2), 171–180.

Brinchi, L.; Cotana, F.; Fortunati, E.; Kenny, J. M. Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications. Carbohydr. Polym. 2013, 94 (1), 154–169.

Brito, B. S. L.; Pereira, F. V.; Putaux, J.-L.; Jean, B. Preparation, Morphology and Structure of Cellulose Nanocrystals from Bamboo Fibers. Cellulose 2012, 19 (5), 1527–1536.

Cadena, E. M.; Garcia, J.; Vidal, T.; Torres, A. L. Determination of Zeta Potential and Cationic Demand in ECF and TCF Bleached Pulp from Eucalyptus and Flax. Influence of Measuring Conditions. Cellulose 2009, 16 (3), 491–500.

Charreau, H.; Foresti, M. L.; Vázquez, A.; Vazquez, A.; Vázquez, A. Nanocellulose Patents Trends: A Comprehensive Review on Patents on Cellulose Nanocrystals, Microfibrillated and Bacterial Cellulose. Recent Pat. Nanotechnol. 2013, 7 (1), 56–80.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Chen, X.; Deng, X.; Shen, W.; Jiang, L. Controlled Enzymolysis Preparation of Nanocrystalline Cellulose from Pretreated Cotton Fibers. BioResources 2012, 7 (3), 4237–4248.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Filson, P. B.; Dawson-Andoh, B. E. Characterization of Sugars from Model and Enzyme-Mediated Pulp Hydrolyzates Using High-Performance Liquid Chromatography Coupled to Evaporative Light Scattering Detection. Bioresour. Technol. 2009, 100 (24), 6661–6664.

Filson, P. B.; Dawson-Andoh, B. E.; Schwegler-Berry, D. Enzymatic-Mediated Production of Cellulose Nanocrystals from Recycled Pulp. Green Chem. 2009, 11 (11), 1808-1814.

Fraschini, C.; Chauve, G.; Berre, J.-F. Le; Ellis, S.; Méthot, M.; Connor, B. O. Critical Discussion of Light Scattering and Microscopy Techniques for CNC Particle Sizing. Nord. Pulp Pap. Res. J. 2014, 29 (1), 31–40.

Garcia-Ubasart, J.; Torres, A. L.; Vila, C.; Pastor, F. I. J.; Vidal, T. Biomodification of Cellulose Flax Fibers by a New Cellulase. Ind. Crops Prod. 2013, 44, 71–76.

Genung, L. Analysis of Cellulose Derivatives. Anal. Chem. 1950, 14 (12), 935–940.

Gu, J.; Catchmark, J. M.; Kaiser, E. Q.; Archibald, D. D. Quantification of Cellulose Nanowhiskers Sulfate Esterification Levels. Carbohydr. Polym. 2013, 92 (2), 1809–1816.

Habibi, Y. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Habibi, Y.; Lucia, L. A.; Rojas, O. J. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Hornig, S.; Heinze, T. Efficient Approach to Design Stable Water-Dispersible Nanoparticles of Hydrophobic Cellulose Esters. Biomacromolecules 2008, 9 (5), 1487–1492.

Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. Angew. Chemie - Int. Ed. 2011, 50 (24), 5438–5466.

Lin, N.; Dufresne, A. Nanocellulose in Biomedicine: Current Status and Future Prospect. Eur. Polym. J. 2014, 59, 302–325.

Lu, P.; Hsieh, Y.-L. Preparation and Properties of Cellulose Nanocrystals: Rods, Spheres, and Network. Carbohydr. Polym. 2010, 82 (2), 329–336.

Lu, Z.; Fan, L.; Zheng, H.; Lu, Q.; Liao, Y.; Huang, B. Preparation, Characterization and Optimization of Nanocellulose Whiskers by Simultaneously Ultrasonic Wave and Microwave Assisted. Bioresour. Technol. 2013, 146, 82–88.

Majoinen, J.; Kontturi, E.; Ikkala, O.; Gray, D. G. SEM Imaging of Chiral Nematic Films Cast from Cellulose Nanocrystal Suspensions. Cellulose 2012, 19 (5), 1599–1605.

Morais, J. P. S.; Rosa, M. D. F.; de Souza Filho, M. D. S. M.; Nascimento, L. D.; do Nascimento, D. M.; Cassales, A. R. Extraction and Characterization of Nanocellulose Structures from Raw Cotton Linter. Carbohydr. Polym. 2013, 91 (1), 229–235.

Nelson, M. L.; O'Connor, R. T. Relation of Certain Infrared Bands to Cellulose Crystallinity and Crystal Lattice Type. Part II. A New Infrared Ratio for Estimation of Crystallinity in Celluloses I and II. J. Appl. Polym. Sci. 1964, 8 (3), 1325–1341.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part I: On a Fully Bleached, Commercial Acid Sulfite Dissolving Pulp. Cellulose 2015a, 2067–2079.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part II: On a Biobleached Acid Sulphite Pulp. Cellulose 2015b, 22 (3), 2081–2093.

Revol, J. F.; Bradford, H.; Giasson, J.; Marchessault, R. H.; Gray, D. G. Helicoidal Self-Ordering of Cellulose Microfibrils in Aqueous Suspension. Int. J. Biol. Macromol. 1992, 14 (3), 170–172.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Široký, J.; Blackburn, R. S.; Bechtold, T.; Taylor, J.; White, P. Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy Analysis of Crystallinity Changes in Lyocell Following Continuous Treatment with Sodium Hydroxide. Cellulose 2010, 17, 103–115.

Teixeira, E. D. M.; Corrêa, A. C.; Manzoli, A.; de Lima Leite, F.; de Ribeiro Oliveira, C.; Mattoso, L. H. C.; Teixeira, E. D. M.; Corre, A. C.; Leite, F. D. L.; Henrique, L.; et al. Cellulose Nanofibers from White and Naturally Colored Cotton Fibers. Cellulose 2010, 17 (3), 595–606.

Xu, Q.; Gao, Y.; Qin, M.; Wu, K.; Fu, Y.; Zhao, J. Nanocrystalline Cellulose from Aspen Kraft Pulp and Its Application in Deinked Pulp. Int. J. Biol. Macromol. 2013, 60, 241–247.

Chapter 5

Optimizing acid hydrolysis conditions for NCC isolation from enzymatically-pretreated fibers

Abstract

In this chapter, NCC preparation using sulfuric acid hydrolysis from cellulase pretreated fibers was optimized in order to obtain the highest possible yield with 62% and 65% wt. sulfuric acid throughout two statistical plans. At optimal conditions (10 U/g odp cellulase, 25 min hydrolysis, 47 ºC and 62 % wt. H_2SO_4) high yields were obtained (≥80 %) including an increase produced by enzyme of ≈9 %. Optimal conditions produced nanosized particles of around ≈200 nm with a reduced surface charge and sulfur content. The performed optimization allowed reducing the hydrolysis time in a 44%, and also increasing yield in more than 10% compared to results exposed in chapter 4. The effects of cellulase pretreatment were noticeable even under aggressive hydrolysis conditions, emphasizing its possibilities. Zeta potential and polydispersity indexes indicated that all studied conditions leaded to good quality final products, with values among -50 mV and 0.2, respectively. TEM analysis confirmed the presence of NCC. Finally FTIR analysis provided evidence that cellulase pretreatment increased crystallinity of both cellulose fibers and NCC, and also increased accessibility to fibers, supporting data obtained from NCC.

5.1. Introduction

As been mentioned in previous chapters, from the chemical perspective, cellulose consists on a linear homopolymer of β-D-glucopyranose units linked by glycosidic bonds, while its repeating subunit consists of two glucose units linked corkscrewed 180º respect each other (Klemm et al., 2005). Cellulose chains aggregate onto larger structures, *i.e.* microfibrils. These microfibrils present a crystalline ordering which is disrupted by amorphous regions. Degradation of these defects leads to the release of needle-like particles consisting of crystalline regions, denominated nanocrystalline cellulose (NCC) or cellulose nanocrystals (CNC) (Habibi et al., 2010). The interest in NCC lies in that it presents outstanding mechanical properties at the nano-scale, making it a very interesting sustainable reinforcing agent for a variety of materials. NCC chemical properties, which strongly depend of the preparation method

used, determine their physicochemical behavior when incorporated onto polymeric matrixes or other composites (Klemm et al., 2011).

In literature, isolation of NCC has been carried out by diverse methods, which have traditionally been characterized by low yields, reducing their economic and environmental efficiency. Studies such as those reported by Fan and Li (2012) and Chen et al. (2015) addressed this topic studying ways to increase sulfuric acid hydrolysis yield, the most extended preparation method. The study exposed in chapter 4 demonstrated that the combination of an enzymatic pretreatment with sulfuric acid hydrolysis could increase the yield of NCC isolation while influencing other properties and leading to well-stable suspensions of electrically charged nanoparticles. Also, it was observed that the noticeability of enzymatic pretreatment effects on NCC is largely dependent on the hydrolysis conditions used for isolation. Experimental designs have been used in literature in order to optimize process conditions (Fillat and Roncero, 2009; Valls and Roncero, 2009; Fillat et al., 2010; Valls et al., 2010a). In this chapter two experimental plans were carried out with two different acid doses for studying the influence of three variables: the presence of an enzymatic pretreatment, hydrolysis time and hydrolysis temperature on NCC preparation. To our best knowledge, an optimization of this kind was being performed on enzymatically pretreated fibers for the first time. The aim of this chapter was to both maximize NCC yield from enzymatically pretreated fibers and also to assess the relation between the effects of enzymatic pretreatment and the intensity of acid hydrolysis.

5.2. Materials and methods

For details concerning materials and methods used in the present chapter, please refer to **chapter 2** "Materials and methods".

5.2.1. Fibers, enzyme and enzymatic treatment

Cotton linters, provided by Celsur (Spain), were used as cellulose source (cellulose content 97.7 ± 0.3 %), and named initial fibers. A cellulase ("C") provided by Fungal Bioproducts (Spain) was used for treatments. Activity, as U per gram of enzyme

stock was 1700 U/g CMCase units, that is to say, the amount of enzyme degrading 1 μmol of CMC (carboxymethilcellulose) per minute. Enzymatic treatment (C) was performed with a 10 U/g oven-dried pulp (odp) dose for 24h in a 4 L cylindrical reactor with agitation produced by rotating blades at 30 rpm, 55 ºC, 5% consistency and pH 5 maintained using 50 mM acetate buffer. Control fibers, named "KC" (0 U/g odp) were obtained using the same conditions as for enzymatic treatment, but without enzymatic addition.

5.2.2. Experimental designs

For the purpose of studying the interactions between hydrolysis process variables and the enzymatic pretreatment, three independent variables were studied via two 2^3 factorial designs. X1 (cellulase) with 0 U/g odp, *i.e.* absence, and 10 U/g odp *i.e.* presence; X2 (acid hydrolysis time) being it 25 or 50 min; X3 (acid hydrolysis temperature) being it 47 ºC or 60 ºC. When the cellulase dose corresponded to "0 U/g odp", KC (control fibers) were used as cellulose source. These independent variables were coded as -1 or +1; both for direct comparison of coefficients and to better understand the effect of each variable on the responses (Table 5-1). Due to the nature of X1 variable, not allowing a central point, no curvatures were analyzed and only linear saturated models were studied. Therefore, the experimental designs were two 2^3 complete factorial designs requiring 8 experiences each. The purpose of this was to only determine individual effects of each of the three variables and their interactions, as described in literature (Valls et al., 2010a). Runs in factorial designs were randomized in order to reduce the impact of bias on the results. Data was then analyzed using a Microsoft Excel spreadsheet to implement the stepwise backward regression method and discard all terms with a probability (p-value) less than 0.05 (Table 5-2).

Table 5-1: Experiences of both statistical plans with their conditions.

Sulfuric acid 62% wt.						
	X1	X2	X3	Cellulase (U/g odp)	Time (min)	Temperature (ºC)
Y1	-1	-1	-1	0	25	47
Y2	1	-1	-1	10	25	47
Y3	-1	1	-1	0	50	47
Y4	1	1	-1	10	50	47
Y5	-1	-1	1	0	25	60
Y6	1	-1	1	10	25	60
Y7	-1	1	1	0	50	60
Y8	1	1	1	10	50	60
Sulfuric acid 65% wt.						
	X1	X2	X3	Cellulase (U/g odp)	Time (min)	Temperature (ºC)
Y1	-1	-1	-1	0	25	47
Y2	1	-1	-1	10	25	47
Y3	-1	1	-1	0	50	47
Y4	1	1	-1	10	50	47
Y5	-1	-1	1	0	25	60
Y6	1	-1	1	10	25	60
Y7	-1	1	1	0	50	60
Y8	1	1	1	10	50	60

5.2.3. NCC preparation

Nanocrystalline cellulose was obtained by a controlled hydrolysis via sulfuric acid, using a protocol proposed by Dong et al. 1998, throughfully exposed in chapter 2. Previous to acid hydrolysis fibers were fluffed and oven-dried. Typically, 1.5 g of fibers weighted immediately from desiccator were hydrolyzed using 62% or 65% wt. sulfuric acid. An acid-to-fibers ratio of 10:1 (10 mL/1g cellulose) was used and reaction was conducted with magnetic stirring. Other reaction conditions were different for each sample and indicated in Table 5-1. Final samples were filtered through a Whatman® 41 membrane.

Table 5-2: p-values of each variable for the different obtained models using each acid dose.

	Yield		Z average		Surface charge	
	62 % wt.	65 % wt.	62 % wt.	65 % wt.	62 % wt.	65 % wt.
X1	0.0196	0.0055	-	0.0024	-	0.0047
X2	0.0059	-	0.0014	0.0231	0.0269	-
X3	0.0352	0.0022	0.0005	0.0012	0.0012	0.049
X1X2	-	0.0047	-	0.0164		0.0054
X1X3	0.0339	-	-	0.0433	0.044	0.0161
X2X3	0.0047	0.0008	0.0018	-	-	-
X1X2X3	-	0.0219	-	-	-	-

5.2.4. Samples characterization

Cellulose fibers samples were characterized in length in accordance to TAPPI T271 in a Kajaani Fiber analyzer FS300 (Metso automation, Finland).

NCC samples were characterized in terms of yield, particle size, surface charge, sulfur content and electrophoretic mobility according to the protocols described in chapter 2.

Transmission electron microscopy (TEM) was used to examine NCC morphology using a similar protocol to that described elsewhere (Chen et al., 2015). Briefly, carbon-coated Cu-grids were firstly glow-discharged for 30 seconds and then floated on 5 μL drops of NCC suspensions (0.1-0.5 % w/v) for 5 minutes. After that, NCC was negatively stained by floating grids consecutively into two 50 μL drops of 2% aqueous uranyl acetate for 30 seconds. Excess stain was removed by capillary action and gentle blotting. Samples were analyzed using a JEOL JEM-1010 transmission electron microscope operating at 80 kV.

Fourier transformed infrared spectroscopy (FTIR) spectra of cellulose fibers and NCC samples were recorded at room temperature using a Spectrum 100 ATR-FTIR spectrophotometer (Perkin Elmer, USA). FTIR spectral analysis was conducted within the wavenumber range of 600-4000 cm^{-1}. A total of 64 scans were run to collect each spectrum at a 1 cm^{-1} resolution. Lateral order index (LOI) and Total crystallinity index (TCI), proposed by O'connor (O'Connor et al., 1958) and Nelson and O`Connor (Nelson and O'Connor, 1964), were estimated from the ratio between the absorption peaks at 1430 cm^{-1} and 890 cm^{-1} bands, and 1370 cm^{-1} and 2900 cm^{-1}, respectively.

5.3. Results and discussion

5.3.1. Starting fibers and enzymatic treatment

Fiber length data exposed in Figure 5-1 showed that cellulase pretreatment modified cotton linters length distribution compared to initial and control fibers, as it reduced the amount of longer fibers (*i.e.* between 3.2 and 7.6 mm) in a 40%, and increased the amount of shorter ones. In fact, this effect was also suspected to occur in the study developed in chapter 4, where a reduction in viscosity and fiber length produced by the same enzyme was observed. Also, a higher homogeneity in fiber length was observed, as more fibers were counted in the middle lengths (groups between 0.5 and 2 mm), highlighting an increase in raw material quality due to a more homogeneous size distribution. Control treatment (KC) by its side did not seem to affect fiber length. These macroscopic modifications of fibers together with other chemical modifications such as in viscosity or crystallinity observed in chapter 4 are assumed to be the causes of the modifications in acid-fiber interaction during acid hydrolysis produced by enzymatic pretreatment.

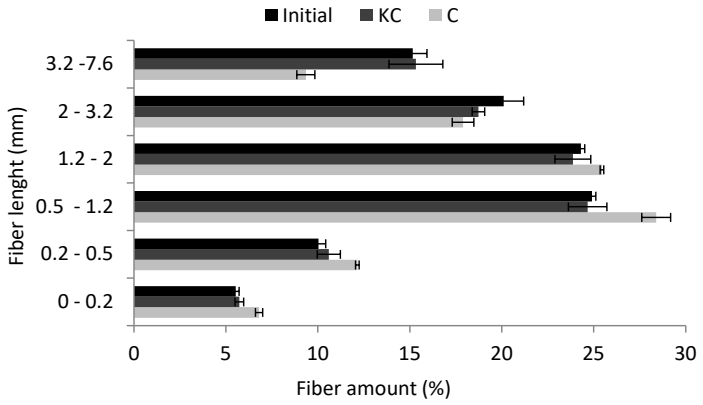

Figure 5-1: Fiber length (mm) distribution of initial, cellulase treated (C) and control (KC) fibers indicated as % of total.

5.3.2. Models for yield, average particle size and surface charge of NCC.

The experimental results obtained for NCC yield, average particle size and surface charge are shown in Table 5-3. Statistical models were built fitting experimental data.

Yield of the NCC preparation process constitutes a key aspect to be analyzed due to the implication it has on the overall economic cost of the process, as low yields suppose a higher biomass and reactants consumption (Klemm et al., 2011; Wang et al., 2012). In this direction, the optimal yield of the process corresponds to the highest possible. Models relating yield and other process variables for both 62% and 65% wt. H_2SO_4 fitted Equation 5-1 and Equation 5-2, respectively. Equation 5-1 shows that yield was positively influenced by X2X3 and by X1 and negatively by X2, X3 and X1X3. Equation 5-2 indicates that with the stronger acid dose, reaction time (X2) did not independently influence yield, but it interacted with cellulase presence and temperature, conditioning their influence.

Table 5-3: Experimental results of the statistical plans

	Sulfuric acid 62% wt.			Sulfuric acid 65% wt.		
	Yield (%)	Z average (nm)	Surf. charge (meq/g)	Yield (%)	Z average (nm)	Surf. charge (meq/g)
Y1	73.5 ± 0.2	184.8 ± 5.6	0.164 ± 0.02	24.4 ± 0.1	89.2 ± 1.6	0.227 ± 0.005
Y2	84.1 ± 0.5	214.7 ± 34.3	0.188 ± 0.009	25.8 ± 0.6	92.3 ± 3.6	0.216 ± 0.009
Y3	54.5 ± 0.3	97.5 ± 1	0.190 ± 0.002	27.4 ± 0.4	81.6 ± 5.7	0.245 ± 0.01
Y4	62.2 ± 0.2	98.9 ± 2.8	0.222 ± 0.001	27.7 ± 0.3	93.4 ± 1.9	0.188 ± 0.011
Y5	63.3 ± 0.2	76.7 ± 1.2	0.265 ± 0.014	27.8 ± 0.2	72.6 ± 0.6	0.222 ± 0.01
Y6	64.9 ± 0.2	79.7 ± 2.3	0.225 ± 0.013	30.3 ± 0.3	81.5 ± 1.4	0.232 ± 0.011
Y7	64.8 ± 0.5	69.3 ± 2.3	0.269 ± 0.008	26.8 ± 0.2	66.6 ± 1.6	0.241 ± 0.004
Y8	65.8 ± 0.5	80.2 ± 2	0.271 ± 0.016	26.3 ± 0.4	81.6 ± 1.5	0.216 ± 0.014

$$Yield_{62\%}\ (\%) = 66.65 + 2.61\ X1 - 4.82\ X2 - 1.93\ X3 + 5.4\ X2X3 - 1.96\ X1X3 \quad R^2 = 0.996$$

Equation 5-1

$$Yield_{65\%}\ (\%) = 27.06 + 0.47\ X1 + 0.74\ X3 - 0.51\ X1X2 - 1.24\ X2X3 - 0.23\ X1X2X3 \quad R^2 = 0.999$$

Equation 5-2

Based on the obtained models, with 62% sulfuric acid yields between 60-84% were obtained (Figure 5-2A), while 65% wt. produced considerably smaller values (among 24 – 30%) (Figure 5-2B). This difference was probably consequence of the stronger depolymerization of cellulose produced by a larger acid concentration (Chen et al., 2015). Time and temperature had little influence when observing data with 65 % wt., fact that did not occur with 62 % wt. acid, with which an increase of any of both variables produced a significant reduction of yield, particularly in presence of cellulase. Importantly, cellulase presence produced a top increase of yield of up to 9 points with the 62 % wt. dose. Moreover, this effect could even be observed under strong hydrolysis conditions (65 % acid). Under these conditions, cellulase showed to be capable of increasing yield up to ≈ 2.4 points, strongly highlighting the benefits of this pretreatment. As commented in chapter 4, this increase produced by cellulase was speculated to be caused by its preferential attack on amorphous cellulose regions on fibers, increasing their crystallinity and also facilitating the interaction with acid, reducing undesired cellulose mass loss.

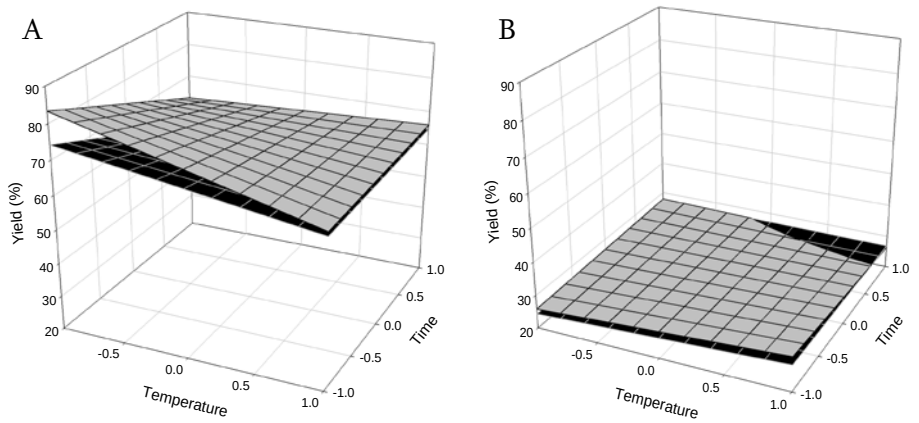

Figure 5-2: Yield data predicted by models with 62% wt. sulfuric acid (A), and with 65% wt. sulfuric acid (B). Grey and black charts represent data in presence and absence of cellulase, respectively.

Concerning average particle size, as previously stated, DLS measurements do not provide a real measurement of particle size, particularly when considering rod-like structures such as nanocrystalline cellulose. Nevertheless, these measurements provide a useful approximation to average particle size in order to establish comparisons between similar samples (Fraschini et al., 2014). Size of nanocrystalline cellulose is a key aspect to analyze in order to ensure the quality and characteristics of the obtained final product. NCC morphology could affect its performance when used on a determined application. These affectations could be, for instance, variations in permeability of membranes formed by NCC (Thielemans et al., 2009) or toxicity, as it has been found that smaller NCC particles showed greater toxicity than larger ones (Yanamala and Farcas, 2014). Also, it was previously reported that size of NCC and their yield seemed to be related parameters (chapter 4).

Equation 5-3 and Equation 5-4 indicate the models fitting Z average data with 62% and 65% wt. H_2SO_4, respectively. For 62% acid, it was observed that particle size was only affected (negatively) by reaction time and temperature and positively by their double interaction. Similar influences were found for 65% acid, although in this case, cellulase positively influenced this parameter, as well as it interacted with both time and temperature.

$$Z\ average_{62\%}\ (nm) = 112.7 - 26.2\ X2 - 36.2\ X3 + 24.5\ X2X3 \quad R^2 = 0.976$$

Equation 5-3

$$Z\ average_{65\%}\ (nm) = 82.4 + 4.8\ X1 - 1.6\ X2 - 6.8\ X3 + 1.9\ X1X2 + 1.1\ X1X3\ R^2 = 0.998$$

Equation 5-4

With 62% sulfuric acid particle size was statistically not influenced by cellulase presence, being it only affected by reaction time and temperature (Figure 5-3A). These other two variables reduced average particle up to a 60% by enhancing cellulose degradation. With 65% acid (Figure 5-3B), average particle size was strongly reduced,

consequence of the greater cellulose depolymerization produced by acid, as observed by other authors (Fan and Li 2012). With this stronger acid dose, cellulase presence increased particle size up to 15 nm, while reaction time and temperature only seemed to produce an effect in absence of enzymatic pretreatment.

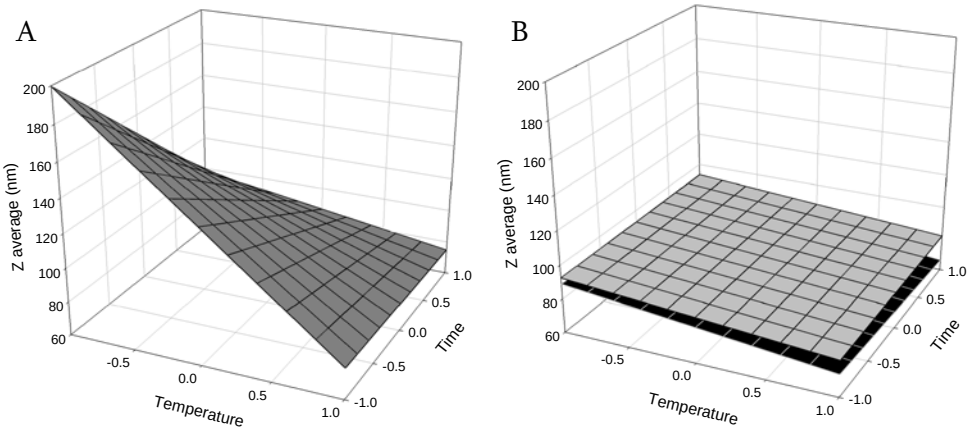

Figure 5-3: Particle size data predicted by models with 62% wt. sulfuric acid (A), and with 65% wt. sulfuric acid (B). Grey and black charts represent data in presence and absence of cellulase, respectively.

Surface charge of cellulose NCC is mainly responsibility of sulfate groups esterified onto free superficial OH- groups of cellulose during hydrolysis with sulfuric acid (Habibi et al., 2010; Abitbol et al., 2013). Because of this, NCC obtained with different acids, such as hydrochloric or hydrobromic result mainly uncharged (Habibi et al., 2010). Thus, NCC surface charge and their sulfur content are expected to be related. Equation 5-5 and Equation 5-6 fitted surface charge data for 62% and 65% wt. acid, respectively. In the first case, surface charge showed to be positively influenced by reaction time and temperature, and negatively by interaction of cellulase and temperature. In the second case influences were different, with cellulase reducing surface charge and interacting with the other two variables.

$$\text{Surface charge}_{62\%}\ (\text{meq/g}) = 0.224 + 0.014\ X2 + 0.033\ X3 - 0.012\ X1X3\ R^2 = 0.956$$

Equation 5-5

$$\text{Surface charge}_{65\%}\ (\text{meq/g}) = 0.223 + 0.01X1 + 0.004X3 - 0.01X1X2 + 0.007X1X3\ R^2 = 0.980$$

Equation 5-6

Observing charts in Figure 5-4 it could be observed that all surface charge values were between 0.16 and 0.29 meq/g, similar values as those observed in chapter 4. Generally, smaller values were observed with the lower acid dose (62 % wt.) and also at the lower temperature and reaction time probably due to smaller levels of sulfate esterification at milder hydrolysis conditions, as was also observed by Chen et al. (2015). In any case, the interpretation of modelized surface charge data appeared to be difficult, possibly due to the necessity of studying central points of variables.

The problem of the assessment of central points in a statistical plan will be addressed in the following chapter in which another experimental design will be performed optimizing enzymatic treatment parameters.

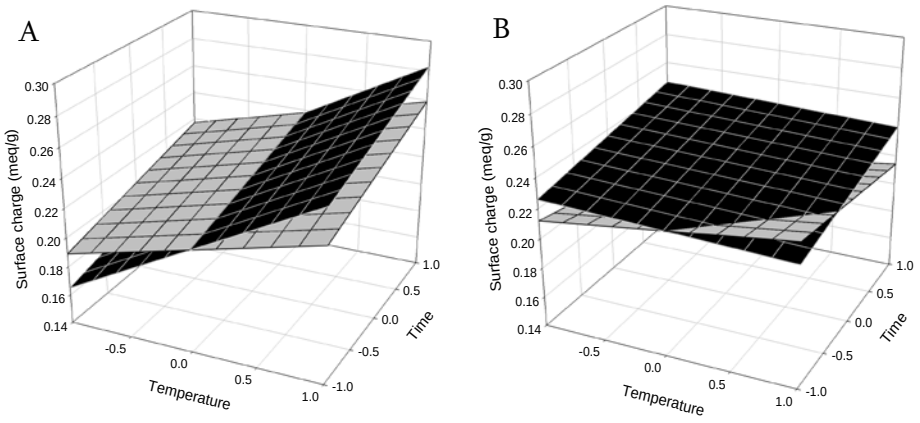

Figure 5-4: Surface charge data predicted by models with 62% wt. sulfuric acid (A), and with 65% wt. sulfuric acid (B). Grey and black charts represent data in presence and absence of cellulase, respectively.

5.3.3. Sulfur content, stability and polydispersity of NCC.

Sulfate groups could influence several characteristics of NCC, such as their dispersibility in water suspensions (Klemm et al., 2011) or their thermodegradability (Roman and Winter, 2004). They could also influence the properties conferred to composites if used as fillers (Moon et al., 2011). Sulfate groups on NCC surface would allow their use in biomedical applications, due to the possibility of electrostatically absorb enzymes or proteins (Lin and Dufresne, 2014). NCC with no SO_4^{2-} groups on its surface, such as those obtained with enzymes (Filson et al., 2009; Anderson et al., 2014; Teixeira et al., 2015) or non-sulfuric acids (*e.g.* hydrobromic or hydrochloric) (Habibi et al., 2010) aggregate and flocculate on water, hindering their applicability. NCC with large contents in sulfur, on the other hand, would be very susceptible to thermal degradation (Roman and Winter, 2004), hindering their use as fillers in polymeric matrixes, which are usually manipulated at high temperatures (Hubbe et al., 2008). Sulfur content of samples is indicated in Figure 5-5. Some observations aroused: firstly, the larger acid concentration led to higher content in sulfurs, result of the larger extent of esterification, as previously observed in chapter 4 and reported in literature (Chen et al., 2015). Secondly, with 62 % wt. acid, no significant effect was produced by any of the studied variables in sulfate esterification. Thirdly, with 65% wt. acid reaction time and temperature showed to reduce NCC sulfur content when increased. This observed desulfation was attributed to the degradation of NCC to sugars due to excessive depolymerization (Chen et al., 2015). Noteworthy, with 65 % wt. acid and 25 min of hydrolysis at 47 ºC, cellulase produced the biggest increase in sulfur content of NCC, also coinciding with the largest yield increase produced by the enzymatic pretreatment with this acid concentration (Figure 5-2B). Possibly, cellulase effect partially reduced acid-driven degradation of cellulose, increasing yield and neutralizing the previously cited sulfur content reduction by an excessive depolymerization.

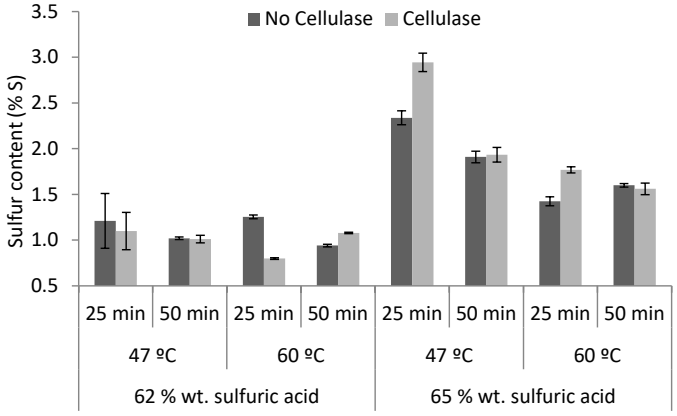

Figure 5-5: Sulfur content (as % S) of samples at studied conditions in presence and absence of cellulase.

Zeta potential, measured as electrophoretic mobility, is an indicator of the stability of colloidal suspensions (Filson et al., 2009; Alves et al., 2015). As indicated in Figure 5-6A, all the studied hydrolysis conditions led to values between -45 mV and -60 mV, considered to be indicators of very high stability (Alves et al., 2015). Little differences were found among samples, only being able to observe an increase in stability by increasing hydrolysis severity, corresponding to NCC with a higher electrical charge. These results indicated that well stable NCC suspensions were obtained independently of hydrolysis conditions and highlighted the benefit of using combined treatments with enzymes and sulfuric acid hydrolysis.

Finally, polydispersity index (PDI) is a measure of the heterogeneity in particle sizes within a sample, where smaller values indicate a higher homogeneity. It is known that having a NCC sample with a high homogeneity in particle size, *i.e.* a narrow particle size distribution is an indicator of good quality and thereafter a desirable feature. Also, this homogeneity constitutes a necessary characteristic for NCC application as a standardized component (Moon et al., 2011). As illustrated (Figure 5-6B), all samples had PDI values around 0.2 indicating particle size distribution of samples was homogeneous.

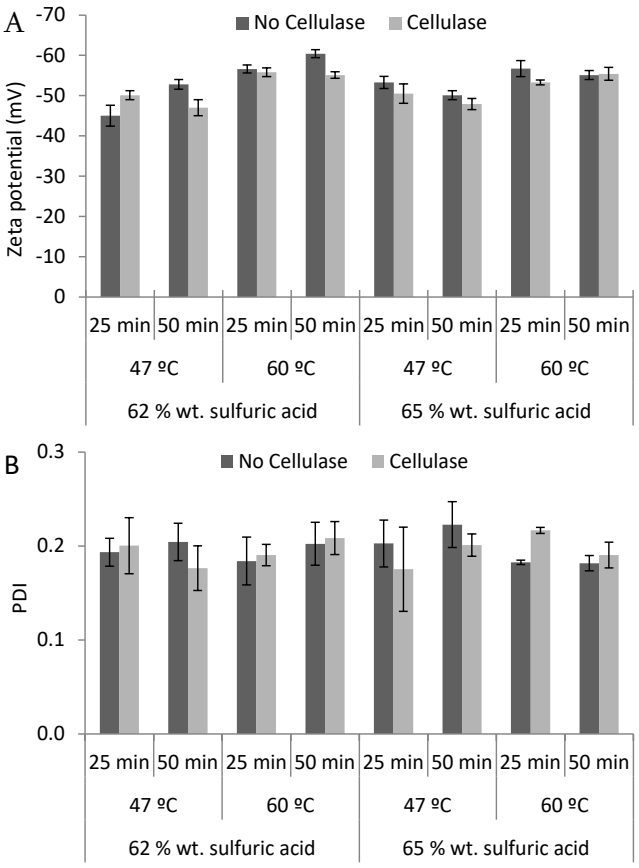

Figure 5-6: Zeta Potential (A) and Polydispersity Index (PDI) (B) of samples at studied conditions in presence and absence of cellulase

5.3.4. Optimal point and model verification

The objective of the present chapter was to find the hydrolysis conditions producing the highest possible NCC yield in combination with the enzymatic pretreatment. Thus, the optimal point was defined as the one providing the maximal yield. From the obtained statistical models, these conditions were found to be: cellulase presence (X1 = 1), 25 minutes of hydrolysis (X2 = -1) and 47 ºC (X3 = -1) and 62 % wt. H_2SO_4. At these conditions a yield of 83.4 % was predicted by the model. These

conditions also provided a maximal yield value of 74.2% in absence of cellulase, which was smaller than the former and thereafter considerably less interesting. It is also interesting that comparing these conditions with those used in chapter 4 a reduction of 20 min, *i.e.* 44% in acid hydrolysis time was achieved. Importantly, these optimized conditions led to a maximum yield more than 10 points higher than that obtained in chapter 4, possibly by reducing unnecessary cellulose depolymerization.

Optimal yield was also significantly higher than other reported values. Recently, Fan and Li 2012 reported a ≈62% optimal yield from cotton fibers and Martínez-Sanz et al. 2014 reported ≈77% yield for NCC also derived from pure cellulosic sources. Other innovative preparation methods such as ultrasonic assisted hydrolysis (Tanaka et al., 2014) offered a ≈40% yield.

At optimal conditions, models also predicted the larger NCC size among those observed, around 200 nm, fact that would reduce their toxicity (Yanamala and Farcas, 2014). Also, at this point surface charge was among the lower ones, indirectly indicating a low sulfate presence on NCC surface which would reduce their thermodegradability (Roman and Winter, 2004). Finally, zeta potential and polydispersity index provided evidence that optimal point generated a sample with high stability and a narrow size distribution.

