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Enzymatic and chemical treatments to obtain pulps with high-cellulose content

Doctoral Dissertation

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CERTIFIQUEN:

Que **Elisabet Quintana Vilajuana**, Enginyera Tècnica en Química Industrial i Màster en Enginyeria Tèxtil, Paperera i Gràfica, ha realitzat sota la seva direcció el treball d'investigació titulat **"Enzymatic and chemical treatments to obtain pulps with high-cellulose content"** que presenta per optar al títol de Doctor.

I perquè així consti expedeix el present certificat a Terrassa, 25 de Juliol de 2016.

Cristina Valls Vidal
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Als meus pares,

a la meva germana Anna,

al Jordi

Agraïments

La present tesi s'ha dut a terme en el grup de recerca Celbiotech, del Departament d'Enginyeria Textil i Paperera, de l'Escola Tècnica Superior d'Enginyeries Industrial i Aeronàutica de Terrassa, de la Universitat Politècnica de Catalunya (UPC-BarcelonaTech). El treball ha estat finançat per els següents projectes nacionals:

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Moltes són les persones que han ajudat i col·laborat en aquesta tesi, i a les quals vull expressar el meu agraïment:

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Abstract

Cellulose is the most abundant polymer in the Earth and recently has gained wide interest as a source for new products with distinct processing and quality requirements compared to conventional processes for obtaining pulp and paper. The restrictions on cotton cultivation and the increase of dissolving pulp manufacturing provide new opportunities to cellulose polymer. In addition, compared to petroleum based chemicals, cellulose enables sustainable approaches and environmentally friendly products. This is the context that framed the present doctoral thesis. The work focused on two different research lines, namely: **conversion of sulfite pulp to dissolving-grade with the aid of enzymatic and chemical treatments**, and the **(bio)modification of high-cellulose content pulps**. The approach of two research lines, justifies the use of two pulps with different characteristics, as a feedstock.

The first part of the thesis involved the use of enzymes to bleach softwood sulfite pulp. The aim was to explore the **potential** of **natural and synthetic compounds** for lignin removal and provide an improvement in the bleaching process. The effluents resulting from each stage in the sequence were analyzed with a view to assessing the environmental impact of the laccase treatment. Afterwards, the mediator with best efficiency to promote delignification and enhance the bleaching effect, was used to **develop a totally chlorine free (TCF) sequence** composed of laccase-mediator system (LMS), a chelating (Q) stage and a hydrogen peroxide stage reinforced with pressurized oxygen (PO). The resulting pulp exhibited an ISO brightness of 90% and a satisfactory degree of polymerization. In order to understand the effects of enzymatic treatments caused in crystallinity and the surface of the cellulose chain, samples were analyzed by thermogravimetry and X-ray diffraction techniques.

The second part of the thesis was based on the conversion of these biobleached sulfite pulps to dissolving-grade. **Purification** was carried out with the aid of **chemical and enzymatic treatments**, specifically employing **strong alkaline extractions and hydrolytic enzymes (endoglucanases)**. The combination of both procedures led to reduce the amount of hemicelluloses and increase the solubility behavior of cellulose, desired aspects for its final application.

Moreover, **pure cellulose modification** (commercial dissolving-grade pulp) by means of **enzymatic systems**, but with the interest to bring and develop new properties to the final products was also studied. With the purpose of improving reactivity and accessibility, the study gains knowledge on understanding the role of endoglucanases in combination with strong alkaline extraction to cellulose structure. On the other side, **laccase-TEMPO system** was used to evaluate the improvement of strength properties through oxidative modification of cellulose. A significant improvement in wet strength attributed to the formation of aldehyde groups in cellulose chains that facilitated inter-fiber bonding through hemiacetal linkages was observed. The ability of laccase-TEMPO system was improved by introducing a pre-refining step.

The last part of the thesis investigates the suitability of **dissolving grade-pulp obtained by chemo-enzymatic processes for heterogeneous- and homogeneous-phase acetylation reactions (chemical modification)**. The results were compared with those from a commercially-available dissolving fiber grade, obtained by traditional chemical methods, which was used as reference. Surface acetylated handsheets and transparent cellulose acetate films were achieved.

To conclude, "**bioconversion**" was explored from the initial stages, with the need to bleach pulp resulted from a sulfite cooking; to the transformation stages, where dissolving pulps characteristics were achieved; until the preparation of acetate films as a final product.

Resumen

La celulosa es el polímero más abundante en la Tierra y en los últimos años ha ganado un amplio interés como fuente de nuevos productos con requisitos de procesamiento y de calidad diferentes a los procesos convencionales de obtención de pulpa y papel. Las restricciones en el cultivo de algodón y el aumento de la producción de las llamadas pulpas para disolver (*dissolving pulps*) ofrecen nuevas oportunidades al polímero de celulosa. Además, en comparación con los productos químicos derivados del petróleo, la celulosa permite el desarrollo de procesos y productos sostenibles y amigables con el medio ambiente. Este es el contexto que enmarca la presente tesis doctoral. El trabajo se desarrolló siguiendo básicamente dos líneas de investigación: por un lado la **conversión de pulpas al sulfito a pulpas aptas para su disolución mediante la ayuda de tratamientos enzimáticos y químicos**, y por otro lado la (**bio)modificación de pulpas con alto contenido en celulosa**. El planteamiento de estas dos líneas de investigación justificó el uso como materia prima de dos pulpas con distintas características.

La primera parte de la tesis se centra en la aplicación de enzimas para el blanqueo de pulpa de conífera procedente de una cocción al sulfito. El objetivo era explorar el **potencial** de algunos **compuestos naturales y sintéticos** para la eliminación de lignina, así como aportar una mejora en el proceso de blanqueo. Los efluentes resultantes de cada etapa se caracterizaron con el fin de evaluar el impacto ambiental de los sistemas basados en el empleo de lacasas, un aspecto poco explorado en los estudios de bioblanqueo. A continuación, el mediador con mejor eficiencia para promover la deslignificación y potenciar el efecto de blanqueo, se utilizó para **desarrollar** una **secuencia totalmente libre de cloro (TCF)**, formada por el sistema lacasa-mediador (LMS), una etapa quelante (Q) y finalmente una etapa de peróxido de hidrógeno reforzado con oxígeno presurizado (PO). La pulpa obtenida presentó una blancura ISO del 90% y un satisfactorio grado de polimerización. Con el fin de comprender los efectos de los tratamientos enzimáticos en la cristalinidad y en la superficie de la cadena de celulosa, las muestras se analizaron mediante técnicas de termogravimetría y difracción de rayos X. En la segunda parte de la tesis, se estudió la conversión de estas pulpas bioblanqueadas en pulpas aptas para disolver. La **purificación** y

modificación se llevó a cabo con la ayuda de **tratamientos químicos y enzimáticos**, concretamente con **extracciones alcalinas fuertes** y **enzimas hidrolíticas (endoglucanasas)**. La combinación de ambos procedimientos permitió disminuir el contenido de hemicelulosas así como aumentar la solubilidad de la celulosa, aspectos deseados para su aplicación final. También se estudió la modificación de **celulosa pura** (fibras comerciales para disolver) mediante **sistemas enzimáticos**, pero enfocando la modificación en la necesidad de conferir nuevas propiedades. En la línea de mejorar la reactividad y la accesibilidad, el estudio proporciona conocimiento para entender el papel que juegan las **endoglucanasas** en **combinación** con **extracciones alcalinas fuertes** en la estructura de la celulosa. Por otro lado, se evaluó el uso del sistema **lacasa-TEMPO** para oxidar la estructura de la celulosa y conseguir una mejora de las propiedades de resistencia mecánica. Esta modificación aportó una importante mejora de la **resistencia en húmedo** en la pasta tratada, atribuida a la presencia de grupos aldehídos en las cadenas celulósicas capaces de generar enlaces hemiacetales (inter-fibras) con los hidroxilos libres. El potencial del sistema lacasa-TEMPO se mejoró introduciendo una etapa de pre-refino.

La última parte del trabajo ilustra la **modificación química** mediante **reacciones de acetilación** (heterogénea o homogénea) de las **pulpas para disolver** obtenidas a partir de **procesos enzimáticos**. Los resultados se compararon con una fibra para disolver disponible comercialmente, obtenida por métodos químicos tradicionales.

Para concluir, la "**bioconversión**" se llevó a cabo des de las etapas iniciales del blanqueo de la pulpa procedente de una cocción al sulfito hasta la determinación de las características propias de las pulpas para disolver, consiguiendo preparar films de acetato de celulosa, como producto final.

Resum

La cel·lulosa és el polímer més abundant de la Terra i en els últims anys ha guanyat un ampli interès com a font de nous productes amb requisits de processament i de qualitat diferents als processos convencionals per obtenir pasta i paper. Les restriccions en el cultiu del cotó i l'augment de la producció de les anomenades pastes per dissoldre (*dissolving pulps*) ofereixen noves oportunitats al polímer de cel·lulosa. A més a més, en comparació amb els productes químics derivats del petroli, la cel·lulosa permet el desenvolupament de processos i productes sostenibles i amigables amb el medi ambient. Aquest és el context que emmarca la present tesi doctoral. El treball es va desenvolupar seguint bàsicament dues línies d'investigació: **conversió de pasta al sulfit a pastes aptes per a la seva dissolució mitjançant tractaments enzimàtics i químics**, i **(bio)modificació de pastes amb alt contingut en cel·lulosa**. El plantejament d'aquestes dues línies d'investigació va justificar l'ús de dues pastes amb característiques diferents, com a matèria primera.

La primera part de la tesi es centra en l'aplicació d'enzims per al blanqueig de pasta de conífera procedent d'una cocció al sulfit. L'objectiu era explorar el **potencial** d'alguns **compostos naturals i sintètics** per a l'eliminació de lignina així com aportar una millor en el procés de blanqueig. Els efluents resultants de cada etapa es van caracteritzar per tal d'avaluar l'impacta ambiental dels sistemes basats en l'ús de lacases, un aspecte poc explorat en els estudis de bioblanqueig. A continuació, el mediador amb millor eficiència a promoure la deslignificació i a potenciar l'efecte de blanqueig, es va utilitzar per **desenvolupar una seqüència totalment lliure de clor (TCF)**, integrada pel sistema lacasa-mediador (LMS), una etapa quelant (Q) i una etapa de peròxid d'hidrogen reforçat amb oxigen pressuritzat (PO). La pasta resultant va exhibir una blancor ISO del 90% i un satisfactori grau de polimerització. Per tal de comprendre els efectes dels tractaments enzimàtics en la cristal·linitat i a la superfície de la cadena de cel·lulosa, les mostres es van analitzar mitjançant tècniques de termogravimetria i difracció de raigs X.

La segona part de la tesi es base en la conversió d'aquestes pastes bioblanquejades a pastes aptes per ser dissoltes. La **purificació** i **modificació** es va

dur a terme amb l'ajuda de **tractaments químics i enzimàtics**, específicament emprant **extraccions alcalines fortes i enzims hidrolítics (endoglucanases)**. La combinació d'ambdós procediments va permetre disminuir el contingut d'hemicel·luloses així com augmentar la solubilitat de la cel·lulosa, aspectes altament desitjats per a la seva aplicació final. També es va estudiar la **modificació de cel·lulosa pura** (fibres comercials per dissoldre) mitjançant **sistemes enzimàtics**, però enfocant la (bio)modificació a la necessitat de conferir noves propietats. En la línia de millorar la reactivitat i l'accessibilitat, l'estudi proporciona coneixement per entendre el paper que juguen les **endoglucanases** en **combinació amb extraccions alcalines fortes** en l'estructura de la cel·lulosa. D'altra banda, es va avaluar l'ús del **sistema lacasa-TEMPO** per oxidar la cadena de cel·lulosa i aconseguir millorar les propietats de resistència mecànica. Aquesta modificació va aportar una important millora de la **resistència en humit** a la pasta tractada, atribuïda a la presència de grups aldehids en les cadenes cel·lulòsiques amb la capacitat de generar enllaços hemiacetals (inter-fibres) amb els grups hidroxils lliures. El potencial del sistema lacasa-TEMPO es va millorar introduint una etapa de pre-refí.

L'última part de la tesi il·lustra la **modificació química** mitjançant **reaccions d'acetilació** (heterogènia o homogènia) **de les pastes per dissoldre obtingudes a partir de processos enzimàtics.** Els resultats es van comparar amb una pasta per dissoldre disponibles comercialment, obtinguda per mètodes químics tradicionals.

Per concloure, la "**bioconversió**" es va portar a terme des de les etapes inicials del blanqueig de la pasta procedent d'una cocció al sulfit fins la determinació de les característiques pròpies de les pastes per dissoldre, i aconseguint la preparació de films d'acetat de cel·lulosa com a producte final.

List of abbreviations and symbols

A	
A	Acid stage
(DMA)/LiCl	dimethylacetamide-lithium chloride
a*	red-green coordinate
AA	Active Alkali
ABTS	2,2'-azinobis-(3-ethylbenzenethiazoline-6-sulphonic acid)
AGU	Anydro-β-D-glucopyranose
AS	Acid sulfite
AV	Acetovanillone
B	
B	Endoglucanase from *Paenibacillus barcinonensis*
b*	yellow-blue coordinate
B120	B endoglucanase at 120 U/g
B120_CCE	B endoglucanase at 120 U/g + Cold Caustic Extraction
BLI	Brightness Loss Index
BOD	Biological Oxygen Demand
C	
C*	Chroma coordinate
CA	Cellulsoe Acetate
CBD	Cellulose-binding domain
CBH	Cellobiohydrolases
CCE	Cold Caustic Extraction
CCE_B120	Cold Caustic Extraction + B endoglucanase at 120 U/g
CCE_F12	Cold Caustic Extraction + F endoglucanase at 12 U/g
CHO	Aldehydes
CMC	Carboxymethylcellulose
COD	Chemical Oxygen Demand
Com	Bleached commercial dissolving pulp
COOH	Carboxylic acids
CS	Chain Scission
Cuen	Copper II-ethlenediamine
D	
D	Chlorine Dioxide
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide-formaldehyde
DP	Degree of polymerization

DS	Degree of substitution
DTI	Dry Tensile Index
DTPA	Diethylenetriaminepentaacetic acid
E	
E	Alkaline extraction
ECF	Elemental Chlorine Free
EHEC	Ethyldroxyethylcellulose
F	
F	Endoglucanase from *Cerrena unicolor*
F12	F endoglucanase at 12 U/g
F60	F endoglucanase at 60 U/g
FAO	Food Agricultural Organitzation
FTIR	Fourier Transform Infrared Microscopy
G	
G	Guayacylpropane
H	
H	Hydroxyphenylpropane
HBT	1-hydroxybenzotriazole
HexA	Hexenuronic acid
HPLC	High Performance Liquid Chromatography
I	
IL	Ionic Liquids
K	
K	Control buffer
K_CCE	Control buffer + Cold Caustic Extraction
KL	Control laccase
KN	Kappa Number
KQPO	Control buffer + Chelating stage + Hydrogen peroxide
L	
L (l)	Length-weighted average of fiber length
L (n)	Numercial average of fiber length
L*	Lightness coordinate
L_B120	Biobleached sulfite pulp + B endoglucanase at 120 U/g
L_CCE	Biobleached sulfite pulp + Cold Caustic Extraction
L_CCE_B120	Biobleached sulfite pulp + Cold Caustic Extraction + B Endoglucanase at 120 U/g
L_CCE_F	Biobleached sulfite pulp + Cold caustic extraction + F endoglucanase (*Paenibacillus barcinonensis*)