In order to check the accuracy of the obtained models, new samples were prepared using the optimal hydrolysis conditions in presence and absence of cellulase. Table 5-4 indicates these new experimental values and also those predicted by models. As can be observed, models successfully predicted experimental data for yield, surface charge and average size within confidence intervals, only observing a small bias in the latter (<5 nm). Concerning non-modelized parameters, new values were also similar to former ones, with only small discrepancies in parameters showing a higher variability among samples, such as sulfur content or zeta potential.

Table 5-4: Models verification. New experimental values and those predicted by models are indicated for optimal hydrolysis conditions (25 min, 47 ºC and sulfuric acid 62% wt.). *When no model was found fitting data, previous experimental value is indicated.

	Optimal conditions			
	10 U/g odp cellulase		0 U/g odp cellulase	
	Experimental	Predicted*	Experimental	Predicted*
Yield (%)	82.8 ± 1.1	83.4	72.4 ± 1.2	74.2
Z average (nm)	186.4 ± 9.5	199.6	174.2 ± 19.1	199.6
Surface charge (meq/g)	0.185 ± 0.02	0.165	0.180 ± 0.018	0.189
Sulfur content (% S)*	0.82 ± 0.04	1.1 ± 0.21	0.93 ± 0.03	1.21 ± 0.3
Zeta potential (mV)*	-49.6 ± 1.1	-50.1 ± 1.1	-49.8 ± 0.8	-45 ± 2.6
PDI*	0.17 ± 0.02	0.20 ± 0.03	0.19 ± 0.03	0.19 ± 0.02

5.3.5. TEM analysis

Transmission electron microscope (TEM) images of individual NCC particles are shown in Figure 5-7. Firstly, images confirmed the achievement of rod-shaped nanostructures (NCC) at optimal and also at other studied conditions. Secondly, agreeing with DLS data, images of NCC obtained with optimal conditions in presence and absence of cellulase pretreatment (Figure 5-7A and B) seemed to show the largest particles, not observing noticeable differences between both samples. Images in Figure 5-7C, D and E by their side seemed to expose NCC particles with a smaller size compared to the former ones. This evidence was predicted by DLS data and was explained by the stronger hydrolysis severity caused either by longer hydrolysis, a higher temperature or a higher acid concentration, as previously exposed.

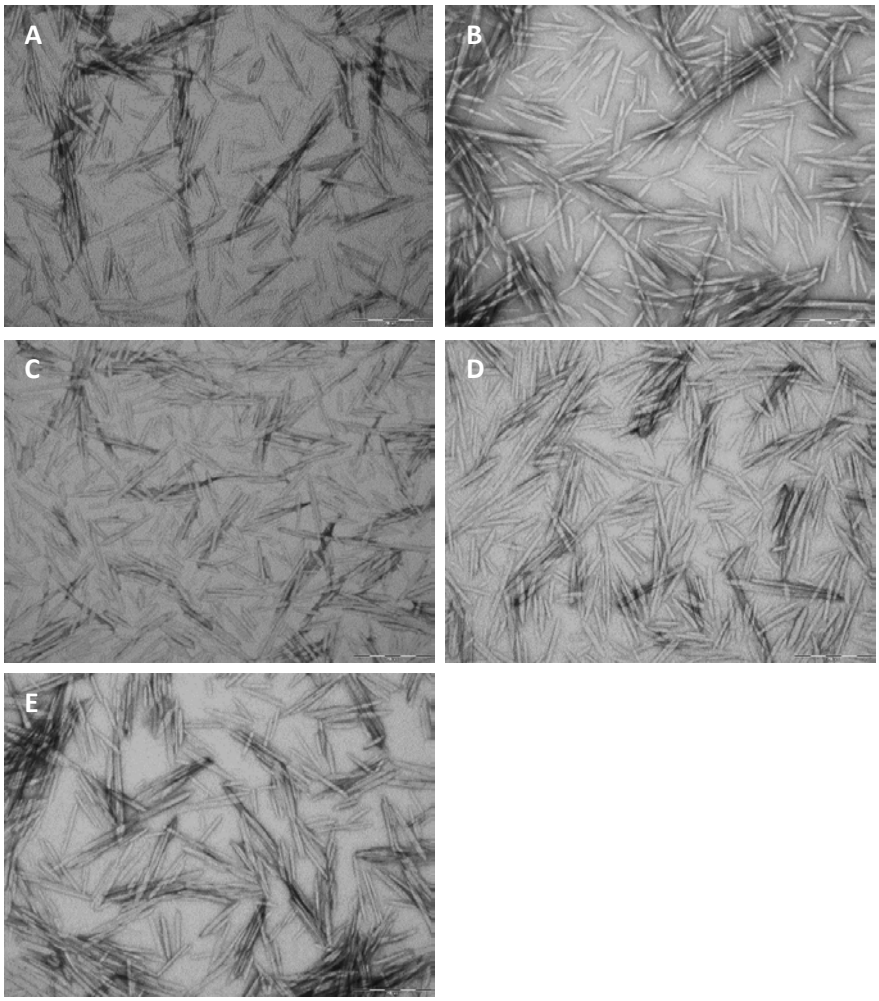

Figure 5-7: TEM images of NCC. Images correspond to: cellulase, 25 min, 47 ºC 62% acid (optimal point, A); control, 25 min, 47 ºC, 62% acid (B); cellulase, 50 min, 60ºC, 62% acid (C); cellulase, 25 min, 47 ºC, 65% acid (D); cellulase, 50 min, 47 ºC, 65% acid (E). Scale bar: 100 nm.

5.3.1. FTIR analysis

In Figure 5-8 examples of FTIR spectra of cellulose fibers and extracted NCC are shown. In the three cases spectra appeared to be very similar, attending the fact that chemical composition of samples (pure cellulose) remained unchanged during all studied processes. Main differences found were related to peaks intensity. Typical

bands of cellulose were observed (Široký et al., 2010). The broad absorption band in the range of 3600-3100 cm^{-1} was mainly due to the stretching of the –OH groups of cellulose, with typical sharpening around 3400 cm^{-1} (Široký et al., 2010; Alves et al., 2015). The peak at 2900 cm^{-1} appeared due to C-H stretching of cellulose (Fahma et al., 2010). Band at 1650-1600 cm^{-1} originated from the bending mode of water absorbed on cellulose (*i.e.* moisture water) (Fahma et al., 2010). From 1800 to 600 cm^{-1} the anhydroglucopyranose vibration modes were shown (deformation, wagging and twisting) (Alves et al., 2015). Among them, the absorption at 1044 cm^{-1} was mainly attributed to C-O stretching on C-O-C linkages, and the band at 895 cm^{-1} to C-H deformation of β-glyosidic linkages between glucose units (Alves et al., 2015). Presence of sulfate groups on NCC, not present on original fibers is illustrated by a small peak at 1205 cm^{-1}, which can be attributed to S=O linkage vibration, as previously described (Lu and Hsieh, 2010; Flauzino Neto et al., 2013). This tiny peak appears at NCC samples spectra but seems to be inexistent at original fibers.

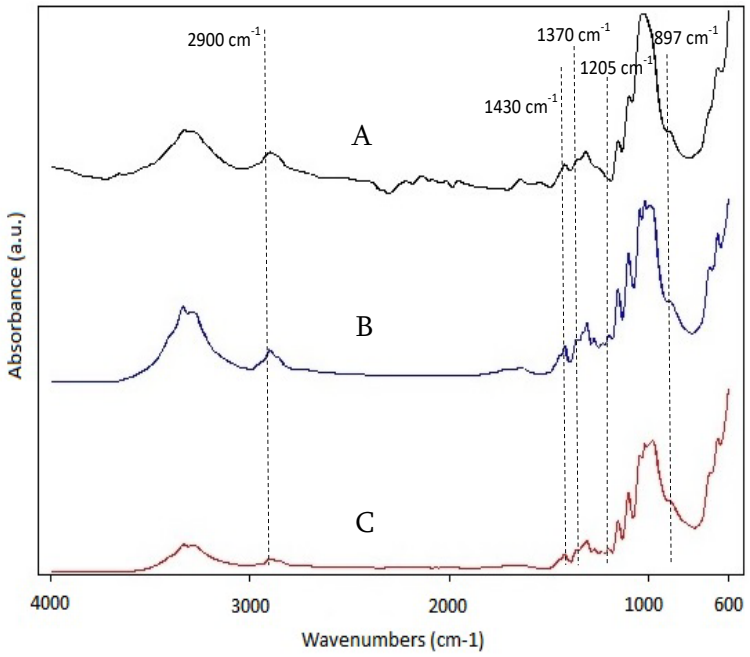

Figure 5-8: FTIR spectra of (A) cellulase pretreated fibers, (B) NCC (no cellulase, 62% wt. acid, 25 min), (C) NCC (cellulase, 65% wt. acid, 50 min).

Cellulose crystallinity determination is important due to its impact on cellulose practical characteristics. This parameter is determined by the proportion between crystalline and amorphous regions in cellulose, while is also affected by the different possible spatial arrangements of the polymer. Likewise, cellulose crystallinity index has been related to the size of crystalline domains, matter of great importance when considering NCC preparation (French and Santiago Cintrón, 2013). It has been reported that segments in cellulose polymer will vibrate differently in well-ordered crystalline regions compared to amorphous phases, permitting then the assignation of absorption bands to crystalline and amorphous regions and allowing the calculation of two different crystallinity indexes.

Lateral Order Index (LOI), being the absorption ratio of bands at 1430 cm^{-1} (characteristic of crystalline areas) and 890 cm^{-1} band (representing amorphous regions) is a parameter generally correlated to overall degree of order in cellulose (O'Connor et al., 1958). Concerning cellulose fibers, in Table 5-5 the decrease in LOI of fibers consequence of enzyme action could be observed. This reduction, meaning a reduction in the overall order degree of cellulose, could be related to an increase in accessibility to cellulose surface (Spiridon et al., 2010). This modification in LOI value was consistent with previous observations, and could help explaining the improved outcome of sulfuric acid hydrolysis of enzymatically pretreated fibers.

Total Crystallinity Index (TCI), by its side, is calculated from the ratio of absorption peaks at 1370 and 2900 cm^{-1} and claimed to be proportional to cellulose crystallinity index (Nelson and O'Connor, 1964). Hence, TCI corresponds to the crystallinity degree of cellulose. In Table 5-5 TCI values for fibers and NCC are shown. Concerning fibers, it can be seen how crystallinity increased after the enzymatic pretreatment, possibly consequence of cellulase preferential attack on amorphous regions (Ahola et al., 2008). Regarding NCC firstly, we observed TCI increasing in all samples compared to their original fibers, consequence of amorphous regions removal during acid hydrolysis. Secondly, it was observed that the larger cellulose depolymerization produced by the greatest acid dose reduced TCI values, probably because of the notorious reduction in crystals size (French and Santiago Cintrón, 2013). Lastly, enzymatic pretreatment seemed to increase TCI of NCC, possibly due to a larger presence of crystalline cellulose and/or a size increase of these regions in fibers and also to a more efficient hydrolysis.

FTIR data suggested that a more accessible and crystalline cellulose structure was obtained on cellulose fibers as a consequence of cellulase action. These modifications might have produced a cleaner and more rapid access of sulfuric acid to more abundant crystalline regions, reducing sample loss on the acid hydrolysis process and providing larger NCC particles with a higher crystallinity. This evidence was also in accordance with the proposed mechanism underlying the NCC yield increase produced by cellulase.

Table 5-5: Total crystallinity index (TCI) and Lateral order index (LOI) of fibers and NCC. Data for NCC was obtained at 25 minutes and 60 ºC.

	Cellulose fibers		NCC			
Cellulase dose	0 U/g odp	10 U/g odp	0 U/g odp		10 U/g odp	
Acid dose	-	-	62%	65%	62%	65%
LOI	1.02	0.67	0.33	0.31	0.36	0.5
TCI	0.69	0.92	1.46	0.79	2.01	1.29

5.4. Conclusions

Results presented in this chapter allowed finding the optimal chemical conditions maximizing yield for NCC isolation from enzymatically pre-treated fibers, obtaining yields higher than 80% and with a top enzyme-assisted gain of 9 points. Cellulase effects were noticeable even with a large acid concentration, highlighting the potential of this pretreatment. Optimal conditions (25 min of hydrolysis at 47ºC with 62 % wt. H_2SO_4) permitted to substantially reduce hydrolysis time (a 44%), and to increase yield in more than 10 points compared to the conditions used in chapter 4. It was found that at optimal conditions particle size was still on nano-scale and surface charge was reduced, the same that happened with sulfur content. Results also indicated that all NCC suspensions had good stability values and a narrow particle size distribution. TEM analysis confirmed the presence of NCC even under the milder hydrolysis conditions and FTIR measurements indicated that cellulase pretreatment increased crystallinity in fibers and in NCC and also accessibility to fibers. The optimal

conditions exposed in this chapter will permit a better usage of raw materials for NCC production, reducing unnecessary biomass consumption and allowing a better exploitation of the benefits of enzymatic pretreatment.

5.5. References

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. Enzymatic Hydrolysis of Native Cellulose Nanofibrils and Other Cellulose Model Films: Effect of Surface Structure. Langmuir 2008, 24 (20), 11592–11599.

Alves, L.; Medronho, B.; Antunes, F. E.; Fernández-García, M. P.; Ventura, J.; Araújo, J. P.; Romano, A.; Lindman, B. Unusual Extraction and Characterization of Nanocrystalline Cellulose from Cellulose Derivatives. J. Mol. Liq. 2015, 210, 106–112.

Anderson, S. R.; Esposito, D.; Gillette, W.; Zhu, J. Y.; Baxa, U.; McNeil, S. E. Enzymatic Preparation of Nanocrystalline and Microcrystalline Cellulose. Tappi J. 2014, 13 (5), 35–42.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Fahma, F.; Iwamoto, S.; Hori, N.; Iwata, T.; Takemura, A. Isolation, Preparation, and Characterization of Nanofibers from Oil Palm Empty-Fruit-Bunch (OPEFB). Cellulose 2010, 17, 977–985.

Fan, J. S.; Li, Y. H. Maximizing the Yield of Nanocrystalline Cellulose from Cotton Pulp Fiber. Carbohydr. Polym. 2012, 88 (4), 1184–1188.

Fillat, A.; Colom, J. F.; Vidal, T. A New Approach to the Biobleaching of Flax Pulp with Laccase Using Natural Mediators. Bioresour. Technol. 2010, 101 (11), 4104–4110.

Fillat, U.; Roncero, M. B. Effect of Process Parameters in Laccase-Mediator System Delignification of Flax pulp. Part I. Pulp Properties. Chem. Eng. J. 2009, 152 (2-3), 322–329.

Filson, P. B.; Dawson-Andoh, B. E.; Schwegler-Berry, D. Enzymatic-Mediated Production of Cellulose Nanocrystals from Recycled Pulp. Green Chem. 2009, 11 (11), 1808-1814.

Flauzino Neto, W. P.; Silvério, H. A.; Dantas, N. O.; Pasquini, D. Extraction and Characterization of Cellulose Nanocrystals from Agro-Industrial Residue - Soy Hulls. Ind. Crops Prod. 2013, 42, 480–488.

Fraschini, C.; Chauve, G.; Berre, J.-F. Le; Ellis, S.; Méthot, M.; Connor, B. O. Critical Discussion of Light Scattering and Microscopy Techniques for CNC Particle Sizing. Nord. Pulp Pap. Res. J. 2014, 29 (1), 31–40.

French, A. D.; Santiago Cintrón, M. Cellulose Polymorphy, Crystallite Size, and the Segal Crystallinity Index. Cellulose 2013, 20 (1), 583–588.

Habibi, Y.; Lucia, L. A.; Rojas, O. J. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Hubbe, M. A.; Rojas, O. J.; Lucia, L. A.; Sain, M. Cellulosic Nanocomposites: A Review. BioResources 2008, 3 (3), 929–980.

Klemm, D.; Heublein, B.; Fink, H. P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie - Int. Ed. 2005, 44 (22), 3358–3393.

Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. Angew. Chemie - Int. Ed. 2011, 50 (24), 5438–5466.

Lin, N.; Dufresne, A. Nanocellulose in Biomedicine: Current Status and Future Prospect. Eur. Polym. J. 2014, 59, 302–325.

Lu, P.; Hsieh, Y.-L. Preparation and Properties of Cellulose Nanocrystals: Rods, Spheres, and Network. Carbohydr. Polym. 2010, 82 (2), 329–336.

Martínez-Sanz, M.; Vicente, A. A.; Gontard, N.; Lopez-Rubio, A.; Lagaron, J. M. On the Extraction of Cellulose Nanowhiskers from Food by-Products and Their Comparative Reinforcing Effect on a Polyhydroxybutyrate-Co-Valerate Polymer. Cellulose 2015, 22 (1), 535–551.

Moon, R. J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose Nanomaterials Review: Structure, Properties and Nanocomposites. Chem. Soc. Rev. 2011, 40 (7), 3941–3994.

Nelson, M. L.; O'Connor, R. T. Relation of Certain Infrared Bands to Cellulose Crystallinity and Crystal Lattice Type. Part II. A New Infrared Ratio for Estimation of Crystallinity in Celluloses I and II. J. Appl. Polym. Sci. 1964, 8 (3), 1325–1341.

O'Connor, R. T.; DuPré, E. F.; Mitcham, D. Applications of Infrared Absorption Spectroscopy to Investigations of Cotton and Modified Cottons Part I: Physical and Crystalline Modifications and Oxidation. Text. Res. J. 1958, 28 (5), 382–392.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Široký, J.; Blackburn, R. S.; Bechtold, T.; Taylor, J.; White, P. Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy Analysis of Crystallinity Changes in Lyocell Following Continuous Treatment with Sodium Hydroxide. Cellulose 2010, 17, 103–115.

Spiridon, I.; Teaca, C.-A.; Bodîrlau, R. Structural Changes Evidenced By Ftir Pre-Treatment With Ionic Liquid and Enzymatic. BioResources 2010, 6 (1), 400–413.

Tanaka, R.; Saito, T.; Ishii, D.; Isogai, A. Determination of Nanocellulose Fibril Length by Shear Viscosity Measurement. Cellulose 2014, 21, 1581–1589.

Teixeira, R. S. S.; Silva, A. S. Da; Jang, J.-H.; Kim, H.-W.; Ishikawa, K.; Endo, T.; Lee, S.-H.; Bon, E. P. S. Combining Biomass Wet Disk Milling and Endoglucanase/β-Glucosidase Hydrolysis for the Production of Cellulose Nanocrystals. Carbohydr. Polym. 2015, 128, 75–81.

Thielemans, W.; Warbey, C. R.; Walsh, D. a. Permselective Nanostructured Membranes Based on Cellulose Nanowhiskers. Green Chem. 2009, 11 (4), 531–537.

Valls, C.; Roncero, M. B. Using Both Xylanase and Laccase Enzymes for Pulp Bleaching. Bioresour. Technol. 2009, 100 (6), 2032–2039.

Valls, C.; Colom, J. F.; Baffert, C.; Gimbert, I.; Roncero, M. B.; Sigoillot, J.-C. Comparing the Efficiency of the laccase–NHA and laccase–HBT Systems in Eucalyptus Pulp Bleaching. Biochem. Eng. J. 2010, 49 (3), 401–407.

Wang, Q. Q.; Zhu, J. Y.; Reiner, R. S.; Verrill, S. P.; Baxa, U.; McNeil, S. E. Approaching Zero Cellulose Loss in Cellulose Nanocrystal (CNC) Production: Recovery and Characterization of Cellulosic Solid Residues (CSR) and CNC. Cellulose 2012, 19 (6), 2033–2047.

Yanamala, N.; Farcas, M. In Vivo Evaluation of the Pulmonary Toxicity of Cellulose Nanocrystals: A Renewable and Sustainable Nanomaterial of the Future. ACS Sustain. Chem. Eng. 2014, 2, 1691–1698.

Chapter 6

Optimizing enzymatic pretreatment conditions for NCC isolation

Abstract

In this chapter conditions for the enzymatic pretreatment prior to NCC isolation were assessed. Different cellulase doses and reaction times were studied within an experimental design and NCC were obtained using optimal hydrolysis conditions from chapter 5 (45 min, 47ºC and 62% wt. H_2SO_4). At optimal enzymatic conditions, a total yield greater than 80% was achieved and the necessary enzymatic treatment time was reduced to 2h (a 90%) compared to studies in chapters 4 and 5. Different intensities of enzymatic treatments led to proportional decreases in fiber length and viscosity and also were inversely proportional to the amount of released oligosaccharides. These differences within fibers were acknowledged to lead to quantitative differences in NCC: acid hydrolysis yield was increased up to 90%, NCC surface charge was reduced and their crystallinity increased. Benefits produced by enzymatic treatments did not have influence over other NCC characteristics such as their sulfur content (≈1%), size (≈200 nm), zeta potential (≈ -50 mV) or degree of polymerization (≈200). Evidence presented in this chapter would increase the industrial feasibility of this greener technology while reducing the use of harsh corrosive sulfuric acid and generating a cleaner stream of profitable oligosaccharides.

6.1. Introduction

Research in nanocrystalline cellulose (NCC), a material also named cellulose nanocrystals, has generated a huge interest in recent years due to the promising features this material holds (Sun et al., 2014). Typically, it consists on a rigid rod-like monocrystalline cellulose domain with dimensions oscillating among 1-100 nm in width and up to several hundred nanometers in length (Lin and Dufresne, 2014). Also, they are extracted from cellulose fibers, a very abundant and inexpensive raw material. NCC has a high degree of crystal structure, a very high aspect ratio (length-to-diameter, up to 300), a large surface area (above 150 m^2/g), a very high elastic moduli, (estimated to be over 130-150 GPa) and a low thermal expansion coefficient (6 ppm/K) (Tanaka et al., 2014). This material finds many potential applications in diverse fields

such as an additive for composite materials (Moon et al., 2011), optical applications (Lin et al., 2012), or diverse uses in biomedicine (Lin and Dufresne, 2014), to name a few.

Biotechnology has been used for several applications in cellulose industry, such as biobleaching, biorefining, or even pulp quality upgrades (Valls and Roncero, 2009; Garcia-Ubasart et al., 2013; Quintana et al., 2013). Generally, the use of enzymes as a green technology allows reducing the pollution generated by traditional chemical processes, providing a solution for an enormous social concern. However, introduction of enzymes into nanocellulose production has produced limited literature up to date. Cellulases, enzymes degrading cellulose include three different enzymatic activities (Teixeira et al., 2015). Endoglucanases (E.C. 3.2.1.4) catalyze the hydrolysis of the 1, 4-glycosidic linkages of the amorphous regions of cellulose. In nature, they hydrolyze cellulose in synergy with cellobiohydrolases (E.C. 3.2.1.91), which act upon the reducing and non-reducing ends of cellulose chains. Finally, β-glucosidases (E.C. 3.2.1.21), catalyze the hydrolysis of cellobiose into glucose. Generally, this enzymatic cellulose degrading activity is capable of participating into NCC preparation, fact that is reflected in some examples of authors successfully introducing enzymes (cellulases) into nanocellulose preparation process (Zhang et al., 2012b; Anderson et al., 2014; Teixeira et al., 2015). Furthermore, enzymatic preparation of NCC has been related with an improved quality of final product compared to pure chemical processes (George et al., 2011).

One of the main drawbacks associated with NCC preparation is the low yield presented by the typical acid hydrolysis with sulfuric acid used for its preparation (Chen et al., 2015). Considering this evidence, in chapter 4 it was demonstrated that a cellulase pretreatment on cotton linters could increase the yield of NCC as well as to influence other characteristics of them. In a subsequent study, exposed in chapter 5, conditions of sulfuric acid hydrolysis in order to maximize NCC yield from cellulase-pretreated fibers were optimized using a factorial design. Maximal yield was achieved with 25 minutes of hydrolysis at 47ºC and using 62% wt. H_2SO_4. Optimizations via factorial designs have been widely used in literature for optimizing enzymatic and chemical treatments for diverse applications (Bondeson et al., 2006; Valls and Roncero, 2009). Now, in this chapter, the whole enzymatic pretreatment and sulfuric acid hydrolysis system was considered, varying cellulase dose and reaction time within a 2^2 complete factorial design. We focused into the assessment of quantitative effects of

these pretreatments of different intensity and their relations in both cellulose fibers and NCC. The purpose of this study was to find the conditions for enzymatic treatment providing the highest NCC yield in combination with optimal conditions for sulfuric acid hydrolysis described in chapter 5.

6.2. Materials and methods

For details concerning all materials and methodologies used in the present study, please refer to **chapter 2**.

6.2.1. Cellulose source and enzyme

Cotton linters provided by Celsur (Spain) were used as a raw material for experiments. Composition of fibers was: glucans content (cellulose) 97.7% ± 0.3; xylans content 2% ± 0.2; Ramnans 0.2% ± 0.15; acetyl groups 0.1% ± 0.1. Fibers, as received from provider, were beated in a valley mill for 90 minutes for reducing average length. Obtained fibers were named as "initial". A cellulase preparation (named "C"), provided by Fungal Bioproducts (Spain) and obtained from *Cerrena sp.* fungus was used for treatments. Activity as U/g from enzyme stock was 1700 and was expressed as CMCase units *i.e.* the amount of enzyme degrading 1 μmol of CMC (carboxymethilcellulose) per minute.

6.2.2. Enzymatic treatments

Enzymatic treatments were held using cellulase C on an Ahiba Easydye (Datacolor, USA) apparatus having independent 250 mL vessels with agitation consisting on upside-down inversions at 20 oscillations per minute. Treatments were performed at 55ºC, 5% consistency and pH 5 maintained with a 50 mM sodium acetate buffer solution. Enzyme dose and reaction time were variables chosen in accordance to an experimental design (Table 6-1). After reactions a liquor sample was recovered for residual enzymatic activity determination and enzyme was deactivated by heating samples to 105ºC during 15 min. Fibers were then filtered using a filter with pore size Nº2 and reaction liquor was passed through fibers 3 times in order to recover fines. No

washing was performed after treatments in order to avoid sample loss and samples of reaction liquor were saved for sugar content analysis. A control for enzymatic treatments was also performed on fibers, applying the same conditions as for treatments during 2h, but with no enzyme addition.

6.2.3. Experimental design

Enzymatic treatments were applied in accordance to a 2^2 statistical factorial plan involving two levels and two variables plus three repetitions in the central point, which required a total of 7 experiences (Table 6-1). Variables were: X1(enzyme dose), varied within 2 – 20 $U.g^{-1}$ odp (oven-dried pulp) range and X2 (reaction time) varied within 2 – 24 h. These independent variables were coded as -1 or +1; both for direct comparison of coefficients and to better understand the effect of each variable on the responses. The results of the three repetitions at the central point and their variance were used in combination with the variance of the saturated model to calculate Snedecor's F-value in order to determine whether the variance was homogeneous or heterogeneous. Since the variance was homogeneous in all cases, a linear model was constructed, its significant terms identified and potential curvature detected. Two additional points were required for solving quadratic terms confounding. Linear multiple regression technique was applied by using an Microsoft Excel spreadsheet to implement the stepwise backward regression method and discard all terms with a probability (p-value) less than 0.05.

Table 6-1: Experiences of the statistical plan with their conditions

Y	X1	X2	Cellulase dose (U/g odp)	Enzymatic treatment time (h)
Y1	-1	-1	2	2
Y2	1	-1	20	2
Y3	-1	1	2	24
Y4	1	1	20	24
Y5	0	0	11	13
Y6	0	0	11	13
Y7	0	0	11	13
Y8	1	0	20	13
Y9	0	-1	11	2

6.2.4. Nanocrystalline cellulose preparation

Nanocrystalline cellulose (NCC) was obtained from initial, control and enzymatically pretreated fibers by a controlled sulfuric acid hydrolysis, using the protocol proposed by Dong et al., 1998. Fibers were fluffed prior to hydrolysis, oven dried and cooled in a desiccator. Typically, 1.5 g of sample weighted immediately from desiccator was hydrolyzed with 62 % (w/w) sulfuric acid for 25 min at 47 ºC with an acid-to-fibers ratio of 10:1 (*i.e.* 10 mL/1 g cellulose), optimal hydrolysis conditions obtained in chapter 5. Final samples were filtered through Whatman ashless paper filters, Nº 41 (pore size 20-25 μm).

6.2.5. Samples characterization

6.2.5.1. Cellulose fibers

Enzymatic treatment yield was calculated by determining the solid residue (treated fibers) after treatments and was indicated as % of recovered fibers mass.

Initial and enzymatically treated fibers were characterized in terms of viscosity, degree of polymerization and fiber length.

Infrared spectra of fibers samples were recorded at room temperature using a Perkin Elmer Spectrum 100 ATR-FTIR spectrophotometer. Fourier transformed infrared spectroscopy (FTIR) spectral analysis was conducted within the wavenumber range of 600-4000 cm^{-1}. A total of 64 scans were run to collect each spectrum at a 1cm^{-1} resolution. Total crystallinity index (TCI) as proposed by Nelson and O`Connor (Nelson and O'Connor, 1964) was estimated from the ratio between the absorption peaks at 1370 cm^{-1} and 2900 cm^{-1}, respectively.

6.2.5.2. Enzymatic treatment effluents

Released reducing sugars on enzymatic reaction effluents were analyzed using a 1100 Agilent HPLC instrument (Agilent technologies, USA) furnished with a BIO RAD Aminex HPX-42A ion-exchange column.

Residual enzymatic activity on effluents was determined using an adapted version of Somogyi-Nelson method to determine reducing sugar concentrations on a solution (Spiro, 1966).

6.2.5.3. Nanocrystalline cellulose

Nanocrystalline cellulose was characterized in terms of yield, sulfur content, particle size, zeta potential, viscosity (degree of polymerization) and crystallinity (FTIR).

6.3. Results and discussion

6.3.1. Modelling enzymatic treatment response on fibers

Due to the degrading nature of cellulase action, a loss of cellulose mass is associated to these enzymatic treatments, fact that must be taken into account when considering process yield. In the same direction, cellulase action strongly reduced average fiber length. For studying this, values of enzymatic treatment yield and fiber length are shown in Table 6-2 and were found to fit Equation 6-1 and 6-2, respectively. As shown by equations, both responses were affected by both individual variables and also by the quadratic term of reaction time, being it the most influential one. Data predicted by models showed that enzymatic yield and fiber length suffered a great variation from 2 hours to ≈11 hours, in which a yield loss of ≈10 points (Figure 6-1A) and a ≈1 mm reduction of fiber length (Figure 6-1B) were produced. On the other hand, enzyme dose had a smaller influence than reaction time in both parameters, particularly in enzymatic treatment yield, as at 2 h of treatment, the reduction in fiber length produced by increasing enzyme dose did not produce a noticeable loss in fiber mass.

$$\text{Enzymatic treatment yield (\%)} = 88.4 - 1.4\,X1 - 3.8\,X2 - 1.6\,X1X2 + 5.7\,X2^2 \quad R^2 = 0.93$$

Equation 6-1

$$\text{Fiber length (mm)} = 0.71 - 0.25\,X1 - 0.33\,X2 + 0.48\,X2^2 \quad R^2 = 0.95$$

Equation 6-2

Table 6-2: Fiber characterization. Enzymatic treatment yield, average length, viscosity, degree of polymerization (DP), crystallinity (as total crystallinity index, TCI) and total released glucose after enzymatic treatments. *Sugar concentration as molar addition of all glucose oligosaccharides, expressed as glucose equivalents.

		Enzymatic yield (%)	Fiber length (mm)	Viscosity (mL/g)	DP	Released glucose* (mg/mL)	TCI
Initial		-	1.95 ± 0.1	777 ± 37	2813 ± 87	n/a	1.16 ± 0.02
Control 2h		96.6	1.75 ± 0.04	767 ± 17	2769 ± 74	n/a	1.18 ± 0.02
2 U	2 h	97.8	1.70 ± 0.06	564 ± 12	1931 ± 49	1.07 ± 0.04	1.24 ± 0.06
	24 h	93.2	1.26 ± 0.04	456 ± 16	1504 ± 63	2.57 ± 0.04	1.21 ± 0.03
11 U	2 h	97.5	1.47 ± 0.02	539 ± 21	1828 ± 85	1.47 ± 0.06	1.28 ± 0.07
	13 h	88.3	0.66 ± 0.03	381 ± 15	1218 ± 54	3.36 ± 0.04	1.24 ± 0.04
	13 h	88.2	0.69 ± 0.04	411 ± 9	1329 ± 34	3.25 ± 0.22	1.29 ± 0.03
	13 h	89.4	0.65 ± 0.01	415 ± 7	1346 ± 27	3.39 ± 0.08	1.22 ± 0.02
20 U	2 h	98.3	1.38 ± 0.04	444 ± 11	1456 ± 42	2.87 ± 0.02	1.28 ± 0.03
	13 h	86.4	0.57 ± 0.01	378 ± 3	1205 ± 11	3.39 ± 0.04	1.34 ± 0.03
	24 h	87.5	0.45 ± 0.01	281 ± 33	849 ± 118	4.12 ± 0.03	1.36 ± 0.07

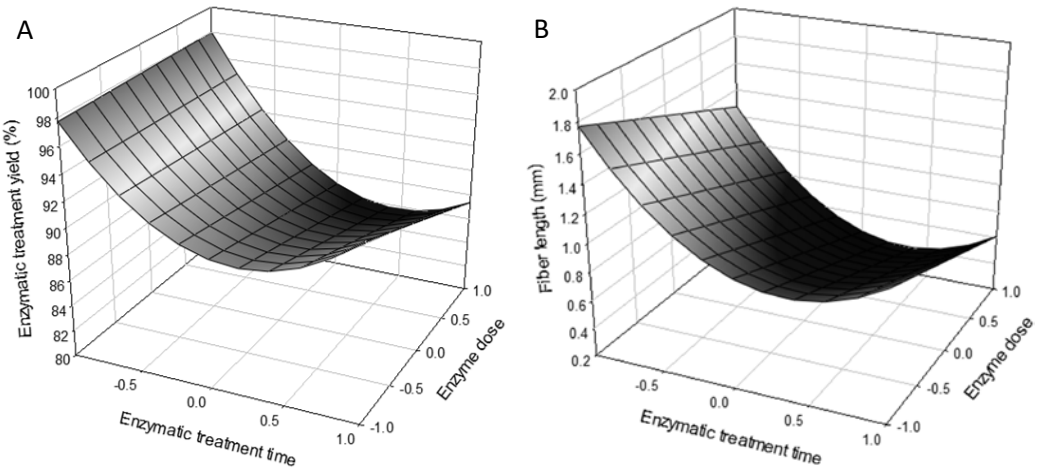

Figure 6-1: Models relating enzymatic treatment yield (A) and fiber length (B) to enzyme dose and enzymatic treatment time.

In order to fully understand the effects of enzymatic treatments in fiber length, the distribution among different measures was studied and illustrated in Figure 6-2. Comparing samples at 2 h of treatment, increase in enzyme from 2 to 20 U/g odp dose slightly reduced the amount of fibers above 1.2 mm while it increased the amount of the ones below this length. In turn, reaction time produced a major effect, as previously observed, strongly reducing the presence of fibers longer than 1.2 mm and thereafter increasing the presence of shorter ones. The action pattern of enzyme in the reduction of fiber length seemed to be the same for increases in enzyme dose or reaction time. However, the magnitude of the effects of the increase in the former was much smaller than the one of the latter. This fact could explain that no loss in cellulose mass was associated to increases in enzyme dose although a small reduction in length was observed.

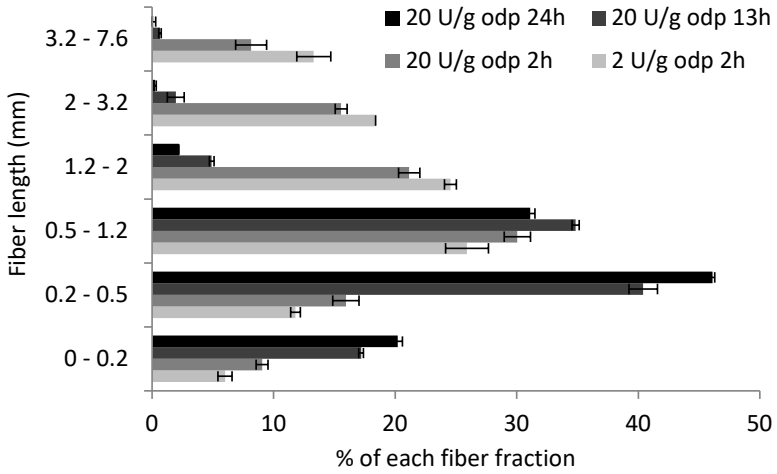

Figure 6-2: Fiber length distribution of samples after enzymatic treatments.

Oligosaccharides released as a consequence of enzymatic treatments were expressed as glucose equivalents after the molar addition of each oligosaccharide multiplied by their number of glucose units. These values are indicated in Table 6-2 and fitted Equation 6-3. For this response a similar effect to that of yield and fiber length was observed (Figure 6-3A). A large increase in glucose concentration was observed from 2 hours to ≈11 hours of treatment, up to ≈4 mg/mL, observing stabilization after this period. In this case, enzyme dose had a linear effect, smaller than

that of reaction time and independent of it, increasing sugar concentration all along enzymatic treatment. On the other hand, fibers viscosity values (Table 6-2) fitted Equation 6-4. In it, the quadratic term of reaction time was not found to affect the response and a linear surface was obtained (Figure 6-3B). Viscosity decreased as enzymatic treatment intensity increased with a minimum value obtained at the point of the most intensive enzymatic conditions, *i.e.* 20 U/g odp and 24 hours, accounting for a 50% reduction of viscosity.

$$\text{Released glucose (mg/mL)} = 3.17 + 0.71\,X1 + 0.77\,X2 - 0.59\,X2^2 \quad R^2 = 0.93$$

Equation 6-3

$$\text{Viscosity (mL/g)} = 429 - 69\,X1 - 76\,X2 \quad R^2 = 0.93$$

Equation 6-4

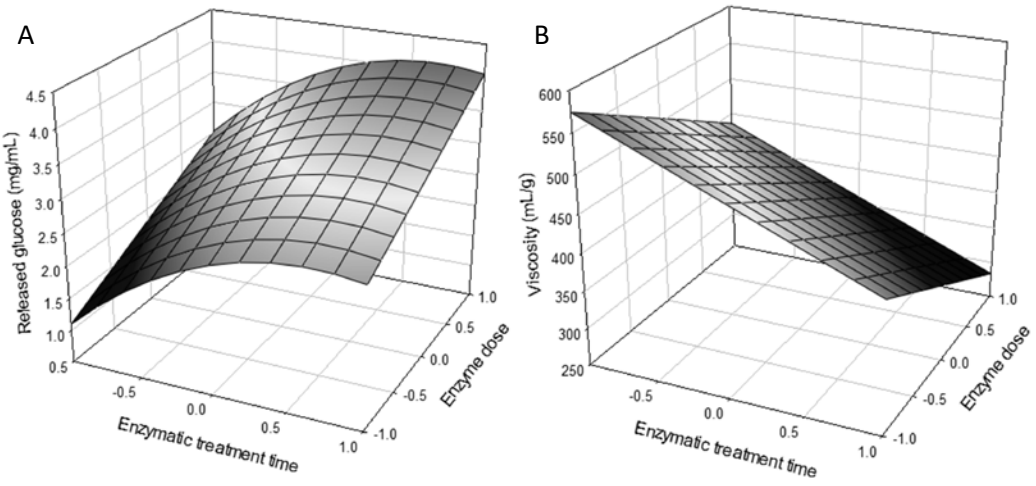

Figure 6-3: Models relating total released glucose (A) and fiber viscosity (B) to enzyme dose and enzymatic treatment time.

Enzymatic treatments also increased cellulose crystallinity, expressed as total crystallinity index (TCI). Data indicated that fibers TCI (Figure 6-4) increased as a consequence of higher enzyme doses, while reaction time did not seem to produce any effect. Generally, this crystallinity increase indicates that a higher amount of

crystalline cellulose was present on fibers after enzymatic treatments. The explanation of this might be found in the reduction in amorphous cellulose regions caused by cellulase preferential attack on them. This preference is due to the larger accessibility presented by β-1,4 glycosidic bonds in these domains (Tąta et al., 2015).

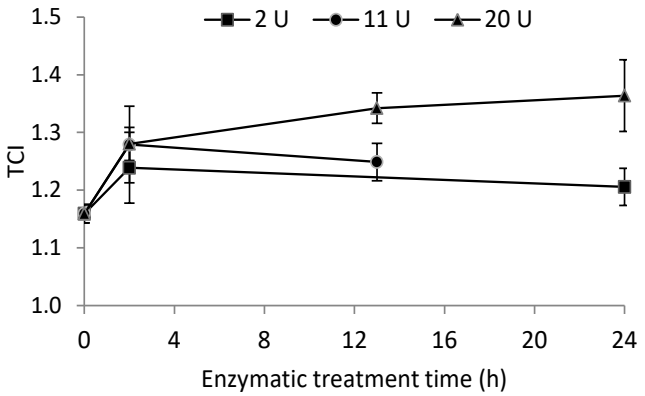

Figure 6-4: Total crystallinity index of fibers (TCI) of fibers during enzymatic treatments.

6.3.2. Enhancing enzymatic effects on nanocrystalline cellulose

6.3.2.1. Modelling enzymatic treatment response on nanocrystalline cellulose

Low yields traditionally attributed to NCC isolation raised interest in the study of ways for its increase, in order to increment the industrial feasibility of this process (Fan and Li, 2012; Chen et al., 2015). NCC yield values from sulfuric acid hydrolysis (Table 6-3) fitted the model indicated in Equation 6-5, showing that it was positively influenced by both independent variables studied in this chapter. Cellulase pretreatment increased the yield of sulfuric acid hydrolysis up to a 90%, with a larger effect produced by reaction time (Figure 6-5). NCC yield model revealed an linear inverse correlation to the model presented by fibers viscosity. The minimal and maximal values of NCC yield were shown by 2 U/g odp and 2 h and 20 U/g odp and 24 h samples, respectively. These samples also showed the maximal and minimal fiber viscosity and fiber length values, respectively. This suggested that a higher depolimerization and shortening of fibers by cellulase was the cause for the increase in the yield of NCC hydrolysis.

Table 6-3: NCC characterization. Sulfuric acid yield, total yield, surface charge and crystallinity (as TCI).

		NCC yield (%)	Total yield (%)	Surface charge (meq/g)	TCI
Initial		61.1 ± 3.3	61.1	0.156 ± 0.010	1.38 ± 0.05
Control 2h		71.1 ± 1.3	68.7	0.190 ± 0.004	1.30 ± 0.12
2 U	2 h	78.4 ± 2.1	76.7	0.171 ± 0.006	1.41 ± 0.15
	24 h	85.4 ± 3.6	79.6	0.152 ± 0.006	1.39 ± 0.09
11 U	2 h	81 ± 2.8	79	0.163 ± 0.003	1.65 ± 0.14
	13 h	85.5 ± 1.3	75.4	0.151 ± 0.005	1.47 ± 0.04
	13 h	84 ± 1	74.1	0.142 ± 0.004	1.44 ± 0.06
	13 h	83.6 ± 3.7	74.7	0.145 ± 0.003	1.45 ± 0.08
20 U	2 h	83.9 ± 0.9	82.5	0.160 ± 0.002	1.66 ± 0.07
	13 h	87.2 ± 2.1	75.4	0.158 ± 0.009	1.50 ± 0.18
	24 h	90.1 ± 0.8	78.8	0.153 ± 0.006	1.69 ± 0.13

$$\text{NCC yield (\%)} = 84.4 + 2.6\,X1 + 3.3\,X2 \quad R^2 = 0.97.$$

Equation 6-5

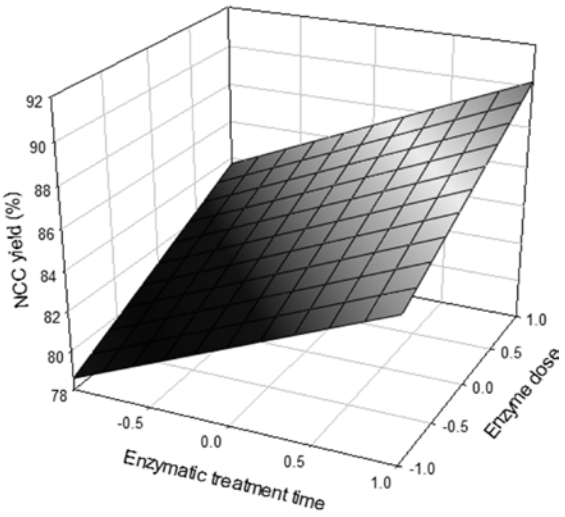

Figure 6-5: Model relating NCC hydrolysis yield to enzyme dose and enzymatic treatment time.

As stated in introduction and considering evidence previously exposed, calculation of total yield, as combined enzymatic and acid hydrolysis yields becomes crucial for acknowledging a real value of the outcome of the NCC isolation process. A compromise solution between the gain in the NCC yield and the loss of fibers mass both due to enzymatic pretreatment must be found. Total yield values are indicated in Table 6-3 and were found to fit Equation 6-6. Compared to the model expressed in Equation 6-5, individual influence of enzyme dose decreased, while treatment time now influenced only in the quadratic term and double-interacting with the former. Total yield (Figure 6-6) had a minimum value at around 11 h of treatment, coinciding with the point of stabilization of enzyme effect on fibers, showing higher values with shorter and longer times. This was explained by yields of both enzymatic and sulfuric acid hydrolysis (Figure 6-1A and Figure 6-5). At short reaction times the loss in cellulose mass by enzymatic treatments was small, while with extended treatments, cellulose mass loss was compensated by higher gains in NCC hydrolysis yield.

$$\text{Total yield (\%)} = 74.6 + 1.2\ X1 - 1.6\ X1X2 + 4.7\ X2^2 \quad R^2 = 0.98$$

Equation 6-6

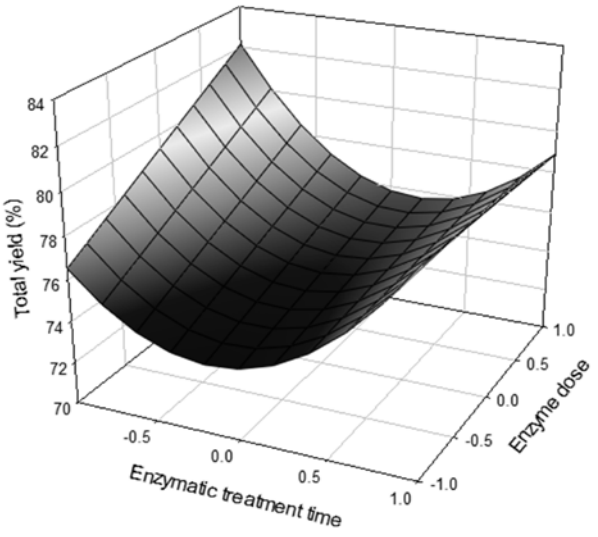

Figure 6-6: Model relating total yield to enzyme dose and enzymatic treatment time.

Surface charge of NCC (Table 6-3) was found to fit Equation 6-7. As can be observed, it was negatively influenced by enzymatic reaction time and positively by the quadratic term of enzyme dose. It was observed that surface charge of NCC was slightly reduced with longer enzymatic pretreatments (Figure 6-7), while enzyme dose produced no significant affectation. This charge reduction was in accordance with observations from chapters 4 and 5 where enzymatic effects seemed to reduce this parameter.

$$\text{Surface charge (meq/g)} = 0.152 - 0.007\ X2 + 0.007\ X1^2 \quad R^2 = 0.79$$

Equation 6-7

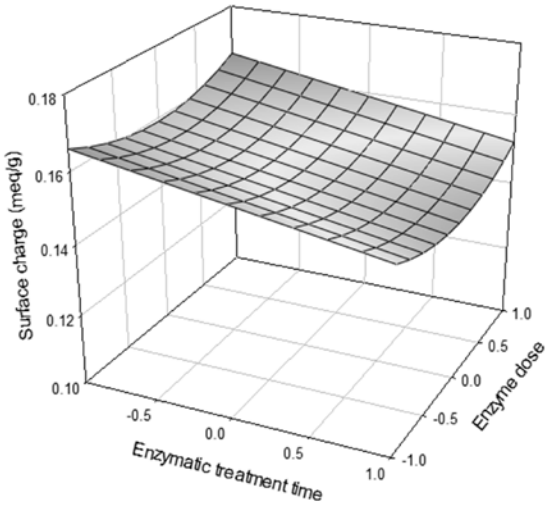

Figure 6-7: Model relating NCC surface charge to enzyme dose and enzymatic treatment time.

Crystallinity values of NCC, as TCI (Table 6-3) fitted Equation 6-8. TCI of NCC was affected by enzyme dose linearly and by quadratic terms of both variables. In Figure 6-8 it can be observed how enzymatic pretreatment on fibers led to NCC with a higher crystallinity. Data shows that TCI was majorly increased by enzyme dose with values tending to stabilize after a ≈10 U/g odp dose. However, no significant effect was found to be produced by enzymatic reaction time, a similar behavior to that observed

in TCI of fibers. Also, it is important to remark that the optimal point of the process concerning total yield (20 U/g odp, 2h) corresponded to NCC with the higher crystallinity, providing further evidence of the quality increase produced by this enzymatic-aided process.

$$TCI = 1.46 + 0.14\,X1 - 0.1\,X1^2 + 0.18\,X2^2 \quad R^2 = 0.99$$

Equation 6-8

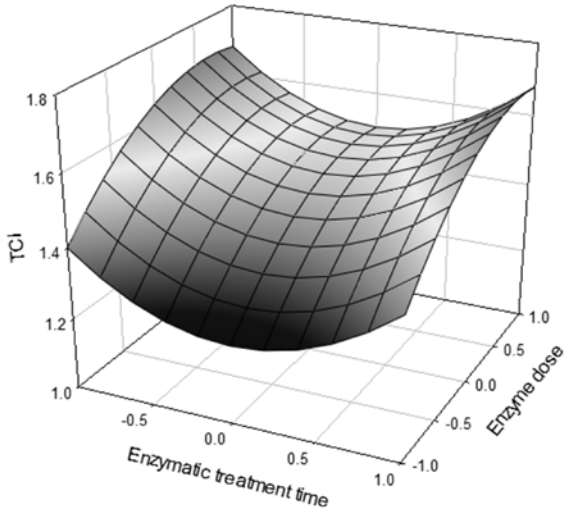

Figure 6-8: Model relating NCC total crystallinity index (TCI) to enzyme dose and enzymatic treatment time.

The observation of former data also foregrounded the fact that quantitative differences in enzymatic treatment intensity led to quantitative differences in NCC features. This statement is well illustrated in Figure 6-9A, where it can be observed how NCC hydrolysis yield is linearly correlated to fibers viscosity (inverse correlation) and also to total released glucose (as glucose equivalents). Also, with the aim of further illustrating this, Figure 6-9B correlates chain scission number (CSN), i.e. the average number of cuts produced in cellulose chains with the increase in yield derived from enzymatic action. The correlation between both parameters indicated again that a higher number of cuts, *i.e.* a stronger enzymatic action, corresponded to a greater

increase in yield. Finally, the reduction of NCC electrical charge produced by the enzyme is well illustrated in Figure 6-9C, where larger NCC yields (*i.e.* larger enzymatic effects) led to smaller values of surface charge, agreeing with data exposed in Figure 6-7.

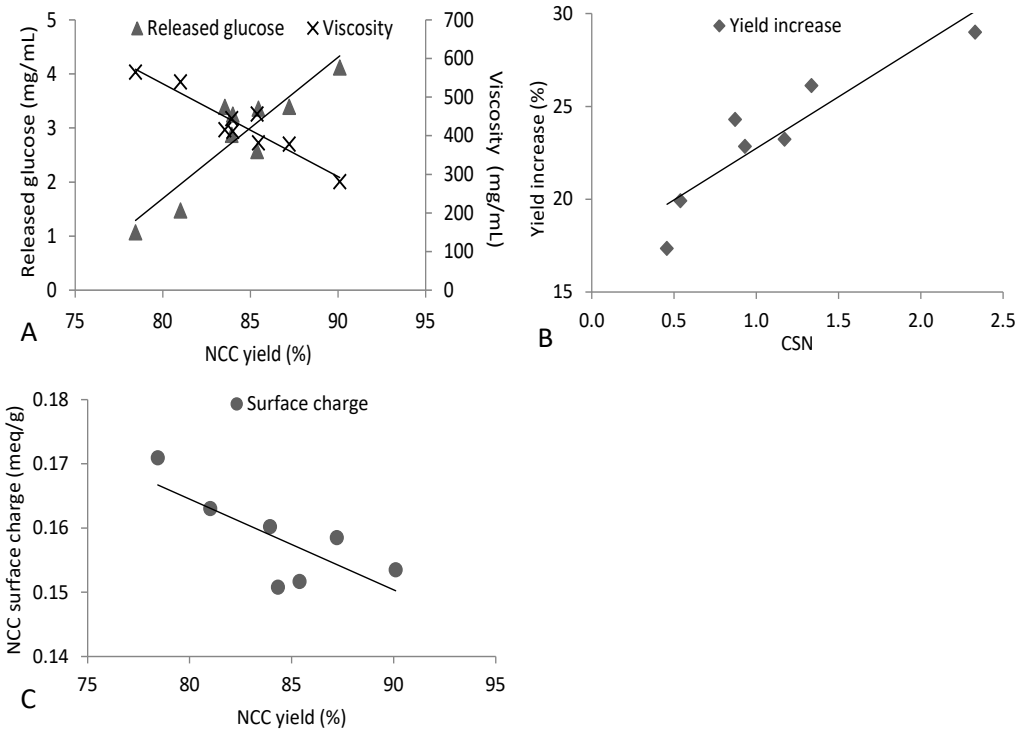

Figure 6-9: Cellulase quantitative effects. Total released sugars (as glucose equivalents) and fibers viscosity expressed in front of NCC yield (A), NCC yield increase expressed versus chain scission number (CSN), both calculated from initial fibers (B) and NCC surface charge expressed versus NCC yield (C).

6.3.3. Studying enzymatic reaction effluents

Enzymatic hydrolysis rate, calculated dividing the total glucose equivalents produced during each enzymatic treatment by the total duration of the treatment (in minutes), is illustrated in Figure 6-10 in front of the hydrolysis yield obtained from each sample. For all enzymatic doses, highest hydrolysis rates were found at 2 h, with

higher values at a higher dose. From this point, extending treatment up to 24 h time seemed to reduce hydrolysis rate. This reduction was possibly due to the increase in oligosaccharides concentration on reaction media, compounds which are known to be capable of act as cellulase inhibitors (Nguyen et al., 2015). Interestingly, the maximal hydrolysis rate, *i.e.*, the point of maximal hydrolytic efficiency, was found at 2 h of treatment and with 20 U/g odp. This point was also found to be the optimal for cellulase application as it offered the highest total yield, showing a correlation between efficiency of the entire process and of enzymatic catalysis. Furthermore, in order to validate these results, residual activity (as % of initial dose) was measured. After 24 hours of treatment, 2 U/g odp and 20 U/g odp samples showed activity conservation values of 55% ± 10 and 24% ± 4, confirming that the enzyme was still active after 24 h and thereafter validating data shown in Figure 6-10.

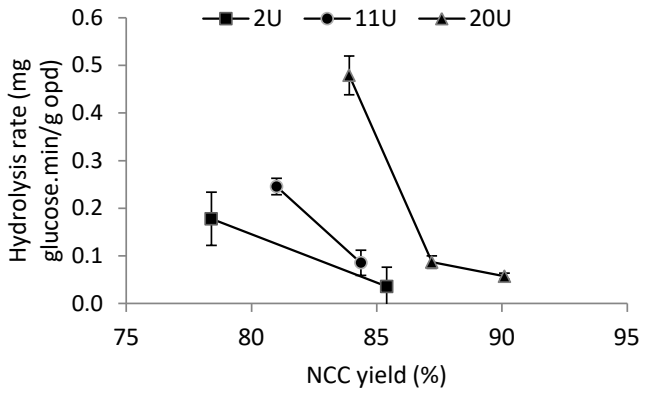

Figure 6-10: Enzymatic hydrolysis rate, as mg glucose released per minute as a consequence of enzymatic treatments expressed in front NCC hydrolysis yield.

Concerning the different sugar species found in effluents (Table 6-4), in the first place, a small amount of xylose was found. This presence was product of a xylanolytic activity present on cellulase preparation and proceeded of the hydrolysis of the small amount of xylans initially present on fibers. In the second place, concerning glucose-oligosaccharides, glucose was found to be the main released sugar, followed by cellobiose and cellotriose, in a decreasing amount. Proportions among glucose-oligosaccharides are also illustrated in Figure 6-11. Differences in enzyme dose led to a

variation among these proportions. Generally, higher enzymatic doses led to the release of longer oligosaccharides within two hours of treatment, fact well illustrated by the finding of cellotetraose only in one sample. Meanwhile, reaction time seemed to tend to reestablish the original proportions among oligosaccharides, *i.e.* ≈67% glucose, ≈30% cellobiose and ≈3% cellotriose.

Table 6-4: Reducing sugar concentration (in mg/mL) on enzymatic treatment effluents. X1, C4, C3, C2 and C1 stand for xylose, cellotetraose, cellotriose, cellobiose and glucose respectively. *Sugar concentration as molar addition of all glucose oligosaccharides, expressed as glucose equivalents.

		X1	C4	C3	C2	C1
2U	2 h	0.095 ± 0.02	0	0.011 ± 0.007	0.39 ± 0.01	0.645 ± 0.041
	24 h	0.502 ± 0.012	0	0.122 ± 0.024	1.171 ± 0.039	1.208 ± 0.027
11U	2 h	0.208 ± 0.032	0	0.016 ± 0.03	0.676 ± 0.017	0.744 ± 0.043
	13 h	0.300 ± 0.09	0	0.089 ± 0.023	1.407 ± 0.002	1.780 ± 0.067
	13 h	0.204 ± 0.031	0	0.080 ± 0.014	1.423 ± 0.115	1.663 ± 0.111
	13 h	0.329 ± 0.085	0	0.091 ± 0.006	1.393 ± 0.009	1.827 ± 0.059
20U	2 h	0.373 ± 0.046	0.061 ± 0.002	0.128 ± 0.02	1.216 ± 0.05	1.389 ± 0.03
	13 h	0.452 ± 0.003	0	0.098 ± 0.014	1.138 ± 0.012	2.088 ± 0.013
	24 h	0.602 ± 0.022	0	0.103 ± 0.002	1.214 ± 0.013	2.728 ± 0.001

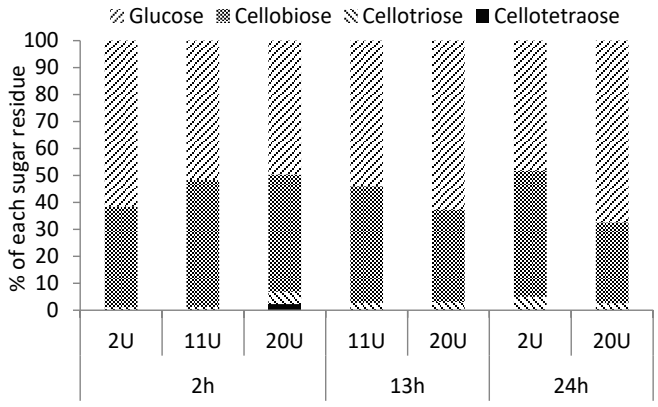

Figure 6-11: Proportion of each oligosaccharide released during enzymatic hydrolysis.

6.3.3.1. NCC sulfur content, size and stability

NCC surface charge is responsibility of charged sulfate moieties introduced on their surface during sulfuric acid hydrolysis (Abitbol et al., 2013; Peyre et al., 2015). Sulfur content data of NCC (Table 6-5) failed to show quantitative reductions produced by cellulase pretreatment, as observed for surface charge. Nevertheless, it could be observed that compared to initial fibers, enzymatic pretreatment on fibers led to NCC with lower sulfur content. In addition, sulfate groups on NCC are known to increase the thermodegradability of the material (Roman and Winter, 2004) making the reduction produced by cellulase a positive modification for NCC quality.

Another NCC parameter, suspension stability, is critical in the preparation of nanocomposites (Filson et al., 2009). This stability is indicated by the absolute value of zeta potential (electrophoretic mobility) of suspensions and is promoted by the negative charge of sulfate groups on crystals surface (Peyre et al., 2015). Table 6-5 shows zeta potential values, being all them among -50 mV indicating high suspension stability, which seemed to be maintained regardless of the enzymatic treatment performed. A similar behavior was shown by polydispersity index (PDI), as all suspensions showed a narrow particle size distribution.

Concerning NCC dimensions, it was observed that different intensities of enzymatic pretreatment did not produce any modification in average particle size of resulting NCC (Table 6-5). With this, it was highlighted that the benefits of cellulase pretreatment did not result in deleterious modifications in the morphology of NCC. Also, this fact was already observed in chapter 5, when cellulase pretreatment produced no affectation on NCC size with 62% wt. sulfuric acid. However, in chapter 4 a slight size increase in NCC was produced by enzymatic pretreatment. This evidence remarks again the fact that the effects of enzymatic pretreatment are largely dependent on the acid hydrolysis conditions, which were modified within these studies.

Finally, the degree of polymerization (DP) of cellulose chains in NCC was calculated from viscosity values (Table 6-5). DP of NCC did not seem to be modified by enzymatic treatments, observing in all cases that cellulose chains were formed by ≈200 glucose units. These values were similar to those reported by other authors for NCC obtained via sulfuric acid hydrolysis (Satyamurthy et al., 2011).

Table 6-5: NCC sulfur content, average size, electrophoretic mobility, polydispersity index (PDI) and degree of polymerization (DP).

		Sulfur content (% S)	Zeta Potential (mV)	PDI	Z average (nm)	DP
Initial		1.21 ± 0.03	-47.2 ± 0.6	0.18 ± 0.04	205 ± 4	183 ± 17
Control 2h		1.12 ± 0.06	-49.1 ± 0.7	0.19 ± 0.03	184 ± 19	200 ± 25
2 U	2 h	1.22 ± 0.02	-46.7 ± 0.7	0.19 ± 0.01	191 ± 25	188 ± 6
	24 h	1.12 ± 0.04	-48.2 ± 0.4	0.18 ± 0.03	206 ± 7	193 ± 17
11 U	2 h	0.99 ± 0.01	-49.3 ± 0.7	0.18 ± 0.04	199 ± 4	193 ± 12
	13 h	1.03 ± 0.04	-49.4 ± 0.5	0.19 ± 0.01	206 ± 5	179 ± 12
	13 h	0.92 ± 0.01	-48.7 ± 0.4	0.17 ± 0.02	204 ± 13	173 ± 21
	13 h	0.87 ± 0.01	-48.5 ± 0.6	0.19 ± 0.02	209 ± 24	210 ± 12
20 U	2 h	0.99 ± 0.05	-48.9 ± 0.7	0.20 ± 0. 01	206 ± 10	208 ± 26
	13 h	1.03 ± 0.03	-48.9 ± 0.6	0.19 ± 0.02	195 ± 9	203 ± 14
	24 h	1.05 ± 0.01	-48.2 ± 0.7	0.19 ± 0.02	183 ± 14	198 ± 41

6.3.4. Optimal point and models verification

As stated in previous sections, the objective of this chapter was to find the optimal conditions for enzymatically pretreating fibers in order to produce the largest possible total NCC yield. Thus, the optimal point of the cellulase combined with acid hydrolysis was found at: 20 U/g odp cellulase dose and 2 h of treatment, producing a ≈82 % total yield, 21 points higher than that of NCC obtained from initial fibers. This total yield was similar to that reported by Tang et al., (2013) using a non-conventional preparation procedure obtaining NCC esterified with acetic acid. Also, it was noticeably bigger than other optimal values reported using sulfuric acid hydrolysis (Fan and Li, 2012; Chen et al., 2015). Moreover, although it involved duplicating the enzyme dose, this point allowed reducing in a 90% the required enzymatic treatment time compared to chapters 4 and 5. In addition, if increasing enzyme dose resulted unaffordable, a total yield of ≈79% was obtained using a ≈11 U/g odp dose and 2 hours of treatment, representing a loss of 3 points in total yield but a smaller enzyme dose. This strong reduction would increase the industrial feasibility of this greener process, as industry is usually reluctant to long treatments. Accordingly, enzyme showed the largest hydrolysis rate *i.e.* the one using more efficiently its potential, at 20 U/g odp and at 2 hours of treatment (Figure 6-10), conditions defined as optimal. In other words,

this optimization meant a reduction of the hydrolysis of biomass mediated by sulfuric acid in benefit of an efficient enzymatic catalysis. Furthermore, sugars present on effluents as a result of NCC manufacture could be used as a feedstock, for example, for bioethanol conversion (Brinchi et al., 2013). In this case, enzymatic hydrolysis effluents permit an easier usage than those produced by sulfuric acid hydrolysis, due to the absence of sulfuric acid in them, highlighting another benefit of the proposed enzymatic-assisted process.

Finally, with the aim of verifying the obtained models, new samples were prepared using the optimal cellulase conditions plus another sample with a 20 U/g odp dose and 24 h of treatment, which led to a total yield of ≈79% and thereafter was also interesting. Table 6-6 shows data obtained from these new samples and also the predicted data by models. As can be observed, new values were in all cases in accordance with those predicted by models or similar to previous experimental data.

Table 6-6: Characterization of samples for models verification. New experimental values and those predicted by models are indicated. *When no model was found fitting data, previous experimental data is indicated.

	20U 2h		20U 24h	
Fibers	Predicted*	Observed	Predicted*	Observed
Enzymatic treatment yield (%)	98	96.3	87.4	85.8
Fiber length (mm)	1.27	1.28 ± 0.04	0.61	0.48 ± 0.02
Viscosity (mL/g)	436.1	457 ± 28	283.7	296 ± 12
Released glucose (mg/mL)	2.52	2.62 ± 0.09	4.06	4.3 ± 0.19
TCI*	1.28 ± 0.03	1.25 ± 0.02	1.36 ± 0.06	1.30 ± 0.06
NCC				
NCC yield (%)	83.7	84.5 ± 0.8	90.2	89.8 ± 0.8
Total yield (%)	82.2	81.4	78.9	77.1
Surface charge (meq/g)	0.166	0.172 ± 0.005	0.152	0.156 ± 0.004
TCI	1.68	1.61 ± 0.1	1.68	1.65 ± 0.16
Sulfur content (% S)*	0.99 ± 0.05	1.1 ± 0.09	1.05 ± 0.01	0.94 ± 0.05
Z average (nm)*	206 ± 10	186 ± 11	183 ± 14	204 ± 8
Z potential (mV)*	-48.9 ± 0.7	-49.6 ± 0.5	-48.2 ± 0.7	-50.7 ± 0.8
PDI*	0.2 ± 0.1	0.21 ± 0.02	0.19 ± 0.02	0.2 ± 0.02
DP*	208 ± 26	198 ± 18	198 ± 41	203 ± 50

6.4. Conclusions

Evidence presented in this chapter allowed finding the optimal enzymatic conditions for NCC isolation in combination with optimal sulfuric acid hydrolysis ones from chapter 5 (25 minutes of hydrolysis at 47ºC and 62% wt. H_2SO_4). Now, an enzyme dose of 20 U/g odp and 2h of hydrolysis allowed reaching a total NCC yield of ≈82%. This outcome was found to be 12 points higher to that of NCC from control fibers and 21 points higher than that of NCC obtained from initial ones. Also, this optimization reduced the necessary enzymatic treatment time in a 90% (from 24h to 2h) compared to previous chapters, boosting the industrial feasibility of this greener technology. Furthermore, enzymatic pretreatment showed to increase NCC crystallinity and to slightly reduce their surface charge, not affecting other characteristics. We found that quantitative differences in enzymatic effects on fibers led to proportional differences in NCC. The use of optimal enzymatic conditions, together with those obtained in chapter 5 would permit to reduce the use of harsh corrosive sulfuric acid for NCC production while generating a more easily exploitable stream of oligosaccharide-rich effluents.

6.5. References

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Anderson, S. R.; Esposito, D.; Gillette, W.; Zhu, J. Y.; Baxa, U.; McNeil, S. E. Enzymatic Preparation of Nanocrystalline and Microcrystalline Cellulose. Tappi J. 2014, 13 (5), 35–42.

Bondeson, D.; Mathew, A.; Oksman, K. Optimization of the Isolation of Nanocrystals from Microcrystalline Cellulose by Acid Hydrolysis. Cellulose 2006, 13 (2), 171–180.

Brinchi, L.; Cotana, F.; Fortunati, E.; Kenny, J. M. Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications. Carbohydr. Polym. 2013, 94 (1), 154–169.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Fan, J. S.; Li, Y. H. Maximizing the Yield of Nanocrystalline Cellulose from Cotton Pulp Fiber. Carbohydr. Polym. 2012, 88 (4), 1184–1188.

Filson, P. B.; Dawson-Andoh, B. E.; Schwegler-Berry, D. Enzymatic-Mediated Production of Cellulose Nanocrystals from Recycled Pulp. Green Chem. 2009, 11 (11), 1808-1814.

Garcia-Ubasart, J.; Torres, A. L.; Vila, C.; Pastor, F. I. J.; Vidal, T. Biomodification of Cellulose Flax Fibers by a New Cellulase. Ind. Crops Prod. 2013, 44, 71–76.

George, J.; Ramana, K. V.; Bawa, a. S.; Siddaramaiah. Bacterial Cellulose Nanocrystals Exhibiting High Thermal Stability and Their Polymer Nanocomposites. Int. J. Biol. Macromol. 2011, 48 (1), 50–57.

Lin, N.; Dufresne, A. Nanocellulose in Biomedicine: Current Status and Future Prospect. Eur. Polym. J. 2014, 59, 302–325.

Lin, N.; Huang, J.; Dufresne, A. Preparation, Properties and Applications of Polysaccharide Nanocrystals in Advanced Functional Nanomaterials: A Review. Nanoscale 2012, 4 (11), 3274–3294.

Moon, R. J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose Nanomaterials Review: Structure, Properties and Nanocomposites. Chem. Soc. Rev. 2011, 40 (7), 3941–3994.

Nelson, M. L.; O'Connor, R. T. Relation of Certain Infrared Bands to Cellulose Crystallinity and Crystal Lattice Type. Part II. A New Infrared Ratio for Estimation of Crystallinity in Celluloses I and II. J. Appl. Polym. Sci. 1964, 8 (3), 1325–1341.

Nguyen, L. T.; Neo, K. R. S.; Yang, K.-L. Continuous Hydrolysis of Carboxymethyl Cellulose with Cellulase Aggregates Trapped inside Membranes. Enzyme Microb. Technol. 2015, 78, 34–39.

Peyre, J.; Pääkkönen, T.; Reza, M.; Kontturi, E. Simultaneous Preparation of Cellulose Nanocrystals and Micron-Sized Porous Colloidal Particles of Cellulose by TEMPO-Mediated Oxidation. Green Chem. 2015, 17 (2), 808–811.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. An Enzyme-Catalysed Bleaching Treatment to Meet Dissolving Pulp Characteristics for Cellulose Derivatives Applications. Bioresour. Technol. 2013, 148, 1–8.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Satyamurthy, P.; Jain, P.; Rudrapatna, H. B.; Vigneshwaran, N. Preparation and Characterization of Cellulose Nanowhiskers from Cotton Fibres by Controlled Microbial Hydrolysis. Carbohydr. Polym. 2011, 83 (1), 122–129.

Spiro, R. G. R. Analysis of Sugars Found in Glycoproteins. Methods Enzymol. 1966, 566 (C), 3–26.

Sun, Q.; Mandalika, A.; Elder, T.; Nair, S. S.; Meng, X.; Huang, F.; Ragauskas, A. J. Nanocomposite Film Prepared by Depositing Xylan on Cellulose Nanowhiskers Matrix. Green Chem. 2014, 16 (7), 3458–3462.

Tanaka, R.; Saito, T.; Ishii, D.; Isogai, A. Determination of Nanocellulose Fibril Length by Shear Viscosity Measurement. Cellulose 2014, 21, 1581–1589.

Tang, L.; Huang, B.; Lu, Q.; Wang, S.; Ou, W.; Lin, W.; Chen, X. Ultrasonication-Assisted Manufacture of Cellulose Nanocrystals Esterified with Acetic Acid. Bioresour. Technol. 2013, 127, 100–105.

Tąta, A.; Sokołowska, K.; Świder, J.; Konieczna-Molenda, A.; Proniewicz, E.; Witek, E. Study of Cellulolytic Enzyme Immobilization on Copolymers of N-Vinylformamide. Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 2015, 149, 494–504.

Teixeira, R. S. S.; Silva, A. S. Da; Jang, J.-H.; Kim, H.-W.; Ishikawa, K.; Endo, T.; Lee, S.-H.; Bon, E. P. S. Combining Biomass Wet Disk Milling and Endoglucanase/β-Glucosidase Hydrolysis for the Production of Cellulose Nanocrystals. Carbohydr. Polym. 2015, 128, 75–81.

Valls, C.; Roncero, M. B. Using Both Xylanase and Laccase Enzymes for Pulp Bleaching. Bioresour. Technol. 2009, 100 (6), 2032–2039.

Zhang, Y.; Xue, G. X.; Zhang, X. M.; Zhao, Y. Enzymatic Preparation of Nanocrystalline Cellulose from Bamboo Fibers. Adv. Mater. Res. 2012, 441, 754–758.

Chapter 7

High-cellulose content fibers preparation from a non-wood alkaline pulp

Abstract

In this chapter, treatments with a xylanase (X) and carbohydrases mixture (Cx) were applied on a TCF bleached sisal pulp in order to obtain high-cellulose content fibers for NCC preparation. TCF sisal fibers were chosen in accordance to data exposed in chapter 3. Concerning hemicelluloses, a limit of ≈12% w/w final content was found regardless of the enzymatic treatment assessed either with X or Cx+X treatments. Then, an extraction with 4% and 9% w/v NaOH was performed for further removal. It was found that NaOH dose could be strongly reduced if combined with Cx or Cx + X treatments. Also, if necessary, a stronger reduction could be obtained with 9% w/v NaOH, which was found to be boosted in a 14% if performed after a treatment with Cx. Finally, an end-product with a low content in xylans (≈2.9% w/w) and in HexA (5.8 μmol/odp), low Kappa Number (≈ 0.7) and high ISO brightness (≈ 86.2 % ISO) was obtained. HPLC analysis of effluents provided useful information of enzymatic catalytic mechanisms.