L_CCE_F12	Biobleached sulfite pulp + Cold Caustic Extraction + F Endoglucanase at 12 U/g
L_CCE_F+Lac-T	Biobleached sulfite pulp + Cold caustic extraction + F endoglucanase (*Paenibacillus barcinonensis*) + Laccase-TEMPO treatment at 8%, for 8h with O_2
L_F	Biobleached sulfite pulp + F endoglucanase (*Paenibacillus barcinonensis*)
L_F12	Biobleached sulfite pulp + F endoglucanase at 12 U/g
L_K	Biobleached sulfite pulp + endoglucanase control
Lac-T	Laccase-TEMPO treatment
Lac-T 2%, 8h, no O_2	Laccase-TEMPO treatment with 2% TEMPO, for 8h with no oxygen pressure
Lac-T 8%, 20h, O_2	Laccase-TEMPO treatment with 8% TEMPO, for 20h with oxygen pressure
Lac-T 8%, 8h, no O_2	Laccase-TEMPO treatment with 8% TEMPO, for 8h with no oxygen pressure
Lac-T 8%, 8h, O_2	Laccase-TEMPO treatment with 8% TEMPO, for 8h with oxygen pressure
LCC	Lignin-carbohydrate complexes
LMS	Laccase-mediator system
LQPO	Laccase treatment + chelating treatment + hydrogen peroxide treatment reinforced with oxygen
M	
MAS	Magic Angle Spinning
ML	Middle Lamella
N	
NHA	N-hydroxyacetanilide
Nitren	Tris(2-aminoethyl)amine nickel complex
NMMO	N-methylmorpholine oxide
NMR	Nuclear Mass Ressonance
O	
O	Oxygen delignification
odp	Oven-dried pulp
P	
P	Hydrogen peroxide
*p*CA	Para-Coumaric acid
PFI mill	Laboratory beating
PHK	Pre-Hydrolysis Kraft
PO	Hydrogen peroxide treatment reinforced with oxygen

PW	Primary Wall
Q	
Q	Chelating treatment
R	
R	Refined pulp
R + Lac-T 8%, 8h, O_2	Refined dissolving pulp + Laccase-TEMPO treatment with 8% TEMPO, for 8h with oxygen pressure
R10	Alkali resistance 10% NaOH
R18	Alkali resistance 18% NaOH
R_{FS}	Fock solubility
S	
S	Syringylpropane
S1	Primary layer
S2	Middle layer
S3	Third layer
SA	Syringaldehyde
SEM	Scanning Electron Microscope
T	
t, tn	ton
TC	Terminal enzyme complexes
TCF	Totally Chlorine Free
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
TGA	Thermogravimetric analysis
TLC	Thin Layer Chromatography
TvL	*Trametes villosa*
Tween 20®	Polyethylene glycol sorbitan monolaurate
U	
U	Enzyme activity Unit
V	
V	Vanillin
VA	Violuric acid
W	
W(n)	Fiber width
W/D	Wet-to-dry strength ratio
WCA	Water Contact Angle
WRV	Water Retention Value
wt	Weight
WTI	Wet Tensile Index

WZSTI	Wet zero-Span Tensile Index
WZSTS	Wet Zero-Span Tensile Strength
X	
X	Xylanase
XPS	X-ray photoelectron spectroscopy
XRD	X-Ray Diffraction
Z	
Z	Ozone

List of figures

List of tables

List of schema

Table of contents

Chapter 1

Introduction

1.1 Biomass components

Cellulose

Cellulose is a linear homopolymer consisting of anhydro-β-D-glucopyranose units (AGU) that are linked together by (1-β-4) glycosidic bonds. Cellobiose is the repeating unit, consisting of two AGUs (Fengel and Wegener, 1989; Sjöström, 1993). In the solid state, AGU units are rotated by 180° with respect to each other due to the constraints of β-linkage. The number of AGUs determines the chain length, i.e. degree of polymerization (DP), but the DP of cellulose is greatly dependent on the origin and the method of isolation. Native cellulose has a degree of polymerization (DP) between 5000 - 15 000 (Klemm et al., 2005) but the cellulose used in practice has an average DP of between 800-3000 (Krässig, 1996). Every AGU unit has three hydroxyl groups in the positions C2 and C3 (secondary hydroxyl groups) and C6 (primary hydroxyl groups). One end contains an alcoholic OH group at C4 (non-reducing end), while on the other end C1 is part of an aldehyde group with reducing activity (Figure 1-1).

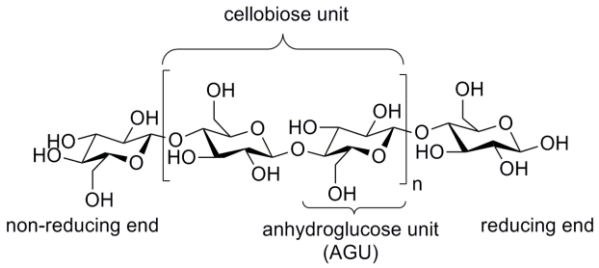

Figure 1-1 Molecular structure of cellulose with repeating cellobiose units showing reducing (right) and nonreducing (left) end-groups (from Olsson and Westman 2013)

The reactivity and chemical character of cellulose is determined by reactivity of the primary and two secondary OH groups in the AGU (Klemm et

al., 1998). These hydroxyl groups are responsible for forming both intra- and intermolecular hydrogen (H)-bonding network. Intramolecular H-bonds can be formed between the C6 hydroxyl and the C2 hydroxyl and, between the C5 oxygen and C3 hydroxyl within the same molecule, stabilizing the glycosidic bond and making the structure stiff. H-bonds can also be formed between two neighboring cellulose chains which interact via their C3-OH and C6-OH groups. Those interactions are called intermolecular H-bonds (Figure 1-2).

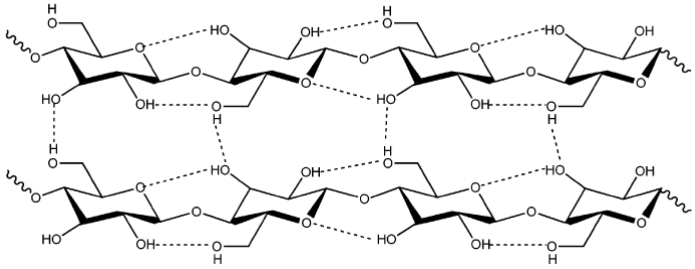

Figure 1-2 Intermolecular and intramolecular hydrogen bonds in cellulose (from Wang et al. 2012)

The H-bonds are responsible for cellulose structure and for its insolubility in water and common organic solvents. The characteristic rigidity is via co-crystallization of multiple chains into parallel structures forming elementary fibrils, which are further organized into microfibrils (Fengel and Wegener, 1989; Kontturi et al., 2006; Gandini, 2011; Moon et al., 2011). However, the fundamental reason behind this is still on debate. One hypothesis states that the main reason of cellulose almost non-dissolving properties lies in the fact that the hydroxyl groups are responsible for the extensive hydrogen bonding network which suppresses the solubility (Klemm et al., 1998). Recently, Lindman et al. (2010) asserts that the solubility characteristics of cellulose are based upon amphiphilic and hydrophobic molecular interactions, which have been extensively discussed by authors such as Glasser et al. (2012). Also Johansson et al. (2011) showed important evidence that the reactivity of the surface hydroxyl groups governs the behavior of cellulose in different media.

As mentioned earlier, the supramolecular model of cellulose is based on the organization of cellulose chains. Parallel synthesis of cellulose in the biosynthesis of wood or other non-wood plants leads to noncovalent association

between multiple chains, which results in substructures termed microfibrils. Wood microfibrils consist of elementary fibrils which start formation from the terminal enzyme complexes (TC) that take shape of a six-lobed "rosette", where β-D-glucose chains are held together via van der Waals forces. Those microfibrils are micrometers long and few nanometers thick. They contain both ordered and disordered components (Atalla and VanderHart, 1999; Vietor et al., 2002) but their structure and exact size are still under discussion (Figure 1-3). Cellulose fibrils are thus ordered 3-dimensional crystals. Thus, it may be interpreted as the crystals have different bonds in each dimension. The first dimension is given by the covalent bonds (enforced by some hydrogen bonds) along the cellulose chains, giving the final length of the fibril. The second dimension constitutes the hydrogen bonds, holding the cellulose chains together in sheets. The Van der Waals bonds and x-interactions bridging the cellulose sheets in the fibril form the third dimension (Ek et al., 2009).

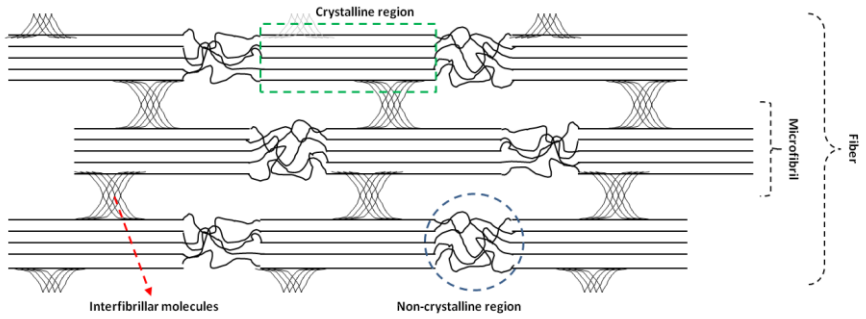

Figure 1-3 Crystalline and non-crystalline regions (from Börjesson and Westman 2015)

There are several polymorphs of crystalline cellulose (I, II, III, IV) (O'sullivan, 1997). Cellulose I (native cellulose) is the form found in nature, produced by trees, tunicates, algae, plants and bacteria. Native cellulose (cellulose I) occurs in two allomorphs, Iα and Iβ, with different parallel crystalline structures. Cellulose Iα (triclinic structure) is the dominant crystalline structure for native bacterial cellulose and *Valonia* (algae) cellulose, whereas cellulose Iβ (monoclinic structure) dominates in the higher plants such as wood and cotton. These two different crystal forms differ in cellulose chain conformation, hydrogen bonding and different arrangement of cellulose molecules in the unit cell (Nishiyama et al., 2003). Cellulose I is

thermodynamically metastable and can be converted to either cellulose II or III. Cellulose II has been the most stable structure of technical relevance and can be produced by regeneration (solubilization and precipitation) or by mercerization (aqueous sodium hydroxide treatments) (Klemm et al., 2005). Mercerization is considered to begin at NaOH concentrations above 8-9% (Sixta, 2006) and the conversion occurs rapidly (Crawshaw et al. 2002). Under these concentrations, the most severe changes materialize in the crystallinity (Sixta; Ek et al., 2009). The changes during the process depend not only on the alkalinity but also on the treatment time (Borysiak and Doczekalska, 2008). Cellulose II has antiparallel chains, i.e. that every second chain has opposite polarity to the next. Thus, the hydrogen pattern is different, and there is one more hydrogen bond per glucose residue, compared to cellulose I. Cellulose III can be formed from Cellulose I or II through liquid ammonia or organic amine treatments, and subsequent thermal treatments lead to Cellulose IV. The crystalline regions of cellulose are interspersed with less ordered paracrystalline or amorphous areas, arising from imperfect packing and interactions with other non-cellulosic polysaccharides. Those amorphous areas were shown to be distributed on the surface (Larsson et al., 1997) as well as along the cellulose microfibrils (Nishiyama et al., 2003). Depending on the source, the degree of crystallinity in native cellulose is typically 50-70%, but can also be over 94% (Olszewska, 2013).

Hemicelluloses

The monosaccharides that constitute hemicelluloses are pentosans (D-xylose and L-arabinose), hexosans (D-glucose, D-galactose, L-galactose, D-mannose, L-rhamnose, L-fucose) and uronic acids (D-glucuronic acid, D-galacturonic acid) (Figure 1-4). Generally, the units of main chain are linked together by β-(1→4) linkages, although some species combine β-(1→4) and β-(1→3) linkages. The composition and relative amount of hemicelluloses in the cell wall depends on the wood species. The main hemicellulose in hardwood is xylan, more specifically a 4-O-methylglucuronoxylan (15 - 30% in wood, 4-O-MeGlcA:Xyl = 1:10) and glucomannan (about 2 - 5% in wood, Glc:Man = 1:1-2). The main softwood hemicelluloses are galactoglucomannan (about 20% in wood, Gal:Glc:Man = 0.1-1:1:3-4) and arabino-4-Omethylglucuronoxylan (5-10% in wood, Ara:4-OMeGlcA:Xyl = 1.3:2:10) (Fengel and Wegener, 1989; Sjöström, 1993; Iakovlev, 2011). Some other polysaccharides present in both softwood and

hardwood biomass are glucans (starch, callose, located in parenchyma cells), pectins (polyrhamnogalactouronides), arabinans, galactans (mostly located in the compound middle lamella) (Fengel and Wegener, 1989). Hemicelluloses are associated with cellulose microfibrils via hydrogen bonding and with other cell wall constituents via covalent linkage. Hemicelluloses contribute to the strength formation of cell wall in association with cellulose microfibrils. Another function of hemicelluloses is to bring flexibility to the structural assembly of cell wall components. The presence of hemicelluloses also inhibits the coalescence of cellulose microfibrils, which improve the fibrillation of cellulose into nanosized fibrill. Hemicelluloses possess side groups on the chain molecules (amorphous form), therefore are easy to dissolve and degrade in acid and alkaline solutions, and their DP ranges from 200 to 300 (Olszewska, 2013).

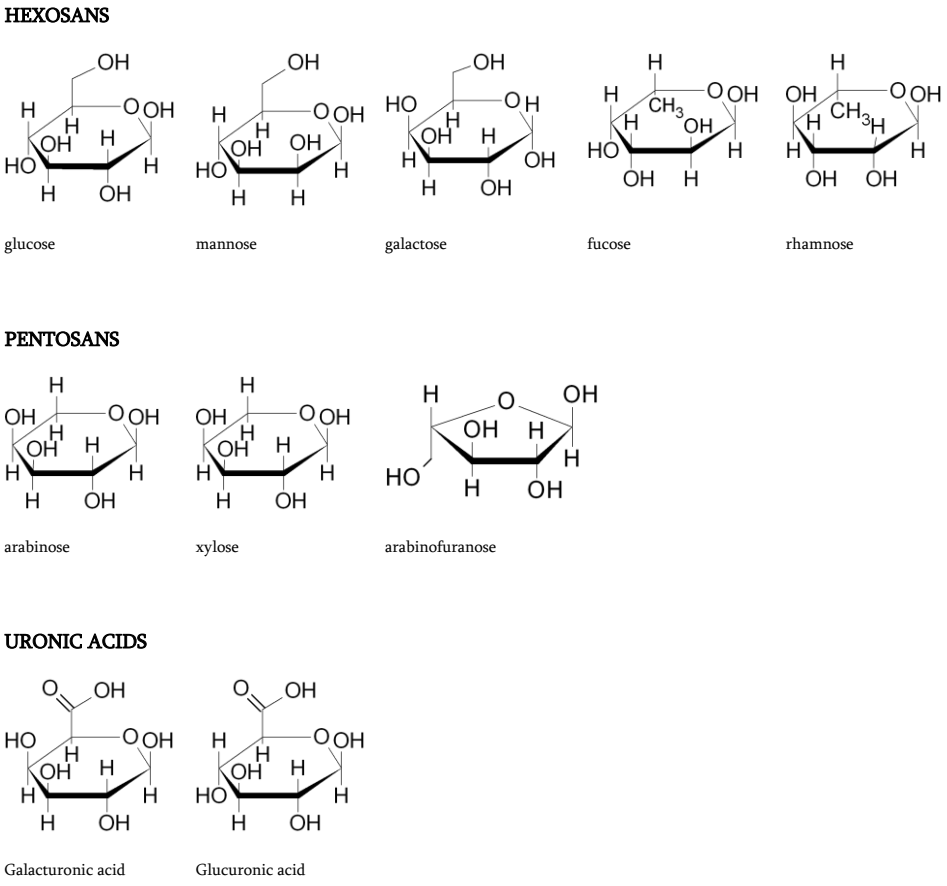

Figure 1-4 Monosaccharide constituents of hemicelluloses (from Fengel and Wegener 1989)

Lignin

Lignin is an aromatic heteropolymer built from 4-phenylpropane structural units (guaiacylpropane, G; syringylpropane, S; and hydroxyphenylpropane, H) bound by ether and carbon-carbon bonds (Figure 1-5).