7.1. Introduction

As stated in chapter 1, the objective of the present thesis was to introduce biotechnology in the NCC preparation process, also stating that high-cellulose content fibers were the preferential cellulose source for this purpose. In chapter 3 it was exposed that TCF sisal fibers presented the highest content in hemicelluloses among all raw materials, being it then the best target for treatments intended for their removal. Then, the objective of this chapter was to be able to convert any bleached cellulosic raw material into a high-quality source for NCC production. Quality improvement from paper grade to high cellulose content fibers has attracted plenty of interest in recent years (Li et al., 2015). Traditionally, pulps with low hemicelluloses content have been obtained through acid sulphite or pre-hydrolysis Kraft process (Li et al., 2015). On these processes, hemicelluloses that are present on pulp suffer a greater attack than during alkaline processes such as Kraft or NaOH-AQ, reducing their presence on final product. However, pulps obtained through these processes have some drawbacks related to quality of final product or the pollution they generate. Also, these pulping processes imply higher costs than alkaline ones in terms of chemical consumption,

production rate, inventories and storage space (Barlow and Hillman, 2006). For these reasons, several methods have been studied in order to carry out the selective elimination of hemicelluloses from alkaline pulps (Jackson et al., 1998; Bajpai and Bajpai, 2001; Kopcke et al., 2008). These methods include nitren, cuen and alkaline extraction. Besides them, enzymatic hydrolysis of different components of lignocellulose has attracted special attention because of its potential as a "green" process. It is well known that biomass availability to enzymes is hindered by diverse factors (Zhu et al., 2008). Because of this, several methods have been studied for enhancing enzymatic biomass conversion, including physical, chemical, biological or thermophysical pretreatments (Maache-Rezzoug et al., 2011; Pierre et al., 2011b). Among enzymes, xylanases have been traditionally used in pulp and paper industry for pulp bleaching (Valls and Roncero, 2009; Fillat et al., 2011). In this work, however, they are applied on bleached pulps with the purpose of removing hemicelluloses. Other enzymes, such as endoglucanases (cellulases), have been mainly used by authors for fibers biorefining (Garcia-Ubasart et al., 2013), biomass saccharification (Pierre et al., 2011b; Zhang et al., 2012a; Pihlajaniemi et al., 2014) or increasing cellulose reactivity (Kopcke et al., 2008; Pierre et al., 2011a; Quintana et al., 2013; Miao et al., 2014). In this study, a cellulase is applied with the objective of increasing cellulose accessibility for enhancing the action of xylanase enzyme and also for adjusting cellulose viscosity, a crucial feature of fibers intended for NCC manufacture.

This study focuses on the possibility of using bleached non-wood fibers from sisal (*Agave sisalana*), to obtain high cellulose-content (HCC) fibers by means of enzymatic treatments combined with alkaline extractions. Bleached sisal fibers were used and different enzymatic treatments with new carbohydrases were applied in order to modify them. Although similar works have been published in literature (Henriksson et al., 2005; Ibarra et al., 2009; Wang et al., 2014), this study introduced new enzymes, a xylanase and a carbohydrases mixture (containing endoglucanase and xylanase activities, not still commercially available) together with a NaOH extraction. Furthermore, possibilities of enzymatic treatments were studied by assessing different ways of applications including a newly focused evaluation of xylanolytic treatments. Also, a comprehensive study of enzymatic effects on fibers was performed for better understanding the effects of these new catalysts.

7.2. Materials and methods

For details concerning all materials and methodologies used in the present study, please refer to **chapter 2**.

7.2.1. Pulp and enzymes

A totally chlorine free (TCF) bleached pulp from sisal (*Agave sisalana*) was used as a raw material. Pulp was provided by Celesa (Spain), and was obtained by an alkaline NaOH-AQ process. Pulp initial parameters were: Content in hemicelluloses (Xylans, % w/w) = 16.1 ± 0.3; Kappa Number (KN) = 4.7 ± 0.2; ISO Brightness (%) = 82.1 ± 0.3; Viscosity (mL/g) = 616 ± 41; HexA content (μmol/g odp) = 45.1 ± 1.5; Fock solubility (%) 13.2 ± 0.2.

A xylanase (X) and a carbohydrases mixture (Cx) were used for treatments, both provided by Fungal Bioproducts (Spain) and obtained from *Cerrena sp* fungus. Carbohydrases mixture (Cx) had both Carboxymethylcellulase (CMCase) and xylanase activities. Activities as U/g dried enzyme powder were: 11000 U/g for the xylanase (X), 1700 U/g and 680 U/g for the cellulase and xylanase activity on the mixture (Cx), respectively. Enzymatic activity was determined at application conditions (50 ºC and pH 7 for X; and 55 ºC and pH 5 for Cx). An activity unit (U) is defined as the amount of enzyme capable of converting 1 μmol of substrate per minute. Enzymatic activity was determined using Spiro method to quantify released reducing sugars (Spiro, 1966) after a microscale enzymatic reaction carried out for 15 minutes.

7.2.2. Enzymatic treatments

Enzymatic treatments were held using X enzyme (Figure 7-1A) and combined treatments using Cx and X enzymes (Figure 7-1B). Letter "K" in a sample name indicates a control (*e.g.* KX indicate control for X treatments, and KCx for Cx). Control samples were prepared using the same conditions as in enzymatic treatments, but with no enzyme addition. After treatments, an aliquot of effluents was saved for analysis. Enzymatic reactions were then stopped washing pulps with decalcified water three times and one time with deionized water.

7.2.2.1. Treatments with xylanase (X) (Figure 7-1A)

They were applied in plastic bags on a thermostatic bath with a 10 U/g oven-dried pulp (odp) dose at 50 ºC, 10 % consistency, pH 7 (adjusted with 50 mM Tris-HCl buffer) and manual agitation every 10 min according to two different procedures:

Direct (X/KX): Reaction was carried out up to 5h, and samples were collected after each hour for characterization (X_1, X_2, X_3, X_4, X_5).

Stepwise addition (Xs/KXs): 2U/g odp xylanase were added 1h periods which were immediately followed by washing with deionized water (Xs_1, Xs_2, Xs_3, Xs_4, Xs_5). At the end of treatment (5h) a final dose of 10 U/g odp was applied, equal to that used on "direct" treatment.

7.2.2.2. Treatments with carbohydrases mixture (Cx) and xylanase (X) (Figure 7-1B)

Treatments with Cx: Performed at 55 ºC and pH 5 adjusted with 50mM sodium acetate buffer. Applied dose was 10 CMCase U/g odp. Two different conditions were assessed:

- Cx treatment: Carried out for 2 h in plastic bags at 10 % consistency, with manual agitation every 10 min.
- Cx24h treatment: Carried out in an Ahiba Easydye oscillating reactor for 24 h at 5 % consistency with agitation set at 20 rpm.

Combined treatments: After Cx treatment (2 h), X was applied for 2 and 5 h with a 10 U/g odp dose, 50ºC, 10% consistency and pH 7 (Tris-HCl), as for X2, X5.

7.2.3. Alkaline extraction (E stage)

An alkaline extraction with NaOH was performed after enzymatic treatments for further hemicelluloses removal. Two different conditions were assessed: 4 % (w/v) NaOH for 2 h, 25 ºC and 5 % consistency; and 9 % (w/v) NaOH for 1 h, 25 ºC and 5 % consistency. Pulps were extensively washed with deionized water after alkaline extraction.

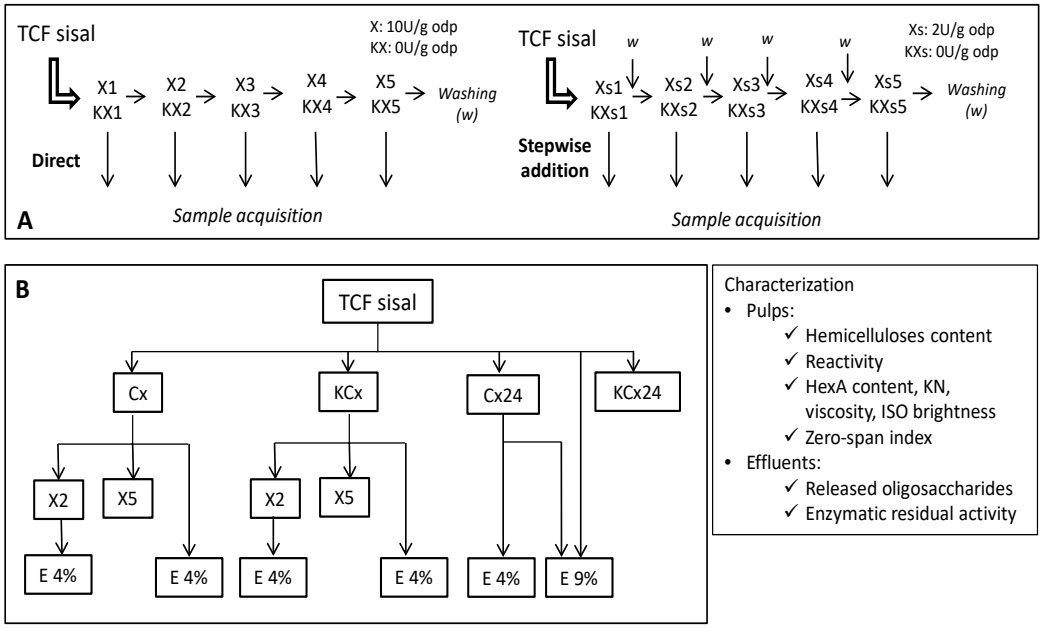

Figure 7-1: Work scheme of the present chapter.

7.2.4. Pulp properties

Kappa number (KN), ISO brightness, viscosity, wet zero span index and hexenuronic acid (HexA) content of initial and treated fibers were determined according to ISO 302:2004, ISO 3688:1999, ISO 5351:2010, ISO 15361:2000 and TAPPI T282 om-13, 2013, respectively.

KN_{lignin}/KN_{HexA}: An estimate of the actual lignin content of samples was obtained by determining KN due to lignin (KN_{lignin}). This involved determining KN after HexA removal with mercury acetate and efficient washing with distilled water. KN_{HexA} was estimated by the difference of total KN and KN_{lignin}. This procedure was used in literature to clarify enzymes action over pulp (Valls et al., 2010b).

Chain scission number: Pulp degradation was also assessed via the number of scissions in the cellulose strain.

Carbohydrate composition: Carbohydrate composition of initial and treated fibers was determined using high performance liquid chromatography (HPLC) following a modified version of TAPPI T 249 cm-09 method (Aracri and Vidal, 2011). Chromatographic analysis was performed using an 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-87H ion-exchange column. Data was collected by the refractive index detector (RID).

Moist heat ageing: Fibers were aged in an ageing vessel at 80 ºC and 65 % of relative humidity (RH) during 72 h according to ISO 5630-3:1996.

Cellulose Fock Solubility (reactivity): Reactivity values of samples was determined according to slightly modified version of Fock's method (Kopcke et al., 2008). Prior to analysis, samples were dried overnight in a controlled atmosphere (25 ºC and 50% RH). Reactivity measurements were expressed as the regenerated cellulose yield (Ibarra et al., 2010).

SEM microscopy: Small pieces of paper of each sample were used for SEM analysis with a JEOL JSM-6400 microscope operating at 10 kV. Samples were first coated with a very thin layer (14 nm thick) of gold–palladium in a sputter coater SCD005 in order to obtain a conductive surface.

7.2.5. Effluent properties

Residual enzymatic activity: It was determined using an adapted version of Somogyi-Nelson method to determine reducing sugar concentrations on a solution (Spiro, 1966).

Released carbohydrates: They were identified and quantified on effluents using an 1100 Agilent HPLC instrument furnished with a BIO RAD Aminex HPX-42A ion-exchange column. Identification and quantification of compounds was done by interpolation into calibration curves run from standards. Data was collected by the refractive index detector (RID).

Protein content: Protein content in effluents was measured using Bradford's micromethod (Bradford, 1976).

7.3. Results and discussion

7.3.1. Xylanase treatments

This work was focused on the removal of the highest possible amount of xylans (hemicelluloses) from TCF bleached sisal fibers with high hemicelluloses content (16.1 ± 0.2% w/w). For this purpose a higher dose of xylanase (10 U/g odp), than those commonly applied on bleaching processes (3 U/g odp) (Valls and Roncero, 2009) was applied. In order to find the best option, it was decided to administrate the same dose according to two different procedures (Figure 7-1A), while only after 5 treatment hours xylanase dose was equivalent. On one hand, the "*direct*" (X) treatment allowed the evaluation of xylanase effects on this higher dosage. On the other hand, "Stepwise addition" (Xs) of xylanase was carried out with the intention of studying the performance of the enzyme in a fresh environment for reaction each hour. As described elsewhere, this application procedure could led to better results in enzymatic hydrolysis of biomass (Pihlajaniemi et al., 2014). All fiber characterization values after xylanase treatments are indicated in Table 7-1 and Table 7-2.

Carbohydrate composition was determined for samples, with special interest on xylans content. As shown in Figure 7-2A, X enzyme produced deeper effects removing hemicelluloses on X (direct) treatments than did on Xs (stepwise addition). Fibers with xylans content close to 12% w/w were obtained already at 3 h of interaction with enzyme, suggesting that the maximal limit of accessible hemicelluloses was hydrolyzed. Xs treatment, by its side, did not provide any evidence of reaching this availability limit as a final value of xylans of 13.9% w/w was reached (Xs5). Enzymatic hydrolysis of biomass is limited by factors traditionally divided into two groups: biomass structural features and enzyme mechanisms (Zhu et al., 2008). Regarding physical structural features, it has been proposed that accessibility problems could be caused by: xylanase size (being too big to enter into fiber structure), fiber pores being too small or too few and/or the existence of an insufficient surface area, with a lower ratio than optimal (Ibarra et al., 2010). Chemical structural features of xylans such as composition, ramification or acetylation (Zhu et al., 2008); or their linkage to cellulose or lignin (Dammström et al., 2009) could also affect their digestibility. Finally, enzyme features such as their diffusion or product inhibition could also hinder these reactions.

Carbohydrate composition of samples provided evidence that direct addition of 10 U/g odp of X enzyme was more efficient for xylans removal than the stepwise admixture.

Table 7-1: Xylan content, HexA content, ISO brightness and viscosity values of initial fibers and after xylanase treatments (X and Xs) up to 5h. Values of control samples are also indicated.

Xylans (%)						
Time (h)	Starting	1	2	3	4	5
X	16.1 ± 0.3	13.7 ± 0.4	13.3 ± 0.1	12.1 ± 0.8	11.9 ± 0.5	11.9 ± 0.2
Xs	16.1 ± 0.3	15.1 ± 0.3	14.6 ± 0.2	14.3 ± 0.1	14 ± 0.1	13.8 ± 0.4
KX	16.1 ± 0.3	-	15.3 ± 0.2	-	-	15.3 ± 0.3
KXs	16.1 ± 0.3	16.1 ± 0.1	16 ± 0.1	15.6 ± 0.2	15.2 ± 0.2	15 ± 0.3
HexA (µmol/g odp)						
Time (h)	Starting	1	2	3	4	5
X	45.1 ± 1.5	36.7 ± 2.3	36.5 ± 0.9	34.1 ± 2.7	36.4 ± 1.1	34.8 ± 2.6
Xs	45.1 ± 1.5	43.2 ± 1.8	41.3 ± 3.5	40.6 ± 0.4	40.5 ± 0.7	40.4 ± 0.4
KX	45.1 ± 1.5	-	40.3 ± 0.7	-	-	40.2 ± 0.2
KXs	45.1 ± 1.5	44.8 ± 0.9	44.9 ± 2.6	44.5 ± 0.2	44.3 ± 2.5	44.2 ± 0.2
ISO Brightness (%)						
Time (h)	Starting	1	2	3	4	5
X	82.1 ± 0.3	82.8 ± 0.2	83.9 ± 0.3	84 ± 0.2	84 ± 0.1	83.9 ± 0.4
Xs	82.1 ± 0.3	82.2 ± 0.2	83.1 ± 0.2	83 ± 0.2	82.9 ± 0.2	83.3 ± 0.2
KX	82.1 ± 0.3	-	81.8 ± 0.3	-	-	81.4 ± 0.1
KXs	82.1 ± 0.3	82.6 ± 0.3	82.1 ± 0.2	82.1 ± 0.2	82.3 ± 0.1	82.5 ± 0.4
Viscosity (mL/g)						
Time (h)	Starting	1	2	3	4	5
X	616 ± 41	644 ± 7	657 ± 20	644 ± 24	657 ± 33	692 ± 22
Xs	616 ± 41	610 ± 27	628 ± 29	646 ± 27	642 ± 22	651 ± 2
KX	616 ± 41	-	617 ± 20	-	-	641 ± 8
KXs	616 ± 41	642 ± 26	640 ± 15	652 ± 16	646 ± 41	650 ± 17

Besides the removal of hemicelluloses, X and Xs treatments influenced other pulp characteristics. A 2.5 % ISO brightness increase was obtained with X treatment and a 1 % ISO after Xs, representing an improvement in pulp quality (Figure 7-2B). KN (Figure 7-3) decreased a 31 % with X treatment and a 24 % after Xs treatment (Samples X5 and Xs5, respectively). KN can be influenced not only by lignin, but also by any

other component capable of being oxidized by potassium permanganate (Gellerstedt and Li, 1996). Because of this, KN values were also expressed as produced by lignin or HexA, while KN_{lignin} + KN_{HexA}= Total KN (Figure 7-3). In this case, data suggested that the reduction in KN was due to HexA removal and not due to an actual delignification.

Table 7-2: KN values of initial and xylanase treated fibers up to 5h. Values after treatments (X and Xs), control samples, and KN produced by HexA and by lignin are indicated.

KN Time (h)	Starting	1	2	3	4	5
X	4.3 ± 0.2	3.6 ± 0.1	3.4 ± 0.2	3.4 ± 0.3	3 ± 0.1	2.9 ± 0.1
Xs	4.3 ± 0.2	4.1 ± 0.3	4.1 ± 0.2	3.6 ± 0.3	3.4 ± 0.2	3.2 ± 0.3
KX	4.3 ± 0.2	-	4.8 ± 0.1	-	-	4.7 ± 0.1
KXs	4.3 ± 0.2	4.8 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.1
(KN_{HexA}) X	3.5	2.7	2.5	2.6	2.1	2
(KN_{HexA}) Xs	3.5	3.4	3.2	2.9	2.7	2.4
(Kn_{lignin}) X	0.8	0.9	0.9	0.8	0.9	0.9
(Kn_{lignin}) Xs	0.8	0.7	0.9	0.7	0.7	0.8

Figure 7-2C showed a reduction of about 25 % in HexA content on X5 pulp compared to starting pulp, supporting data from KN_{HexA}. This reduction was similar to reductions reported by other authors (≈27 %) (Aracri and Vidal, 2011), observed on a non-bleached sisal pulp also with xylanase treatments. HexA removal importance is due to their negative effects on some pulp properties, such as brightness reversion (Cadena et al., 2010a). Finally, a linear correlation seemed to exist between xylan content of and HexA content of fibers (Figure 7-2A and C), which was logical attending the fact that HexA are contained in hemicelluloses (Valls et al., 2010b). Regarding viscosity, no statistically significant difference was observed between starting, treated pulps and controls (Table 7-1). However, a small increasing tendency was observed, which could have been due to the fact that removal of hemicelluloses, short polysaccharides, increased average degree of polymerization and thereafter, viscosity (Roncero et al., 2003; Fillat et al., 2011).

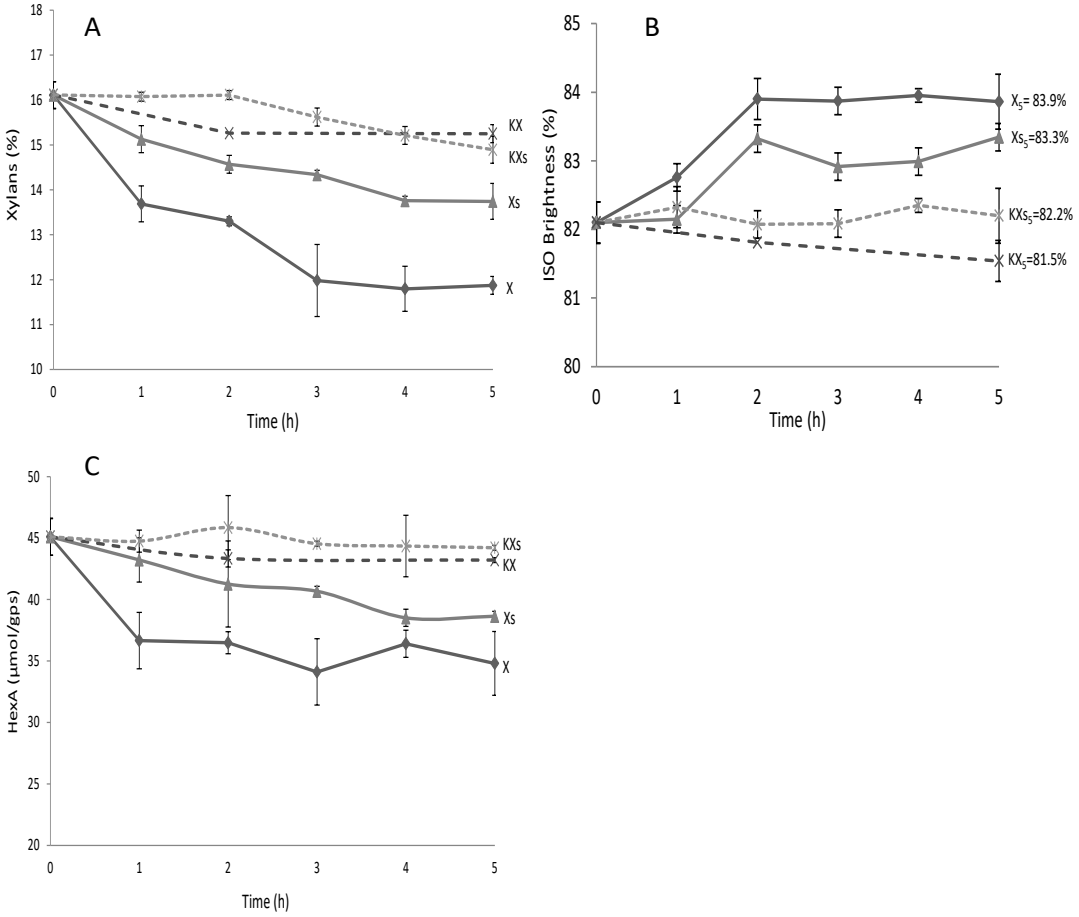

Figure 7-2: A- Xylan content (%); B- ISO Brightness (%) and C – HexA content (µmol/g odp). Mean values are represented, error bars indicate confidence intervals.

Ageing of pulps, measured as ISO brightness loss (%) is a process associated with many compounds present on pulp, such as lignin and HexA (Cadena et al., 2010a). A reduction in pulp brightness loss implies an increase in pulp quality and durability. Treatments with X seemed to reduce brightness loss (Table **7-3**), accounting for higher reductions after longer treatments, suggesting that these treatments were useful increasing fibers quality.

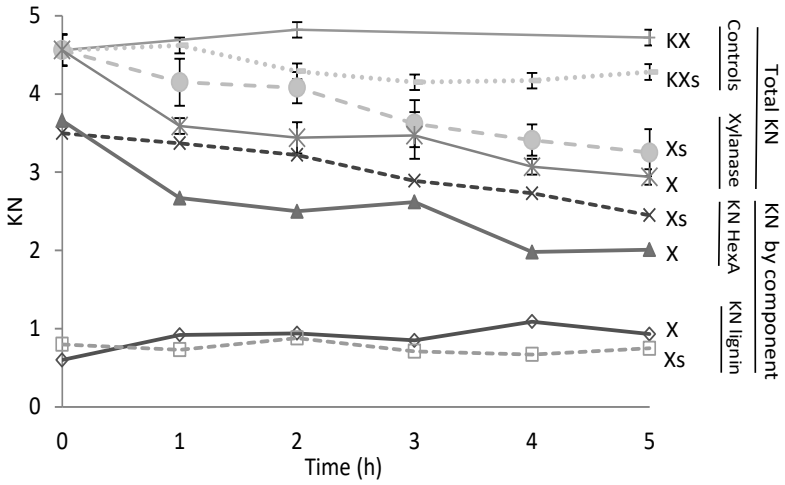

Figure 7-3: KN values along xylanolytic treatments. Specific values of KN due to HexA and to lignin are also indicated.

Table 7-3: Brightness loss index (BLI, %) after 72h of moist heat ageing

Time (h)	1	2	3	4	5
X	37.9 ± 0.3	17.5 ± 0.1	14.2 ± 0.1	11.8 ± 0.1	11.8 ± 0.2
KX	-	31.6 ± 0.4	-	-	32.5 ± 0.6

After treatments with xylanase, enzymatic activity was measured in effluents and it was expressed as a % of the initial dose that remained active. Re-using effluents for treating fibers instead of using fresh enzyme would reduce the economic cost related to their application, increasing their industrial feasibility. Results indicated that a high proportion of activity was conserved even after 5h of treatment, as in all cases more than 60 % of initial activity was still present on effluents (Table 7-4). Noteworthy, at the end of xylanolytic treaments (X and Xs), the same protein amount as initially administrated for treatments was found on effluents by Bradford method, confirming that X enzyme did no remain attached to fibers after reaction (Table 7-4).

Table 7-4: Xylanase residual activity (as % of initially administrated dose), and protein content in effluents for direct (X) and stepwise addition (Xs) treatments.

	Residual activity (%)		Protein in effluent (μg/mL)	
Time (h)	X	Xs	X	Xs
0	-	-	3.50 ± 0.21	0.75 ± 0.12
1	77 ± 10	78 ± 21	3.65 ± 0.42	0.79 ± 0.07
2	80 ± 6	74 ± 24	3.70 ± 0.19	0.74 ± 0.1
3	94 ± 12	106 ± 32	3.35 ± 0.28	0.61 ± 0.28
4	77 ± 26	102 ± 18	3.06 ± 0.59	0.63 ± 0.28
5	91 ± 11	69 ± 19	3.74 ± 0.27	0.74 ± 0.18

For a better understanding of enzymatic effects, oligosaccharides released from fibers were measured on reaction effluents following a similar protocol as reported by other authors (Zhang et al., 2012a; Garcia-Ubasart et al., 2013). Sugar concentration on effluents increased with treatment time (Figure 7-4A and B). Regarding enzyme action mechanism, Figure 7-4A and B provided evidence that this enzyme might be cleaving xylans chains preferably by 2 -3 subunits. This evidence showed that X enzyme produced xylo-oligosaccharides of lower molecular weight than some novel xylanases applied by Valls et al. (2010b). Xylans were also cut into xylose (X1 on chart), but in a lower amount than the other two sugars. For both treatments, the release pattern, by concentrations was Xilobiose> Xilotriose> Xilose. Data on charts supported the evidence that direct treatment (X) was more efficient to our purposes than the stepwise admixture (Xs), as higher final concentrations of xylooligomers were released from pulp on the former. This differs from other works where stepwise addition of enzymes provided better results than direct one (Pihlajaniemi et al., 2014), showing that each enzyme behavior is unique.

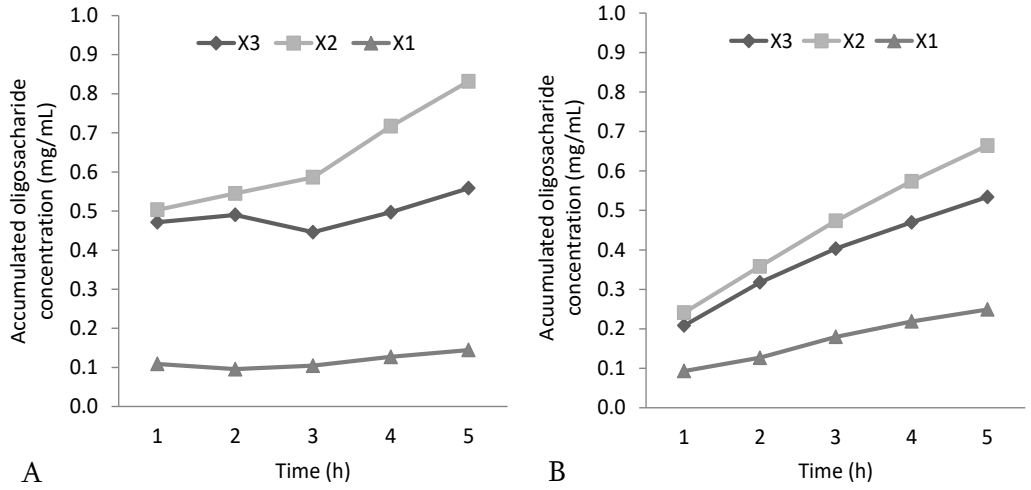

Figure 7-4: Accumulated concentration (*i.e.* adding the amount released each hour) of reducing sugars on effluents of treatments with X (A: X treatment, B: Xs treatment). X3, X2 and X1 stand for xilotriose, xilobiose and xylose, respectively.

7.3.2. Combined enzymatic treatments

A carbohydrase mixture (Cx) was applied on fibers alone and followed by X enzyme with the intention of further reducing fibers xylans content, obtaining samples with characteristics exposed in Table 7-5.

In Figure 7-5A, it can be seen that a 25% reduction was achieved combining Cx and X5 treatments, reaching a final content of xylans of 12 % w/w, the same obtained with X5 treatment alone. Again, a limit to enzymatically degradable xylans was found. Also, it was observed that X enzyme applied after Cx produced a lower effect than when applied alone. Observing data at Figure 7-5A, it was noticed that xylanase contained into Cx seemed to be more efficient than X enzyme. Cx was applied based on CMCase activity, and when 10 CMCase U/g odp were applied for 2 h, the applied equivalent xylanase dose (of Cx) was 4 U/g odp. Attributing all hemicelluloses removal to this xylanase activity, we assumed that this 4 U/g caused 11% elimination in 2 h, compared to the 15 % elimination provoked by 10 U/g of X produced within 2 h. Calculating the effect *per U*, it can be seen that Cx xylanase activity seemed to be twice as effective X, applied alone or after KCx. Because of this, a very prolonged treatment

(24 h) was performed with Cx in order to study the full potential of this enzyme. Enzymatic residual activity confirmed that Cx was still active at the end of this treatment. Interestingly, a 12.4 % w/w final content in hemicelluloses was reached after Cx24h treatment, while Cx treatment leaded to a final value of 13.5 % w/w, removing 23 % and 16 % of xylans from initial pulp, respectively (Figure 7-5A). This showed that Cx alone was capabale of producing a similar removal of hemicelluloses to that produced in combination with X enzyme. Regarding other characteristics, it can be observed that Cx produced similar effects to X enzyme: Removing HexA, decreasing KN and increasing ISO brightness (Figure 7-5B, C and D).

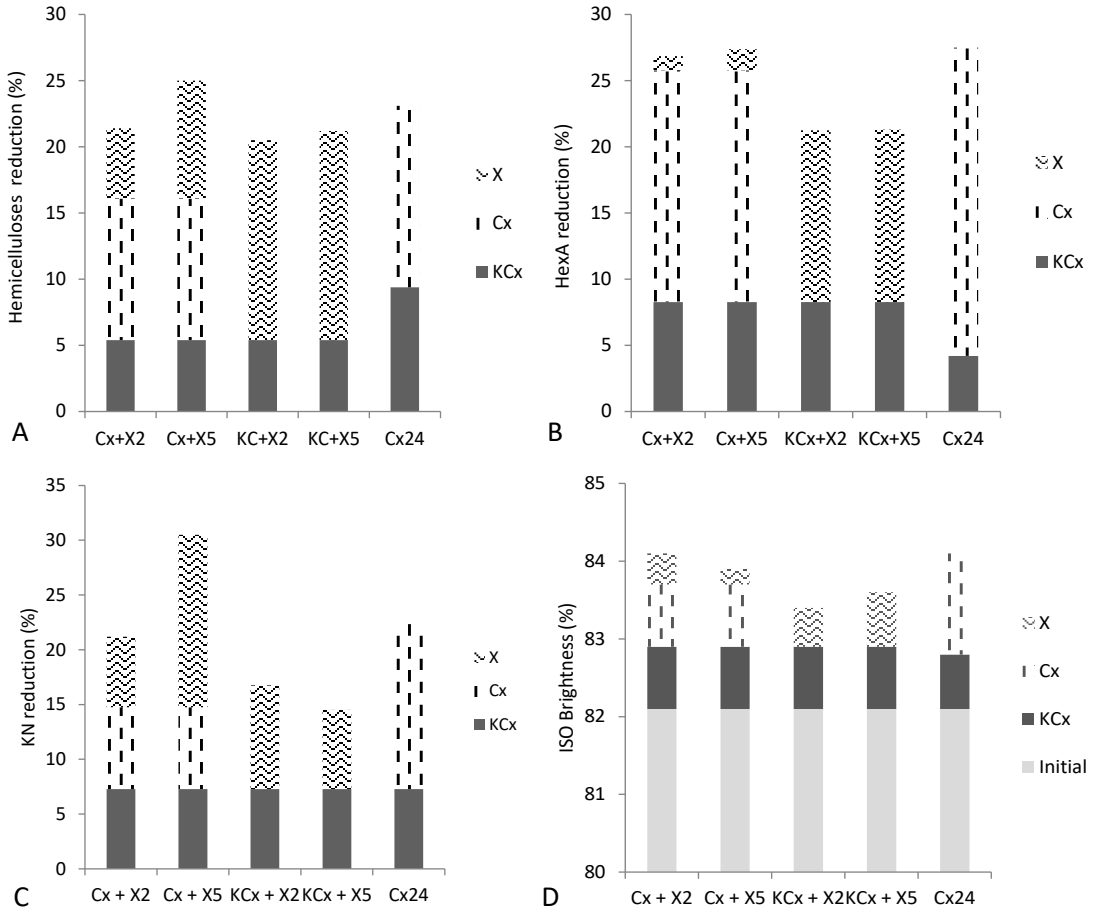

Figure 7-5: Reduction in hemicelluloses content (A), HexA content (B) and KN (C) compared to initial pulp, and increase in brightness (D) produced by combined treatments, indicated as a contribution of each enzymatic step.

Table 7-5: Fibers characterization after combined treatments with Cx and X enzymes.

	KCx	Cx	Cx + X2	Cx+ X5	Cx + KX2	Cx + KX5
Xylans (%)	15.3 ± 0.1	13.5 ± 0.2	12.7 ± 0.3	12.1 ± 0.1	12.8 ± 0.1	12.7 ± 0.1
Brightness (% ISO)	81.9 ± 0.1	83.7 ± 0.1	84.1 ± 0.2	83.9 ± 0.2	84.2 ± 0.2	83.4 ± 0.1
Viscosity (mL/g)	628 ± 1	431 ± 6	486 ± 3	475 ± 16	495 ± 23	442 ± 17
HexA (μmol/g odp)	41.4 ± 2.8	33.5 ± 2.5	33 ± 1.1	32.8 ± 2.5	35.4 ± 3.1	35.2 ± 1.2
KN	4.7 ± 0.1	4 ± 0.3	3.7 ± 0.3	3.3 ± 0.1	3.8 ± 0.1	3.7 ± 0.4
	KCx + X2	KCx + X5	KCx + KX2	KCx + KX5	Cx24	KCx24
Xylans (%)	13.1 ± 0.1	12.7 ± 0.1	14.6 ± 0.1	14.9 ± 0.2	12.4 ± 0.1	14.6 ± 0.1
Brightness (% ISO)	83.4 ± 0.2	83.6 ± 0.1	82.8 ± 0.3	82.7 ± 0.1	84.1 ± 0.2	82.5 ± 0.1
Viscosity (mL/g)	647 ± 23	665 ± 6	633 ± 7	639 ± 18	365 ± 8	607 ± 27
HexA (μmol/g odp)	35.5 ± 2.1	35.5 ± 0.8	41.1 ± 2.3	41.7 ± 2.5	32.7 ± 1.6	43.2 ± 1.5
KN	3.9 ± 0.1	4 ± 0.3	4.4 ± 0.1	4.4 ± 0.3	3.7 ± 0.1	4.5 ± 0.1

Cx treatment also produced a decrease in fibers viscosity (Table 7-6) as other authors have reported for these type of enzymes (Wang et al., 2014). Cleavage of cellulose chains by Cx leaded to a reduction on degree of polymerization (DP) of cellulose, reducing the viscosity of pulp. By its side, again, X enzyme action produced a slight increase on this parameter (Table 7-6). Wet zero-span fiber resistance represents a measure of fibers intrinsic strength, *i.e.*, in independence of fibers network (Hägglund et al., 2004). It indirectly indicates the physical integrity of fibers, and can provide more information of treatments influence on their mechanical performance. It was observed that X treatments did not affect fibers mechanical resistance (Figure 7-6A), while Cx application produced a reduction in zero-span index (Figure 7-6B). This reduction was probably provoked by cellulolytic action, as previously reported (Suchy et al., 2009; Garcia-Ubasart et al., 2013), and went accompanied with a reduction in cellulose viscosity. Figure 7-6 suggested that fibers intrinsic strength and their viscosity were two related parameters.