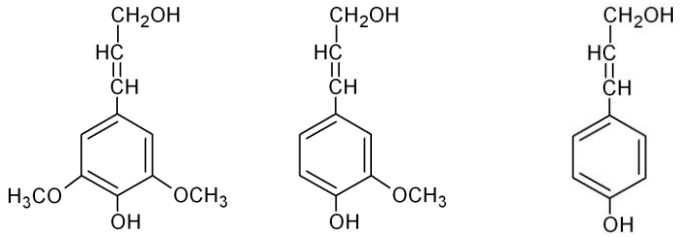

Figure 1-5 Phenylpropane precursors of lignin (from Sjöstrom 1981)

Lignin composition depends on its botanical origin. Softwood lignin has mainly G units, hardwood lignin G and S units and nonwood lignin H, G and S units, at different proportions. S units are known to be more reactive than G units, hence lignin rich in S units is easier to remove by pulp delignification (del Río et al., 2001). The S/G ratio has a direct effect on delignification efficiency: the higher S/G, the higher the delignification rate is. As a consequence, less alkali is needed and thus resulting in a higher pulp yield.

Lignin represents between 25-33% of dry softwood biomass and about 18-34% of hardwood biomass. It is produced by maturing cells and it is placed between fibrous walls mainly in intercellular regions (middle lamella), creating a stiff and cohesive structure. Moreover, lignin is responsible for transportation of water, nutrients and metabolites in the vascular system. Also, it plays an important role in the system of plant defense against degradation by microbial enzymes (Fengel and Wegener, 1989). Because of its hydrophobicity, lignin inhibits water absorption and fiber swelling, which can make fibers less responsive to mechanical refining. However, because it is thermoplastic, lignin possesses a feature that can be used to advantage in mechanical pulping to soften it at high temperature. The covalent union between lignin, cellulose and hemicellulose give the well-known lignin-carbohydrate complexes (LCC). As a result, lignocellulosic materials are considered as a lignocellulosic matrix rather

than individual fractions, and as a consequence the removal of lignin implies degradation of carbohydrates.

Extractives

Wood also contains small quantities of extractives, between 2-10% depending on wood species. These compounds are mainly resin and fatty acids and esters (Gullichsen, 1999). They are partly soluble in water or in organic solvents but may cause major difficulties in wood pulping since extractives are released from fibers and can form colloidal pitch and cause production problems such as deposits in water circuits.

1.2 Wood cell wall structure and ultrastructure

Wood cells are composed of a number of cells. The walls of cells are structured in layers (Figure 1-6). The outermost amorphous and lignin-rich layer, called the middle lamella (ML), connects individual cells together. It is the area between neighbouring cells, and not actually a part of the cell wall. The true cell wall is divided into two main layers: primary and secondary. The primary wall (PW) is thin; it is built of an open network of microfibrils embedded in an amorphous material. The secondary wall is subdivided into three separate layers: S1, S2, and S3. The S2 layer is the thickest layer representing about 80% of the total cell wall thickness (Fengel, 1971). Its main function is mechanical reinforcement and the layer is considered as the main load-bearing element in wood fibers. The microfibrils in the secondary walls are arranged in spiral patterns along the axis of the cell wall (vertical orientation), and impart the major mechanical and physical properties to wood products (Ek et al., 2009).

The three major components that comprise wood cell walls are namely cellulose, lignin and hemicelluloses. Cellulose is the main component of cell wall. It represents about 40-60% in softwood, 41-50% in hardwood and 40-60% in nonwood. Most of the cellulose in wood (between 80 and 85% of the total cellulose matter in wood) is presented in the middle layer (S2 layer) of the cell wall. Hemicelluloses are found along the cell wall, from the middle layer to S3 layer of secondary wall, being more abundant in S1 and S3 layers. Cell walls are also surrounded by lignin, which is mainly located in middle lamella (ML) in the cell wall structure. Lignin concentration is higher in the middle lamella and

lower in the S2 and S3 layers but, because of the differing thicknesses of the layers, there is more lignin in the secondary wall. To liberate the cellulose fibers from other cell wall constituents, a pulping process is used. The main purpose of chemical pulping is to selectively remove lignin. However, it is difficult to be achieved due to lignin-carbohydrate complexes (LCC) and poor selectivity. After pulping, bleaching is applied and helps to remove lignin, but also hemicelluloses and cellulose are partly depolymerized. All these treatments can strongly affect the properties and composition of the cellulose fibers produced (Ek et al., 2009).

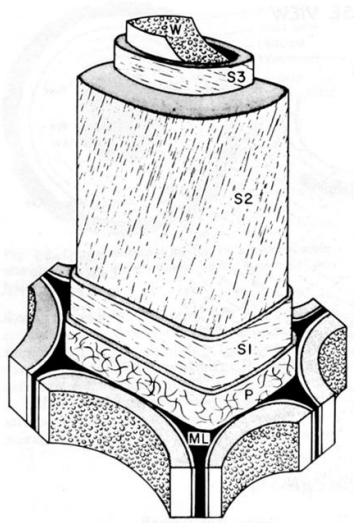

Figure 1-6 Simplified structure of a typical tracheid cell wall (from Cote 1967)

1.3 Separation of cellulosic fibers from lignocellulosic materials

Cellulosic fibers are separated from lignocellulosic materials to produce paper and board. The separation can be mechanical, chemical, or a combination of these methods (Sixta, 2006). In **mechanical pulping**, fibers are separated by defibrating the wood or wood chips mechanically into fibers and fiber fragments and bundles. The process uses very little or no chemicals, but highly energy consuming. Mechanical pulps, thus, contain the chemical components nearly in the same proportion as they are present in wood. In addition, mechanical pulps contain significant amounts of fines and fiber fragments, which influence the possible pulp applications.

The fundamental of **chemical pulping** is to remove the lignin from wood or other lignocellulosic material by chemical reactions to separate the cellulosic fibers containing mainly cellulose and hemicelluloses (Sixta, 2006). This lignin dissolution is conducted at high temperature, but some associated carbohydrate (mainly hemicellulose and some cellulose in soluble form) are also removed. Chemical pulp typically possesses a high mechanical strength. The most abundantly applied chemical pulping method is kraft pulping, followed by sulphite pulping. Several variations, such as the use of cooking additives, have been introduced to kraft pulping in order to produce pulps with different qualities or to improve the process.

Kraft pulping

Kraft pulping is based on the reactions of hydroxide and hydrosulfide ions (pH~14) with lignin structures under elevated temperature (Pönni, 2014). Apart from the active species, white liquor also contains small amounts of Na_2CO_3, Na_2SO_4, $Na_2S_2O_3$, NaCl and $CaCO_3$. Active alkali content (AA) is an important parameter of kraft pulping; it refers to the hydroxide ion concentration in the cooking liquor taking into account the hydroxide ions originating from the dissociation of sodium sulphide into hydrogen sulphide and hydroxide ions (Sixta, 2006). The alkaline conditions also promote swelling which enhances the cooking chemical penetration. Application of softwood and hardwood chips is possible, but the wood species influences the selection of the pulping parameters due to the differences in the chemical composition (Sjöström, 1993).

Lignin reactions in kraft pulping can be divided into three distinct phases: initial delignification, bulk delignification, and residual delignification. Initial delignification takes place mainly in the impregnation phase (T< 140 °C) and very little lignin is dissolved (20-25% of total). The rate of delignification increases dramatically when the cooking temperature is elevated above 140 °C, and 70-80% of all lignin dissolves during this phase. The dissolution begins in the S2 layer of the cell wall and progresses into the middle lamella. The bulk delignification phase will continue until about 90% of all lignin has been dissolved. The three precursors of lignin, namely *p*-coumaryl, coniferyl, and sinapyl alcohols react differently with the cooking chemicals. Generally, the phenolic subunits are more reactive than the non-phenolic units (Sixta, 2006). Simultaneously to lignin removal, some carbohydrate will be dissolved,

especially during the initial delignification phase. Due to peeling reactions (i.e. alkaline induced end-wise degradation) the DP of carbohydrates is reduced by removal of anhydrosugar units from the reducing end of the carbohydrate chain (Sixta, 2006). In the final stages of kraft pulping, the alkalinity of the cooking liquor decreases which leads to the precipitation of hemicelluloses on the fibers. Cellulose will also dissolve to some extent (10-15%) during alkaline cooking, especially during the residual delignification phase.

A complete lignin removal by kraft cooking is not possible in order to achieve a high yield; therefore, further treatments are conducted with the purpose of reducing lignin content by means of oxygen delignification and bleaching stages. During these processing steps, lignin is removed more selectively by applying oxidants. The bleaching stages use both alkaline and acidic conditions under elevated temperatures (Gullichsen, 1999; Alén, 2000).

Acid sulfite

The acid sulfite (AS) process employs aqueous $SO_2/M(HSO_3)n$ as its cooking liquor, where the cation M is calcium, sodium, magnesium or ammonium. Acid processes are those in which the pH is 2-3, bisulfite process operate at pH range 3-5, neutral sulfite processes cover the pH range 6-9, and alkaline sulfite processes are above pH~11. Calcium requires pH~2 to stay in solution, while magnesium allows operation up to pH~4. The sodium and ammonium sulfite solutions can be strongly alkaline without precipitation. The composition of sulfite cooking liquors is generally expressed in terms of total SO_2 and combined SO_2. The AS process includes two phases: impregnation at about 110-120 °C in order to uniformly distribute the cooking chemicals within the biomass structure, and the actual cooking which is performed at 125-150 °C (Sixta, 2006). Lignin reactions in sulfite pulping can be divided into three distinct phases: sulfonation, hydrolysis and condensation. Sulfonation makes lignin more hydrophilic, and hydrolysis breaks lignin bonds so that new and smaller dissolvable lignin fragments are formed. Lignin condensation darkens the pulp, decreases the homogeneity of delignification, promotes shive formation, and makes bleaching difficult. However, lignin condensation can be avoided by keeping the bound sulfite concentration high enough (Iakovlev, 2011).

An important drawback of AS cooking is the very high cover-to-cover cooking times (up to 12 hours) due to the very slow (up to 6 hours) low-temperature impregnation stage (Fengel and Wegener, 1989); but a significant advantage with respect to alkaline processes, is the absence of peeling reactions that leads to higher carbohydrate preservation. The common products of acidic biomass fractionation processes are cellulosic fibers, dissolved carbohydrates, lignin, acetic acid, furfural and lignosulfonic acids. Lignosulfonic acids are used in concrete admixtures, road base, oil drilling muds, among other applications. The dissolved carbohydrates are an especially important feedstock for chemical and biochemical treatment leading to a various valuable products including biofuels (Sixta, 2006).

Kraft process is the dominant pulping system and the main reasons are as follows: excellent pulp strength properties, low demands on wood species and wood quality; well-stablished recovery of cooking chemicals, energy and by-products; and short cooking times. However, the sulfite process is still important at least in certain countries and for some pulp qualities, such as speciality paper grades, tissue and dissolving pulp (Fengel and Wegener, 1989). In terms of fiber quality, sulfite pulps have higher yields at a given kappa number and higher brightness of unbleached pulp due to absence of strong chromophores such as quinones and stilbenes. In addition, it is characterized by covering the whole range of pH (versatility), lower odor problems and lower investment costs (Gullichsen, 1999; Alén, 2000).

1.4 Bleaching process

The purpose of the bleaching is to dissolve (chemical pulp) or modify (mechanical pulp) the brown-coloured lignin that was not removed during pulping while preserving the integrity of the pulp (García Hortal et al., 1984; Dence, 1996). Chlorine-based chemicals were typically used in order to control cellulose degradation and achieve a desired viscosity level, but in response to environmental concerns (Brooks et al., 1994) these technologies were largely abandoned. The new bleaching processes such as the elemental chlorine free (ECF) and the totally chlorine free (TCF) are based on oxygen-derived compounds. A typical ECF sequence comprises multi-stages, oxygen delignification (O), chlorine dioxide (D), alkaline extraction (E) and hydrogen peroxide (P) stages in any combinations, while a conventional TCF sequence

includes an oxygen delignification (O) stage, an acid (A) stage, an ozone (Z) stage and a hydrogen peroxide (P) stage in different multi-positions.

The pulp and paper industry is under steady, ever-increasing pressure from global competition, stringent environmental regulations and new market demands. In this scenario, the use of biotechnology has emerged as a very promising approach not only to developing cleaner processes, but also to obtaining novel, high-value products.

1.5 Dissolving-grade pulp

Most of the wood pulp – $1.86 \cdot 10^8$ ton (FAO, 2012) – is used in the papermaking and packaging industry but a substantial part ($4.22 \cdot 10^6$ ton) is employed to obtain cellulose-based products with important applications in industrial sectors such as, pharmaceutical, textile, food and printing, among others. Dissolving-grade pulps are high quality pulps with specific characteristics and used in various cellulose derivatives and regenerated cellulose. Dissolving pulps are mainly produced by two different processes: the acid sulfite process, which is the dominant process that covers approximately 65% of the total dissolving pulp production, and the prehydrolysis kraft process, which covers approximately 25% (Sixta, 2006). In the sulfite process, hemicelluloses and lignin are removed from the wood chips in the same process step, which impedes the recovery of hemicelluloses from cooking liquor. The pre-hydrolysis kraft process instead uses an alkaline cooking step, where hemicelluloses are extracted from the wood chips in big quantities prior to cooking. Other methods such as organosolv have been investigated although are not used at industrial scale. In organosolv pulping, wood is delignified with systems based on organic solvents, mostly alcohol-water.