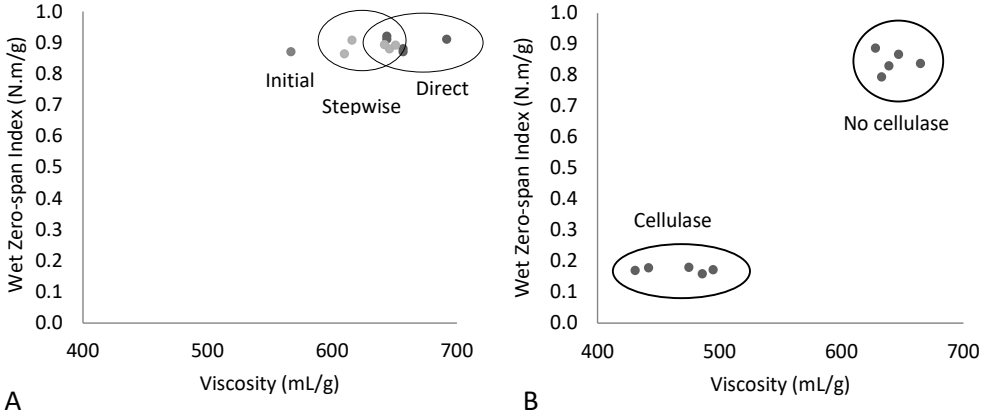

Figure 7-6: Wet zero-span index values expressed in front of viscosity for (a) xylanase treatments (X, Xs) and (b) Carbohydrase mixture (Cx) + xylanase (X) treatments.

Table 7-6: Fock reactivity (as % of reacted cellulose) and viscosity values of samples.

	Cx	KCx	Cx24	KCx24	Cx + X2
Fock reactivity (%)	56.1 ± 2.4	26.7 ± 1.4	61.3 ± 1	30 ± 1.3	53.9 ± 0.4
Viscosity (mL/g)	431 ± 6	628 ± 1	365 ± 8	607 ± 27	486 ± 3

Concerning released sugars, Cx treatments produced both glucose and xylose oligosaccharides (Figure 7-7), which were now expressed in relation to pulp, not as concentration in effluents because of the existing differences in consistency among treatments. Cx xylanase activity released xylose in the first place and xylobiose in a lower amount after 2 h of reaction, while only xylose was found after 24 h. This finding suggests the existence of a beta-xylosidase activity on Cx enzyme. Higher amounts of xylo-oligosaccharides were released after 24 h compared to 2 h (expressed as xylose-equivalents), results that were consistent with the higher xylan elimination from fibers. Regarding glucose oligosaccharides, proportions between released oligosaccharides were maintained during both treatments, with larger releases after 24h compared to 2h. Of total released, cellotriose represented ≈2%, cellobiose a 31% and glucose a 67% after Cx and Cx24 treatments (Figure 7-7), similar proportions to those observed in chapter 6. Cx cellulolytic activities removal pattern was then

glucose> cellobiose> cellotriose. This pattern was different from others reported for other cellulolytic enzymes (Garcia-Ubasart et al., 2013), where cellobiose> glucose> cellohexose> cellotriose was found. Data from Cx treatment effluents suggested that this enzymatic preparation contained a β-glucosidase activity converting larger oligosaccharides to glucose. Cellotriose and cellobiose could act as a cellulase inhibitors (Philippidis et al., 1993), so the existence of this activity would improve the efficiency of the enzyme. Finally, X enzyme applied after Cx had a similar behavior than when applied alone.

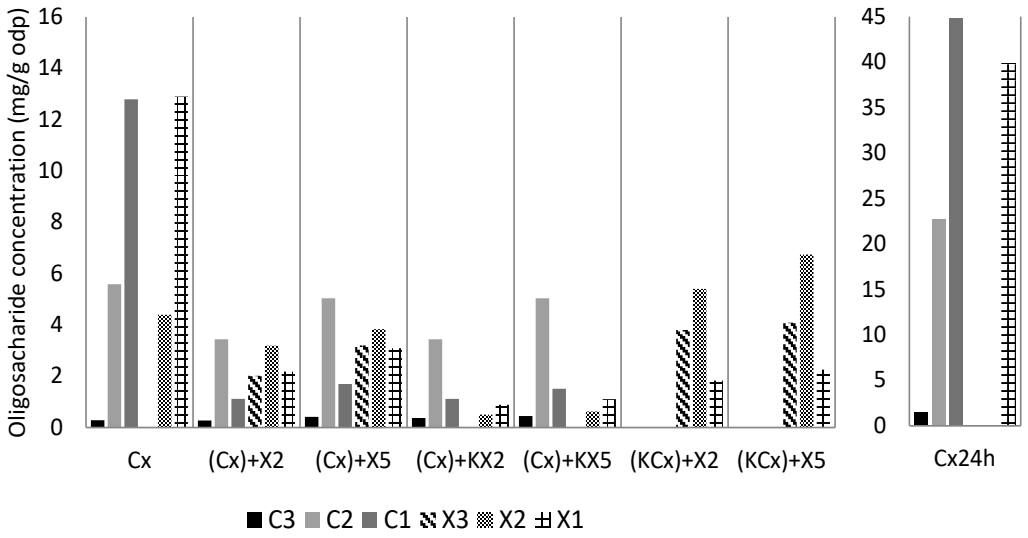

Figure 7-7: Concentration of sugars on effluents (expressed in relation to pulp mass) of combined Cx and X treatments. Previous treatment, when available, is indicated between parenthesis and only data of effluents of the second treatment is shown. X3, X2 and X1 stand for xilotriose, xilobiose and xylose, respectively; C3, C2 and C1 stand for cellotriose, cellobiose and glucose, respectively.

7.3.3. Achieving a better pulp quality

As commented in previous sections, the used enzymes seemed to have found a limit of degradable xylans in sisal fibers. For a further reduction, some authors proposed a treatment with NaOH (Ibarra et al., 2010; Hakala et al., 2012). In the present chapter, two different NaOH concentrations were assessed. It is proposed that

the two types of xylans previously mentioned (Dammström et al., 2009) were being preferably attacked by either enzymes or by NaOH.

Data in Figure 7-8 showed that xylans and HexA elimination caused by 4% w/v NaOH (E4%) was similar in all cases (≈30%) achieving total reductions of ≈50% of initial content after both enzymatic and chemical stages. Previous works using an enzymatic treatment combined with 4% w/v NaOH reported a similar total reduction in xylan content, around 50% (Hakala et al., 2012). For a greater reduction, a stronger extraction with NaOH 9 % w/v was performed on Cx24 treated and also on initial fibers. With it, total reductions of 82 % and 45% of xylan content were achieved, respectively (Figure 7-8A). Combination of Cx24 and E 9% treatments led to final xylan content in fibers of 2.9% w/w and a cellulose content of ≈96%, a satisfactory value for HCC fibers. Furthermore, it was found that Cx + X2 and Cx24 treatments, combined with E 4% produced higher xylans removal than E 9% alone, allowing a reduction of 55% in the amount of NaOH needed to reach a given purity degree. Also, E 9% removed 59% of hemicelluloses after Cx24 treatment, achieving a greater reduction than when performed on initial fibers. This evidence shows that combining enzymatic treatments with NaOH not only provided a higher total reduction (≈82%), but also seemed to produce a synergetic effect, as Cx treatment boosted E 9% effect by increasing its effect in 14 points. This synergy would also permit a reduction in the need of NaOH for reaching a specific purity, highlighting the potential of enzymes as green catalysts. By its side, Figure 7-8B showed that HexA content was reduced in similar proportions to xylan content, observed in previous samples

E stage also influenced other pulp properties. It reduced KN up to 3 units, it increased ISO brightness, with gains from 1.5 % to 3 % ISO and it did not produce remarkable effects on viscosity (Table 7-8). Finally, E stage did not affect mechanical resistance of fibers either, as very similar zero-span values as those obtained after enzymatic stages were obtained (Table 7-7).

Table 7-7: Zero-span tensile strength of NaOH extracted samples.

Wet zero-span index (N.m/g)				
X2+E 4%	KX2+E 4%	Cx+E 4%	KCx+E 4%	Cx+X2+E 4%
0.93 ± 0.11	0.89 ± 0.1	0.20 ± 0.04	0.92 ± 0.1	0.20 ± 0.04

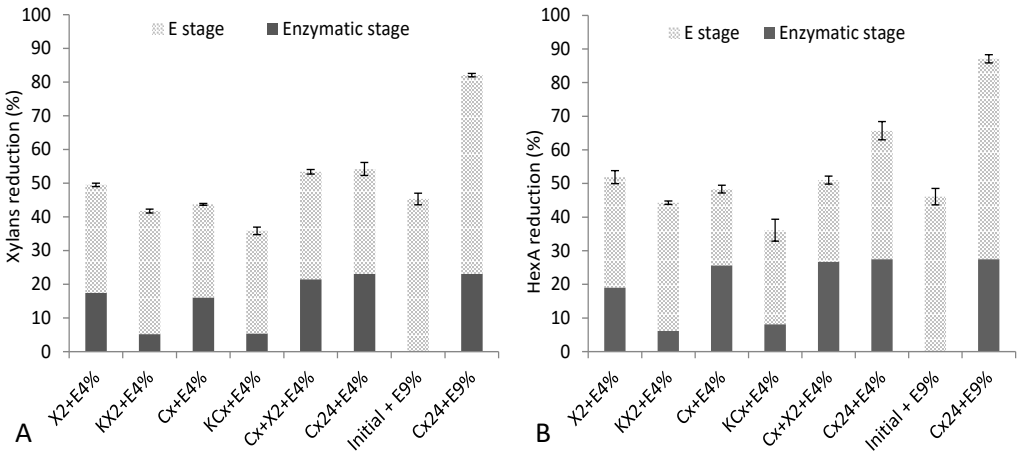

Figure 7-8: Xylans (A) and HexA content (B) reduction from initial pulp after different enzymatic steps and alkaline extractions with NaOH. Error bars indicate confidence intervals.

Observing SEM images (Figure 7-9) no major change seemed to be produced by X enzyme on the surface of fibers but the apparition of small fibrils on surface (Figure 7-9A and B). Cx treatment seemed to lead to the apparition of breaking points on fibers after 2h (Figure 7-9C and D). These spots probably represent enzyme placement site where hydrolysis started (Igarashi et al., 2011). After 24 h (Figure 7-9E), Cx effects were more noticeable than after 2h as a reduction in fiber length is clearly appreciable in comparison to KCx24 sample (Figure 7-9F), being this effect also observable macroscopically. These weaker fragments produced by cellulase might be the responsible for the loss in zero-span resistance (Figure 7-6B). Finally, NaOH did not seem to produce any noticeable change on fibers surface (Figure 7-9G and H).

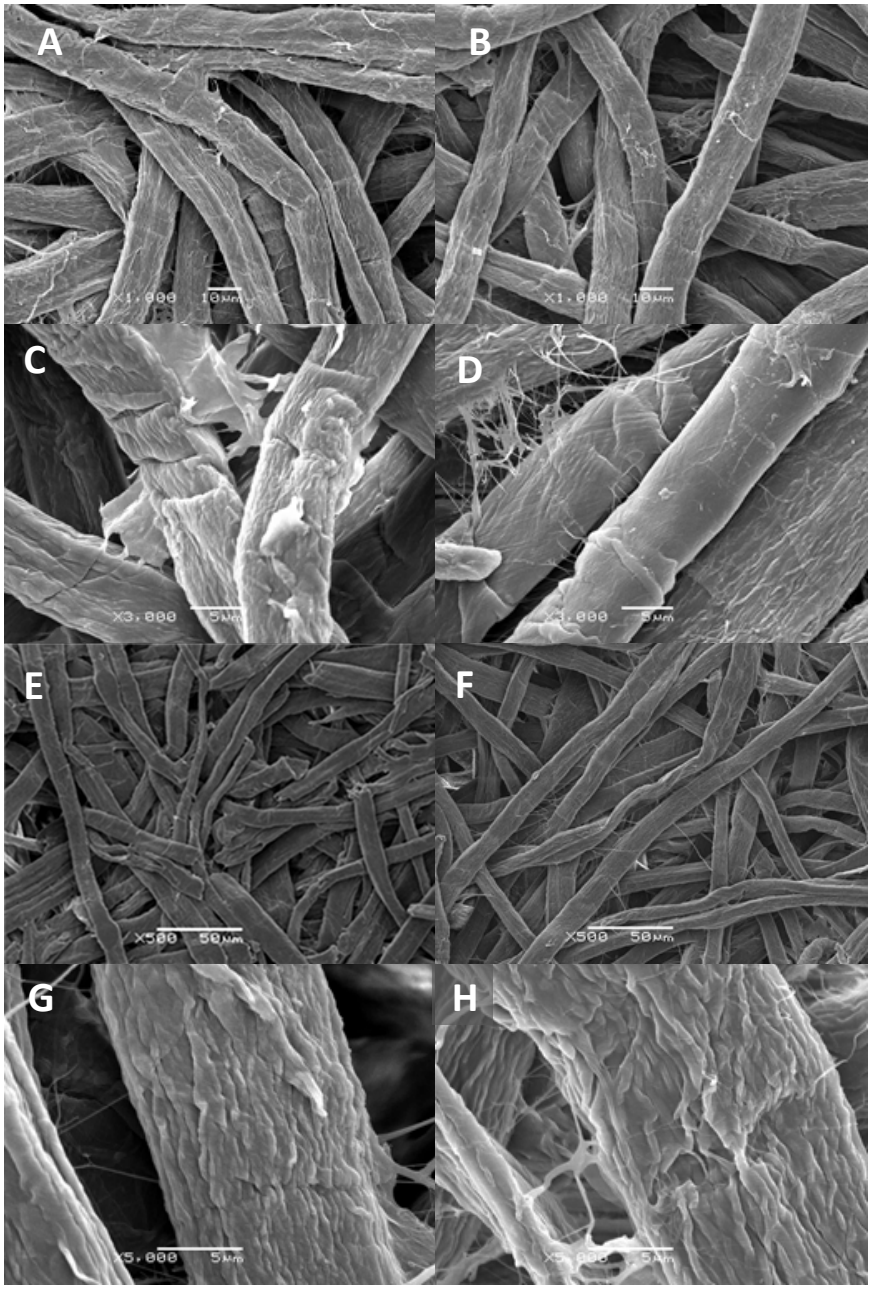

Figure 7-9: SEM images of treated fibers. Pictures represent following samples: A) X2, B) KX2, C) Cx, D) KCx, E) Cx24, F) KCx24, G) X2 and H) X2+E

7.3.4. Other applications of high-cellulose content fibers

High-cellulose content fibers have a variety of potential uses requiring a high-purity cellulose source. They could be used for nanocellulose production (Fortunati et al., 2013) or other cellulose derivatives, such as viscose rayon (Ibarra et al., 2009). For this last application, besides the low content of hemicelluloses, high reactivity values are also required (Ibarra et al., 2010). Pulp reactivity provides an idea of the capability of pulps to be transformed into its derivative during viscose process (Ibarra et al., 2009; Quintana et al., 2015c). Fock solubility is a convenient method to provide insight of the performance a pulp will have in this further process. Treatments with cellulases and particularly endoglucanases could increase pulps Fock solubility (Henriksson et al., 2005; Kvarnlöf et al., 2007; Hakala et al., 2012; Miao et al., 2014; Quintana et al., 2015a, 2015b). Two mechanisms were proposed to explain this increase: degradation of amorphous zones of cellulose could lead to a reduction of structural diversity on fibrils surface, increasing its swelling and therefore, its contact with reagents; while degradation of amorphous regions causes a cleavage on fibrils reducing their size and increasing its swellability (Henriksson et al., 2005). In the present study, Cx treatment provoked an increase of Fock solubility of ≈30 points, reaching a final value of 61% (Table 7-6). This gain was similar to that reported with the application of an endoglucanase on sisal pulp, also of ≈30 points (Ibarra et al., 2010).

Concerning viscosity, it has been reported that fibers intended for viscose manufacture should have viscosities around 300 mL/g, as too high values could affect cellulose processability during this process (Henriksson et al., 2005; Batalha et al., 2011). As can be observed in Table 7-6, a ≈ 250 mL/g reduction produced by Cx24, bigger than the ≈ 180 mL/g accounted with Cx treatment, leaded to a more convenient final value for this application.

Chain scission number (CSN) is defined as the average chain cuts produced by a certain process (Bouchard et al., 2000). This parameter is calculated using DP values, which experienced slight increases after some treatments (X or E stage) and because of this, some small negative CSN values were obtained (Figure 7-10A). Data showed that X enzyme and E stage did not modify pulp CSN or pulp reactivity unlike Cx treatment, which produced an increase in both parameters (Figure 7-10A). This evidence fitted very well with previous explanations, as the increase in scission points provoked by

cellulolytic activity could have increased cellulose swellability and thereafter, its reactivity towards viscose process. In relative terms, compared to control samples, reactivity increases of about 100-110 % were obtained, reaching a top value of ≈61%. These values were in the good direction to meet specialty cellulose requirements, considering the 65-70 % reactivity presented by commercial dissolving pulps (Kopcke et al., 2008).

Concerning hemicelluloses, Figure 7-10B shows that both X and E stages were effective removing them, but produced no effect in pulp reactivity. However, Cx enzyme showed effectivity removing hemicelluloses, boosting further alkaline extraction and increasing reactivity, making it a very promising single catalyst for carring out the whole studied process. After treatments, Cx enzyme and NaOH 9%, allowed reaching a final product with low xylan and HexA content, low KN, high ISO brightness and increased reactivity (Table 7-8).

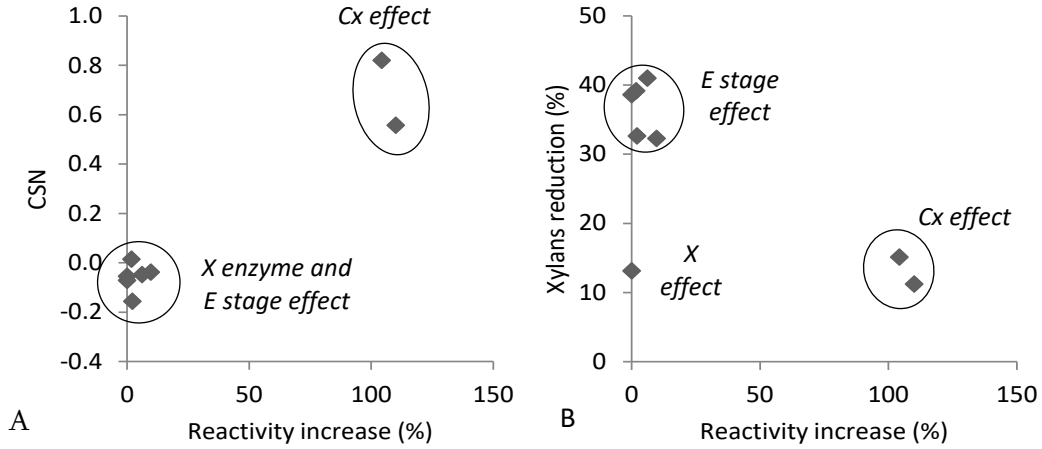

Figure 7-10: CSN (A) and xylans content reduction (as %) (B), represented in front of reactivity increase. Variations were calculated in comparison to the previous stage, not initial pulp.

Table 7-8: Final properties of pulps after enzymatic and chemical treatments.

	X2 + E 4%	Cx + E 4%	Cx + X2 + E 4%	Cx24 + E 4%	Cx24 + E 9%
Xylans (%)	8.1 ± 0.1	9.1 ± 0.1	7.5 ± 0.1	7.4 ± 0.3	2.9 ± 0.1
HexA (μmol/g odp)	21.7 ± 0.9	23.3 ± 0.5	22.1 ± 0.5	15.5 ± 1.2	5.8 ± 1.1
KN	2.5 ± 0.1	2.5± 0.3	2.2 ± 0.2	2.1 ± 0.1	0.7 ± 0.1
Brightness (% ISO)	85.4 ± 0.4	86.6 ± 0.2	87.1 ± 0.1	86.1 ± 0.1	86.2 ± 0.2
Viscosity (mL/g)	649 ± 1	498 ± 13	507 ± 18	450 ± 7	503 ± 18

7.4. Conclusions

Evidence in chapter 7 has shown that treatments with new carbohydrases were effective modifying sisal fibers to produce high cellulose content fibers. Firstly, xylanase (X) treatments were found to work better with the complete dosage compared to the stepwise addition. Secondly, carbohydrase mixture (Cx) appeared as a new very promising catalyst, capable itself of removing hemicelluloses and increase pulp reactivity. Thirdly, only 25% of initially present hemicelluloses could be removed by X or even Cx + X treatments, reaching a final content in fibers of ≈12%. This problem was solved by the application of an alkaline extraction (9% w/v NaOH) in combination with a single enzymatic treatment with Cx, which led to a high-quality end product with a high content in cellulose (≈96%). Also, a synergetic effect was observed, as Cx treatments boosted NaOH effects by 14 points, reducing the amount of it necessary to reach a given cellulose purity level, and making them suitable treatments for green processes. Evidence presented in this chapter would allow using non-pure cellulosic sources such as sisal fibers or other non-woods for the preparation of NCC, converting any raw material into a high-quality cellulose source.

7.5. References

Aracri, E.; Vidal, T. Xylanase- and Laccase-Aided Hexenuronic Acids and Lignin Removal from Specialty Sisal Fibres. Carbohydr. Polym. 2011, 83 (3), 1355–1362.

Bajpai, P.; Bajpai, P. K. Development of a Process for the Production of Dissolving Kraft Pulp Using Xylanase Enzyme. Appita J. 2001, 54 (4), 381–384.

Barlow, F.; Hillman, D. Do Dissolving Pulps Really Dissolve? Pap. Asia 2006, 22 (7), 12–18.

Batalha, L.; Colodette, J. L.; Gomide, J. L.; Barbosa, Luiz, C. A.; Maltha, C. R. A.; Borges Gomes, F. J. Dissolving Pulp Production from Bamboo. BioResources 2011, 7 (1), 640–651.

Bouchard, J.; Morelli, E.; Berry, R. M. Gas-Phase Addition of Solvent to Ozone Bleaching of Kraft Pulp. J. Pulp Pap. Sci. 2000, 26 (1), 30–35.

Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 1976, 72, 248–254.

Cadena, E. M.; Vidal, T.; Torres, A. L. Influence of the Hexenuronic Acid Content on Refining and Ageing in Eucalyptus TCF Pulp. Bioresour. Technol. 2010, 101 (10), 3554–3560.

Dammström, S.; Salmén, L.; Gatenholm, P. On the Interactions between Cellulose and Xylan, a Biomimetic Simulation of the Hardwood Cell Wall. BioResources 2009, 4 (1), 3–14.

Fillat, A.; Roncero, M. B.; Vidal, T. Assessing the Use of Xylanase and Laccases in Biobleaching Stages of a TCF Sequence for Flax Pulp. J. Chem. Technol. Biotechnol. 2011, 86 (12), 1501–1507.

Fortunati, E.; Puglia, D.; Monti, M.; Peponi, L.; Santulli, C.; Kenny, J. M.; Torre, L. Extraction of Cellulose Nanocrystals from Phormium Tenax Fibres. J. Polym. Environ. 2013, 21, 319–328.

Garcia-Ubasart, J.; Torres, A. L.; Vila, C.; Pastor, F. I. J.; Vidal, T. Biomodification of Cellulose Flax Fibers by a New Cellulase. Ind. Crops Prod. 2013, 44, 71–76.

Gellerstedt, G.; Li, J. An HPLC Method for the Quantitative Determination of Hexeneuronic Acid Groups in Chemical Pulps. Carbohydr. Res. 1996, 294, 41–51.

Hägglund, R.; Gradin, P. A.; Tarakameh, D. Some Aspects on the Zero-Span Tensile Test. Exp. Mech. 2004, 44 (4), 365–374.

Hakala, T. K.; Liitiä, T.; Suurnäkki, A. Enzyme-Aided Alkaline Extraction of Oligosaccharides and Polymeric Xylan from Hardwood Kraft Pulp. Carbohydr. Polym. 2012, 93 (1), 102–108.

Henriksson, G.; Christiernin, M.; Agnemo, R. Monocomponent Endoglucanase Treatment Increases the Reactivity of Softwood Sulphite Dissolving Pulp. J. Ind. Microbiol. Biotechnol. 2005, 32 (5), 211–214.

Ibarra, D.; Kopcke, V.; Ek, M. Exploring Enzymatic Treatments for the Production of Dissolving Grade Pulp from Different Wood and Non-Wood Paper Grade Pulps. Holzforschung 2009, 63 (6), 721–730.

Ibarra, D.; Kopcke, V.; Larsson, P. T.; Jaaskelainen, A.-S.; Ek, M. Combination of Alkaline and Enzymatic Treatments as a Process for Upgrading Sisal Paper-Grade Pulp to Dissolving-Grade Pulp. Bioresour. Technol. 2010, 101 (19), 7416–7423.

Igarashi, K.; Uchihashi, T.; Koivula, A.; Wada, M.; Kimura, S.; Okamoto, T.; Penttilä, M.; Ando, T.; Samejima, M. Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface. Science. 2011, 333 (6047), 1279–1282.

Jackson, L. S.; Heitmann Jr., J. A.; Joyce, T. W. Production of Dissolving Pulp from Recovered Paper Using Enzymes. Tappi J. 1998, 81 (3), 171–178.

Kopcke, V.; Ibarra, D.; Ek, M. Increasing Accessibility and Reactivity of Paper Grade Pulp by Enzymatic Treatment for Use as Dissolving Pulp. Nord. Pulp Pap. Res. J. 2008, 23 (4), 363–368.

Kvarnlöf, N.; Germgärd, U.; Jönson, L. J.; Carl-Axel, S. Optimization of the Enzymatic Activation of a Dissolving Pulp before Viscose Manufacture. Tappi J. 2007, 6 (6), 14–19.

Li, J.; Zhang, H.; Duan, C.; Liu, Y.; Ni, Y. Enhancing Hemicelluloses Removal from a Softwood Sulfite Pulp. Bioresour. Technol. 2015, 192, 11–16.

Maache-Rezzoug, Z.; Pierre, G.; Nouviaire, A.; Maugard, T.; Rezzoug, S. A. Optimizing Thermomechanical Pretreatment Conditions to Enhance Enzymatic Hydrolysis of

Wheat Straw by Response Surface Methodology. Biomass and Bioenergy 2011, 35 (7), 3129–3138.

Miao, Q.; Chen, L.; Huang, L.; Tian, C.; Zheng, L.; Ni, Y. A Process for Enhancing the Accessibility and Reactivity of Hardwood Kraft-Based Dissolving Pulp for Viscose Rayon Production by Cellulase Treatment. Bioresour. Technol. 2014, 154, 109–113.

Philippidis, G. P.; Smith, T. K.; Wyman, C. E. Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by the Simultaneous Saccharification and Fermentation Process. Biotechnol. Bioeng. 1993, 41 (9), 846–853.

Pierre, G.; Sannier, F.; Goude, R.; Nouviaire, A.; Maache-Rezzoug, Z.; Rezzoug, S. A.; Maugard, T. Evaluation of Thermomechanical Pretreatment for Enzymatic Hydrolysis of Pure Microcrystalline Cellulose and Cellulose from Brewers' Spent Grain. J. Cereal Sci. 2011a, 54 (3), 305–310.

Pierre, G.; Maache-Rezzoug, Z.; Sannier, F.; Rezzoug, S. A.; Maugard, T. High-Performance Hydrolysis of Wheat Straw Using Cellulase and Thermomechanical Pretreatment. Process Biochem. 2011b, 46 (11), 2194–2200.

Pihlajaniemi, V.; Sipponen, S.; Sipponen, M. H.; Pastinen, O.; Laakso, S. Enzymatic Saccharification of Pretreated Wheat Straw: Comparison of Solids-Recycling, Sequential Hydrolysis and Batch Hydrolysis. Bioresour. Technol. 2014, 153, 15–22.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. An Enzyme-Catalysed Bleaching Treatment to Meet Dissolving Pulp Characteristics for Cellulose Derivatives Applications. Bioresour. Technol. 2013, 148, 1–8.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part I: On a Fully Bleached, Commercial Acid Sulfite Dissolving Pulp. Cellulose 2015a, 2067–2079.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part II: On a Biobleached Acid Sulphite Pulp. Cellulose 2015b, 22 (3), 2081–2093.

Quintana, E.; Valls, C.; Barneto, A. G.; Vidal, T.; Ariza, J.; Roncero, M. B. Studying the Effects of Laccase Treatment in a Softwood Dissolving Pulp : Cellulose Reactivity and Crystallinity. Carbohydr. Polym. 2015c, 119, 53–61.

Roncero, M. B.; Colom, J. F.; Vidal, T. Influence of the Xylanase Enzymatic Treatments on the Carbohydrate Composition of Pulp for Paper Manufacture. Afinidad 2003, 60 (503), 8–15.

Spiro, R. G. R. Analysis of Sugars Found in Glycoproteins. Methods Enzymol. 1966, 566 (C), 3–26.

Suchy, M.; Hakala, T.; Kangas, H.; Kontturi, E.; Tammelin, T.; Pursula, T.; Vuorinen, T. Effects of Commercial Cellobiohydrolase Treatment on Fiber Strength and Morphology of Bleached Hardwood Pulp. Holzforschung 2009, 63 (6), 731–736.

Valls, C.; Roncero, M. B. Using Both Xylanase and Laccase Enzymes for Pulp Bleaching. Bioresour. Technol. 2009, 100 (6), 2032–2039.

Valls, C.; Gallardo, O.; Vidal, T.; Ignacio, F.; Pastor, J.; Díaz, P.; Roncero, M. B.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B.; et al. New Xylanases to Obtain Modified Eucalypt Fibres with High-Cellulose Content. Bioresour. Technol. 2010, 101 (19), 7439–7445.

Wang, H.; Pang, B.; Wu, K.; Kong, F.; Li, B.; Mu, X. Two Stages of Treatments for Upgrading Bleached Softwood Paper Grade Pulp to Dissolving Pulp for Viscose Production. Biochem. Eng. J. 2014, 82, 183–187.

Zhang, J.; Tang, M.; Viikari, L. Xylans Inhibit Enzymatic Hydrolysis of Lignocellulosic Materials by Cellulases. Bioresour. Technol. 2012, 121, 8–12.

Zhu, L.; O'Dwyer, J. P.; Chang, V. S.; Granda, C. B.; Holtzapple, M. T. Structural Features Affecting Biomass Enzymatic Digestibility. Bioresour. Technol. 2008, 99 (9), 3817–3828.

Chapter 8

NCC preparation from high-cellulose content fibers

Abstract

The purpose of the work exposed in this chapter was to obtain NCC from high-cellulose content (HCC) sisal fibers obtained in chapter 7. For this objective optimal chemical and enzymatic conditions obtained in chapters 5 and 6 were used for NCC preparation. Removing hemicelluloses from fibers increased hydrolysis efficiency, increasing NCC yield in up to 12 points and reducing particle size in ≈40 nm. Then, untreated sisal and industrial dissolving-grade fibers were also analyzed as a source for NCC. For this, different acid concentrations and also different enzymatic pretreatment intensities were assessed. Both materials proved to be useful NCC sources, providing yields among 50 % and also allowing top yield increases of ≈10 points produced by enzymatic pretreatments. Evidence presented in this work increases the robustness of the enzymatic-assisted NCC preparation procedure presented in previous chapters, as it has been proved to work in different cellulose sources and also using different enzymes.

8.1. Introduction

Nanocrystalline cellulose (NCC), one of the existing nanocellulose types, consists of rod-like nanoparticles formed by individualized crystalline domains naturally occurring in cellulose fibers (Klemm et al., 2011). The relevance of the research concerning NCC is originated in their nano-scale outstanding physical properties, which could strongly enhance the mechanical performance of nanocomposites (Habibi et al., 2010). The most extended NCC preparation procedure is through a controlled hydrolysis using sulfuric acid for which several cellulosic sources have been used in literature, including plants, animals (tunicates), bacteria and algae. Virtually, NCC could be extracted from almost any cellulosic material (Brinchi et al., 2013). Also, the morphological characteristics of NCC are known to vary depending on the cellulose source and extraction conditions (Klemm et al., 2011).

Concerning the composition of the raw material used for isolation, high-cellulose content fibers are the preferential source for NCC preparation through

sulfuric acid hydrolysis due to a series of reasons. Firstly, other components of lignocellulosic biomass such as hemicelluloses or lignin are known to hinder the acid-cellulose interaction, modifying the kinetics of the acid hydrolysis reaction and thereafter reducing its efficiency (Yoon et al., 2014). Secondly, hemicelluloses or lignin presence in biomass could reduce the NCC yield of the reaction as non-cellulosic components. Lastly, hemicelluloses presence in NCC has been related to a decrease in their quality, for example, by increasing their thermal degradability (Jonoobi et al., 2015).

As stated in previous chapters the main objective of the present thesis was to study the effects and possibilities of an enzymatic pretreatment previous to nanocrystalline cellulose (NCC) isolation. With this aim, chapters 4, 5 and 6 studied different aspects of an enzymatic pretreatment with a cellulase using cotton linters, a natural source of pure cellulose. Then, in chapter 7 a combined enzymatic and chemical procedure for obtaining high-cellulose content (HCC) fibers from TCF sisal was studied. Finally, in this chapter the objective was to isolate NCC from these HCC fibers prepared in chapter 7 using optimal conditions obtained in chapters 5 and 6. With this, the intention was to achieve a combined enzymatic and chemical system for obtaining high yields of NCC from any source.

Then, for a proper assessment of the benefits of the initial cellulose purification step, NCC was obtained from enzymatically pretreated sisal fibers using different hydrolysis conditions and a different enzyme to that used in previous chapters. Finally, commercial dissolving-grade fibers (high-cellulose content) were also used for NCC preparation in combination with a cellulase pretreatment. These final trials allowed validating the effects of the cellulase pretreatment by assessing its effects on different raw materials and using an enzyme from a different origin than in the first studies of this thesis.

8.2. Materials and methods

For details concerning all materials and methodologies used in the present study, please refer to **chapter 2**.

8.2.1. Cellulose source

Totally chlorine-free (TCF) bleached sisal (*Agave sisalana*) fibers (coded as "S"), provided by Celesa (Spain); TCF bleached sulphite dissolving-grade fibers (coded as "D") provided by Domsjö Fabriker (Sweden), being it a mixture of 60 % Norway spruce (*Picea abies*) and 40 % Scots pine (*Pinus sylvestris*); and also cotton linters (*Gossypium sp.*) were used as cellulose sources.

Initial fiber composition was:

TCF sisal fibers (S): 82.7 ± 0.6 % glucan, 16.1 ± 0.3 % xylan, 0.2 ± 0.2 % ramnman, 0.7 ± 0.1 % glucoronic acid, and 0.3 ± 0.2 % acetic acid (chapter 3).

HCC TCF sisal fibers (S HCC): 95.8 ± 1.8 % glucan, 2.9 ± 0.1 % xylan, 0.4 ± 0.1 % glucoronic acid, and 0.9 ± 0.4 % acetic acid (chapter 7).

Cotton linters (C): 97.7 ± 0.3 % glucan, 2 ± 0.2 % xylan, 0.1 ± 0.1 % ramnan, 0.1 ± 0.05 % glucuronic acid and 0.1 ± 0.1 % acetic acid (chapter 3).

TCF dissolving fibers (D): 95.1 ± 0.3 % glucan, 2.8 ± 0.2 % mannan, 0.8 ± 0.1 % xylan, 0.2 ± 0.2 % ramnan, 0.2 ± 0.2 % arabinan, 0.3 ± 0.1 % glucuronic acid, and 0.2 ± 0.1 % acetic acid (Quintana et al., 2015a).

8.2.2. Enzymatic treatments

Two different commercial cellulases were used for treatments: A cellulase preparation (coded as "F") provided by Fungal Bioproducts (Spain) and also another one named "Celluclast 1.5L" (coded as "N"), provided by Novozymes (Denmark). Activities of enzymes were 1700 U/g and 640 U/mL for the Fungal Bioproducts cellulase and Celluclast 1.5L, respectively. Activity was expressed as CMCase units *i.e.* the amount of enzyme degrading 1 μmol of CMC (carboxymethilcellulose) per minute.

Enzymatic treatments were held in an Ahiba easydye reactor (Datacolor, USA) furnished with 250 mL independent vessels with agitation consisting in upside-down inversions at 20 rpm. Consistency was 5% and pH set to 5 using 50 mM acetate buffer. Temperature was 50 ºC and 55 ºC for treatments with Celluclast and Fungal bioproducts cellulase, respectively. Enzyme dose as U/g oven-dried pulp (odp), treatment time and the treated raw materials were different among treatments and indicated in Table 8-1 and schematized in Figure 8-1. After treatments enzyme was deactivated increasing temperature to 105 ºC for 15 min. Fibers were filtered using an

Nº2 borosilicate filter in order to eliminate aqueous phase. Fines were recovered by recirculating effluents through fibers several times. No washing was performed on fibers to avoid sample loss. Control fibers (coded as "K") were obtained using the same conditions as for enzymatic treatments but without enzyme addition.

Table 8-1: Conditions of enzymatic treatments with each cellulase (F and N) and the cellulose source to which they were applied, sisal (S), cotton linters (C) or dissolving-grade fibers (D). Fa treatment corresponds to the optimal pretreatment conditions obtained in chapter 6.

Enzyme	Treatment name	Enzyme dose (U/g odp)	Treatment time (h)	Applied in
Fungal bioproducts (F)	Fa (optimal chapter 6)	20	2	S/C
	Fc	10	24	S/C
Celluclast (N)	Na	20	2	S/D
	Nb	10	2	S/D
	Nc	10	24	D

8.2.3. Cold caustic extraction (E stage)

Initial and enzymatically treated sisal (S) fibers were submitted to an alkaline extraction (E) as described in chapter 7 in order to obtain HCC fibers from sisal. 9% w/v NaOH was used for 1h at 25 ºC. Fibers were extensively washed with deionized water after treatments (Figure 8-1).