Dissolving-grade pulp is a highly pure pulp that exhibits high cellulose content (over 90%) and low levels of hemicelluloses, lignin and extractives. Traditionally, dissolving pulps and cotton linters have been used as raw materials for the production of regenerated cellulose and cellulose derivatives. Population growth and an improved standard of living have accelerated the demand for textile fibers, but an increase of cotton production is difficult to achieve on because of the large land area required for farming and the amount of water required for irrigation, which results in higher costs. Until the 1940s, it

was believed that only cotton and softwoods were appropriate for dissolving pulps. However, it was observed that vast quantities of hardwoods could also be successfully utilized, making their use economically attractive (Köpcke, 2010). Nowadays, many researches focus on the development of new technologies to obtain high-cellulose content pulps (Köpcke, 2008; Köpcke et al., 2008; Ibarra et al., 2010a; Gehmayr and Sixta, 2011; Schild and Sixta, 2011; Sixta et al., 2013; Roselli et al., 2014; Sixta, 2015). Dissolving pulps are utilized in the production of rayon and acetate. It includes viscose rayon staple and filament yarn used especially for textiles. Another example are tire cords, acetate staple and filament yarn used for textiles and acetate fiber for cigarette filters (Durbak, 1993). Low content of hemicelluloses is a very important requirement since hemicelluloses can affect the filterability of viscose, the xanthation of cellulose, and the strength of the end product during the production of viscose (Croon et al., 1968; Christov and Prior, 1993).

During the production of cellulose derivatives, cellulose is modified by substitution at the hydroxyl groups, whereas, for regenerated cellulose, cellulose is, in addition chemically dissolved and then regenerated. The accessibility and reactivity of cellulose are key parameters in the manufacturing of cellulose derivatives and regenerated cellulose since an inhomogeneous substitution of the hydroxyl groups of the cellulose chain might lead to the production of low-quality derivatives.

1.6 Production of regenerated cellulose

Regenerated cellulose which is produced as rayon fibers, cellophane and Lyocell fibers are obtained from cellulose and it is the largest production among the wide range of derivatives. The viscose process was developed in the 1800s but most production facilities are based on technology and equipment that was developed many years ago (LaNieve and Richard, 2007). The major developments in recent years have been focused on the installation of facilities to protect the environment. The most complex challenge in the production of regenerated cellulose is achieving the complete dissolution of the cellulose structure. Unfortunately, this dissolution cannot be done with cheap and common solvents. For this reason, it is important to enhance cellulose accessibility and reactivity to obtain homogeneous products (Köpcke, 2010).

The viscose process involves the following steps: cellulose is first steeped in an aqueous solution of strong sodium hydroxide, which causes the fibers to swell and converts the cellulose to sodium cellulosate, commonly called alkali cellulose. This step is named mercerization and converts the cellulose I to cellulose II. After steeping, the swollen mass is pressed to obtain a precise ratio of alkali to cellulose and then shredded to provide adequate surface area for uniform reaction in subsequent process steps. The alkali cellulose is aged under controlled conditions of time and temperature to depolymerize the cellulose by oxidation to the desired DP prior to reacting with carbon disulfide (CS_2) to form sodium cellulose xhantate. The xhantate, which is a yellow to orange crumb, is dissolved in dilute sodium hydroxide to yield a viscous orange-colored solution called viscose (Figure 1-7).

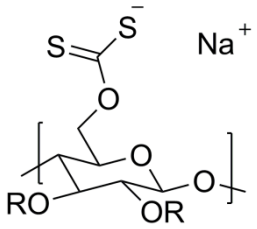

Figure 1-7 Cellulose xhantogenate with R = H or xhantogenate group (from Olsson and Westman 2013)

The rayon filaments are formed when the viscose solution is extruded through very small holes of a spinnerette into a spin-bath consisting basically of sulfuric acid, sodium sulfate, zinc sulfate, surfactant, and water. Coagulation of the filaments occurs immediately upon neutralizing and acidifying the cellulose xhantate followed by simultaneous controlled stretching and decomposition of the cellulose xhantate to cellulose. These latter steps are important for obtaining the desired tenacity and other properties of the rayon. Finally, the newly formed rayon, either in the form of continuous filament (yarns or tow) or cut into staple, is washed and chemically treated (desulfurized) to remove impurities before applying a processing finish and packaging (LaNieve and Richard, 2007).

When viscose filaments are produced clogging of the viscose filters usually occurs. In order to avoid this problem, it is important to achieve maximum cellulose dissolution. This dissolution can be achieved by increasing the amount

of CS_2, but unfortunately, CS_2 is an expensive and highly toxic (malodorous, highly volatile, and flammable) liquid with harmful effects on the environment (Treiber, 1985). Consequently, several studies aimed at developing methods for reducing the consumption of CS_2 while maximizing cellulose activation for further modification. Alternative solvents have been successfully tested and present environmentally acceptable conditions although at higher costs. Among these new processes, the NMMO (N-methylmorpholine oxide) industrial process appears to be the most promising replacement for conventional viscose processes. It basically involves the use of polar, aqueous NMMO solvent for dissolution of cellulose. The fibers produced by using this approach are called NMMO fibers with the generic name of Lyocell. NMMO is an oxidizing agent that can dissolve cellulose due to its strong N-O dipole that can form hydrogen bonds with the hydroxyl groups of cellulose. The main advantage of the NMMO solvent is the lack of toxicity. Lyocell fibers have some improvements over the conventional viscose fibers in the properties such as wet and dry strength, modulus of elasticity, wearing properties, gloss and touch, but inferior in wet fibrillation.

Recently, the dissolution of cellulose in ionic liquids, the so-called “green solvents”, has attracted significant attention because of their chemical and thermal stability, non-flammability and low vapor pressure compared with traditional volatile organic solvents (Laus et al., 2005; Wang et al., 2012; Sixta et al., 2013; Roselli et al., 2014). Furthermore, they are almost completely recyclable (around 99.5%), and the amount of wastewater produced is much lower than that produced by the viscose process.

1.7 Production of cellulose derivatives

The three major pathways of functionalizing cellulose to cellulose derivatives are the reaction types altering the three available hydroxyl groups of the glucose unit, mainly esterification and etherification (Figure 1-8).

Carboxymethylcellulose (CMC) is used as a thickener and stabilizer; ethylhydroxyethylcellulose (EHEC) is employed as a water-retaining agent and as a thickener; cellulose nitrate is used in the production of explosives, and cellulose acetate is used in cigarette filters, photographic films, lacquers and

fabrics. These are some examples of cellulose derivatives available in the current market.

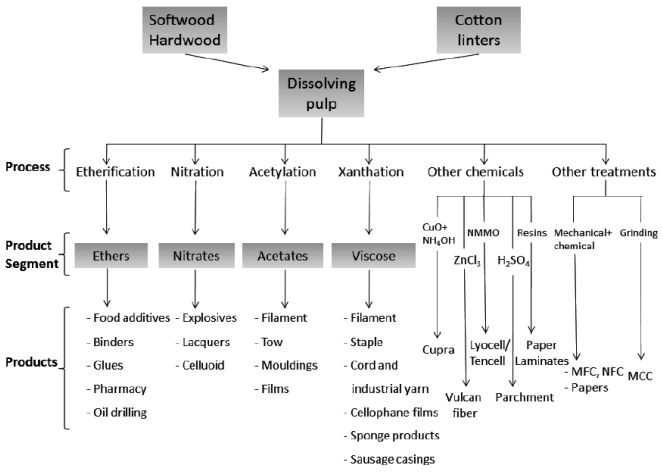

Figure 1-8 Overview of some processes and products obtained from dissolving pulps (from Peter Strunk, 2012 thesis disertation)

Camille and Henry Dreyfus developed the first commercial process to manufacture cellulose acetate in 1905 and commercialized the spinning of cellulose acetate fibers in 1924 in the United States. Cellulose triacetate textile fiber was commercialized later in the 1950s (LaNieve and Richard, 2007).

There are two methods for acetylation, namely, heterogeneous (in fiber dispersions) and homogeneous (in solution). The heterogeneous acetylation process is performed in the presence of a non-solvent, such as toluene, benzene, amyl acetate or carbon tetrachloride. The reaction product (cellulose acetate) is insoluble and thereby, this process preserves the morphological structure of the fiber. In the homogeneous acetylation process, cellulose acetate is solubilized in the reaction medium and cellulose fibers undergo substantial morphological changes. Therefore, solvents capable of deconstructing the crystalline network and interacting with the anhydro-glucose units of cellulose are required (Luo et al., 2013).

Acetic acid and excess of acetic anhydride (typically 5-15 wt %) is the most common sytem used for the homogenous acetylation. Adequate shredding of the

cellulose sheet and adequate swelling of the cellulose through a pretreatment step are necessary operations prior to the acetylation. The acetic anhydride reacts with the moisture in the pretreated cellulose, giving acetic acid and a completely anhydrous reaction medium. Then cellulose begins to react with acetic anhydride, the initial reaction is mainly in the amorphous regions of the cellulose. Sulfuric acid is used as a catalyst and it combines with the cellulose, forming sulfate linkages; but, most of these are removed during acetylation via an exchange with acetyl groups. It is important that the final cellulose triacetate or cellulose acetate contains only a very small amount of sulfate groups because adversely affect the properties, especially the color. When acetylation is virtually complete, the reaction mixture is viscous and clear. Excess acetic anhydride is then killed by adding stop acid (aqueous acetic acid) that helps to desulfate the residual sulfate linkages and the presence of water reduces chain degradation (LaNieve and Richard, 2007).

Methylene chloride is the second most common reagent used for homogeneous acetylation process and it replaces the acetic acid as solvent. The solvent is a mixture of methylene chloride, acetic anhydride and acetic acid during and at the end of the acetylation. Perchloric acid is frequently used as catalyst.

Other cellulose solvents used for the homogeneous derivatization are Dimethyl sulfoxide-formaldehyde (DMSO), dimethylacetamide-lithium chloride ((DMA)/LiCl), and N-methylmorpholine oxide (NMMO), N,N-dimethylformamide (DMF). These solvents permit nondegradative dissolution of the cellulose but cannot be defined as true ionic liquids (ILs) because they melt above 100 °C. These solvents have been studied intensively for a long time and they have showed very promising results, but their use is restricted to lab-scale, mostly due to their high costs and poor recyclability (LaNieve and Richard, 2007; Gericke et al., 2012)

1.8 Cellulose accessibility

Cellulose accessibility and reactivity have been a topic of great interest for years. Due to the arrival of novel processes requiring high reactivity of cellulose and its hydroxyl groups, the topic has recently gained further interest. Moreover

it is the most important quality parameter for dissolving pulps (Köpcke, 2010; Pönni, 2014).

The accessibility of these hydroxyl groups for the reacting chemicals (reagent and solvent) determines the concept of cellulose accessibility (Pönni, 2014). It has been suggested that cellulose reactivity is more related to the dissociation of fibril aggregates into elementary fibrils than to crystallinity. The accessibility of cellulose depends mainly on the number and size of the pores in the cellulose structure, the size and type of solvent or reagent, the internal surface that is accessible (as determined by the size of fibrils or fibril aggregates), and the structure of the cellulose molecules (which determines which hydroxyl groups are accessible). Therefore, to increase cellulose accessibility, the pores must be opened and both the fibril aggregates and the highly ordered regions must be altered (Krässig, 1993).

The accessibility and reactivity are commonly affected by microfibril coalescence, i.e., aggregation, during processing of the cellulosic material. The most commonly accepted explanation for the coalescence is the formation of exceptionally strong hydrogen bonds between the adjacent cellulose microfibrils (Newman, 2004; Pönni, 2014). Predominantly, the hydrogen bond formation is considered to occur in the amorphous regions of cellulose. However, crosslinking between crystalline cellulose domains in adjacent cellulose microfibrils is considered another possible mechanism for the irreversible hydrogen bonding (Newman, 2004). Another approach to cellulose microfibril coalescence has been the lactone bridge formation, i.e., the formation of bonds between hydroxyl and carboxyl groups (Fernandes Diniz et al., 2004; Pönni, 2014).

The term hornification describes the changes in chemical pulp fibers during drying (Fernandes Diniz et al., 2004). The removal of water from the cell wall of chemical pulp fibers causes the collapse of almost all of the pores and allows the formation of irreversible bonds between the microfibrils. The internal fiber volume is shrunk and a reduction in its reactivity is observed. There are many variables and treatments that induce cellulose microfibril coalescence. For example, during chemical pulping, cell wall components are removed from the cell wall. The dissolution of lignin and hemicelluloses creates larger pores that

collapse when water is removed and are significant for hornification (Maloney and Paulapuro, 1999). Hemicelluloses have shown to hinder the hornification effect (Oksanen et al., 1997). Important differences were found between low and high yield pulps during drying. Cotton, which is close to pure cellulose, is hornified strongly during drying due to the absence of hemicelluloses. Dissolving pulp is characterized by low amount of hemicelluloses and therefore when hemicelluloses are removed hornification is likely to occur. The changes induced by hornification, relevant for the papermaking industry, include reduced swelling and altered strength properties (Maloney and Paulapuro, 1999, 2000). The loss of strength is due to the stiffening of the fibres which decreases the fibre-fibre bonding area (Maloney and Paulapuro, 1999).

In recent years, several optimal treatments have been proposed to activate cellulose and, therefore, increase its accessibility and reactivity. Activation methods include degradative treatments (e.g., hydrolysis, oxidation and thermal treatments), mechanical treatments (e.g., wet milling and dry milling), and swelling treatments (e.g., interfibrillar and intrafibrillar action) (Krässig, 1993). For instance, it has been reported that different aqueous solvents such as Cuen, Nitren and sodium hydroxide, have been used as swelling solvents (Puls et al., 2006; Janzon et al., 2008a; Arnoul-Jarriault et al., 2014; Beltramino et al., 2015), as well as the combination of sodium hydroxide and urea (Kunze and Fink, 2005; Chen et al., 2015). Dry chemical pulp is known to be reswollen by an alkaline treatment. In addition, enzymatic treatments have been shown to activate cellulose by enhancing cellulose accessibility and reactivity and have become a very promising environmentally friendly alternative to these other treatments (Köpcke, 2010).

1.9 Cellulose interactions with alkali

It is known that swelling improves cellulose accessibility and, thus, enhances its reactivity in many heterogeneous chemical and enzymatic treatments. Alkaline treatments have also been suggested to improve cellulose accessibility by increasing the surface area as the crystallite size decreases (Pönni, 2014).

Since the mercerization process was developed, strong alkaline pretreatments (referred as cold caustic extraction, CCE) of cellulosic raw materials have been studied widely (Mercer, 1851). Currently, alkaline

pretreatments are used, for example, in the production of carboxymethyl cellulose (Ambjörnsson A., 2013) and the production of viscose for textile fibres, to refine dissolving pulp to acetate grade pulp or to remove hemicelluloses in larger quantities from paper grade pulp (Mozdyniewicz et al., 2013). The dissolution of short chain carbohydrates is preceded by inter- and intracrystalline swelling, caused by alkali. In general, swelling refers to the loosening of the intermolecular interactions in cellulose to competing interactions with the swelling agent. For aqueous systems, interactions are limited to the amorphous parts of cellulose and the pores (intercrystalline swelling). Therefore, the solvent only penetrates into the accessible space between the microfibrils and the amorphous zones. At sodium hydroxide concentrations exceeding 8–9 wt% also intracrystalline swelling occurs. There, the solvent also enters the highly organized crystalline regions of the cellulose. Cellulose I is gradually converted to Na-cellulose I (and after neutralization to cellulose II) when the alkali concentration is increased and the intracrystalline swelling proceeds through the cellulose fibrils. The conversion from cellulose I to cellulose II starts at 8–10 wt% alkali concentrations and is quantitative at NaOH concentrations of 17–18 wt% (Klemm et al., 1998), slightly depending on the cellulosic raw material (Hirota et al., 2012). The NaOH concentration should be kept low to selectively extract hemicelluloses and to avoid the formation of cellulose II. As the hemicelluloses are mainly located in the intercrystalline areas alkali concentrations of around 10 wt% are usually sufficient to largely remove the hemicelluloses. CCE can decrease the residual amount of both, xylan and glucomannan, to 0.5 wt% for AS pulp, and to 1.5 wt% of xylan and an unnoticeable amount of glucomannan for PHK pulp. Low temperature is beneficial for swelling due to the stronger interaction between NaOH and cellulose (Porro et al., 2007), but increases the lye viscosity, which may lead to chemical losses during washing (Roselli et al., 2014).