8.2.4. Nanocrystalline cellulose preparation

Nanocrystalline cellulose (NCC) was obtained from initial and enzymatically treated fibers by a controlled sulfuric acid hydrolysis, using the protocol described by Dong et al., 1998. Fibers were fluffed prior to hydrolysis, oven dried and cooled in a desiccator. Firstly, S and C fibers were hydrolyzed using the optimal conditions described in chapter 5, *i.e.* 25 min hydrolysis, 47ºC and an acid to fiber ratio of 1:10 with 62 % wt. acid. Also, 1.5 g of S or D fibers weighted immediately from desiccator were hydrolyzed with 61, 62 or 63 % (w/w) sulfuric acid for 45 min and 45ºC. Specific conditions of hydrolysis used for each cellulose source are indicated in Table 8-2 and schematized in Figure 8-1.

Table 8-2: Conditions for NCC preparation for each cellulose source and enzymatic treatment to which they were applied.

Cellulose source	Enzymatic treatment	Acid dose (% wt.)	Reaction time (min)	Reaction temperature (ºC)
S (HCC)	Fa	62	25	47
		(Optimal NCC conditions chapter 5)		
C	Fa	62	25	47
S	Na	61/62/63	45	45
	Nb	61/62/63	45	45
D	Na	63	45	45
	Nb	63	45	45
	Nc	63	45	45

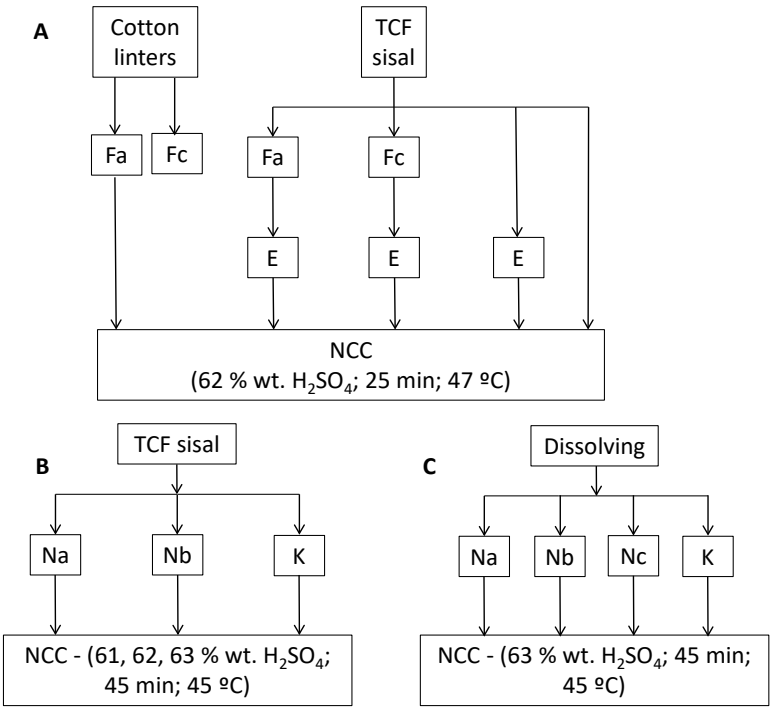

Figure 8-1: Workflow scheme of the different treatments performed. A: NCC from HCC fibers (chapter 7) using optimal conditions (chapters 5 and 6). B: NCC from sisal fibers. C: NCC from dissolving-grade fibers.

8.2.5. NCC characterization

NCC was characterized in terms of yield, average particle size and sulfur content using the same methodologies as in other chapters, described throughfully in chapter 2.

8.3. Results and discussion

8.3.1. Applying optimal conditions on HCC sisal fibers

In agreement with one of the thesis general objectives, this chapter first focused on the application on HCC sisal fibers with optimal enzymatic and chemical conditions for NCC preparation from previous chapters (Figure 8-1A). For this, TCF sisal fibers were treated with cellulase F using two different conditions. First, optimal conditions from chapter 6 were used (20 U/g odp dose, 2 h of treatment, Fa sample), and also conditions used in chapter 7 for maximal hemicelluloses removal (10 U/g odp dose, 24 h of treatment, Fc sample). Then, an alkaline extraction with 9% w/v NaOH was applied on both samples and also on starting fibers. Finally NCC was prepared from these samples and also from initial fibers and alkali-extracted initial fibers. Hydrolysis was performed using conditions previously found to produce the highest yield from enzymatically pretreated fibers in chapter 5 using cotton linters (25 min, using 62 % wt. sulfuric acid at 47 ºC). Results now are compared with results from chapter 6 to assess the effect of using different raw materials.

Firstly, the effects of Fa and Fc treatments in fiber morphology were assessed in both cotton linters and sisal fibers (Figure 8-2). As shown in chart, both enzymatic treatments produced a decrease in the amount of longer fibers, increasing the proportion of shorter ones. Stronger effects were produced by Fc treatment due to the longer exposure to enzyme, predicting the deeper effects produced on NCC. Generally, data suggested that enzymatic treatments led to a more homogeneous raw material for NCC manufacture, as more fibers were counted in a narrower segment of fiber lengths. Comparing between raw materials, initial sisal fibers seemed to be longer compared to initial cotton linters, fact that is probably caused by the pre-refining step to which cotton linters were submitted. Fibers between 3.2 and 7.6 mm showed approximately the same proportion. Fibers between 1.2 and 3.2 mm were more abundant in sisal

while those between 1.2 and 0 mm showed preponderancy in cotton linters. Considering cellulase action, Fc treatment produced different effects in sisal and cotton. Comparing to initial fibers, longer ones (1.2 – 7.6 mm) were reduced in a 90 % in cotton by Fc treatment, a larger shrinkage compared to the 82 % reduction in sisal. On the other hand, shorter fibers (0.2 – 1.2 mm) were sensitively more increased in sisal than in cotton by cellulase treatments, with increases of 250 % and 120 % compared to each initial sample, respectively. This tendency was also observed for fine particles (0 – 0.2 mm), where a huge increase of 3900 % was produced in sisal, markedly bigger than the 220 % increase produced in cotton linters. Finally, fiber length data was useful to corroborate that the type of reactor used for cellulase treatments had a major effect on fiber length, as said in chapter 4. This conclusion arises from the comparison of Figure 8-2A with a similar chart exposed in chapter 5.

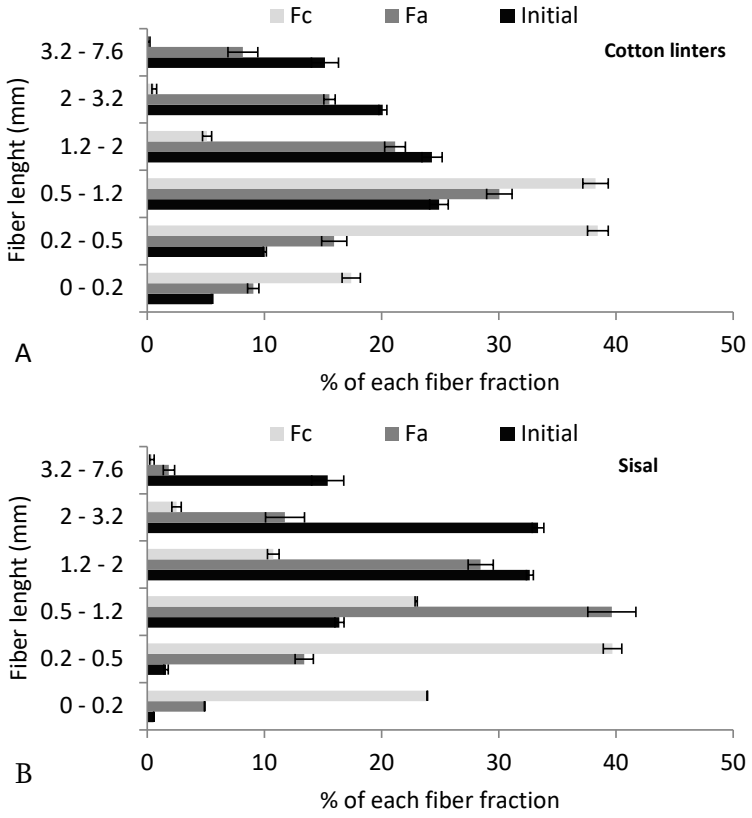

Figure 8-2: Fiber length distribution of initial fibers and after Fa (20 U/g odp, 2 h) and Fc (10 U/g odp 24 h) treatments from cotton linters (A) and sisal (B).

Prior to results evaluation it must be stated that optimal conditions obtained in chapter 6 refer to the compromise solution between the loss of cellulose mass due to cellulase action and the gain in NCC mass for the same reason. In this case, however, only yield of hydrolysis reactions were considered.

Generally, lower hydrolysis yields were obtained with sisal (Figure 8-3) compared to cotton linters (chapter 6). This fact could be probably due to differences in cellulose structure and chemical composition of fibers from both sources. Among samples, firstly, initial sisal fibers yielded the lowest amount of NCC, possibly due to an uncomplete hydrolysis. Then, yield increased in 6 points with the application of the E extraction on initial sisal fibers (Figure 8-3B). Coincidentally, this treatment was capable of increasing in ≈7 points the cellulose content of the sample in chapter 7. Thus, yield increase could be due to this increase in cellulose mass in sample, enhancing effectiveness of hydrolysis by the lower presence of hemicelluloses (Yoon et al., 2014). Concerning enzymatic pretreatments, Fa treatment, described as optimal conditions for the enzymatic pretreatment of cotton liners in chapter 6 was also capable of increasing NCC yield from sisal fibers. Compared to NCC from initial sisal fibers, Fa treatment provided a total gain of 8 points, most of it being due to alkaline extraction. Also, Fc treatment, being more intense than Fa, produced a 12 point yield increase compared to NCC from initial fibers, reaching a total yield of ≈46% (Figure 8-3B). With comparative purposes, when cotton linters were used as cellulose source (chapter 6), Fa treatment produced a higher increase of hydrolysis yield, of ≈22 points (Figure 8-3A). This difference in the effectivity of enzymatic effects could be due to differences in optimal hydrolysis conditions between raw materials, as the optimizations in chapters 5 and 6 were performed with cotton linters. Also, fiber length data indicated that Fc treatment produced a large increase in fines amount. These fines could have been easily reduced to oligosaccharides by acid, indicating that Fc treatment may have been excessively strong, explaining the lower effects it produced on sisal fibers.

With sisal fibers, the treatment which seems to be providing the highest NCC yield was Fc (10 U/g odp 24h). This observation agrees with data exposed in chapter 6, as hydrolysis yield was linearly and positively correlated to the severity of enzymatic pretreatment. Optimal point from chapter 6 (Fa) corresponded to the highest total yield, considering the loss in fiber mass and the gain in NCC yield due to cellulase

pretreatment. In this case, again, only hydrolysis yields were considered. Nevertheless, information provided by these trials was useful for validating the enzymatic pretreatment as a pre-step in NCC preparation showing it could work on different raw materials. Moreover, it was useful to highlight the need of finding the specific optimal enzymatic and chemical conditions for each raw material.

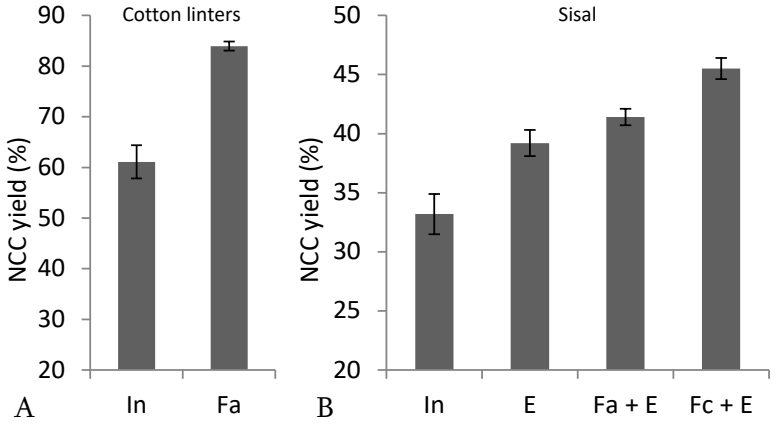

Figure 8-3: Yield of NCC samples obtained from initial (In), enzymatically treated (Fa, Fc) and alkali extracted (E) cotton linters (A) (data reproduced from chapter 6) and sisal fibers (B). All samples were prepared with 62% wt. H_2SO_4 at 47ºC during 25 min.

Concerning particle size (Figure 8-4A), the biggest NCC were obtained from initial fibers, possibly consequence of the uncomplete hydrolysis, also responsible for the low yield. The partial elimination of hemicelluloses showed by alkali-extracted initial fibers strongly reduced NCC particle size, supporting the idea of a more efficient hydrolysis produced by their removal. Enzymatically pretreated samples showed a similar particle size to that of the former sample, with a tendency of producing larger particles with a more intensive treatment, comparing Fa and Fc samples. This evidence was in accordance with the previously observed yield increase, agreeing with data shown in chapter 4 relating yield and particle size increments. Finally, size of NCC particles from sisal seemed to be smaller than those obtained from cotton linters at equivalent conditions. Moreover pretreatment showed no effect in particle size from cotton linters (≈200 nm, chapter 6).

Sulfur content of NCC is an important feature to be analyzed in this material due to the implications sulfate groups esterified on surface have on its physico-chemical behavior (Habibi, 2014). Regarding this characteristic, the studied treatments showed no influence on sulfate esterification in NCC surface, with all values ranging between 1.2-1.4 % sulfur (Figure 8-4B). These values were slightly bigger than those presented by NCC from cotton linters in chapter 6 (1- 1.2 % S).

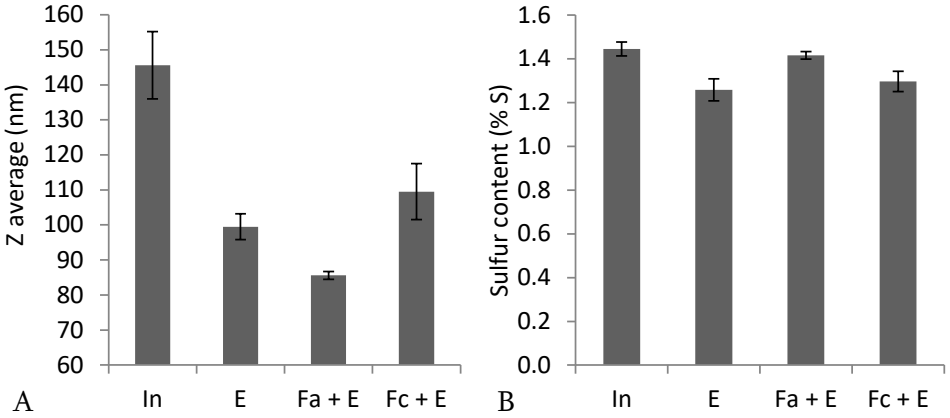

Figure 8-4: Average particle size (A) and sulfur content (B) of NCC samples obtained from initial (In), enzymatically treated (Fa, Fc) and alkali extracted (E) sisal fibers. All samples were prepared with 62% wt. H_2SO_4 at 47ºC during 25 min.

8.3.2. Effect of a different cellulase in NCC from sisal fibers

In the light of the results exposed in previous section, the same raw material was now investigated as received from fiber supplier for evaluating the effect of different enzymatic pretreatments and hydrolysis conditions. The used cellulase now (N) was different to that used in all other studies in this thesis (F). Thus, three different acid doses were used in order to seek for those providing the greatest yield also evaluating the effects of enzymatic treatments (Figure 8-1B).

As shown in Figure 8-5, similar yields were obtained using 61 % wt. and 62 % wt. acid while 63 % showed a lower yield due to an excessive cellulose depolymerization and therefore lacked of interest. Also, sulfuric acid at 61 % wt.

produced insufficient depolymerization when applied to initial, and particularly to control fibers, leading to the production of macroscale particles which were retained during filtration step of NCC preparation, explaining the low yields observed.

Regarding cellulase pretreatments, increases of yield of about a 10 points (Na and Nb samples) in comparison to NCC from initial fibers were obtained with 61% wt. acid, reaching a total yield of 55 % (Figure 8-5). Also, these yields were 10 points higher than when using HCC sisal, again highlighting the importance of adjusting enzymatic and hydrolysis conditions for each raw material. In return, no observable effects were produced by enzyme with 62% wt., showing the same masking effect of enzymatic action due to a more intense hydrolysis observed in previous chapters. This data provided further evidence that enzymatic pretreatments could increase NCC yield regardless of the enzyme and raw material studied.

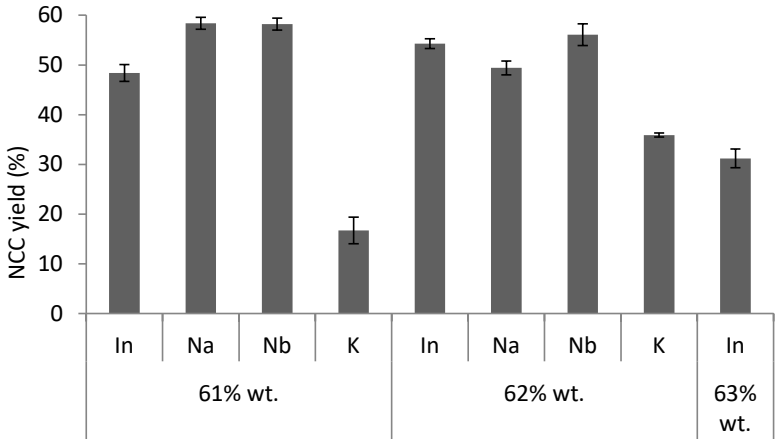

Figure 8-5: NCC from sisal fibers using three different sulfuric acid concentrations and different enzymatic treatments with Celluclast enzyme (N). Samples were prepared with 45 min of acid hydrolysis at 45 ºC using each indicated acid concentration.

Concerning sulfur content, Table 8-3 shows that Na and Nb samples showed a smaller amount of sulfate groups with 61% wt. acid compared to 62%. This evidence is product of a smaller esterification produced by a weaker acid concentration (Roman and Winter, 2004). No statistically significant effect on sulfur content was produced by either enzymatic treatment compared to initial and control samples under this hydrolysis conditions.

Table 8-3: Sulfur content of NCC samples obtained from initial, enzymatically treated and control sisal fibers.

	Sample	Sulfur content (% S)
61% wt.	In	1.5 ± 0.2
	Na	2 ± 0.6
	Nb	1.3 ± 0.7
	K	2.8 ± 2
62% wt.	In	1.1 ± 0.2
	Na	2.5 ± 0.1
	Nb	3.7 ± 0.6
	K	2.2 ± 0.1

8.3.3. NCC from dissolving-grade fibers

After the evaluation of sisal as a source for NCC, the effects of the enzymatic pretreatment were last studied in industrially-manufactured HCC dissolving-grade fibers. Three enzymatic treatments were now assessed in this raw material (Figure 8-1C). Dissolving-grade fibers used in this section had undergone a cooking process intended for the selective elimination of hemicelluloses and a bleaching process, and thereafter constitute high-cellulose content fibers (Quintana et al., 2015a).

NCC was prepared from these fibers obtaining yields among 35-45% (Figure 8-6), similar to those obtained from HCC sisal fibers. These values were generally lower than yields obtained from cotton linters in chapters 4, 5 and 6, showing again differences in the behavior between cellulose sources for NCC, even though being high-cellulose content fibers. Again, cellulase pretreatment increased hydrolysis yields from another raw material. Now, a top yield gain of about 10 points for Nc treatment was achieved compared to NCC yield from control fibers, as shown in Figure 8-6. These results agree with those from HCC sisal fibers in the first section of the present chapter, where the use of these application conditions (10 U/g odp, 24 h) but using a different cellulase also provided the highest yield of all studied samples.

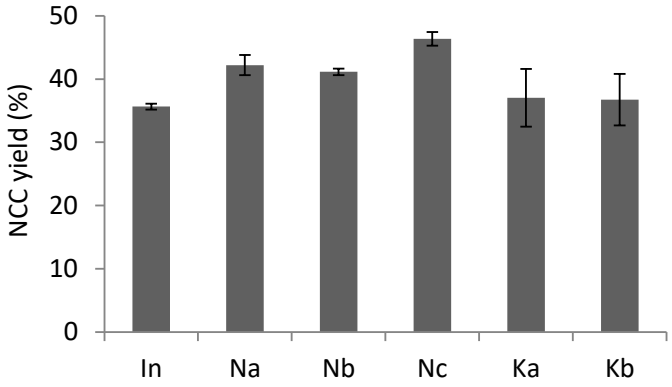

Figure 8-6: NCC extraction yield from dissolving fibers (D) using H_2SO_4 63% wt at 45ºC during 45 min. Initial, and Celluclast (N) treated samples are indicated.

8.4. Conclusions

In this chapter optimal conditions for enzymatic pretreatment and for acid hydrolysis obtained in chapters 5 and 6 were applied to high-cellulose content sisal fibers obtained in chapter 7. Evidence showed that the elimination of hemicelluloses from fibers together with enzymatic pretreatments could increase the efficiency of the hydrolysis reaction. Yields up to 12 points greater and smaller particles were obtained from enzymatically pretreated sisal HCC fibers compared to NCC from initial fibers. Furthermore, enzymatic pretreatments were useful increasing hydrolysis yields using other raw materials. NCC yield increases of ≈10 points were produced by enzymatic pretreatment in untreated sisal and the used dissolving-grade pulp. Generally, these increases were significantly smaller than those obtained with cotton linters which were around 21 points. Concerning total yields, values around ≈45 % were obtained from HCC sisal and dissolving-grade fibers, while the use of untreated sisal under different hydrolysis conditions led to higher yields (≈55 %), clearly reflecting the necessity of finding specific optimal conditions for each raw material. Data presented in this chapter highlighted the utility of the removal of hemicelluloses prior to NCC preparation and validated the enzymatic pretreatment studied in this thesis as it was proven to work in different raw materials and using a different cellulase.

8.5. References

Brinchi, L.; Cotana, F.; Fortunati, E.; Kenny, J. M. Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications. Carbohydr. Polym. 2013, 94 (1), 154–169.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Habibi, Y. Key Advances in the Chemical Modification of Nanocelluloses. Chem. Soc. Rev. 2014, 43 (5), 1519–1542.

Habibi, Y.; Lucia, L. A.; Rojas, O. J. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Jonoobi, M.; Oladi, R.; Davoudpour, Y.; Oksman, K.; Dufresne, A.; Hamzeh, Y.; Davoodi, R. Different Preparation Methods and Properties of Nanostructured Cellulose from Various Natural Resources and Residues: A Review. Cellulose 2015, No. 22, 935–969.

Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. Angew. Chemie - Int. Ed. 2011, 50 (24), 5438–5466.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part I: On a Fully Bleached, Commercial Acid Sulfite Dissolving Pulp. Cellulose 2015, 2067–2079.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Yoon, S. Y.; Han, S. H.; Shin, S. J. The Effect of Hemicelluloses and Lignin on Acid Hydrolysis of Cellulose. Energy 2014, 77, 1–6.

Chapter 9

General discussion

Abstract

In this chapter a general summary of the performed work and the results obtained along this thesis will be presented. Firstly, bleached pulps obtained from diverse sources were characterized in order to choose the one fitting best each application, selecting cotton linters and TCF sisal as the raw materials to be used. Secondly, pure-cellulose cotton linters were used for investigating the effects of pretreating fibers with a cellulase prior to nanocrystalline cellulose (NCC) isolation via sulfuric acid hydrolysis. Enzymatic pretreatment was capable of increasing yield of NCC isolation while influencing other characteristics. Thirdly, sulfuric acid hydrolysis conditions were optimized through an experimental factorial design for maximizing hydrolysis yield and enzyme effect. Fourthly, using optimal chemical conditions, another statistical plan was performed in order to optimize conditions for cellulase pretreatment. With this optimization total yield was considered, including the loss of biomass due to cellulase pretreatment, reaching a total yield higher than 80%, increasing the industrial feasibility of the proposed pretreatment. In order to extend these optimal conditions to other raw materials, enzymatic and chemical treatments were applied on sisal fibers to first obtain a high-cellulose content pulp. Finally NCC was obtained from the previously prepared high-cellulose content fibers from sisal using optimal enzymatic and chemical conditions.

General discussion

The present chapter presents a general summary of the results and findings obtained in all the studies carried out for this thesis. The structure of the discussion is schematized in Figure 9-1.

9.1. Selecting the most suitable raw material

The main objective of the present thesis was to study the possibility of introducing an enzymatic step in the process of isolating nanocrystalline cellulose

(NCC) from cellulose fibers. With this aim in mind, the first part of this thesis consisted in the selection of the raw materials to be used for the different conducted studies. A range of pulps bleached through elementary chlorine free (ECF) and totally chlorine free (TCF) sequences were characterized in several physical and chemical aspects. A woody pulp from eucalyptus and non-woody pulps from sisal, flax and cotton linters were studied.

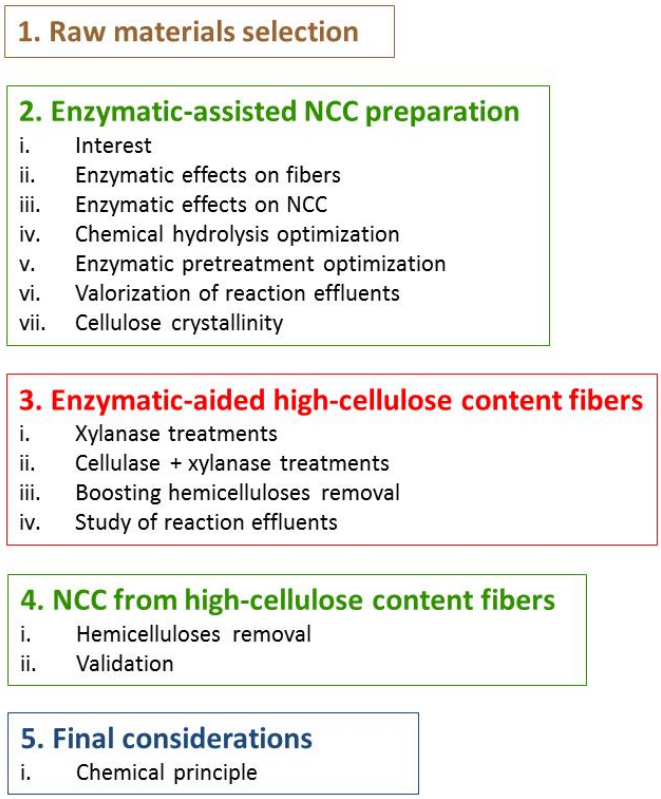

Figure 9-1: Structure of the general discussion.

One of the most influential aspects affecting innovation in the cellulose-related industry is the social and legal pressure to increase its environmental friendliness. In this direction, removing the totality of chlorine reagents from bleaching sequences would prevent the formation of harsh halogenated organic compounds, appearing then as a crucial innovation. Results exposed in chapter 3 generally revealed that ECF

sequences produced fibers with a slightly higher quality, *i.e.* a higher brightness and viscosity; a lower kappa number (KN); and also a lower hexenuronic acid (HexA) and hemicelluloses content. Also, as viscosity and fibers mechanical resistance were found to be linearly correlated, ECF fibers showed a slightly higher mechanical resistance. However, it could be argued that the small loss in fibers quality produced by TCF sequences is widely compensated by the enormous environmental benefits of them. Following this reasoning, TCF bleached fibers appear as the best option in order to develop environmentally friendly processes. The selection of the fiber source was also influenced by this environmental concern. As explained in introduction, non-wood fibers present some advantages over woody ones such as the fact that they are usually obtained from annual crops (*i.e.* renewability), present lower lignin contents allowing milder pulping conditions and are usually obtained as a byproduct of food providing species. Due to these reasons non-wood fibers were preferably chosen for the studies carried out in this thesis.

Generally, high-cellulose content fibers are the preferential raw material for NCC production, fact that will be further discussed in the following sections. This reality brought to us the necessity of using a natural source of high-cellulose content fibers for first studying the effects of the introduction of an enzymatic step into NCC preparation. Thus, cotton linters (≈98 % cellulose) were the chosen non-wood fibers for the studies concerning cellulase pretreatments in NCC isolation process, research exposed in chapters 4, 5 and 6. Subsequently, being aware that the vast majority of natural fibers contain other elements besides cellulose, in chapter 7 an effective combination of enzymatic and chemical treatments for hemicelluloses removal was used. This purification was performed in order to make fibers from any origin a suitable raw material for NCC manufacture. In this case, the selected raw material was a TCF sisal bleached pulp. TCF sisal fibers presented the highest hemicelluloses content of the studied raw materials (≈16%) and thereafter constituted the best target for hemicelluloses-removing treatments. Other characteristics of these fibers such as their high content in HexA or their relatively high KN and also relatively low ISO brightness (considering their bleached status) also allowed an improvement margin.

9.2. Enzymatic-assisted nanocrystalline cellulose preparation

- **Why nanocrystalline cellulose?**

The global interest in the study of NCC is well illustrated by the huge number of research papers published on this issue which also derived in an increasing number of patents describing related technologies. Furthermore, the enormous monetary investment made in a country with a deep tradition of pulp and paper manufacture such as Canada for developing a viable industrial process for its production particularly highlights this interest. The unique features of NCC such as nano-scale dimensions, excellent mechanical properties, ease of chemical modification, high aspect ratio, low density, low energy consumption, inherent renewability, biodegradability and biocompatibility appear as the reasons for this interest. However, the use of a corrosive reactant such as sulfuric acid (the most common reagent for cellulose hydrolysis) poses some treats concerning the environmental compatibility of the process. Entirely enzymatic procedures have been described in bibliography as alternatives to chemical methods for NCC manufacture. Although these procedures could be desirable from an environmental perspective, they usually lead to very low yields and they release uncharged cellulose nanoparticles and unstable suspensions. For this reason, the intention now was to introduce a cellulase pretreatment step in NCC isolation process via acid hydrolysis, always within the framework of Celbiotech research group activity, *i.e.*, replacing traditional chemical processes with enzymatic technologies. One of the main drawbacks classically associated to the preparation of nanocrystalline cellulose is it typically low yield. Low production yields imply a higher biomass and chemical reactants consumption which lead to higher economic costs and an increased environmental impact. For this reason, the introduction of an enzymatic pretreatment was done always focusing on maximizing process outcome. As previously stated, cotton linters were chosen as the cellulose source for the study of this enzymatic-assisted procedure due to their naturally high cellulose content.

- **Which effects does cellulase produce on fibers?**

During the first trials performed in our laboratory for the acquisition of the "know-how" for the lab-scale preparation of NCC, it came to us the observation that treatments with a cellulase had an analogue effect in fiber degradation than that of sulfuric acid. The first trials for the assessment of the effects of a cellulase pretreatment prior to sulfuric acid hydrolysis involved three different experimental setups (chapter 4). The used cellulase was provided by Fungal Bioproducts and contained a predominant cellulolytic activity but also a xilanolytic activity, in a lower proportion. The enzyme, named "Cx" was applied with a 10 U/g odp dose and 24 hours of treatment. The three different setups involved the use of two different reactors. The main difference between these setups was the intensity of their agitation and the absence/presence of a buffer solution for pH control. The two main effects produced by cellulase on fibers were a decrease in cellulose viscosity and also in fiber length. Generally, cellulase treatments produced a higher decrease of cellulose viscosity under the smoother agitation conditions, which in turn produced an inverse effect on fiber length, reduced in a greater extent under a more intensive agitation. These conditions of a smooth agitation led to a viscosity decrease of 67 %, down to ≈250 mL/g and a fiber length reduction of 23 %, down to 1.51 mm. Also, cellulase pretreatments increased the amount of free hydroxyl (OH-) groups in fibers in a ≈12 %. Finally, the adjustment of pH for enzymatic hydrolysis using sulfuric acid instead of acetate buffer did not produce any noticeable effect on fibers.

- **Which effects does cellulase produce on NCC?**

In order to evaluate the effects of these pretreatments, NCC was obtained from fibers from the three different experimental setups. For this first trials, "standard" hydrolysis conditions were used, *i.e.*, 45 minutes of hydrolysis at 45ºC using two different sulfuric acid concentrations (62 % and 64 % wt.) with an acid-fibers ratio of 10:1 (10 mL of acid per gram of dried fibers). Comparison with NCC obtained from initial cotton linters showed the main effects produced in NCC by the cellulase pretreatment: NCC isolation yield increase, NCC size increase and NCC sulfur content reduction. No significant effect in NCC was observed by the use of different reactors.

However, the absence of buffer lead to significantly lower yields, indicating that it could intervene on the hydrolysis reaction.

After these first trials, a further study was performed also in chapter 4 using both acid concentrations to isolate NCC from enzymatically-pretreated, control (no enzyme addition) and initial fibers. These new trials confirmed the previously observed effects of cellulase pretreatment: under the same sulfuric hydrolysis conditions, enzymatic pretreated fibers yielded a larger NCC mass (yields up to a ≈ 9 points higher) than their control counterparts. Also, cellulase increased NCC particle size up to ≈ 30 nm. Other NCC chemical characteristics were also influenced. The typical sulfate group esterification undergoing during sulfuric acid hydrolysis of cellulose was reduced, fact that also led to a reduction in their surface charge. Finally, cellulase showed only little influence either in the very high characteristic suspension stability of sulfated NCC particles (zeta potential ≈ -50 mV) or their hydrophobicity (water contact angle ≈ 45º).

- **The importance of acid concentration**

Besides the effects of cellulase, the experiences performed for the assessment of cellulase effects revealed that kinetics of cellulose hydrolysis was very sensitive to small differences in acid availability. A concentration difference of 2 % wt. produced a huge difference in cellulose degradation. NCC extraction yields among 30 % were obtained using 64 % wt. acid, which were increased in up to 40 points only by reducing sulfuric acid concentration down to 62 % wt. due to a smaller cellulose depolymerization. As happened with cellulase, the reduction in acid concentration had the same effect in particle size, as it increased NCC size in up to 60 nm. Finally, a smaller acid availability reduced the extent of sulfate esterification on NCC surface due to the lower concentration of the reagent (sulfuric acid). This also led to a reduced surface electrical charge. In addition, it was observed that a higher sulfuric acid concentration, *i.e.* a more intensive hydrolysis, could mask enzymatic effects, as almost no significant cellulase-driven yield increases were obtained with 64 % wt. sulfuric acid (≈ 2 points). In conclusion, the lower acid concentration increased overall extraction yield, increased the effects of cellulase pretreatment and reduced the consumption of harsh corrosive sulfuric acid.

- **Which are the best sulfuric acid hydrolysis conditions to be used in combination with the enzymatic pretreatment?**

Aware of that the perceptibility of cellulase effects was strongly dependent on the severity of sulfuric acid hydrolysis, it was intended to seek for those conditions maximizing hydrolysis yield and also enzymatic effects. Hence, two experimental designs were performed in chapter 5 studying the influence of reaction time and temperature with two different sulfuric acid concentrations (62% and 65% wt.) in presence and absence of cellulase. A 2^3 factorial design was performed with each acid concentration, studying the individual effects of the following 3 variables: cellulase (presence/absence), hydrolysis time (25 and 50 minutes) and hydrolysis temperature (47ºC and 60ºC). Given the qualitative nature of "enzyme" variable, no central points could be analyzed in this study. Enzymatic pretreatment was held using the same conditions as those used in chapter 4: 10 U/g odp dose and 24 h of treatment.

Results showed that 62 % wt. acid produced large amounts of NCC, with yield values ranging between 65-82 %, very interesting from an industrial perspective. Agreeing with that previously stated, the use of sulfuric acid at 65% wt. led to lower outcomes, among 20-30% and thereafter it had a lower industrial interest. Regarding cellulase effects, its presence produced a top increase of 9 yield points in comparison to NCC obtained from control fibers using 62 % wt. acid and a 2.5 points increase using 65% wt. acid (Figure 9-2), showing again that enzyme effect was lower with the stronger acid dose, *i.e.* a stronger hydrolysis. The other two studied variables, *i.e.* time and temperature, produced a similar effect: enhancing hydrolysis occurring, a similar effect of a larger acid concentration. Although in different degrees, the increase of temperature and/or hydrolysis time led to a smaller NCC yield, smaller particle size and a larger surface charge. The effect of these variables, however, was considerably less noticeable when using the 65% wt. acid, showing that acid concentration was the most influential variable. Noteworthy, the increase in yield produced by enzyme was still statistically significant in this stronger hydrolysis scenario, fact that enhanced the robustness of the proposed enzymatic-assisted process. Concerning other parameters, cellulase effect generally tended to increase particle size and reduce surface charge with 65 % wt. acid, in accordance to previous experiments. However, with 62 %wt. acid cellulase did not produce any statistically significant effect on these parameters. This non-affectation agrees with the dependence of cellulase effects on hydrolysis

conditions, which were now different from those used in chapter 4. Moreover, cellulase pretreatment neither did not produce any noticeable effect on NCC sulfur content (with values ranging from ≈1% to ≈3 % sulfur, depending on hydrolysis conditions) nor suspension stability, measured as zeta potential (with values around ≈-50 mV).