1.10 Enzymes

Enzymes are protein molecules built of linear heteropolymers consisting of 20 different amino acids. Enzymes are efficient and very specific catalysts. One enzyme normally only catalyses a single reaction, which is determined by the type of the substrate recognized and the type of chemical bond cleaved or

synthesized. Enzymatic reactions proceed via high-energy transition state of the enzyme substrate (Ek et al., 2009).

Biotechnology in the pulp and paper sector has a strong focus on the use of biological products such as enzymes. The fact that the raw materials used to manufacture pulp and paper typically consist of natural fibers provides a large opportunity for applying enzyme technology in the process (Bajpai, 1999). Bleaching has been the subject of much research by the pulp and paper industry into the potential of enzymes for providing more environmentally friendly processes. Enzymes can be used to improve the bleaching process indirectly or directly. Thus, pulp bleachability can be indirectly improved through the action of hemicellulolytic type enzyme. Particularly, xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), cleave internal linkages on the β-1,4-xylose backbone. The application of xylanases let to increase lignin extractability and reduce the consumption of bleaching chemicals as a result (Valls et al., 2010d). Beside the use as a bleach boosting agent, xylanases can also decrease the content of hexenuronic acids (HexA) present in pulp, formed during the alkaline cooking of wood (Cadena et al., 2010a). Xylanases have been used on the industrial scale since let to save important amount of chlorine reagents (up to 35%). However, there are two main inconvenient derived from the use of xylanases: high biological oxygen demand (BOD) and important yield reduction.

Bleachability can also be improved in a direct manner, using oxidative enzymes from white-rot fungi, which can directly attack lignin and have a higher potential than xylanases. The laccase concept is still under development at present (Bajpai 2004).

1.10.1 Laccases

Laccases are phenol-oxidases that are involved in lignin degradation (in white rot fungi) and in lignin synthesis (in trees). Laccases can use a wide range of substrates including phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, inorganic/organic metal compounds and many others, but the preferred substrates are phenols that are oxidized to quinones or phenolic radicals. Their versatility is the main reason for their attractiveness for a number of biotechnological applications (Riva, 2006). Oxygen is used as electron acceptor and is reduced all the way to water. This chemistry is very similar to

the reactions in oxygen delignification. The balance of the reaction is one molecular oxygen to four phenol molecules if phenolic radicals are the product, and one molecular oxygen to two phenols if quinones are the product. Laccase by themselves can only oxidize phenolic lignin units –which account for less than 20% of all lignin in native wood- at the substrate surface. On the contrary, laccases are not able to oxidize non-phenolic structures (redox potential >1.5 V) and therefore require the presence of a low-molecular weight compound known as a "redox mediator" for this purpose (Ek et al., 2009). Direct oxidation of a substrate by laccase may be impossible either because the substrate it is too large to penetrate into the enzyme active site or because its redox potential exceeds that of the enzyme (~0.5–0.8 V). Redox mediators are suitable intermediate substrates for laccase; thus, they are oxidized to radical forms by the enzyme and the resulting radicals are able to oxidize a bulky or high-redox potential substrate via a non-enzymatic mechanism (Riva, 2006), obtaining the well-known laccase-mediator system (LMS). The ability of mediators to extend the effect of laccase to non-phenolic lignin units has been a topic of great interest for aiding pulp bleaching (Bourbonnais and Paice, 1990) (Figure 1-9).

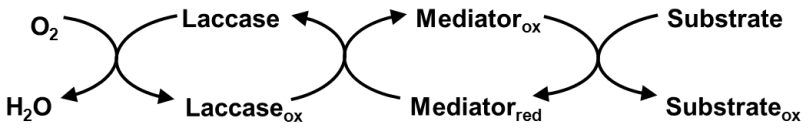

Figure 1-9 Reaction mechanism of Laccase-mediator system

The first synthetic mediator known to oxidize high-redox non-phenolic lignin model compounds is 2,2′-azinobis-(3-ethylbenzenethiazoline-6-sulphonic acid) (ABTS) (Bourbonnais and Paice 1990). The synthetic laccase mediators most widely investigated for use by the pulp and paper sector are the N–OH compounds 1-hydroxybenzotriazole (HBT), violuric acid (VA), and *N*-hydroxyacetanilide (NHA), and the stable 2,2,6,6-tetramethylpiperidine-1-oxyl free radical (TEMPO) (Moldes et al., 2008; Fillat and Roncero, 2009; Valls et al., 2010c; Fillat et al., 2011) (Figure 1-10).

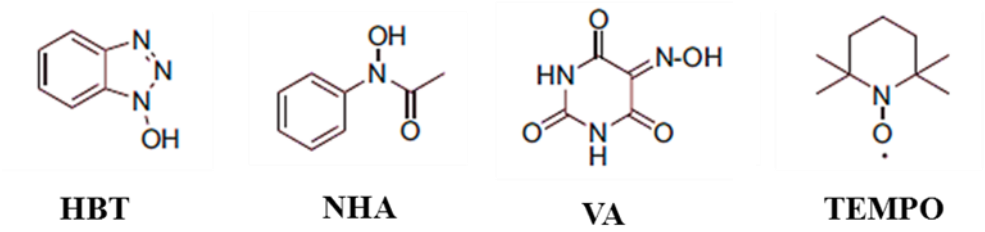

Figure 1-10 Chemical structure of synthetic mediators (from Aracri 2012)

Biobleaching applications

Research has shown that the laccase-HBT (Ibarra et al., 2006; Fillat and Roncero, 2009), laccase-VA (Moldes and Vidal, 2008; Aracri and Vidal, 2011; Fillat et al., 2011) and laccase-NHA systems (Valls et al., 2010b) can substantially reduce the requirements of bleaching chemicals for chemical pulp bleaching, or allow bleaching to smaller kappa numbers and higher brightness. LMS have recently proved efficient in removing sterols, the main culprits of pitch problems, from eucalyptus pulp (Gutiérrez et al., 2006, 2009). The promising results obtained by Valls et al. (2009) demonstrate that sterols are better eliminated by an LMS stage than by a chlorine dioxide stage. Another recently found, beneficial feature of LMS relating to bleaching is their ability to reduce the hexenuronic acid (HexA) content of pulp via an as yet unclear mechanism. HexA presence in pulps is unfavorable due to some negative effects it has on further processes and final quality pulp. Valls et al., (2010c) found the HexA content of eucalyptus pulp to decrease upon treatment with a laccase–HBT system, to an extent dependent on the enzyme and mediator doses, but not on the reaction time. The joint use of laccase and HBT on TCF eucalyptus pulp was found to remove hexenuronic acids by 23% and to significantly reduce brightness reversion (Cadena et al., 2010b). Moreover, it has been demonstrated that the reduction of HexA can be the consequence of the coupling of phenolic mediators to these structures (Cadena et al., 2011).

Despite the associated advantages of LMS, mediators are expensive and can generate toxic derivatives. There have been recent trends to using eco-friendly, potentially cost-effective alternative mediators such as naturally occurring phenols, which can be readily obtained from plants and waste pulping liquors or, directly, from fungal metabolism (Camarero et al., 2005; Moldes et al., 2008;

Vehviläinen et al., 2008; Aracri et al., 2009). Recently, phenolic compounds derived from lignin degradation (*e.g.* acetosyringone, syringaldehyde, ferulic acid, *p*-coumaric acid) have proved highly efficient natural laccase mediators for the removal of recalcitrant substances such as lignin (Camarero et al., 2007) and lipids (Gutiérrez et al., 2007) from pulp.

Cellulose modification

Cellulose oxidation can be induced by very different processes, e.g., radiation, energy impact or the application of oxidizing reagents (Potthast et al., 2007). These chemical oxidants can be divided into non-selective (nitrogen oxides (Butrim et al., 2007), alkali metal nitrites and nitrates (Painter, 1977), ozone (Johansson and Lind, 2005) and permanganates (Manhas et al., 2007); and selective, such as periodates (Fras et al., 2005; Calvini et al., 2006; Potthast et al., 2009) and nitroxyl radicals (de Nooy et al., 1996; Isogai and Kato, 1998; Vignon and Tahiri, 2000; Coseri et al., 2009, 2013; Biliuta et al., 2010). Periodates are specific oxidants able to oxidize the vicinal hydroxyl groups at carbon atoms 2 and 3 in an anhydroglucose unit (AGU) of cellulose, forming thus two aldehyde groups. The carbon carbon bond between the carbon atoms 2 and 3 is broken (Coseri et al., 2013). Nitroxyl radicals have been widely studied for catalytic and selective oxidation of primary hydroxyl groups of polysaccharides under aqueous conditions (Saito and Isogai, 2004). In particular, TEMPO-mediated oxidation is an efficient method for introducing carboxyl and carbonyl functional groups into cellulose in aqueous suspension (de Nooy et al., 1996; Saito and Isogai, 2004).

Two TEMPO-mediated oxidation systems have been reported so far: TEMPO/NaBr/NaClO system at pH 10-11 (de Nooy et al., 1996; Isogai and Kato, 1998; Saito and Isogai, 2006, 2007) and TEMPO/NaClO/NaClO2 system at pH 4-7 (Saito et al., 2009, 2010). NaClO and NaClO2 are used as the primary oxidants in each system. Therefore, reaction starts by the addition of the respective primary oxidants to the system which oxidize TEMPO to the corresponding oxoammonium cation, which is capable of direct and selective oxidation of primary alcohols to produce the corresponding aldehydes, with concomitant reduction of the oxoammonium species to hydroaxylamine. The catalytic cycle is closed by oxidation of the hydroxylamine to the oxoammonium species (de Nooy et al., 1996; Saito and Isogai, 2006, 2007; Isogai et al., 2010, 2011b; Okita et

al., 2010). When TEMPO/NaBr/NaClO is applied to native celluloses significant amounts of sodium carboxylate groups and small amounts of aldehyde groups are formed, which is usually accompanied by important depolymerization. On the other hand, TEMPO/NaClO/NaClO2 system suppressed the depolymerization of the oxidized cellulose, although the efficiency for the formation of carboxyl groups was somewhat lower (Isogai et al., 2011a).

TEMPO-mediated oxidation has been successfully exploited to improve various physical properties of pulp fibres including interfibre bonding —and hence the strength-related properties of the resulting paper. Improved wet strength in paper from TEMPO-oxidized fibres was recently reported in various studies and ascribed to the formation of large amounts of surface aldehyde groups as intermediate structures of the TEMPO-mediated oxidation process. Surface aldehyde groups can form interfibre covalent bonds through hemiacetal linkages with hydroxyl groups of adjacent fibre surfaces (Saito and Isogai, 2005, 2006).

As observed in literature, TEMPO-mediated oxidation is commonly carried out in the presence of NaClO/NaBr as a co-oxidizer system (de Nooy et al., 1996; Isogai and Kato, 1998; Bragd et al., 2001). However, environmental concerns have recently driven research interests into the development of halide-free oxidative systems. One promising approach for this purpose is the use of laccase together with oxygen as primary oxidants. The ability to use laccase to catalyse the regenerative oxidation of TEMPO has been demonstrated (Viikari et al., 1999; Fabbrini et al., 2001; d'Acunzo et al., 2002; Arends et al., 2006; Aracri et al., 2011, 2012; Patel et al., 2011; Aracri and Vidal, 2012; Xu et al., 2013; Jaušovec et al., 2014). Similarly to the NaClO/NaBr process, oxoammonium ion is regenerated *in situ*, so only oxygen is consumed in the course of the reaction. In addition to the environmental benefits associated with the use of an enzyme, this method provides the advantage of operating at near-neutral pH, which reduces the occurrence of β-elimination reactions and hence scission of the polysaccharide chain —a major drawback of the traditional method using pH 10–11 (Patel et al., 2011)(Figure 1-11).

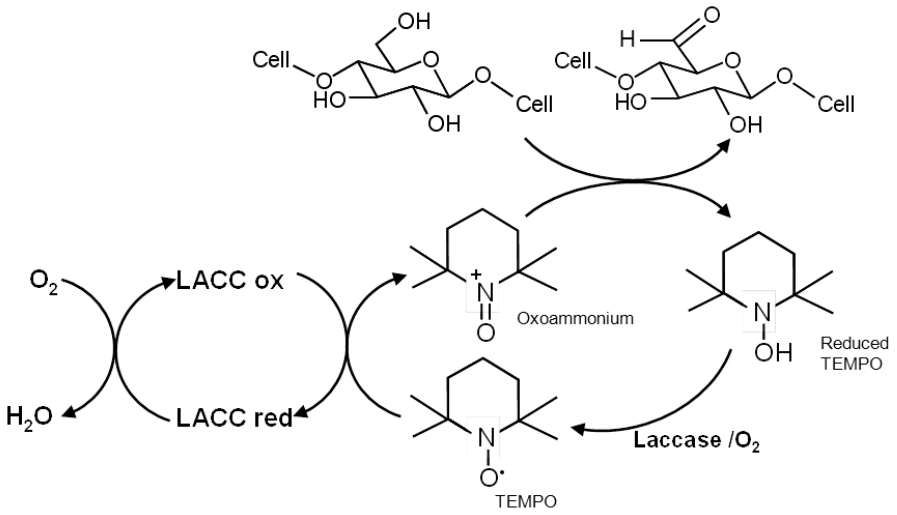

Figure 1-11 Proposed mechanism for the enzymatic production of oxoammonium ion (from Aracri 2012).

1.10.2 Cellulases

Cellulases are enzymes that hydrolyze the 1,4-β-D-glucosidic bonds of the cellulose chains up to glucose oligosaccharides. Microorganisms degrading cellulose usually have a complex enzymatic system generically named cellulase. There are three major groups of cellulases: endoglucanases (EC. 3.2.1.4), cellobiohydrolases or exoglucanases (EC. 3.2.1.91), and glucosidases (EC. 3.2.1.21). Endoglucanase cleave bonds randomly in the amorphous sites of the cellulose, which creates new chain ends and a rapid reduction in the DP (shorter chains). They usually produce a range of oligosaccharides of different size, and glucose. Cellobiohydrolases or exoglucanases (CBH) attack the reducing and non-reducing ends of the cellulose chains, generating mainly cellobiose units. This type of cellulase can also act on microcrystalline cellulose by a peeling mechanism. The action of cellobiohydrolases results in a rapid production of soluble sugars but slow decrease in the DP. Glucosidases act on cellobiose, which is two glucose units linked by a 1,4-β-D-glucosidic bond, generating glucose units (Lynd et al. 2002).

It has been suggested that there are three primary parameters that affect the degree of enzymatic hydrolysis: the crystallinity, the specific surface area and the degree of polymerization of the cellulose. Most cellulases consist of two

domains. The first is a catalytic domain, which is responsible for the hydrolysis of the cellulose chain. The second is a cellulose-binding domain (CBD), which helps the enzyme bind to the cellulose chain and bring the catalytic domain close to the substrate (Ek et al., 2009).

Concerning their application on cellulose fibers, cellulases have been mainly used for energy saving during fibers beating (i.e. biorefining) (Cadena et al., 2010c; Aracri and Vidal, 2012; Garcia-Ubasart et al., 2013a), biomass saccharification for biofuels production (Zhang et al., 2012) or for upgrading fibers through increasing cellulose reactivity. The effect of enzymatic treatments on cellulose reactivity has also been investigated. It has been reported that enzymatic treatments, especially using cellulases, hold great potential for increasing cellulose reactivity in dissolving pulps (Rahkamo et al., 1998; Henriksson et al., 2005; Engström et al., 2006; Kvarnlöf et al., 2006; Cao et al., 2007).

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Chapter 2

Objectives

The objective of this dissertation was to **up-grade unbleached softwood sulfite pulp to biobleached dissolving-grade pulp by means of laccase-based systems and endoglucanase treatments, and to develop cellulose fibers suitable for acetylation reactions.** The motivation of using this type of pulp was justified by the fact that dissolving pulp production is expected to keep building for the next years and also environmental concerns on cotton cultivation play an important role.

The first part of the investigation turns the attention to change the traditional bleaching processes for the introduction of new biotechnological technologies. In **Chpater 4** (**Paper I)**, the potential of natural and synthetic mediators for bleaching softwood sulfite pulp were examined. The best performing mediator was used for developing a biobleaching sequence to meet industrial requirements. **Chapter 5** (**Paper II**, continuation from Paper I) assesses the changes occurring in cellulose during biobleaching treatment and considers the resulted pulp to obtain dissolving-grade pulp.

The latter part of the thesis concentrates on cellulose modification by chemoenzymatic and chemical approaches. Mainly, the study wants to convert sulfite pulp to dissolving-grade by means of hydrolytic enzymes. The action of two different endoglucanases for improving cellulose accessibility and reactivity of biobleached sulfite pulp is reported in **Chapter 6** (**Paper IV)**. Furthermore, understanding cellulose transformation is also included. **Chapter 7** (**Paper III)** also explores the potential of two different endoglucanases on commercial dissolving pulp. Based on cellulose modification, **Chapter 8** introduces a biotechnological approach based on the oxidation of cellulose using the laccase

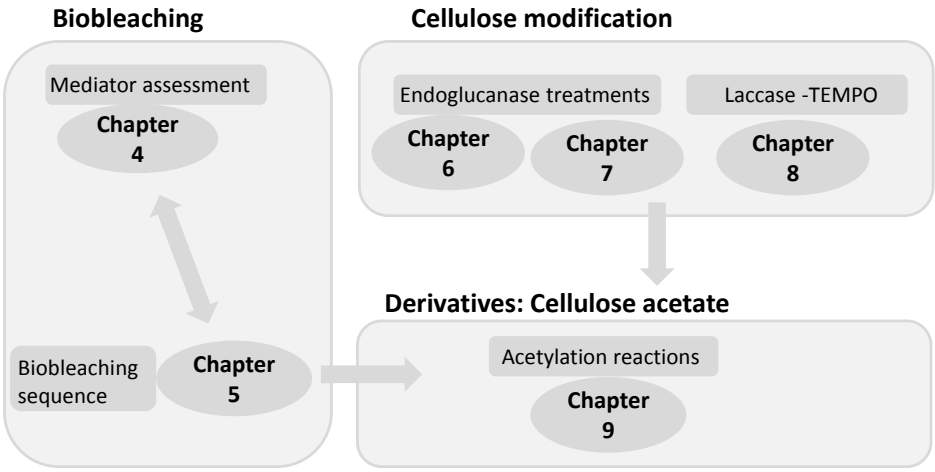
TEMPO-system. In the same line, **Chapter 9** explores cellulose modification by means of acetylation reactions. A comparison in terms of acetylation degree is conducted between commercial dissolving-grade pulp and biobleached dissolving-grade pulp.

As a conclusion, this dissertation presents the complete processing pathway from bleaching, purification and conversion of sulfite pulp to the obtention of a cellulosic product with new functional groups by means of enzymatic treatments.

Thesis format

Schema 2-1 represents an overview of the main themes of this work and summarizes the relations of the papers.

The experimental results of this thesis are presented as chapters based on papers published in scientific journals. This thesis basically consists of six publications:



Schema 2-1 Summary of this compilation dissertation and the publications resulted

Paper I:

Quintana E, Valls C, Vidal T, Roncero MB (2013) An enzyme-catalysed bleaching treatment to meet dissolving pulp characteristics for cellulose derivatives applications. Bioresource Technology 148:1–8. doi: 10.1016/j.biortech.2013.08.104

Paper II:

Quintana E, Valls C, Barneto AG, Vidal T, Ariza J, Roncero MB (2015a) Studying the effects of laccase treatment in a softwood dissolving pulp: Cellulose reactivity and crystallinity. Carbohydrate Polymers 119:53–61. doi: 10.1016/j.carbpol.2014.11.019

Paper III:

Quintana E, Valls C, Vidal T, Roncero MB (2015b) Comparative evaluation of the action of two different endoglucanases. Part I: On a fully bleached, commercial acid sulfite dissolving pulp. Cellulose 22:2067–2079. doi: DOI 10.1007/s10570-015-0623-1

Paper IV:

Quintana E, Valls C, Vidal T, Roncero MB (2015c) Comparative evaluation of the action of two different endoglucanases. Part II: On a biobleached acid sulphite pulp. Cellulose 22:2081–2093. doi: 10.1007/s10570-015-0631-1

Paper V:

Submitted to Carbohydrate Polymers. Quintana, E.; Roncero, M. B.; Vidal, T.; Valls, C. (2015). Cellulose oxidation by Laccase-TEMPO treatments.

Paper VI:

In Draft. Alternative chemo-enzymatic treatment for homogeneous and heterogeneous acetylation of fibers.

Chapter 3

Materials and Methods

3.1 Raw material

The raw material used in the present thesis was unbleached sulphite cellulose, cooked at Domsjö Fabriker mill (Sweden). The pulp was a mixture of 60% spruce (*Picea abies*) and 40% pine (*Pinus sylvestris*). Prior to use, fiber samples were conditioned at pH 4 adjusted with H_2SO_4, stirred at 2% (w/w) pulp consistency for 30 min and washed with de-ionized water in a glass filter funnel. This step was needed to remove contaminants and metals, and also to bring the pulp to the pH required for the enzyme treatments.

As a reference pulp, a commercial dried totally chlorine-free (TCF) bleached dissolving-grade pulp obtained from a mixture of 60% Norway spruce (*Picea abies*) and 40% Scots pine (*Pinus sylvestris*) and also supplied by Domsjö Fabriker mill (Sweden), was used. Characteristics of unbleached sulfite pulp and commercial dissolving pulp, and the chapter where each raw material is used are shown in Table 3-1.

Table 3-1 Characteristics of the raw materials used in the experiments

	Unbleached sulfite pulp	Commercial dissolving pulp
Glucan (%)	92.6 ± 0.04 / 88.5 ± 0.3	95.6 ± 0.3
Xylan (%)	6.6 ± 0.1* / 2.4 ± 0.4	0.8 ± 0.0
Galactan (%)	Nd / 3.0 ± 0.3	0.6 ± 0.3
Mannan (%)	Nd / 6.0 ± 1.3	2.8 ± 0.2
Other monosaccharides (%)	0.8 ± 0.02 / 0.3 ± 0.2	0.2 ± 0.2
Viscosity (mL/g)	511 ± 11	474 ± 0.7
ISO Brightness (% ISO)	61.25 ± 0.6	91.70 ± 0.15
Kappa number	4.2 ± 0.2	< 0.5
Chapter	4, 5 / 6, 9	7, 8, 9

* sum of xylan + mannan / Nd: Non-detected

3.2 Enzymes

3.2.1 Xylanase

A xylanase stage (X) was described as a pre-trial test to assess the efficiency of the enzyme on sulphite cellulose. The enzyme used was a commercial xylanase (Pulpzyme HC) supplied by Novozymes®. The X stage used 3 U/g odp xylanase at 10% consistency adjusted with Tris–HCl buffer at pH 7 at 50 °C for 2 hour in a polyethylene bag. After treatment, liquors were recovered and the resulting pulp was extensively washed as reported elsewhere for eucalyptus pulp (Valls et al., 2010a).

3.2.2 Laccase and mediators

The enzyme used was a commercial laccase from *Trametes villosa* (*Tv*L) which was supplied by Novozymes® (Denmark). Laccase activity was determined by monitoring the oxidation of ABTS 2,2'azino*bis*(3-ethylbenzthiazoline-6-sulphonate) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. One activity unit is defined as the amount of laccase required to convert 1 μmol/min of ABTS to its cation radical (ε_{436} = 29,300 M^{-1} cm^{-1}). Two synthetic compounds and two natural compounds, all purchased from Sigma-Aldrich, were used as mediators. The synthetic mediators were 1-hydroxybenzotriazole (HBT) and violuric acid (VA); and the natural mediators included syringaldehyde (SA) and *p*-coumaric acid (*p*CA).

3.2.3 Endoglucanase

B endoglucanase (EC. 3.2.1.4), produced from the newly identified species *Paenibacillus barcinonensis* by Universitat de Barcelona (Spain) (Chiriac et al., 2010) was used. This endoglucanase is a modular enzyme with the structure GH9-CBM3c-Fn3-CBM3b (E1), a truncated derivative of the cellulase with the structure GH9-CBM3c (E2), and a recombinant cellulose binding module CBM3b (CBD) derived from the enzyme. E1 and E2 exhibit cellulase activity as they contain the catalytic module GH9, whereas CBD has no hydrolytic activity on cellulose (Chiriac et al., 2010, 2013). B is a family 9 enzyme and shows a processive mode of action. A cellulase activity of 60 CMCase U/mL was determined by measuring the amount of reducing sugars released from carboxymethyl cellulose (CMC, Sigma) according to Somogyi–Nelson method (Spiro, 1966). An activity unit (U) is the amount of enzyme capable of

converting 1 μmol of substrate per second. Another endoglucanase (F), supplied by Fungal Bioproducts® (Spain) and produced from *Cerrena unicolor* was used. The activity as U/g dry enzyme powder of the cellulase preparation was: 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the cellulase, respectively. The activity was also determined in our laboratory using the Somogyi–Nelson method.

3.3 Bleaching assays

Bleaching treatments were performed in different chapters of this thesis. Same experimental conditions were applied for enzymatic stage (L), but different experimental conditions were used for hydrogen peroxide stage.

3.3.1 Laccase-mediator treatments

Laccase-mediator treatments, at 5% pulp consistency, were conducted in an oxygen pressurized reactor (0.6 MPa), at stirring rate of 30 rpm, using 50 mM sodium tartrate buffer at pH 4, a dose of 20 U/g odp (oven dried pulp) of laccase and 1.5% odp of each mediator (HBT, VA, SA and *p*CA) at 50 ºC for 4 h. A few drops of the surfactant Tween 20® (0.05% w/v) were also added. Once treated, each pulp was filtered in fritted glass funnel (porosity grade 2, 40-100 μm), its residual liquor collected and the pulp extensively washed with de-ionized water. Control treatment was prepared using the same conditions, but with no enzyme addition.

3.3.2 Bleaching treatment

Different experimental conditions were studied with the purpose to improve the efficiency of hydrogen peroxide stage and obtain better bleaching results. From less to more optimized stage, the conditions are described as follows:

Hydrogen peroxide treatment (P stage)

In Chapter 4, the P stage was carried out at 5% consistency, with 2% odp H_2O_2, 1.5% odp NaOH, 1% odp DTPA (diethylenetriaminepentaacetic acid) and 0.2% odp $MgSO_4$ in a Datacolor Easydye AHIBA oscillating individual reactor at 90 ºC for 2 h. Residual liquors were collected for subsequent analysis, and pulp samples were filtered in fritted glass funnel (porosity grade 2, 40-100 μm) and extensively washed for further processing.

Oxygen-reinforced hydrogen peroxide treatment (PO/P stage)

In Chapter 4 and Chapter 5, the hydrogen peroxide treatment was conducted in multiple sequential steps. PO/P was carried out at 5% consistency in oxygen pressurized (0.6 MPa) reactor, using a stirring rate of 30 rpm under the following conditions: 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$ at 90 ºC for 4 h. This stage was performed in three consecutive steps (PO_1 = 1 h reaction, PO_2 = 1 h reaction, PO_4 = 2 h reaction) each involving the addition of 10% odp H_2O_2 and no interstep washing. In the last P stage, pressure was released and the temperature was maintained at 90 ºC for 2 additional hours without addition of H_2O_2. These last two hours of treatment were performed in order to examine the potential influence of residual hydrogen peroxide on the extent of fiber bleaching. A small amount of pulp was removed after each Po step, filtered, residual liquors collected and then extensively washed with deionized water in a filter funnel for subsequent analysis.

In Chapter 6, PO conditions were slightly modified and an individual stage was run. The treatment was carried out at 5% (w/w) consistency in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm under the following conditions: 10% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$, at 90 ºC for 1 h. Subsequently, the treated pulps were extensively washed with de-ionized water and stored until use.

In Chapter 9, hydrogen peroxide dose was reduced and PO stage was conducted in two sequential steps. PO was carried out at 5% (w/w) consistency in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm under the following conditions: 3% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$, at 90 ºC for 1 h. Treated pulps were extensively washed with deionized water and after that another hydrogen peroxide stage reinforced with oxygen was applied. The treatment was performed under same conditions described above but 2.5% odp H_2O_2 was used and 4 hour of reaction. Chemical stage was finished by washing pulp with deionized water.

Chelating treatment (Q stage)

The Q stage involved the use of chelating agents to reduce the contents in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) capable of degrading the bleaching agents and cellulose during the subsequent peroxide bleaching treatment (Heijnesson et al.,

1995). The Q stage was performed with 1% odp DTPA at 5% consistency at pH 5–6 (adjusted with H_2SO_4 1 N) in polyethylene bags at 85 ºC for 1 h.

A summary of the sequences followed in each chapter is shown in Table 3-2.

Table 3-2 Description of the sequence studied in each chapter

Chapter	Sequence
4	LP, LQPO/P
5	LQPO/P
6	LPO
9	LPOPO

3.4 Endoglucanase assays

Two different endoglucanases, cellulase B and cellulase F, were used at different doses, namely: 120, 12 and 60 U/g (odp), corresponding to samples B120, F12 and F60, respectively. All enzyme treatments were carried out at 10% (w/w) consistency in 0.05 M sodium acetate buffer (pH 5.5) at 55 ºC for 1 h (Cadena et al., 2010c). According to the raw material employed, two different experimental setups were used. Treatments involving commercial dissolving pulp were performed in Ahiba easydye reactor equipped with closed 250 mL vessels. Treatments using biobleached sulphite pulps were conducted in polyethylene bags acting as a reactor in a thermostatic water bath and the agitation was done manually every 10 minutes. In both treatments, the reaction was stopped by washing the pulp with de-ionized water in a porous glass filter funnel of porosity grade 2 (40-100 μm). A control treatment (K) using the same conditions as the enzymatic treatment but in the absence of enzyme was also developed.

A cold caustic extraction (CCE) was performed before or after the enzymatic hydrolytic treatment for further hemicellulose removal. The treatment was conducted in an Easydye Ahiba oscillating individual reactor from Datacolor. The treatment was performed at 10% (w/w) consistency adjusted with 9% (w/v) NaOH at 25 ºC for 1 h. Treated pulps were washed with deionized water in a porous glass filter funnel of porosity grade 2 (40-100 μm), until the filtrate pH was neutral.