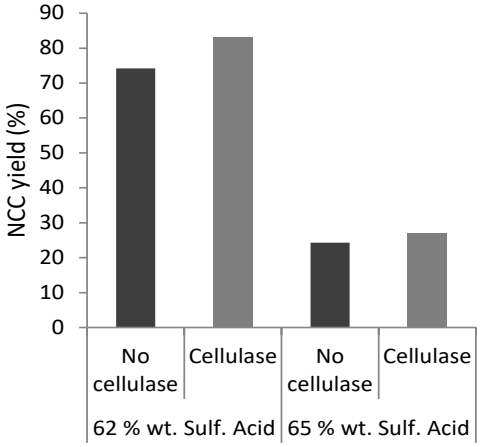

Figure 9-2: NCC yield predicted by models at 25 minutes and 47 ºC in presence and absence of cellulase pretreatment and with 62 % wt. (optimal point) and 65 % wt. H_2SO_4. Data reproduced from chapter 5.

This study allowed us to determine the best acid hydrolysis conditions for their application on cellulase pretreated fibers. It was observed that 25 minutes of hydrolysis at 47ºC and using 62 % wt. sulfuric acid produced a hydrolysis yield of ≈ 82 %, 9 points higher than that of control fibers. With these conditions, NCC of ≈200 nm and ≈1.2 % sulfur content was obtained, the maximum and minimum values observed for each parameter, respectively. As a consequence of this optimization, NCC hydrolysis yield was increased in ≈ 10 points compared to the maximal yield reached in chapter 4, basically due to the reduction of unnecessary cellulose degradation. Also the adjustment of conditions allowed reducing the necessary hydrolysis time down to 25 min, a 44 % reduction compared to previous conditions. It was observed that these conditions were the minimal necessary to achieve a sufficient cellulose degradation to reach NCC level. If any of the variables was reduced, *i.e.* a lower temperature, a shorter reaction time or a lower acid concentration, the "hydrolysis severity" would become insufficient for reaching NCC level.

- **Optimizing the enzymatic pretreatment**

After finding the optimal conditions for the hydrolysis of enzymatically pretreated fibers using sulfuric acid, the effect of different conditions for the enzymatic pretreatment were studied. For this objective, in chapter 6 a 2^2 experimental factorial design was carried out assesing cellulase dose (2-20 U/g odp) and enzymatic treatment time (2-24 hours) in the pretreatment step, always with the aim of maximizing process yield. In this case, central points were studied for variance assessment and also for identifying potential curvatures on response surfaces. After this, NCC was prepared with sulfuric acid using the optimal conditions obtained in chapter 5.

The effects of the different intensities of enzymatic effects in fibers were first assessed. Enzymatic yield *i.e.* the cellulose mass loss due to cellulolytic treatments and average fiber length showed a similar behavior. They were reduced from 2 to 13 hours of treatment in ≈10% and ≈1 mm, respectively, with smaller cellulase effects after this period. The other studied variable, enzyme dose, also increased these reductions, having a lower effect than treatment time. Another variable, fiber viscosity, was reduced all along treatment, showing a linear surface both affected by enzyme dose and reaction time with a final reduction of 65% (down to ≈ 280 mL/g).

Concerning NCC, it was observed that a higher intensity of the cellulase pretreatment, *i.e.* a higher cellulase dose and/or a longer treatment time, led to a higher hydrolysis yield, with values up to 90%. In other words, a stronger cellulolytic treatment reduced the loss of cellulose mass produced by sulfuric acid down to a 10%, partially replacing it by an enzymatic catalysis. Furthermore, the assessment of total yield, understood as a compromise solution between the loss in fibers mass and the gain in acid hydrolysis yield, produced by enzymatic pretreatment was mandatory for the optimization of this process. Total yield values fitted a parabola when modeled, with its lower values being at middle hydrolysis times and the higher at the ends (Figure 9-3). On one hand, at short enzymatic treatment time (2 h), only small losses of fiber mass were produced, enhancing the benefits of the cellulase-driven gain in yield and leading to the maximal observed total yield. On the other hand, at longer hydrolysis time the loss in fibers mass was compensated by the huge gains in the yield of sulfuric hydrolysis, which did not happen at middle times and thereafter showed a lower outcome. Because of this a second point of maximal yield providing a lower yield than the former was obtained at 24 hours of treatment.

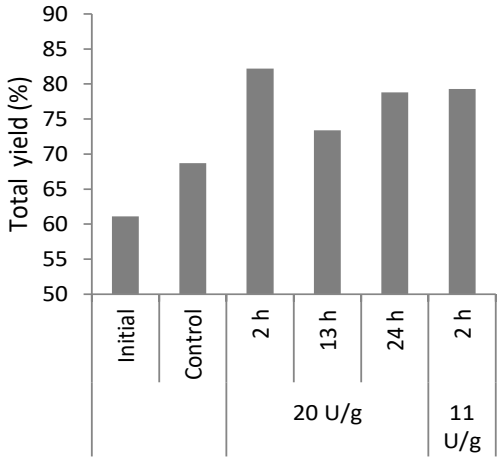

Figure 9-3: Total yield values predicted by models for NCC obtained from initial, control and enzymatically pretreated fibers under different conditions. All samples were hydrolyzed during 25 minutes at 47 ºC with 62 % wt. H_2SO_4. Data reproduced from chapter 6.

Besides the optimization of process yield, these trials showed another important fact: quantitative differences in the intensity of enzymatic pretreatment intensity led to quantitative differences in some of their effects in NCC. A more intensive cellulase pretreatment proportionally increased hydrolysis yield, slightly decreased NCC surface charge and also increased NCC crystallinity, fact that will be further discussed below. Other studied parameters in this chapter resulted unaffected by enzymatic pretreatment: All samples showed ≈1% sulfur content, a ≈200 nm particle size, a ≈-50 mV zeta potential and a cellulose degree of polymerization of ≈200. Again, it was observed that cellulase did not produce any effect on particle size under these hydrolysis conditions, agreeing with data from chapter 5. All these values showed that well-stable, high quality suspensions of NCC could be obtained regardless of the applied cellulase pretreatment.

Finally, this study allowed determining the optimal conditions for the application of cellulase: 20 U/g odp dose and 2 hours of treatment. Using these parameters (chapter 6) and also the optimal acid hydrolysis conditions (chapter 5) a total yield (*i.e.* enzymatic + acid hydrolysis) of 82% was obtained. This high value was 12 and 21 yield points higher than that of NCC from control and initial fibers, respectively (Figure 9-3). Also, data indicated that enzymatic pretreatment could be

shortened to from 24 hours (chapters 4 and 5) to 2 hours, a 90% reduction, dramatically increasing the industrial feasibility of the process. Noteworthy, this optimal point implied duplicating the enzyme dose compared to the studies in previous chapters. However, it could be argued that the strong reduction in treatment time would compensate the higher economic cost of duplicating enzyme dose. Nevertheless, if increasing enzyme dose resulted unaffordable, a total yield of ≈79% was obtained using a ≈11 U/g odp dose and 2 hours of treatment, representing a loss of 3 points in total yield but the same enzyme dose (Figure 9-3).

- **Valorizing reaction effluents**

Besides the improvement in NCC extraction, the benefits of the enzymatic-assisted method could be analyzed from another perspective. It has been said elsewhere that NCC preparation, a process consisting in the selective degradation of cellulose amorphous regions, fits perfectly within the biorefinery concept. This concept advocates for the integral use of biomass, taking profit of each component for a specific application. During NCC isolation, a non-negligible fraction of cellulose biomass is unavoidably transformed to oligosaccharides usable for example, for the production of biofuels. Thus, this parallel saccharification reaction would reduce the economic cost of the process due to the possibility of taking profit of this byproduct. Furthermore, it would also reduce the environmental impact of NCC production, by reducing the current biomass demand for biofuels manufacture as well by preventing these effluents to be spilled.

In chapter 4 the sugar release as a consequence of acid hydrolysis was studied. Sulfuric acid produced the release of glucose-oligosaccharides composed by 1 to 6 monomers. In effluents, glucose constituted the most abundant sugar and the concentration of the longer species decreased with the larger number of subunits. Hydrolysis reaction is normally stopped diluting sulfuric acid, and thereafter a mixture of NCC, released sugars and residual acid is obtained at the end. Recovery of these oligosaccharides after the separation of liquid phase from NCC particles also requires the separation of the sugars from this residual sulfuric acid. In this thesis, the increase in isolation yield produced by the enzymatic pretreatment could also be understood as a reduction in the amount of the sugars released from fibers during acid hydrolysis.

This reduction was consequence of the studied enzymatic pretreatment, which also led to the release of oligosaccharides, studied in chapter 6. It was observed that glucose oligosaccharides of up to 4 subunits were released as a result of enzyme action, being the shorter forms the most abundant. The relative concentrations between the different forms were found to depend on enzyme variables: enzyme dose enhanced the release of longer forms while reaction time promoted the scission into the shorter species. Also, it was noticed that the optimal enzymatic conditions (20 U/g odp, 2h) were those in which the enzyme had the highest catalytic efficiency, measured as mg of sugars released per minute.

Therefore, this displacement from acid-catalyzed to enzyme-catalyzed release of oligosaccharides illustrates another benefit of the enzymatic pretreatment: sugars released by cellulase action do not require any purification. Hence, the application of the enzymatic pretreatment increases the quality and, in consequence, the added-value of the byproduct of NCC production, reducing economic costs and increasing the industrial feasibility of this enzyme-assisted process.

- **What happens with cellulose crystallinity?**

Cellulose crystallinity is a parameter closely related to its physical and chemical characteristics. In the case of NCC a higher crystallinity corresponds to a higher purity due to the lower availability of amorphous cellulose, *i.e.* a higher quality.

Concerning cellulose fibers, changes in their crystallinity during enzymatic treatments were assessed in chapters 5 and 6 via FTIR analysis. For this, total Crystallinity Index (TCI), a parameter analogous to crystallinity index (as calculated from XRD) was studied. Enzymatic treatments were capable of increasing fibers TCI a ≈33 % (Figure 9-4). In addition, data from chapter 6 indicated that only enzyme dose seemed to influence crystallinity, as no change in this characteristic was produced by increasing enzymatic treatment time. At the same time, FTIR analysis also allowed the analysis of Lateral order index (LOI), an index usually claimed to be proportional to overall degree of order in cellulose. The analysis of this index in chapter 5 showed that LOI was reduced in a ≈34 % as a consequence of enzymatic treatments, the same proportion in which crystallinity was increased. This LOI reduction meant that besides the crystallinity increase, enzymatic treatments also produced a more accessible

structure of cellulose in fibers, which could help explaining the benefits of the pretreatment in NCC.

Concerning NCC, firstly, the removal of amorphous regions during acid hydrolysis entailed an increase of crystallinity of up to ≈120 %, comparing to the crystallinity value of its original fibers (Figure 9-4). Then, cellulase pretreatment also increased NCC crystallinity in comparison to NCC from control fibers, as observed in chapter 5. In this section, the enzymatic pretreatment produced a TCI increase of around ≈38 % in NCC obtained with 62 % wt. sulfuric acid. Coincidentally, this increase was similar to that observed in fibers. Data from chapter 6 confirmed this observation, showing a crystallinity increase of ≈30 % (Figure 9-4). Furthermore, data from NCC in this chapter showed again that the only enzymatic variable affecting crystallinity was enzyme dose, as no effect was produced by treatment time.

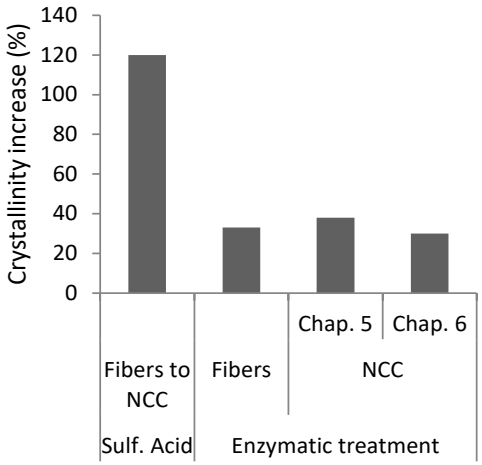

Figure 9-4: Crystallinity increases produced by sulfuric acid during NCC isolation (fibers to NCC) and by enzymatic treatments on fibers and on NCC in studies from chapters 5 and 6. Increases produced by enzyme are calculated from the value showed by each control sample.

9.3. Enzymatic-aided high-cellulose content fibers

For a series of reasons which will be discussed in the following section, high-cellulose content (HCC) fibers are the preferential source for NCC. Because of this, the possibilities of obtaining HCC fibers from a cellulosic source with a natural high

content in hemicelluloses were investigated in this section. The reason for this was to extend to fibers from any plant origin the benefits of the cellulase pretreatment found in natural HCC fibers such as cotton linters. HCC fibers are normally produced in industry by sulfite or pre-hydrolysis kraft processes. These processes are known for some disadvantages regarding final product and environmental friendliness. Because of this, enzymatic-mediated hemicelluloses removal from typical paper-grade fibers appeared as a very promising alternative for the procurement of HCC ones due to its specificity and non-polluting nature. In this section, TCF sisal fibers (≈16 % hemicelluloses) were used. All the results which will be discussed in this section were exposed in chapter 7.

- **Assessing the effects of treatments with xylanases on sisal fibers**

The first attempt to enzymatically remove hemicelluloses (xylans) from sisal fibers was done applying a xylanase (named "X"). A 10 U/g odp dose was used, which could be considered to be high if compared to those normally applied in bleaching processes. Xylanase dose was applied for 5 hours studying the effects after each hour. Also, in order to avoid any possible product inhibition of the enzyme, the same dosage was administrated in 1 hour intervals of 2 U/g odp each hour followed by a washing step (treatment named Xs). In the end, the same dose of 10 U/g odp was used. The first treatment reduced hemicelluloses content down to a final content of 12 % (X5), while the second only to a 13.8 % (Xs5) (Figure 9-5). Hence, better results were obtained applying the entire enzyme dose at one time. However, regardless of the used scheme only 25 % of the total hemicelluloses were enzymatically accessible, being this final content of 12% still too high for our purposes. Moreover, xylanolytic treatments also affected other fiber properties as they reduced their kappa number and HexA content and increased their ISO brightness.

- **Could the hemicelluloses availability to enzymes be enhanced by a cellulase treatment?**

In view of this situation, a treatment was applied using the cellulase from chapters 4, 5 and 6 ("Cx"), being it a mixture of cellulase + xylanase. This treatment was followed by another one using the previously indicated xylanase, with the intention to

have this removal boosted. The accessibility of enzymes to fibers was expected to be increased by cellulolytic treatments.

Concerning Cx enzyme, it was itself already capable of reducing hemicelluloses content of fibers due to the contained xylanase activity (Figure 9-5). After a treatment applying a cellulase dose of 10 U/g odp (and therefore a xylanase dose of 4 U/g odp) for 2h, a final content of 13.5 % xylans was achieved (Cx 2h). Also, it was found that the xylanase contained in Cx was twice as effective as X enzyme. Due to this efficiency, a prolonged treatment was performed during 24 h using the same dose in order to study Cx potential, reaching a final value of 12.4 % xylans in fibers (Cx 24h) (Figure 9-5). Importantly, the application of treatments with X for 2 and 5 h after Cx did not provide any improvement in this removal, reaching the same limit of accessible xylans. At the end, regardless the applied treatment, no enzymatic removal beyond this 12 % limit could be accomplished (Figure 9-5).

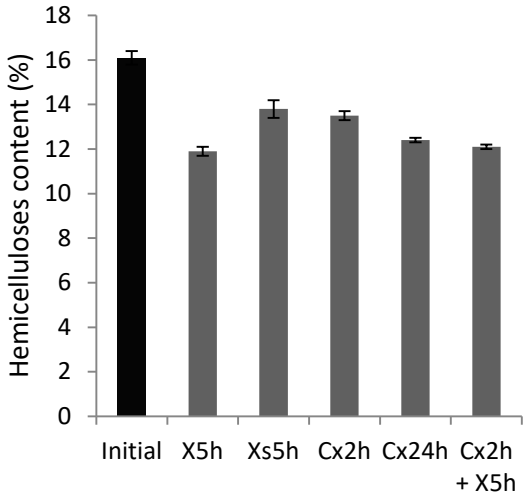

Figure 9-5: Hemicelluloses content of TCF sisal fibers after xylanase treatments (X, Xs), cellulase treatments (Cx2h, Cx24h) and combined treatments (Cx2h + X5h). Data reproduced from chapter 7.

At the same time, Cx enzyme also modified other fiber properties: it reduced fibers KN and HexA content and increased ISO brightness; it strongly reduced fibers viscosity and substantially increased their Fock solubility. One of the most common

applications of high-cellulose content fibers is the production of viscose rayon, a process for which a high Fock solubility is essential and a low viscosity is also desirable. In this thesis, though high-cellulose content fibers were intended as a raw material for preparing NCC. For this application, as it has been previously discussed, a viscosity reduction is also a positive modification. In conclusion, although not capable of enhancing xylanase action, Cx enzyme appeared as a very promising single catalyst both removing hemicelluloses and improving fiber properties.

- **How to boost hemicelluloses removal?**

In order to achieve the goal of producing high-cellulose content fibers from sisal, the limit of 25 % enzymatically removable hemicelluloses needed to be overcame. For this reason, following reports from other authors, a cold caustic extraction using NaOH was applied after enzymatic treatments. Two different NaOH concentrations were assessed: 4 and 9 % w/v. Both concentrations strongly removed hemicelluloses from fibers (Figure 9-6). Also, NaOH effects were boosted by enzymatic treatments: Similar effects were produced when 9% NaOH was applied directly on initial fibers than those obtained when 4% NaOH was applied on Cx treated ones (Figure 9-6). Hence, this could allow a reduction in the necessary NaOH concentration for reaching a specific hemicelluloses content, diminishing the use of chemicals for this purpose. For a maximal hemicelluloses removal, the higher NaOH concentration (9% w/v) was applied in combination with Cx enzyme. This led to fibers with hemicelluloses content below 3% (Figure 9-6), removing more than 80% of the initially present xylans. Furthermore, these combined enzymatic and chemical treatments reduced fibers HexA content up to a 90 %, highlighting the important increase in product quality. These results indicated the accomplishment of one of the main thesis objectives, as fibers with cellulose content of ≈96% *i.e.* HCC fibers, had been obtained.

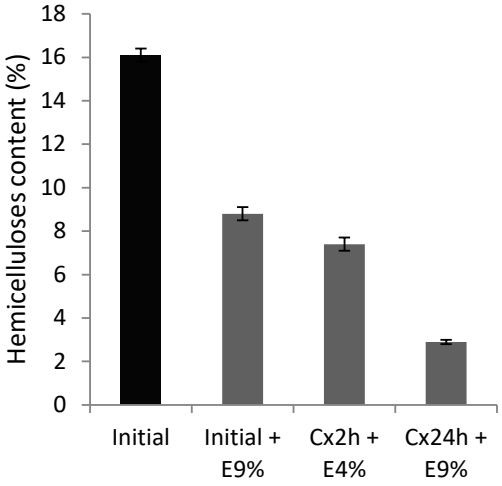

Figure 9-6: Hemicelluloses content of initial or Cx treated TCF sisal fibers after cold alkaline extraction with 4 % (E4%) or 9 % (E9%) w/v NaOH. Data reproduced from chapter 7.

- **What information could be obtained from enzymatic reaction effluents?**

For a deeper assessment of enzymatic effects, sugars present in effluents as a result of their hydrolytic activity were identified and quantified. The release patterns of enzymes, which are known to be specific for each enzyme, were studied. The used xylanase (X) released preferentially oligosaccharides of 2-3 xylose units rather than its monomer. By contrast, the cellulase and xylanase activities present in Cx showed an opposite behavior favoring the release of glucose and xylose monomers, possibly with the action of β-glucosidase and β-xylosidase activities, respectively. The existence of these activities is very useful in order to avoid the widely described inhibition by product, and could be conjectured to be the cause of the higher efficiency of the Cx xylanase compared to X. Moreover, enzymatic effluents indicated that applied enzymes could be re-used as their residual activity was preserved after treatments.

9.4. Obtaining NCC from high-cellulose content fibers

- **Effects of the removal of hemicelluloses in hydrolysis yield**

After the achievement of high-cellulose content fibers via enzymatic and chemical treatments, their performance as an NCC source was investigated. It must be noticed that enzymatic-assisted hemicelluloses elimination combined the necessary cellulose purification with the enzyme-catalyzed cellulose degradation required for NCC yield increase exposed in chapters 4, 5 and 6. The elimination of hemicelluloses prior to NCC isolation is a desirable event due to three causes: firstly, hemicelluloses could hinder the acid-cellulose interaction, modifying cellulose depolymerization kinetics and thereafter reducing its efficiency. Secondly, hemicelluloses presence implied, as a non-cellulosic component, the reduction of process yield. Lastly, hemicelluloses presence in NCC has been related to a decrease in their quality, for example, by decreasing their thermal stability.

In chapter 8 the effects of hemicelluloses elimination in NCC isolation yield were studied. The optimal acid hydrolysis conditions found in chapter 5 were applied to initial sisal fibers, alkali-extracted sisal fibers, high-cellulose content fibers (chapter 7) and also sisal fibers treated using optimal cellulase conditions (chapter 6) and then alkali extracted. In the first place, the application of the alkaline extraction increased isolation yield in 6 points compared to that of initial fibers, certainly most due to the removal of hemicelluloses it produced. In the second place, the inclusion of an enzymatic pretreatment prior to alkaline extraction, *i.e.* the achieved HCC fibers (Cx24 + E9% sample from Figure 9-6) provided an extra increase of 6 yield points, reaching a total yield of ≈45%. Hence, globally, HCC fibers obtained in chapter 7 provided an NCC yield 12 points greater than that offered by initial fibers while it also contributed to enhance their quality. Lastly, optimal conditions from chapter 6 (20 U/g odp, 2h) in combination with the alkali extraction, increased NCC yield in 8 points. The reason for this lower benefit was due to the lower intensity of this pretreatment compared to the former and the fact that only hydrolysis yields were considered in this section.

Importantly, this evidence highlighted the relevance of the hemicelluloses elimination from fibers prior to acid hydrolysis. Also, it demonstrated that fibers from any origin could be a suitable source for high-quality NCC if a cellulose purification step is previously held.

- **Validating the enzymatic-aided process in different raw materials and using a different cellulase**

In order to validate the enzymatic technology proposed in the present thesis it was finally applied in chapter 8 to different raw materials prior to NCC isolation. Given the huge enzymatic diversity existing within a specific activity (*e.g.* cellulolytic activities) it was also necessary to check if the enzymatic preparation used in this thesis (Cx) was replaceable by another enzyme holding the same catalytic nature.

The enzyme used in this case was a commercial cellulase provided by Novozymes and named "Celluclast". This enzyme was applied to sisal fibers and also to commercial dissolving-grade ones (industrially-obtained HCC fibers). After this, NCC was obtained with 45 minutes of hydrolysis at 45ºC and different sulfuric acid concentrations, namely: 61, 62 and 63 % wt. for sisal and 63% wt. for dissolving-grade fibers. In these cases, Celluclast provided top NCC yield increases of ≈10 and ≈11 points in comparison to NCC from initial sisal and dissolving-grade fibers, respectively. Globally, sisal fibers offered a maximum hydrolysis yield of ≈58%, while dissolving grade fibers a ≈46%, similar to that yielded by HCC sisal fibers. This demonstrated that besides the importance of finding optimal conditions for obtaining NCC from each source, the maximum possible yield might be different in each case.

Experimental data presented in this final chapter successfully validated the studied enzymatic-assisted system by demonstrating it could work on different raw materials and using a different cellulase. Thus, cellulase pretreatment was found to be able to increase NCC yield in fibers as different as sisal (non-wood, NaOH-AQ pulped), cotton linters (non-wood, non-pulped fibers) or dissolving grade fibers (wood, sulfite pulped), remarking the utility and the potential of this enzyme-assisted technology.

9.5. Final considerations on the studied process

- **Analyzing the chemical principle behind the enzymatic effects in NCC isolation**

In this final section, after the observation and interpretation of data from cellulose fibers, NCC and reaction effluents, an speculation about the chemical principle underlying behind enzymatic effects in NCC could be done.

Firstly, crystallinity data indicated that cellulase pretreatment preferentially attacked the amorphous regions of fibers, increasing the content of crystalline regions in cellulose (TCI) and also its accessibility (LOI) (chapters 5 and 6). This increase in accessibility was also suggested by free OH- data, as cellulase pretreatment increased the number of free (exposed) hydroxyl groups in fibers (chapter 4). Then, on one hand, the higher abundance of crystalline cellulose in fibers would be a direct cause of the NCC yield increase. On the other hand, these modifications would have also led to a cleaner and more rapid access of sulfuric acid to amorphous cellulose, reducing unnecessary sample loss during acid hydrolysis. As a consequence of both, a higher amount of NCC particles would then be obtained, explaining the observed increases in yield. This hypothesis would also be supported by crystallinity data of NCC as a higher dose of enzyme in chapter 6 (> 10 U/g odp), *i.e.* a stronger enzymatic pretreatment, increased the crystallinity of NCC. This meant that not only a higher mass of NCC was obtained as a consequence of cellulase action; it also led to NCC with a higher proportion of crystalline phases. This might be product of both the more efficient hydrolysis and also of the higher amount of crystalline cellulose available on enzymatically pretreated fibers. Lastly, this modified interaction between cellulose and sulfuric acid could also explain other minor modifications, such as the reduction observed in sulfate esterification also consequence of the enzymatic pretreatment.

Finally, Table 9-1 summarizes the main achievements concerning NCC preparation yield reached in each chapter of the present thesis.

Table 9-1: Summary: Pretreatment conditions; Acid hydrolysis conditions; Top yield reached (hydrolysis yield "NCC", or total yield); and the Yield gain produced by cellulase in each chapter of the thesis.

Chapter	Enzymatic pretreatment	Hydrolysis conditions	Top yield	Enzymatic yield increase (vs control)
4	10 U/g odp 24h (*Cotton linters*)	62 % wt. 45' 45 ºC	71 % (NCC)	8%
5	10 U/g odp 24h (*Cotton linters*)	62 % wt. 25' 47 ºC (optimal)	82 % (NCC)	9%
6	20 U/g odp 2h (optimal) (*Cotton linters*)	62 % wt. 25' 47 ºC	90 % (NCC) 82% (Total)	12%
8	20 U/g odp 2h (*HCC sisal*)	62 % wt. 25' 47 ºC	45 % (NCC)	6%
	10 U/g odp 24h (*Sisal*)	61, 62, 63 % wt. 45' 45 ºC	55 % (NCC)	10%
	10 U/g odp 24h (*Dissolving*)	63 % wt. 45' 45 ºC	45 % (NCC)	11%

Chapter 10

Main conclusions

10.1. Main conclusions

The present memoir describes a series of studies carried out for the introduction of an enzymatic step into nanocrystalline cellulose (NCC) preparation through a sulfuric acid hydrolysis. The **first part** of this thesis consisted in the characterization of several raw materials for the selection of the most suitable for each study. The **second part** consisted in the study of the effects of a cellulase pretreatment on cotton linters prior to NCC preparation through sulfuric acid hydrolysis, highlighting among these effects an increase in reaction yield. The **third part** consisted in the optimization of sulfuric acid hydrolysis conditions for maximizing the NCC yield from enzymatically pretreated fibers. In the **fourth part**, enzymatic pretreatment was optimized, focusing on the maximization of total yield: finding a compromise solution between the loss in cellulose mass due to cellulase action and the further gain in NCC yield for the same reason. In the **fifth part**, an enzymatic treatment followed by an alkaline extraction was performed for the preparation of high-cellulose content fibers from TCF sisal fibers, originally containing a high content in hemicelluloses. Lastly, the **sixth part** consisted in the preparation of NCC from high cellulose content fibers obtained in part 5 and using optimal conditions obtained in previous sections. Finally, the enzymatic-assisted procedure was validated also in this last part through the application of the enzymatic pretreatment in different raw materials and using a different cellulase.

In this thesis, classical analytical methods from the pulp and paper industry were used in combination with innovative techniques such as DLS, WCA, HPLC, FTIR, Z-potential and morphological analysis through SEM and TEM for a comprehensive characterization of samples.

The most relevant conclusions obtained in this thesis were as follows:

- Fibers obtained through TCF bleaching sequences were chosen for this thesis for their enormous environmental benefits although generally, ECF bleaching led to slightly better fiber properties.

- A linear correlation was found linking cellulose viscosity and fibers mechanical strength within the studied raw materials.

- TCF sisal fibers held the highest hemicelluloses content, and thereafter were chosen for the study of hemicelluloses removal, while cotton linters were chosen as natural high-cellulose content fibers for NCC preparation.

- In this thesis an enzymatic pretreatment was performed for the first time on fibers prior to NCC isolation through sulfuric acid hydrolysis.

- Enzymatic pretreatment increased NCC isolation yield up to a 7%, average particle size up to 30 nm and reduced sulfur content without compromising their colloidal stability.

- A higher acid concentration used for hydrolysis largely determined NCC characteristics: reduced yield and particle size; increased sulfur content and surface charge and reduced the effects of the enzymatic pretreatment.

- Reaction time and temperature of acid hydrolysis produced a similar quantitative and qualitative effect on hydrolysis: enhancing its occurring, reducing yield and particle size and increasing sulfur content.

- The optimal acid hydrolysis conditions, *i.e.* those providing the highest yield from cellulase pretreated fibers were: 25 min of hydrolysis at 47ºC using 62% wt. sulfuric acid. At these conditions a hydrolysis (NCC) yield of 82% was obtained, 9 points higher than that obtained in absence of cellulase pretreatment.

- The optimal conditions for enzymatic pretreatment, *i.e.* a compromise solution between the loss in cellulose mass due to cellulase action and the hydrolysis yield gain were found at a 20 U/g odp dose and 2h of treatment.

- Differences in the intensity of enzymatic pretreatment on fibers led to quantitative differences in NCC such as the hydrolysis yield or surface charge.

- Using optimal enzymatic and chemical conditions, a Total yield of 82 % was obtained, 21 and 12 points higher than that of initial and control fibers, respectively, with no deleterious effect in NCC quality.

- Enzymatic pretreatment also reduced the generation of effluents containing sulfuric acid while it produced a clean stream of easily re-usable sugar-rich effluents with a higher added-value.

- Cellulase pretreatment increased fibers crystallinity and accessibility and also increased crystallinity in final NCC.

- Xylanase treatments reached a limit of enzymatically hydrolysable hemicelluloses in sisal fibers, which could not be boosted using a cellulase pretreatment, reaching a final content of 12% hemicelluloses in fibers.

- The enzymatic mixture containing cellulase + xylanase activities ("Cx") was capable of removing hemicelluloses while simultaneously adjusting cellulose viscosity and reactivity in a single treatment.

- A treatment with Cx boosted in 14 points the effect of a NaOH extraction (9% w/v) which all together led to a total hemicelluloses removal of 82% accomplishing the objective of obtaining high-cellulose content fibers (96% cellulose).

- Hemicelluloses removal from fibers prior to acid hydrolysis increased extraction yield and NCC quality.

- The enzymatic pretreatment was validated as a promising first step for NCC preparation as it was capable of increasing yield of NCC extraction regardless of the used raw material and the used cellulase.

- It was speculated that the chemical principle behind the effects of enzymatic pretreatment was both due to the increase in the content of crystalline regions in fibers and also a facilitated interaction between sulfuric acid and cellulose during acid hydrolysis.

General bibliography

Abitbol, T.; Kloser, E.; Gray, D. G. Estimation of the Surface Sulfur Content of Cellulose Nanocrystals Prepared by Sulfuric Acid Hydrolysis. Cellulose 2013, 20 (2), 785–794.

Ahola, S.; Turon, X.; Osterberg, M.; Laine, J.; Rojas, O. J. Enzymatic Hydrolysis of Native Cellulose Nanofibrils and Other Cellulose Model Films: Effect of Surface Structure. Langmuir 2008, 24 (20), 11592–11599.

Alves, L.; Medronho, B.; Antunes, F. E.; Fernández-García, M. P.; Ventura, J.; Araújo, J. P.; Romano, A.; Lindman, B. Unusual Extraction and Characterization of Nanocrystalline Cellulose from Cellulose Derivatives. J. Mol. Liq. 2015, 210, 106–112.

Anderson, S. R.; Esposito, D.; Gillette, W.; Zhu, J. Y.; Baxa, U.; McNeil, S. E. Enzymatic Preparation of Nanocrystalline and Microcrystalline Cellulose. Tappi J. 2014, 13 (5), 35–42.

Andrade, M. F.; Colodette, J. L. Dissolving Pulp Production from Sugar Cane Bagasse. Ind. Crops Prod. 2014, 52, 58–64.

Aracri, E.; Vidal, T. Xylanase- and Laccase-Aided Hexenuronic Acids and Lignin Removal from Specialty Sisal Fibres. Carbohydr. Polym. 2011, 83 (3), 1355–1362.

Aracri, E.; Vidal, T. Enhancing the Effectiveness of a Laccase-TEMPO Treatment Has a Biorefining Effect on Sisal Cellulose Fibres. Cellulose 2012, 19 (3), 867–877.

Araki, J.; Wada, M.; Kuga, S.; Okano, T. Birefringent Glassy Phase of a Cellulose Microcrystal Suspension. Langmuir 2000, 16 (6), 2413–2415.

Atalla, R. H.; VanderHart, D. L. Native Cellulose: A Composite of Two Distinct Crystalline Forms. Science (80-.). 1984, 223, 283–285.

Bajpai, P.; Bajpai, P. K. Development of a Process for the Production of Dissolving Kraft Pulp Using Xylanase Enzyme. Appita J. 2001, 54 (4), 381–384.

Barlow, F.; Hillman, D. Do Dissolving Pulps Really Dissolve? Pap. Asia 2006, 22 (7), 12–18.

Barneto, A. G.; Vila, C.; Ariza, J.; Vidal, T. Thermogravimetric Measurement of Amorphous Cellulose Content in Flax Fibre and Flax Pulp. Cellulose 2010, 18 (1), 17–31.

Batalha, L.; Colodette, J. L.; Gomide, J. L.; Barbosa, Luiz, C. A.; Maltha, C. R. A.; Borges Gomes, F. J. Dissolving Pulp Production from Bamboo. BioResources 2011, 7 (1), 640–651.

Beck, S.; Bouchard, J.; Berry, R. Controlling the Reflection Wavelength of Iridescent Solid Films of Nanocrystalline Cellulose. Biomacromolecules 2011, 12 (1), 167–172.

Beck, S.; Me, M.; Bouchard, J. General Procedure for Determining Cellulose Nanocrystal Sulfate Half-Ester Content by Conductometric Titration. 2015, 101–116.

Beck-Candanedo, S.; Roman, M.; Gray, D. G. Effect of Reaction Conditions on the Properties and Behavior of Wood Cellulose Nanocrystal Suspensions. Biomacromolecules 2005, 6 (2), 1048–1054.

Beg, Q. K.; Kapoor, M.; Mahajan, L.; Hoondal, G. S. Microbial Xylanases and Their Industrial Applications: A Review. Appl. Microbiol. Biotechnol. 2001, 56 (3–4), 326–338.

Boluk, Y.; Lahiji, R.; Zhao, L.; McDermott, M. T. Suspension Viscosities and Shape Parameter of Cellulose Nanocrystals (CNC). Colloids Surfaces A Physicochem. Eng. Asp. 2011, 377 (1–3), 297–303.

Bondeson, D.; Mathew, A.; Oksman, K. Optimization of the Isolation of Nanocrystals from Microcrystalline Cellulose by Acid Hydrolysis. Cellulose 2006, 13 (2), 171–180.

Bouchard, J.; Morelli, E.; Berry, R. M. Gas-Phase Addition of Solvent to Ozone Bleaching of Kraft Pulp. J. Pulp Pap. Sci. 2000, 26 (1), 30–35.

Bouchard, J.; Méthot, M.; Jordan, B. The Effects of Ionizing Radiation on the Cellulose of Woodfree Paper. Cellulose 2006, 13 (5), 601–610.

Bradford, M. M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 1976, 72, 248–254.

Brinchi, L.; Cotana, F.; Fortunati, E.; Kenny, J. M. Production of Nanocrystalline Cellulose from Lignocellulosic Biomass: Technology and Applications. Carbohydr. Polym. 2013, 94 (1), 154–169.

Brito, B. S. L.; Pereira, F. V.; Putaux, J.-L.; Jean, B. Preparation, Morphology and Structure of Cellulose Nanocrystals from Bamboo Fibers. Cellulose 2012, 19 (5), 1527–1536.

Brogniart, A.; Pelonze, A. B.; Dumas, R. Rapport Sur Un Mémoire de M. Payen, Relatif À La Composition de La Matière Ligneuse. Comptes Rendus 1839, 8, 51–53.

Cadena, E. M.; Garcia, J.; Vidal, T.; Torres, A. L. Determination of Zeta Potential and Cationic Demand in ECF and TCF Bleached Pulp from Eucalyptus and Flax. Influence of Measuring Conditions. Cellulose 2009, 16 (3), 491–500.

Cadena, E. M.; Vidal, T.; Torres, A. L. Influence of the Hexenuronic Acid Content on Refining and Ageing in Eucalyptus TCF Pulp. Bioresour. Technol. 2010a, 101 (10), 3554–3560.

Cadena, E. M.; Chriac, a I.; Pastor, F. I. J.; Diaz, P.; Vidal, T.; Torres, A. L. Use of Cellulases and Recombinant Cellulose Binding Domains for Refining TCF Kraft Pulp. Biotechnol. Prog. 2010b, 26 (4), 960–967.

Cao, X.; Chen, Y.; Chang, P. R.; Muir, a. D.; Falk, G. Starch-Based Nanocomposites Reinforced with Flax Cellulose Nanocrystals. Express Polym. Lett. 2008, 2 (7), 502–510.