3.5 Laccase-TEMPO oxidation assays

Prior to oxidative treatments, part of the starting pulp was refined at 5000 revolutions using a PFI mill according to ISO 5264, and the rest was used as received with no refining step.

Laccase-TEMPO oxidation treatments were performed at room temperature, at 5% consistency in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 and 20 U/g odp laccase. Different experimental conditions such as, TEMPO dose, reaction time and presence or absence of oxygen pressure were tested in order to provide the greatest increases in carboxyl and aldehyde groups, and as a result improve wet and dry tensile strength. The specific conditions used for each treatment are described in Table 3-3. Pulp samples treated under identical conditions as chemoenzymatic treatment but in the absence of TEMPO or laccase and TEMPO, were used as controls. After treatment, each pulp was filtered in a porous glass filter funnel of porosity grade 2 (40-100 μm) and washed with de-ionized water until a colourless, neutral filtrate was obtained.

Table 3-3 Conditions used for laccase-TEMPO treatments

Sample ID	Pulp Refined at 5000 rev	TEMPO dose (% odp)	Applied O_2 pressure (MPa)	Time (h)
Lac-T 2%, no O_2, 8h	-	2	-	8
Lac-T 8%, no O_2, 8h	-	8	-	8
Lac-T 8%, 8h	-	8	0.6	8
R+Lac-T 8%, 8h	✓	8	0.6	8
R+Lac-T 8%, 20h	✓	8	0.6	20
R+Cont-Lac, 20h	✓	-	0.6	20
R+Cont-Buffer, 20h	✓	-	0.6	20

3.6 Acetylation reactions

3.6.1 Heterogeneous reactions

2.0 g odp of each type (*Com, L_F* or *L_CCE_F*) was disintegrated and then filtered using a filter paper (Whatman 1) for complete removal of water. The samples were then placed in a glass beaker containing a mixture of 20 mL of acetic acid and 35 mL toluene. The dispersion was stirred for 5 min and 0.2 mL sulfuric acid was added. Then a desired amount of acetic anhydride was added,

and the mixture was stirred for 1 h at room temperature. The specific conditions for acetylation reactions were as follows: 0.53, 2.67, 5.35 and 10.7 g Ac_2O per weight (g) of dried pulp sample (Table 3-4). The reaction was quenched adding 6 mL of distilled water and ethanol at a proportion of 3:7 v/v. The mixture was allowed to stand for 20 min and then washed 3 times with methanol and finally with water until neutral pH.

Table 3-4 Conditions used for acetylation reactions depending on the activity or concentration of acetic anhydride (Ac_2O)

Weight of Ac_2O (g) / weight of dried sample (g)	Nomenclature	*L_F*	*L_CCE_F*	*Com*
0.53 (~1mL Ac_2O)	minimal	✓	Not performed	✓
2.67 (~5 mL Ac_2O)	low	✓	✓	✓
5.35 (~10 mL Ac_2O)	medium	✓	✓	✓
10.7 (~20 mL Ac_2O)	high	✓	✓	✓

Com: Dried-commercial dissolving grade pulp
L_F: Biobleaching treatment (Laccase stage + PO/PO stage) + endoglucanase treatment
L_CCE_F: Biobleaching treatment (L stage + PO/PO stage) + cold caustic extraction + endoglucanase

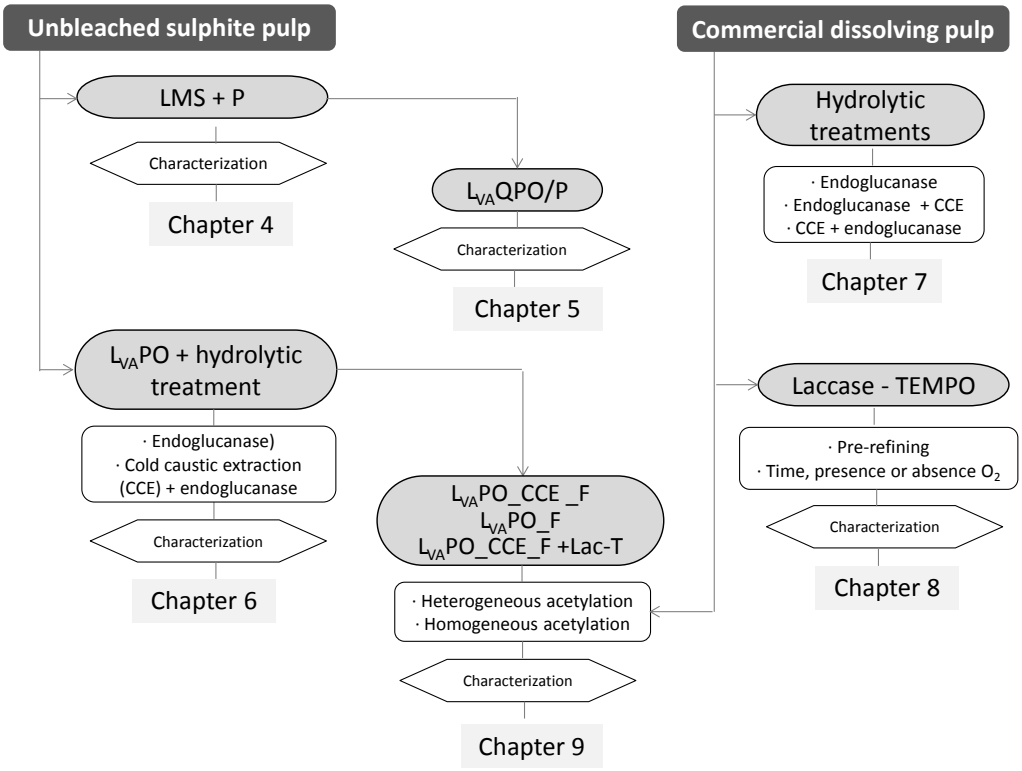
Fibers obtained by surface acetylation were used for preparing handsheets. For sheet manufacture 1g of each sample at 1% solids was disintegrated and poured into an over-pressurized device (< 1 bar pressure difference) allowing few minutes drainage to obtain a web or handsheet of the acetylated fibers. The device was equipped with open mesh fabric screen (Sefar Nitex 03-10/2, mesh opening of 10 µm with open area of 2%) to remove the excess water and retain the fibrils. The webs were pressed between two blotting papers using a metal roller (10 kg) and then dried at 80 °C, for 1 h in a tumble drier. The obtained sheets were then stored in a conditioned room (23 °C and 50% relative humidity) until further use.

3.6.2 Homogeneous reactions

2.5 g odp of respective pulp type (*Com, L_F* or *L_CCE_F, L_CCE_F+Lac-T*) was disintegrated and then filtered using a filter paper for removal of water. Then, 50 mL of acetic acid was added to the sample, stirred 5 min and then filtered. This step was done by duplicate. After filtration, 45 mL of acetic acid and 0.25 mL sulfuric acid was drop off to the sample and stirred 1 min. Then,

5.35 g Ac_2O/g dried pulp (~12.5 mL Ac_2O) was added and continuously stirred for 30 min at room temperature. The reaction was quenched with the addition of 6.25 mL of distilled water and acetic acid at a ratio of 3:7 v/v, respectively. Finally, cellulose acetate (CA) was obtained by pouring the viscous reaction mixture into distilled water obtaining a continuous droplet and with constant stirring. With precipitation, cellulose acetate was regenerated. The obtained product was washed with distilled water until neutrality and subsequently dried using a freeze-drying.

A summary of the treatments conducted in each chapter are shown in Schema 3-1.



Schema 3-1 General overview of the work plan of the thesis

3.7 Cellulosic fiber characterization

3.7.1 Fiber chemical properties

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).

Viscosity

Viscosity measurements were used to assess changes in the degree of cellulose polymerization. Pulp viscosity (as intrinsic viscosity of a sample of cellulose dissolved in a dilute solution of cupriethylenediamine) was determined in accordance with ISO 5351:2001. In the tests described in Chapter 7, borohydride viscosity was measured in addition to standard viscosity. This involved treating oxidized pulp samples with sodium borohydride prior to measurement in order to inactivate carbonyl groups formed by reduction to hydroxyl groups in cellulose and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium. The treatment was performed in polyethylene bags, using pulp at 5% consistency in the presence of 2% $NaBH_4$ at room temperature for 30 min (Roncero et al., 2003), with manual stirring. Viscosity measurements were made in duplicate.

The degree of polymerization (DP) was calculated from intrinsic viscosity values, using the equation of Evans & Wallis (1987) (SCAN-CM 15:88):

$$DP^{0.85} = 1.1 \cdot [\eta]$$

Eq. 3-1

Pulp degradation can also be assessed via the number of scissions in the cellulose chain (CS), which is defined mathematically as (Bouchard et al., 2000):

$$CS = \frac{DPo - DP}{DP}$$

Eq. 3-2

where DPo is the degree of polymerization of the initial pulp or previous stage and DP that at the end of any chemical or enzymatic treatment.

Carbohdyrate composition

Carbohydrate composition of pulps was determined using high performance liquid chromatography (HPLC). Samples were studied on a duplicate basis using a modified version of TAPPI 249 cm-09 test method. Hydrolysis was carried out in two steps: i) A strong hydrolysis step with concentrated sulfuric acid. Approximately 50 mg of sample with known moisture content were treated with 5mL of H_2SO_4 72% and kept at 30 ºC for 1h with gentle stirring. ii) A mild acid step at high temperature. Tube contents were putted into 250 mL-flasks and diluted to 4% H_2SO_4. Flasks were putted into an autoclave for 1 h at 103 kPa. Solution was then cooled and passed through a glass filter to remove insoluble lignin. Prior to HPLC analysis samples were filtered using a 0.45 μm pore size Whatman membrane. Chromatographic analysis was performed using a 1200 Agilent HPLC instrument furnished with a Biorad Aminex HPX-87H ion-exchange column. Data was collected by the refractive index detector (RID). Concentrations were calculated by interpolation into calibration curves ran from standards of glucose, xylose, rhamnose and arabinose. In order to resolve xylose, mannose and galactose peaks, the hydrolyzed effluents were neutralized with barium carbonate ($BaCO_3$), then were filtered through a membrane of 0.45 μm pore size and then were analyzed with a Biorad Aminex HPX-87P column. The chromatographic determination was performed under the following conditions: mobile phase 6 mmol/L (acid samples) or ultrapure water (neutralized samples); flow rate, 0.7 mL/min; column temperature, 60 ºC (acid samples) or 85 ºC (neutralized samples).

Alpha-, beta- and gamma-cellulose

α-, β- and γ-cellulose was determined according to TAPPI method T 203 cm-09. Biobleached pulp samples were extracted with 17.5% and 9.45% sodium hydroxide solutions at 25 ºC. The soluble fraction, consisting of β- and γ-

cellulose, was analyzed volumetrically by oxidation with potassium dichromate, and the α-cellulose, as an insoluble fraction, was calculated by difference.

Alkali solubility

Alkali resistance (R10 and R18) was determined according to TAPPI standard T 235 cm-09. All determinations were performed in duplicate.

Fock solubility

The cellulose solubility of the pulp samples was determined according to slightly modified version of Fock's method (Fock, 1959; Ibarra et al., 2010a). This is a micro-scale method simulating the industrial viscose process for manufacturing regenerated cellulose. Prior to analysis, the samples were dried at 50 ºC and conditioned in a climate room at 23 ºC and 50% RH overnight. In the first step, cellulose was soaked in an alkali medium and then converted into cellulose xanthate with CS_2, which makes the cellulose polymer soluble. Then, $-CS_2$ groups were removed in dilute sulphuric acid to obtain re-precipitated cellulose fibres. Finally, regenerated cellulose was oxidized with $K_2Cr_2O_7$, refluxed and titrated with $Na_2S_2O_3$. Fock solubility was expressed as regenerated cellulose yield (Eq. 3-3):

$$R_{FS} = 100 \cdot 9.62^{a} \frac{M(V_1 C_1 - (V_2 C_2 100/40^{b}))/6}{4Y}$$

Eq. 3-3

where R_{FS} denotes reacted cellulose (%), Y sample weight (g), M the molecular mass of glucopyranosyl residues ($C_6H_{10}O_5$, 162 g/mol), V_1 the volume of titrant ($Na_2S_2O_3$, L), C_1 the concentration of $K_2Cr_2O_7$ (mol/L), C_2 that of $Na_2S_2O_3$ (mol/L), a the first dilution to 100 g and withdrawal of 10 mL (10.4 g) = 100/10.4 = 9.62, and b the second dilution of the sample to 100 mL and withdrawal of 40 mL = 100/40. The respective reactivity results were calculated from 4 replications per sample.

Metal ions

The determination of calcium, iron and manganese in pulps was conducted by atomic absorption spectroscopy according to SCAN-CM 38:87 and was carried out in duplicate. The samples were incinerated and charred at 575 °C for

a period of 3 h. The charred residue was treated with 6 M hydrochloric acid and the acid-soluble element content in the solution was determined by flame atomic absorption spectroscopy. Previously to the measurements, standard solutions of calcium, iron and manganese at five different concentrations were used for the construction of the calibration graph of each metal.

Zeta potential

Zeta potential of fibers was determined using a streaming potential detector (SZP-06, Mütek, Germany). Samples were adjusted to a consistency ≤ 3% (w/w), then disintegrated and adjusted to 0.1 mS/cm with KCl 1M. Fiber suspension was sucked into the suction tube by applying vacuum and formed a pad of fibers in the measuring cell. The flow past the fiber pad moved mobile charges from the shear plane (viz. 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 fibre pad. Zeta potential was calculated from the Smoluchowski equation (Cadena et al., 2009) (Eq. 3-4):

$$Zeta\ potential\ \ mV\ = \frac{\Delta U}{\Delta p} \cdot \frac{\eta \cdot L}{\varepsilon \cdot \varepsilon_o \cdot Q \cdot R}$$

Eq. 3-4

where ΔU is the streaming potential, Δp the pressure difference, η is the viscosity, L/Q the cell constant, R the electric resistance and ε and ε_0 the dielectric constant and electric permittivity of vacuum, respectively.

NMR

^{13}C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ^{13}C. Samples were immersed in deionized water for at least 2h. All measurements were performed at 290 ± 1K. The magic angle spinning (MAS) rate was 4 kHz. The cross-polarization contact time was 1 ms and the recycle delay time 2.5 s. Acquisition time was 98.3 ms and sweep-width was 31.2 kHz. The number of scans was 5100. The quantification method was based on the evolution of two independent and isolated signal arising from cellulose I and II, respectively: the decrease of the

height of the peak near 65 ppm, assigned to the C6 in the crystalline part of cellulose I and the increase of the peak near 107 ppm, assigned to the C1 in the crystalline part of cellulose II (Janzon et al., 2008a, 2008b; Arnoul-Jarriault et al., 2014).