Chai, X.-S.; Zhu, J. Y.; Li, J. A Simple and Rapid Method to Determine Hexeneuronic Acid Groups in Chemical Pulps. J. Pulp Pap. Sci. 2001, 27 (5), 165–170.

Charreau, H.; Foresti, M. L.; Vázquez, A.; Vazquez, A.; Vázquez, A. Nanocellulose Patents Trends: A Comprehensive Review on Patents on Cellulose Nanocrystals, Microfibrillated and Bacterial Cellulose. Recent Pat. Nanotechnol. 2013, 7 (1), 56–80.

Chen, L.; Wang, Q.; Hirth, K.; Baez, C.; Agarwal, U. P.; Zhu, J. Y. Tailoring the Yield and Characteristics of Wood Cellulose Nanocrystals (CNC) Using Concentrated Acid Hydrolysis. Cellulose 2015, 22 (3), 1753–1762.

Chen, X.; Deng, X.; Shen, W.; Jiang, L. Controlled Enzymolysis Preparation of Nanocrystalline Cellulose from Pretreated Cotton Fibers. BioResources 2012, 7 (3), 4237–4248.

Costa, M. M.; Colodette, J. L. The Impact of Kappa Number Composition on Eucalyptus Kraft Pulp Bleachability. Brazilian J. Chem. Eng. 2007, 24 (1), 61–71.

Council, R. cooperation. Nanotechnology Initiative USA-Canada Work Element 2 , Priority Setting : Development of a Joint Nanomaterials Classification Scheme; 2013.

Cusola, O.; Valls, C.; Vidal, T.; Roncero, M. B. Application of Surface Enzyme Treatments Using Laccase and a Hydrophobic Compound to Paper-Based Media. Bioresour. Technol. 2013, 131, 521–526.

Dammström, S.; Salmén, L.; Gatenholm, P. On the Interactions between Cellulose and Xylan, a Biomimetic Simulation of the Hardwood Cell Wall. BioResources 2009, 4 (1), 3–14.

Dashtban, M.; Maki, M.; Leung, K. T.; Mao, C.; Qin, W. Cellulase Activities in Biomass Conversion: Measurement Methods and Comparison. Crit. Rev. Biotechnol. 2010, 30 (4), 302–309.

Dobón-Lopez, A. NanosafePACK. Development of a Best Practices Guide for the Safe Handling and Use of Nanoparticles in Packaging Industries D4 . 1 Analysis of the Consideration of Physic-Chemical and Toxicological Endpoints in LCA Studies; 2013.

Dong, X. M.; Kimura, T.; Gray, D. G. Effects of Ionic Strength on the Isotropic - Chiral Nematic Phase Transition of Suspensions of Cellulose Crystallites. Langmuir 1996, 12 (8), 2076–2082.

Dong, X. M.; Revol, J.-F.; Gray, D. G. Effect of Microcrystallite Preparation Conditions on the Formation of Colloid Crystals of Cellulose. Cellulose 1998, 5 (1), 19–32.

Drexler, E.; Peterson, C.; Pergamit, G.; Brand, S. Unbounding the Future: The Nanotechnology Revolution; 1993; Vol. 15.

Dugan, J. M.; Gough, J. E.; Eichhorn, S. J. Bacterial Cellulose Scaffolds and Cellulose Nanowhiskers for Tissue Engineering. Nanomedicine 2013, 8 (2), 287–298.

Elazzouzi-Hafraoui, S.; Nishiyama, Y.; Putaux, J. L.; Heux, L.; Dubreuil, F.; Rochas, C. The Shape and Size Distribution of Crystalline Nanoparticles Prepared by Acid Hydrolysis of Native Cellulose. Biomacromolecules 2008, 9 (1), 57–65.

Executive Office of the President of the United States. NSTC, The National Nanotechnology Initiative – Strategic Plan, December 2007; 2007.

Fahma, F.; Iwamoto, S.; Hori, N.; Iwata, T.; Takemura, A. Isolation, Preparation, and Characterization of Nanofibers from Oil Palm Empty-Fruit-Bunch (OPEFB). Cellulose 2010, 17, 977–985.

Fan, J. S.; Li, Y. H. Maximizing the Yield of Nanocrystalline Cellulose from Cotton Pulp Fiber. Carbohydr. Polym. 2012, 88 (4), 1184–1188.

FAO. FAOstat Reports: Jute, Kenaf, Sisal, Abaca, Coir and Allied Fibers.; 2012.

Ferrer, A.; Quintana, E.; Filpponen, I.; Solala, I.; Vidal, T.; Rodríguez, A.; Laine, J.; Rojas, O. J. Effect of Residual Lignin and Heteropolysaccharides in Nanofibrillar Cellulose and Nanopaper from Wood Fibers. Cellulose 2012, 19 (6), 2179–2193.

Feynman, R. P. There's Plenty of Room at the Bottom. Eng. Sci. 1960, 23 (5), 22–36.

Fillat, A.; Colom, J. F.; Vidal, T. A New Approach to the Biobleaching of Flax Pulp with Laccase Using Natural Mediators. Bioresour. Technol. 2010, 101 (11), 4104–4110.

Fillat, A.; Roncero, M. B.; Vidal, T. Assessing the Use of Xylanase and Laccases in Biobleaching Stages of a TCF Sequence for Flax Pulp. J. Chem. Technol. Biotechnol. 2011, 86 (12), 1501–1507.

Fillat, A.; Roncero, M. B.; Vidal, T. Elucidating the Effects of Laccase-Modifying Compounds Treatments on Bast and Core Fibers in Flax Pulp. Biotechnol. Bioeng. 2012, 109 (1), 225–233.

Fillat, U.; Roncero, M. B. Effect of Process Parameters in Laccase-Mediator System Delignification of Flax Pulp. Part I: Pulp Properties. Chem. Eng. J. 2009, 152 (2–3), 322–329.

Filson, P. B.; Dawson-Andoh, B. E. Characterization of Sugars from Model and Enzyme-Mediated Pulp Hydrolyzates Using High-Performance Liquid Chromatography Coupled to Evaporative Light Scattering Detection. Bioresour. Technol. 2009, 100 (24), 6661–6664.

Filson, P. B.; Dawson-Andoh, B. E.; Schwegler-Berry, D. Enzymatic-Mediated Production of Cellulose Nanocrystals from Recycled Pulp. Green Chem. 2009, 11, 1808–1814.

Flauzino Neto, W. P.; Silvério, H. A.; Dantas, N. O.; Pasquini, D. Extraction and Characterization of Cellulose Nanocrystals from Agro-Industrial Residue - Soy Hulls. Ind. Crops Prod. 2013, 42, 480–488.

Fock, W. Eine Modifizierte Methode Zur Bestimmung Der Reaktivität von Zellstoffen Für Die Viskoseherstellung. Das Pap. 1959, 13 (3), 92–95.

Fortunati, E.; Puglia, D.; Monti, M.; Peponi, L.; Santulli, C.; Kenny, J. M.; Torre, L. Extraction of Cellulose Nanocrystals from Phormium Tenax Fibres. J. Polym. Environ. 2013, 21, 319–328.

Fraschini, C.; Chauve, G.; Berre, J.-F. Le; Ellis, S.; Méthot, M.; Connor, B. O. Critical Discussion of Light Scattering and Microscopy Techniques for CNC Particle Sizing. Nord. Pulp Pap. Res. J. 2014, 29 (1), 31–40.

French, A. D.; Santiago Cintrón, M. Cellulose Polymorphy, Crystallite Size, and the Segal Crystallinity Index. Cellulose 2013, 20 (1), 583–588.

García-Hortal, J. A. Fibras Papeleras; UPC, E., Ed.; 2007.

Garcia-Ubasart, J.; Torres, A. L.; Vila, C.; Pastor, F. I. J.; Vidal, T. Biomodification of Cellulose Flax Fibers by a New Cellulase. Ind. Crops Prod. 2013, 44, 71–76.

Garrote, G.; Domínguez, H.; Parajó, J. C. Kinetic Modelling of Corncob Autohydrolysis. Process Biochem. 2001, 36 (6), 571–578.

Gellerstedt, G.; Li, J. An HPLC Method for the Quantitative Determination of Hexeneuronic Acid Groups in Chemical Pulps. Carbohydr. Res. 1996, 294, 41–51.

Genung, L. Analysis of Cellulose Derivatives. Anal. Chem. 1950, 14 (12), 935–940.

George, J.; Ramana, K. V.; Bawa, a. S.; Siddaramaiah. Bacterial Cellulose Nanocrystals Exhibiting High Thermal Stability and Their Polymer Nanocomposites. Int. J. Biol. Macromol. 2011, 48 (1), 50–57.

Gleiter, H. Nanostructured Materials: Basic Concepts and Microstructure. Acta Mater. 2000, 48 (1), 1–29.

Gu, J.; Catchmark, J. M.; Kaiser, E. Q.; Archibald, D. D. Quantification of Cellulose Nanowhiskers Sulfate Esterification Levels. Carbohydr. Polym. 2013, 92 (2), 1809–1816.

Gümüskaya, E.; Usta, M.; Kirci, H. The Effects of Various Pulping Conditions on Crystalline Structure of Cellulose in Cotton Linters. Polym. Degrad. Stab. 2003, 81 (3), 559–564.

Habibi, Y. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Habibi, Y. Key Advances in the Chemical Modification of Nanocelluloses. Chem. Soc. Rev. 2014, 43 (5), 1519–1542.

Habibi, Y.; Lucia, L. A.; Rojas, O. J. Cellulose Nanocrystals: Chemistry, Self-Assembly, and Applications. Chem. Rev. 2010, 110 (6), 3479–3500.

Hägglund, R.; Gradin, P. A.; Tarakameh, D. Some Aspects on the Zero-Span Tensile Test. Exp. Mech. 2004, 44 (4), 365–374.

Hakala, T. K.; Liitiä, T.; Suurnäkki, A. Enzyme-Aided Alkaline Extraction of Oligosaccharides and Polymeric Xylan from Hardwood Kraft Pulp. Carbohydr. Polym. 2012, 93 (1), 102–108.

Hamad, W. On the Development and Applications of Cellulosic Nanofibrillar and Nanocrystalline Materials. Can. J. Chem. Eng. 2006, 84 (5), 513–519.

Hart, P. W.; Santos, R. B. Changing the Face of Short Fiber - A Review of the Eucalyptus Revolution. Tappi J. 2015, 14 (6), 353–359.

Henriksson, G.; Christiernin, M.; Agnemo, R. Monocomponent Endoglucanase Treatment Increases the Reactivity of Softwood Sulphite Dissolving Pulp. J. Ind. Microbiol. Biotechnol. 2005, 32 (5), 211–214.

Henriksson, M.; Henriksson, G.; Berglund, L. A.; Lindström, T. An Environmentally Friendly Method for Enzyme-Assisted Preparation of Microfibrillated Cellulose (MFC) Nanofibers. Eur. Polym. J. 2007, 43 (8), 3434–3441.

Hirai, A.; Inui, O.; Horii, F.; Tsuji, M. Phase Separation Behavior in Aqueous Suspensions of Bacterial Cellulose Nanocrystals Prepared by Sulfuric Acid Treatment. Langmuir 2009, 25 (1), 497–502.

Hori, R.; Wada, M. The Thermal Expansion of Wood Cellulose Crystals. Cellulose 2005, 12 (5), 479–484.

Hornig, S.; Heinze, T. Efficient Approach to Design Stable Water-Dispersible Nanoparticles of Hydrophobic Cellulose Esters. Biomacromolecules 2008, 9 (5), 1487–1492.

Hubbe, M. A.; Rojas, O. J.; Lucia, L. A.; Sain, M. Cellulosic Nanocomposites: A Review. BioResources 2008, 3 (3), 929–980.

Hurter, R. W. TCF Bleached Sisal Market Pulp: Potential Reinforcing Fibre for Commodity Papers .1. Tappi Proc. 1997, 501–512.

Ibarra, D.; Kopcke, V.; Ek, M. Exploring Enzymatic Treatments for the Production of Dissolving Grade Pulp from Different Wood and Non-Wood Paper Grade Pulps. Holzforschung 2009, 63 (6), 721–730.

Ibarra, D.; Kopcke, V.; Larsson, P. T.; Jaaskelainen, A.-S.; Ek, M. Combination of Alkaline and Enzymatic Treatments as a Process for Upgrading Sisal Paper-Grade Pulp to Dissolving-Grade Pulp. Bioresour. Technol. 2010, 101 (19), 7416–7423.

Igarashi, K.; Uchihashi, T.; Koivula, A.; Wada, M.; Kimura, S.; Okamoto, T.; Penttilä, M.; Ando, T.; Samejima, M. Traffic Jams Reduce Hydrolytic Efficiency of Cellulase on Cellulose Surface. Science (80-.). 2011, 333 (6047), 1279–1282.

Jackson, L. S.; Heitmann Jr., J. A.; Joyce, T. W. Production of Dissolving Pulp from Recovered Paper Using Enzymes. Tappi J. 1998, 81 (3), 171–178.

Jeffries, T. W. Introduction of a Special Issue on Biotechnology for the Pulp and Paper Industry. Enzyme Microb. Technol. 2008, 43 (2), 77–77.

Jonoobi, M.; Oladi, R.; Davoudpour, Y.; Oksman, K.; Dufresne, A.; Hamzeh, Y.; Davoodi, R. Different Preparation Methods and Properties of Nanostructured Cellulose from Various Natural Resources and Residues: A Review. Cellulose 2015, No. 22, 935–969.

Kargarzadeh, H.; Ahmad, I.; Abdullah, I.; Dufresne, A.; Zainudin, S. Y.; Sheltami, R. M. Effects of Hydrolysis Conditions on the Morphology, Crystallinity, and Thermal Stability of Cellulose Nanocrystals Extracted from Kenaf Bast Fibers. Cellulose 2012, 19 (3), 855–866.

Kelly, J. a.; Yu, M.; Hamad, W. Y.; Maclachlan, M. J. Large, Crack-Free Freestanding Films with Chiral Nematic Structures. Adv. Opt. Mater. 2013, 1 (4), 295–299.

Kenealy, W. R.; Jeffries, T. W. Enzyme Processes for Pulp and Paper : A Review of Recent Developments. Society 2003, 845, 210–241.

Kissinger, M.; Fix, J.; Rees, W. E. Wood and Non-Wood Pulp Production: Comparative Ecological Footprinting on the Canadian Prairies. Ecol. Econ. 2007, 62 (3–4), 552–558.

Klemm, D.; Heublein, B.; Fink, H. P.; Bohn, A. Cellulose: Fascinating Biopolymer and Sustainable Raw Material. Angew. Chemie - Int. Ed. 2005, 44 (22), 3358–3393.

Klemm, D.; Kramer, F.; Moritz, S.; Lindström, T.; Ankerfors, M.; Gray, D.; Dorris, A. Nanocelluloses: A New Family of Nature-Based Materials. Angew. Chemie - Int. Ed. 2011, 50 (24), 5438–5466.

Kobayashi, S.; Sakamoto, J.; Kimura, S. In Vitro Synthesis of Cellulose and Related Polysaccharides. Prog. Polym. Sci. 2001, 26 (9), 1525–1560.

Kopcke, V.; Ibarra, D.; Ek, M. Increasing Accessibility and Reactivity of Paper Grade Pulp by Enzymatic Treatment for Use as Dissolving Pulp. Nord. Pulp Pap. Res. J. 2008, 23 (4), 363–368.

Kuhad, R. C.; Gupta, R.; Singh, A. Microbial Cellulases and Their Industrial Applications. Enzyme Res. 2011, 2011, 1–10.

Kvarnlöf, N.; Germgärd, U.; Jönson, L. J.; Carl-Axel, S. Optimization of the Enzymatic Activation of a Dissolving Pulp before Viscose Manufacture. Tappi J. 2007, 6 (6), 14–19.

Leponiemi, A. Non-Wood Pulping Possibilities - A Challenge for the Chemical Pulping Industry. Appita J. 2008, 61 (3), 234–243.

Li, J.; Gellerstedt, G. The Contribution to Kappa Number from Hexeneuronic Acid Groups in Pulp Xylan. Carbohydr. Res. 1997, 302 (3–4), 213–218.

Li, J.; Zhang, H.; Duan, C.; Liu, Y.; Ni, Y. Enhancing Hemicelluloses Removal from a Softwood Sulfite Pulp. Bioresour. Technol. 2015, 192, 11–16.

Lin, N.; Dufresne, A. Nanocellulose in Biomedicine: Current Status and Future Prospect. Eur. Polym. J. 2014, 59, 302–325.

Lin, N.; Huang, J.; Dufresne, A. Preparation, Properties and Applications of Polysaccharide Nanocrystals in Advanced Functional Nanomaterials: A Review. Nanoscale 2012, 4 (11), 3274–3294.

Liu, Y.; Wang, H.; Yu, G.; Yu, Q.; Li, B.; Mu, X. A Novel Approach for the Preparation of Nanocrystalline Cellulose by Using Phosphotungstic Acid. Carbohydr. Polym. 2014, 110, 415–422.

López, F.; Alaejos, J.; Díaz, M. J.; García, M. M.; Alfaro, A. Vegetales No Madereros Para La Fabricación de Pasta Celulósica. Revisión de Su Utilización Actual. Ing. Química 2004, 36, 141–144.

Lu, P.; Hsieh, Y.-L. Preparation and Properties of Cellulose Nanocrystals: Rods, Spheres, and Network. Carbohydr. Polym. 2010, 82 (2), 329–336.

Lu, Z.; Fan, L.; Zheng, H.; Lu, Q.; Liao, Y.; Huang, B. Preparation, Characterization and Optimization of Nanocellulose Whiskers by Simultaneously Ultrasonic Wave and Microwave Assisted. Bioresour. Technol. 2013, 146, 82–88.

Maache-Rezzoug, Z.; Pierre, G.; Nouviaire, A.; Maugard, T.; Rezzoug, S. A. Optimizing Thermomechanical Pretreatment Conditions to Enhance Enzymatic Hydrolysis of Wheat Straw by Response Surface Methodology. Biomass and Bioenergy 2011, 35 (7), 3129–3138.

Madakadze, I. C.; Masamvu, T. M.; Radiotis, T.; Li, J.; Smith, D. L. Evaluation of Pulp and Paper Making Characteristics of Elephant Grass (Pennisetum Purpureum Schum) and Switchgrass (Panicum Virgatum L.). African J. Environ. Sci. Technol. 2010, 4 (July), 465–470.

Maddern, K. N.; French, J. The Potential Application of Nonwood Fibers in Papermaking - an Australian Perspective. Appita J. 1995, 48 (3), 191–196.

Majoinen, J.; Kontturi, E.; Ikkala, O.; Gray, D. G. SEM Imaging of Chiral Nematic Films Cast from Cellulose Nanocrystal Suspensions. Cellulose 2012, 19 (5), 1599–1605.

Maki, M.; Leung, K. T.; Qin, W. The Prospects of Cellulase-Producing Bacteria for the Bioconversion of Lignocellulosic Biomass. Int. J. Biol. Sci. 2009, 5 (5), 500–516.

Marchessault, R.; Morehead, F.; Walter, N. Liquid Crystal Systems from Fibrillar Polysaccharides. Nature 1959, 184, 632–633.

Mariano, M.; El Kissi, N.; Dufresne, A. Cellulose Nanocrystals and Related Nanocomposites: Review of Some Properties and Challenges. J. Polym. Sci. Part B Polym. Phys. 2014, 52 (12), 791–806.

Marques, G.; Rencoret, J.; Gutierrez, A.; del Jose C., R. Evaluation of the Chemical Composition of Different Non-Woody Plant Fibers Used for Pulp and Paper Manufacturing. Open Agric. J. 2010, 4, 93–101.

Martínez-Sanz, M.; Vicente, A. A.; Gontard, N.; Lopez-Rubio, A.; Lagaron, J. M. On the Extraction of Cellulose Nanowhiskers from Food by-Products and Their Comparative Reinforcing Effect on a Polyhydroxybutyrate-Co-Valerate Polymer. Cellulose 2015, 22 (1), 535–551.

Miao, Q.; Chen, L.; Huang, L.; Tian, C.; Zheng, L.; Ni, Y. A Process for Enhancing the Accessibility and Reactivity of Hardwood Kraft-Based Dissolving Pulp for Viscose Rayon Production by Cellulase Treatment. Bioresour. Technol. 2014, 154, 109–113.

Moon, R. J.; Martini, A.; Nairn, J.; Simonsen, J.; Youngblood, J. Cellulose Nanomaterials Review: Structure, Properties and Nanocomposites. Chem. Soc. Rev. 2011, 40 (7), 3941–3994.

Morais, J. P. S.; Rosa, M. D. F.; de Souza Filho, M. D. S. M.; Nascimento, L. D.; do Nascimento, D. M.; Cassales, A. R. Extraction and Characterization of Nanocellulose Structures from Raw Cotton Linter. Carbohydr. Polym. 2013, 91 (1), 229–235.

Mukherjee, S. M.; Woods, H. J. X-Ray and Electron Microscope Studies of the Degradation of Cellulose by Sulphuric Acid. Biochim. Biophys. Acta 1953, 10, 499–511.

Nelson, M. L.; O'Connor, R. T. Relation of Certain Infrared Bands to Cellulose Crystallinity and Crystal Lattice Type. Part II. A New Infrared Ratio for Estimation of Crystallinity in Celluloses I and II. J. Appl. Polym. Sci. 1964, 8 (3), 1325–1341.

Nguyen, L. T.; Neo, K. R. S.; Yang, K.-L. Continuous Hydrolysis of Carboxymethyl Cellulose with Cellulase Aggregates Trapped inside Membranes. Enzyme Microb. Technol. 2015, 78, 34–39.

Nickerson, R.; Habrle, J. Cellulose Intercrystalline Structure. Ind. Eng. Chem. 1947, 1507–1512.

O'Connor, R. T.; DuPré, E. F.; Mitcham, D. Applications of Infrared Absorption Spectroscopy to Investigations of Cotton and Modified Cottons Part I: Physical and Crystalline Modifications and Oxidation. Text. Res. J. 1958, 28 (5), 382–392.

Pääkko, M.; Ankerfors, M.; Kosonen, H.; Nykänen, a.; Ahola, S.; Österberg, M.; Ruokolainen, J.; Laine, J.; Larsson, P. T.; Ikkala, O.; et al. Enzymatic Hydrolysis Combined with Mechanical Shearing and High-Pressure Homogenization for Nanoscale Cellulose Fibrils and Strong Gels. Biomacromolecules 2007, 8 (6), 1934–1941.

Paavilainen, L. European Prospects for Using Nonwood Fibers. Pulp Pap. Int. 1998, No. 40, 61–66.

Payen, A. Mémoire Sur La Composition Du Tissu Propre Des Plantes et Du Ligneux. Comptes Rendus 1838, 7, 1052–1056.

Pepió, M.; Polo, C. Apunts de L'assignatura de Tercer cicle: "Disseny D'experiments I Optimització de Processos." Dep. d'estadistica i Investig. Oper. Univ. Politècnica Catalunya 2000.

Peyre, J.; Pääkkönen, T.; Reza, M.; Kontturi, E. Simultaneous Preparation of Cellulose Nanocrystals and Micron-Sized Porous Colloidal Particles of Cellulose by TEMPO-Mediated Oxidation. Green Chem. 2015, 17 (2), 808–811.

Philippidis, G. P.; Smith, T. K.; Wyman, C. E. Study of the Enzymatic Hydrolysis of Cellulose for Production of Fuel Ethanol by the Simultaneous Saccharification and Fermentation Process. Biotechnol. Bioeng. 1993, 41 (9), 846–853.

Pierre, G.; Sannier, F.; Goude, R.; Nouviaire, A.; Maache-Rezzoug, Z.; Rezzoug, S. A.; Maugard, T. Evaluation of Thermomechanical Pretreatment for Enzymatic Hydrolysis of Pure Microcrystalline Cellulose and Cellulose from Brewers' Spent Grain. J. Cereal Sci. 2011a, 54 (3), 305–310.

Pierre, G.; Maache-Rezzoug, Z.; Sannier, F.; Rezzoug, S. A.; Maugard, T. High-Performance Hydrolysis of Wheat Straw Using Cellulase and Thermomechanical Pretreatment. Process Biochem. 2011b, 46 (11), 2194–2200.

Pihlajaniemi, V.; Sipponen, S.; Sipponen, M. H.; Pastinen, O.; Laakso, S. Enzymatic Saccharification of Pretreated Wheat Straw: Comparison of Solids-Recycling, Sequential Hydrolysis and Batch Hydrolysis. Bioresour. Technol. 2014, 153, 15–22.

Postek, M. T.; Vladár, A.; Dagata, J.; Farkas, N.; Ming, B.; Wagner, R.; Raman, A.; Moon, R. J.; Sabo, R.; Wegner, T. H.; et al. Development of the Metrology and Imaging of Cellulose Nanocrystals. Meas. Sci. Technol. 2011, 22 (2), 1–10.

Potocnik, J. Commission Recommendation of October 2011 on the Definition of Nanomaterial. Of. J. Eur. Union 2011, L275, 38–40.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. An Enzyme-Catalysed Bleaching Treatment to Meet Dissolving Pulp Characteristics for Cellulose Derivatives Applications. Bioresour. Technol. 2013, 148, 1–8.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part I: On a Fully Bleached, Commercial Acid Sulfite Dissolving Pulp. Cellulose 2015a, 2067–2079.

Quintana, E.; Valls, C.; Vidal, T.; Roncero, M. B. Comparative Evaluation of the Action of Two Different Endoglucanases. Part II: On a Biobleached Acid Sulphite Pulp. Cellulose 2015b, 22 (3), 2081–2093.

Quintana, E.; Valls, C.; Barneto, A. G.; Vidal, T.; Ariza, J.; Roncero, M. B. Studying the Effects of Laccase Treatment in a Softwood Dissolving Pulp : Cellulose Reactivity and Crystallinity. Carbohydr. Polym. 2015c, 119, 53–61.

Rånby, B. G. III. Fibrous Macromolecular Systems. Cellulose and Muscle. The Colloidal Properties of Cellulose Micelles. Discuss. Faraday Soc. 1951, 11, 158–164.

Revol, J. F.; Bradford, H.; Giasson, J.; Marchessault, R. H.; Gray, D. G. Helicoidal Self-Ordering of Cellulose Microfibrils in Aqueous Suspension. Int. J. Biol. Macromol. 1992, 14 (3), 170–172.

Roman, M.; Winter, W. T. Effect of Sulfate Groups from Sulfuric Acid Hydrolysis on the Thermal Degradation Behavior of Bacterial Cellulose. Biomacromolecules 2004, 5 (5), 1671–1677.

Roncero, M. B.; Torres, A. L.; Colom, J. F.; Vidal, T. Effects of Xylanase Treatment on Fibre Morphology in Totally Chlorine Free Bleaching (TCF) of Eucalyptus Pulp. Process Biochem. 2000, 36 (1–2), 45–50.

Roncero, M. B.; Colom, J. F.; Vidal, T. Influence of the Xylanase Enzymatic Treatments on the Carbohydrate Composition of Pulp for Paper Manufacture. Afinidad 2003, 60 (503), 8–15.

Sakurada, I.; Nubushina, Y.; Nukushina, Y.; Ito, T. Experimental Determination of the Elastic Modulus of Crystalline Regions in Oriented Polymers. J. Polym. Sci. 1962, 57, 651–660.

Satyamurthy, P.; Jain, P.; Rudrapatna, H. B.; Vigneshwaran, N. Preparation and Characterization of Cellulose Nanowhiskers from Cotton Fibres by Controlled Microbial Hydrolysis. Carbohydr. Polym. 2011, 83 (1), 122–129.

Saxena, A.; Ragauskas, A. J. Water Transmission Barrier Properties of Biodegradable Films Based on Cellulosic Whiskers and Xylan. Carbohydr. Polym. 2009, 78 (2), 357–360.

Sczostak, A. Cotton Linters: An Alternative Cellulosic Raw Material. Macromol. Symp. 2009, 280 (1), 45–53.

Shaw, D. J. Introduction to Colloid and Surface Chemistry (4th Edition). 1993, 26 (3), 7506.

Široký, J.; Blackburn, R. S.; Bechtold, T.; Taylor, J.; White, P. Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy Analysis of Crystallinity Changes in Lyocell Following Continuous Treatment with Sodium Hydroxide. Cellulose 2010, 17, 103–115.

Sjöström, E.; Westermark, U. Chemical Composition of Wood and Pulps: Basic Constituents and Their Distribution. In Analytical Methods in Wood Chemistry, Pulping, and Papermaking; Springer Berlin Heidelberg, 1999; pp 68–82.

Spiridon, I.; Teaca, C.-A.; Bodîrlau, R. Structural Changes Evidenced By Ftir Pre-Treatment With Ionic Liquid and Enzymatic. BioResources 2010, 6 (1), 400–413.

Spiro, R. G. R. Analysis of Sugars Found in Glycoproteins. Methods Enzymol. 1966, 566 (C), 3–26.

Staudinger, H. Über Polymerisation. Berichte der Dtsch. Chem. Gesellschaft 1920, 53 (6), 1073–1085.

Stone, V.; Nowack, B.; Baun, A.; van den Brink, N.; von der Kammer, F.; Dusinska, M.; Handy, R.; Hankin, S.; Hassellov, M.; Joner, E.; et al. Nanomaterials for Environmental Studies: Classification, Reference Material Issues, and Strategies for Physico-Chemical Characterisation. Sci. Total Environ. 2010, 408 (7), 1745–1754.

Suchy, M.; Hakala, T.; Kangas, H.; Kontturi, E.; Tammelin, T.; Pursula, T.; Vuorinen, T. Effects of Commercial Cellobiohydrolase Treatment on Fiber Strength and Morphology of Bleached Hardwood Pulp. Holzforschung 2009, 63 (6), 731–736.

Sun, Q.; Mandalika, A.; Elder, T.; Nair, S. S.; Meng, X.; Huang, F.; Ragauskas, A. J. Nanocomposite Film Prepared by Depositing Xylan on Cellulose Nanowhiskers Matrix. Green Chem. 2014, 16 (7), 3458–3462.

Swedish EU Presidency. Lund Declaration, “Europe Must Focus on the Grand Challenges of Our Time”; 2009.

Tanaka, R.; Saito, T.; Ishii, D.; Isogai, A. Determination of Nanocellulose Fibril Length by Shear Viscosity Measurement. Cellulose 2014, 21, 1581–1589.

Tang, L.; Huang, B.; Lu, Q.; Wang, S.; Ou, W.; Lin, W.; Chen, X. Ultrasonication-Assisted Manufacture of Cellulose Nanocrystals Esterified with Acetic Acid. Bioresour. Technol. 2013, 127, 100–105.

Tang, L. R.; Huang, B.; Ou, W.; Chen, X. R.; Chen, Y. D. Manufacture of Cellulose Nanocrystals by Cation Exchange Resin-Catalyzed Hydrolysis of Cellulose. Bioresour. Technol. 2011, 102 (23), 10973–10977.

Tąta, A.; Sokołowska, K.; Świder, J.; Konieczna-Molenda, A.; Proniewicz, E.; Witek, E. Study of Cellulolytic Enzyme Immobilization on Copolymers of N-Vinylformamide. Spectrochim. Acta Part A Mol. Biomol. Spectrosc. 2015, 149, 494–504.

Teixeira, E. D. M.; Corrêa, A. C.; Manzoli, A.; de Lima Leite, F.; de Ribeiro Oliveira, C.; Mattoso, L. H. C.; Teixeira, E. D. M.; Corre, A. C.; Leite, F. D. L.; Henrique, L.; et al. Cellulose Nanofibers from White and Naturally Colored Cotton Fibers. Cellulose 2010, 17 (3), 595–606.

Teixeira, R. S. S.; Silva, A. S. Da; Jang, J.-H.; Kim, H.-W.; Ishikawa, K.; Endo, T.; Lee, S.-H.; Bon, E. P. S. Combining Biomass Wet Disk Milling and Endoglucanase/β-Glucosidase Hydrolysis for the Production of Cellulose Nanocrystals. Carbohydr. Polym. 2015, 128, 75–81.

Thielemans, W.; Warbey, C. R.; Walsh, D. a. Permselective Nanostructured Membranes Based on Cellulose Nanowhiskers. Green Chem. 2009, 11 (4), 531–537.

Tsuzuki, T. Commercial Scale Production of Inorganic Nanoparticles. Int. J. Nanotechnol. 2009, 6 (5), 567–578.

Turbak, A. F.; Snyder, F. W.; Sandberg, K. R. Microfibrillated Cellulose, a New Cellulose Product: Properties, Uses, and Commercial Potential. J. Appl. Polym. Sci. Symp. 1983, 37, 815–837.

Valls, C.; Roncero, M. B. Using Both Xylanase and Laccase Enzymes for Pulp Bleaching. Bioresour. Technol. 2009, 100 (6), 2032–2039.

Valls, C.; Molina, S.; Vidal, T.; del Río, J. C.; Colom, J. F.; Martínez, Á. T.; Gutiérrez, A.; Roncero, M. B. Influence of Operation Conditions on Laccase-Mediator Removal of Sterols from Eucalypt Pulp. Process Biochem. 2009, 44 (9), 1032–1038.

Valls, C.; Colom, J. F.; Baffert, C.; Gimbert, I.; Roncero, M. B.; Sigoillot, J.-C. Comparing the Efficiency of the laccase–NHA and laccase–HBT Systems in Eucalyptus Pulp Bleaching. Biochem. Eng. J. 2010a, 49 (3), 401–407.

Valls, C.; Gallardo, O.; Vidal, T.; Ignacio, F.; Pastor, J.; Díaz, P.; Roncero, M. B.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B.; et al. New Xylanases to Obtain Modified Eucalypt Fibres with High-Cellulose Content. Bioresour. Technol. 2010b, 101 (19), 7439–7445.

Valls, C.; Gallardo, Ó.; Vidal, T.; Pastor, F. I. J.; Díaz, P.; Roncero, M. B. Performance of New and Commercial Xylanases for ECF and TCF Bleaching of Eucalyptus Kraft Pulp. Wood Sci. Technol. 2010c, 45 (3), 433–448.

Valls, C.; Vidal, T.; Roncero, M. B. Enzymatic Strategies to Improve Removal of Hexenuronic Acids and Lignin from Cellulosic Fibers. Holzforschung 2014, 68 (2), 229–237.

Ververis, C.; Georghiou, K.; Christodoulakis, N.; Santas, P.; Santas, R. Fiber Dimensions, Lignin and Cellulose Content of Various Plant Materials and Their Suitability for Paper Production. Ind. Crops Prod. 2004, 19, 245–254.

Viikari, L.; Ranua, M.; Kantelinen, A.; Sundquist, J.; Linko, M. Bleaching with Enzymes. In 3rd International conference on Biotechnology in the pulp and paper industry. A challenge for change. Proceedings; 1986; pp 67–69.

Wang, H.; Pang, B.; Wu, K.; Kong, F.; Li, B.; Mu, X. Two Stages of Treatments for Upgrading Bleached Softwood Paper Grade Pulp to Dissolving Pulp for Viscose Production. Biochem. Eng. J. 2014, 82, 183–187.

Wang, Q. Q.; Zhu, J. Y.; Reiner, R. S.; Verrill, S. P.; Baxa, U.; McNeil, S. E. Approaching Zero Cellulose Loss in Cellulose Nanocrystal (CNC) Production: Recovery and Characterization of Cellulosic Solid Residues (CSR) and CNC. Cellulose 2012, 19 (6), 2033–2047.

Xu, Q.; Gao, Y.; Qin, M.; Wu, K.; Fu, Y.; Zhao, J. Nanocrystalline Cellulose from Aspen Kraft Pulp and Its Application in Deinked Pulp. Int. J. Biol. Macromol. 2013, 60, 241–247.

Yanamala, N.; Farcas, M. In Vivo Evaluation of the Pulmonary Toxicity of Cellulose Nanocrystals: A Renewable and Sustainable Nanomaterial of the Future. ACS Sustain. Chem. Eng. 2014, 2, 1691–1698.

Yoon, S. Y.; Han, S. H.; Shin, S. J. The Effect of Hemicelluloses and Lignin on Acid Hydrolysis of Cellulose. Energy 2014, 77, 1–6.

Yoshiharu, N.; Shigenori, K.; Masahisa, W.; Takeshi, O. Cellulose Microcrystal Film of High Uniaxial Orientation. Macromolecules 1997, 30 (20), 6395–6397.

Zhang, J.; Tang, M.; Viikari, L. Xylans Inhibit Enzymatic Hydrolysis of Lignocellulosic Materials by Cellulases. Bioresour. Technol. 2012a, 121, 8–12.

Zhang, Y.; Xue, G. X.; Zhang, X. M.; Zhao, Y. Enzymatic Preparation of Nanocrystalline Cellulose from Bamboo Fibers. Adv. Mater. Res. 2012b, 441, 754–758.

Zhu, J. Y.; Zhou, H. F.; Chai, X. S.; Johannes, D.; Pope, R.; Valls, C.; Roncero, M. B. Inter-Laboratory Comparisons of Hexenuronic Acid Measurements in Kraft Eucalyptus Pulps Using a UV-Vis Spectroscopic Method. Tappi J. 2014, 13 (1), 57–61.

Zhu, L.; O'Dwyer, J. P.; Chang, V. S.; Granda, C. B.; Holtzapple, M. T. Structural Features Affecting Biomass Enzymatic Digestibility. Bioresour. Technol. 2008, 99 (9), 3817–3828.