Bulk acid content

The bulk acid group content was measured by conductimetric titration according to Katz et al., (1984). An amount of 1.50 g odp was stirred in 300 mL of 0.10 M HCl for 1 h, followed by rinsing with de-ionized water in a finely fritted funnel. The sample was then resuspended in 250 mL of 1.00 mM NaCl, spiked with 1.5 of 0.10 M HCl and titrated against 0.05 M NaOH in 0.25 mL increments, with conductivity measurement after each addition. Titration data were plotted in conductivity versus volume graphs in order to determine the milliequivalents of acid groups present in each gram of pulp. The points at which conductance remained constant were quantified on the graph and divided by the weight of pulp sample in order to determine the milliequivalents of acid groups. The carboxylic acid content (COOH) of pulp fibers was obtained from the following equation:

$$COOH = \frac{B - A \ \cdot C_{OH}}{m_{dry}}$$

Eq. 3-5

where (B–A) is the volume of sodium hydroxide solution consumed at the second intersection point, C_{OH} the concentration of the sodium hydroxide and m_{dry} the oven-dry weight of sample. All reported results were the averages of two measurements.

Aldehyde group content

The aldehyde group content of a sample was determined by further oxidation with $NaClO_2$ for selective conversion of aldehyde groups into carboxyl groups. Sample (3.5 g odp) was treated with 350 mL solution of 0.3 M $NaClO_2$ at pH 4–5 at room temperature for 48h. The carboxyl content was determined with the above-described conductimetric titration method. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde

groups originally present in the pulp (Saito and Isogai, 2005; Aracri and Vidal, 2012).

TGA

Thermogravimetric runs were carried out on a Mettler Toledo TGA/SDTA851e/LF1600 thermobalance, using samples of ca. 5 mg. Pyrolysis and combustion runs were performed in nitrogen and synthetic air (N_2:O_2 4:1), respectively. Samples were heated from 25 ºC to 900 ºC at a rate of 5, 10 or 20 ºC/min.

XRD

XRD measurements were performed on a SIEMENS D-500BRAG-BRENTANO θ/2θ geometry X-ray diffractometer with Cu $K\alpha_1$1radiation (λ = 0.15418 nm) at 40 kV and 30 mA. A divergence aperture of 0.3° and a reception aperture of 0.05° were used. Sweeps of 5° to 5° 2θ were made with a step size of 0.05° and a step time of 10 s. The experimental XRD signal was fitted to Gaussian distributions, which include an amorphous background. Pulp crystallinity was assessed from the ratio between the area of the crystalline cellulose peaks and the total area, which included the amorphous background contribution (Andersson et al., 2004).

3.7.2 Physical and morphological properties

Optical properties

Fiber optical properties were measured in terms of brightness (ISO 2470:2009) and in terms of the CIE L^* a^* b^* color coordinates, namely: lightness (L^*), red–green (a^*) and yellow–blue (b*). Chroma (C^*), which is the perpendicular distance of a point from the lightness axis [$C^* = (a^{*2} + b^{*2})^{1/2}$] and represents the amount of color "saturation" of a sample, was also used to characterize the bleaching process.

Moist heat aging

Biobleached fibre samples were subjected to accelerated thermal ageing treatment by moist heating (at 80 ºC and 65% RH) in a HC 2020 Heraeus-Vötsch climatic chamber according to ISO 5630-3:1996. The resulting Brightness Loss Index (BLI) was calculated from the following Eq. 3-6, where Br was the brightness value:

$$BLI \ \% \ = \frac{Br_{0h} - Br_{144h}}{Br_{0h}}$$

Eq. 3-6

Fiber analysis

Morphological properties of fibers such as length, width, and curl, as well as the content of fines in a pulp specimen, were determined with the T 271 om-02 method on a Metso KajaaniFS300 fiber analyzer. Measurements technique is based on the ability of fibers to change the direction of polarized light. An amount of 100 mg of pre-soaked sample was disintegrated with a KajaaniFS300 disintegrator to ensure complete individualization of fibers. The sample was then diluted to a final volume of 4 L in de-ionized water. A volume of 50 mL of diluted suspension was then taken by using a pipette with a tip opening at least 2 mm in diameter and placed in a beaker for analysis. The instruments used provided different types of averages for each target property (numerical, length-weighted and weight-weighted). In this thesis, fiber length is expressed as numerical (a) and length-weighted fiber length (b) (Eq. 3-7):

$$\text{(a) } L = \frac{\sum n_i l_i}{\sum n_i}$$

$$\text{(b) } L_l = \frac{\sum n_i l_i^2}{\sum n_i l_i}$$

Eq. 3-7

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

Water retention value (WRV)

Water retention value was determined according to ISO 23714.

Scanning Electron Microscope (SEM)

Surface and cross-sectional SEM images of the handsheets were taken on a JEOL JSM-6400 microscope. Samples were placed on the SEM sample holding stub with the aid of conductive double side sticky carbon film and coated with Au/Pd alloy prior to analysis.

Light microscopy

Individual fibers were analyzed by light microscopy according to ISO 9184-3:1990. Images were captured with a DeltaPix (Infinity X) digital camera fitted to a microscope. The procedure was as follows: a tiny amount of pulp fibers was stained with 1-2 drops of Herzberg stain (a solution of zinc chloro-iodide) and stained fibers were individualized by means of awls for examination.

Physical properties

Handsheets were prepared according to ISO 5269-2:2004 on a Rapid-Köthen laboratory former. For pulp refining, pulp samples were disintegrated for 30000 revolutions, refined for 5000 revolutions according to ISO 5264-2:2011.

Mechanical properties were performed in accordance with the standards in brackets as follows: dry tensile strength (ISO 1924-3:2005), wet tensile strength (ISO 3781), tearing resistance (ISO 1974:1990), burst strength (ISO 2758:2001) and air permeance (ISO 5636-5:2003). The wet zero span tensile strength (WZSTS) was measured according to T-273 pm-95 and ISO 15361:2000, respectively, on a Zero-Span 1000 Pulmac tester, using strips previously soaked in de-ionized water for 5 s. Wet tensile strength was measured according to ISO 3781:1983, using strips previously soaked in de-ionized water for 5 s.

3.7.3 Acetylated fibers

Handsheets from surface acetylated fibers and tensile strength

Fibers obtained by surface acetylation were used for preparing handsheets. For sheet manufacture 1g of each sample at 1% solids was disintegrated and poured into an over-pressurized device (< 1 bar pressure difference) allowing few minutes drainage to obtain a web or handsheet of the acetylated fibers. The device was equipped with open mesh fabric screen (Sefar Nitex 03-10/2, mesh opening of 10 μm with open area of 2%) to remove the excess water and retain the fibrils. The webs were pressed between two blotting papers using a metal roller (10 kg) and then dried at 80 °C, for 1 h in a tumble drier. The obtained sheets were then stored in a conditioned room (23 °C and 50% relative humidity) until further use.

Wet and dry tensile strength of the surface acetylated sheets were measured on a MTS 400/M Vertical Tensile Tester equipped with a 50 N load cell, in accordance with ISO 1924-3:2005.

Film casting preparation and tensile strength

Cellulose acetate (CA) obtained from homogeneous acetylation reaction was used for preparing transparent films by means of a casting technique. Dried cellulose acetate was dissolved in specific amount of acetone in order to obtain a concentration of 8 wt%. The solutions for film casting were firstly centrifuged at 6000 rpm for 10 min. The supernatant was carefully transferred and centrifuged again at 2000 rpm for 5 minutes. The films were cast by pouring the transparent solution on glass plates, well distributed and followed by drying in a vacuum desiccator for at least 2h. The film samples were finally kept in a desiccator.

Tensile strength tests for CA films resulted from homogeneous acetylation reactions were performed on a MTS 400/M vertical Tensile Tester, with a cross-head speed of 40 mm/min. Specimen strips presented 10 mm width and 40 cm length.

Fourier Transform Infrared Spectroscopy (FTIR)

Acetylated fibers (surface acetylation) were analyzed by Fourier transform infrared spectroscopy (FTIR) by using a Nicolet Avatar 360 spectrophotometer (Nicolet Instrument Corporation). The samples were prepared by mixing 1 mg of fiber mat in a matrix of 300 mg of KBr followed by pressing. The spectrum was recorded in the range of 400–4000 cm^{-1} and 32 scans were run at 4 cm^{-1} resolution. The surface of films (homogeneous acetylation) were directly analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory by performing 32 scans at 1 cm^{-1} intervals over the wavenumber range 4000–400 cm^{-1}. The measuring cell was washed with de-ionized water and ethanol between measurements.

Acetyl group content

The acetyl group content was calculated according to ASTM D871-96. Firstly, the respective acetylated samples were ground and 100 mg odp were weighed accurately and put into 20 mL of 75% v/v of ethanol in an Erlenmeyer flask. The bottle, loosely stoppered, was heated to 50-60 ºC for 30 min for better

swelling of the material. Then, 20 mL of 0.5 N NaOH solution was added to the sample and the mixture was heated to 50-60 ºC for 15 min. A blank was also conducted but in absence of sample. The flasks were stoppered tightly and allowed to stand at room temperature for 72 h. The excess alkali was then titrated with 0.5 N HCl using phenolphthalein as indicator. An excess of about 1 mL of 0.5 N HCl was added and allowed the NaOH to diffuse from the regenerated cellulose overnight. The small excess of HCl was titrated with 0.5 N NaOH to a phenolphthalein end point. The percentage of acetyl groups was calculated as follows:

$$Acetyl\ groups, \% = \quad D - C\ Na + \ A - B\ Nb\ \cdot (F/W)$$

Eq. 3-8

where: A = NaOH solution required for titration of the sample (mL); B = NaOH solution required for titration of the blank (mL); Nb = Normality of the NaOH solution; C = HCl required for titration of the sample (mL); D = HCl required for titration of the blank (mL); Na = Normality of the HCl solution; F = 4.305 for acetyl; W = sample used (g)

Contact angle

The contact angle was determined using the sessile drop method. This method consists in dispensing a water drop of about few microliters from the needle of a precision syringe, and slowly approach the solid material (from which the surface free energy value is required) to the surface of the drop until the contact between them is produced. The contact angle is the initial angle formed between the tangent to the drop's profile and the tangent to the surface at the intersection of the vapor, the liquid and the solid. Water contact angle of surface acetylated sheets was measured using a Dataphysics OCA15EC contact angle goniophotometer (Dataphysics, USA), using an image capture ratio of 25 frames/s. A 4 μL water drop was dropped to the sample surface. A minimum of ten readings were taken on every sample to reduce possible influence of the heterogeneity of the surface. Also, changes in contact angle were monitored until complete absorption of each water drop.

Water drop test (WDT)

The water drop test was performed according to TAPPI standard T835 OM-08. The WDT consists in placing a drop of deionized water on the surface of paper and recording the time needed for complete absorption, which is signaled by the vanishing of the specular gloss of the drop.

3.7.4 Effluent characterization

Chemical Oxygen Demand (COD) and Color

COD and color were measured according to ASTM 1252-06 and 1209-05, respectively, on duplicate samples. Absorbance data were recorded at 600 nm for COD and 465 nm for color. A Thermo Scientific Evolution 600 spectrophotometer was used in both cases.

Toxicity

Effluent toxicity was determined with the Microtox method, using the marine luminescent bacterium *Vibrio fischeri* in accordance with UNE-EN ISO 11348-3: 1999 on a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental). The difference between the amount of light emitted before and after addition of the sample was used to determine its toxicity. In order to prevent pH effects, samples were adjusted to pH 6–8 with NaOH/HCl solution. Ecotoxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed in toxicity units (TU). The reference toxicant $ZnSO_4 \cdot 7H_2O$ was used to control *Vibrio fischeri* batch quality in accordance with the Basic Test procedure. Toxicity measurements were color-corrected as per the recommendations of the equipment manufacturer. Toxicity tests were conducted in duplicate or triplicate.

Residual laccase activity

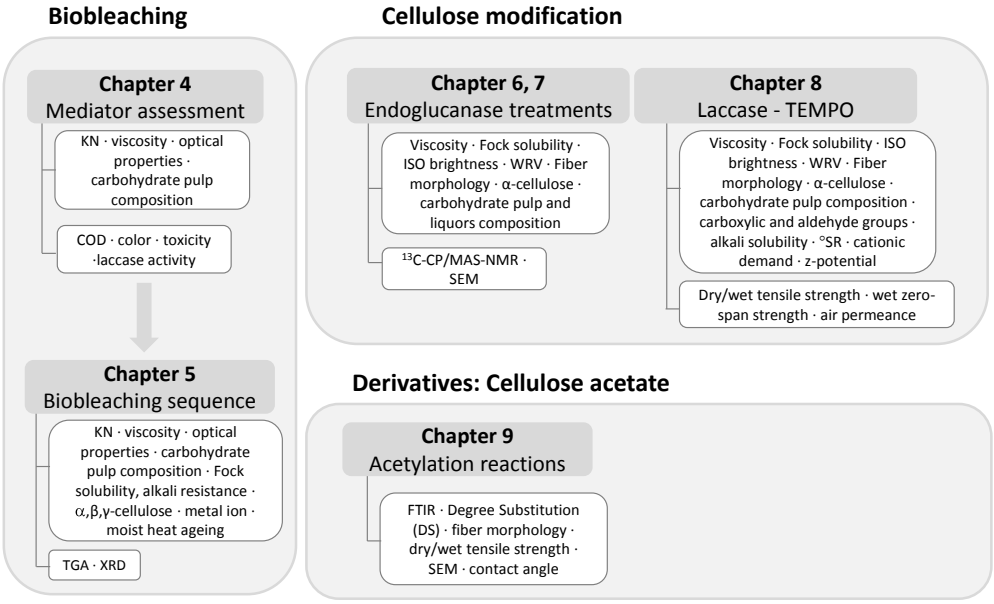
Residual laccase activity in the effluents was measured after filtration of each pulp slurry treated in the reactor and according to the procedure described in section 3.2.2. Residual laccase activity values were corrected for the dilution factor and expressed as percentages of the initial laccase rate.

Released reducing sugars

Dissolved carbohydrates present in the liquors released from the cellulase treatments of biobleached dissolving pulps were quantified by HPLC (Agilent 1200 HPLC instrument), using a Biorad Aminex HPX-87H ion-exchange resin column that affords the separation of glucose, xylose and arabinose. Prior to analysis, the liquors were subjected to acid hydrolysis with 4% H_2SO_4 and the flasks placed in an autoclave at 103 ± 7 kPa for 20 min in order to convert oligomers into monomers for easier on-column separation. Then, the hydrolysed solutions were filtered through Whatman membranes of 0.45 μm pore size and the chromatographic determination was performed under the following conditions: mobile phase, 6 mmol/L sulphuric acid; flow rate, 0.7 mL/min; column temperature, 60 ºC.

The concentration of oligosaccharides present in the treatment liquors was also determined by HPLC, using a Biorad Aminex HPX-42A ion-exchange column (Garcia-Ubasart et al., 2013a). Effluents were filtered using a 0.45 μm pore size Whatman membrane and neutralized to pH 7 using HCl or NaOH solution. The determination was performed under the following conditions: mobile phase, ultrapure water; flow rate, 0.35 mL/min; column temperature, 65 ºC. 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).

The methods used in each chapter are summarized in Schema 3-2.



Schema 3-2 Summary of the different characterization methods used in the thesis.

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Chapter 4

An enzyme-catalyzed bleaching treatment in sulfite cellulose fibers

Abstract

Bleached cellulose with good end-properties (~90% ISO brightness and 62% cellulose preservation) was obtained by using a totally chlorine-free (TCF) biobleaching process. Unbleached sulfite cellulose was treated with *Trametes villosa* laccase in combination with violuric acid. This enzymatic stage (L) was followed by a chelating stage (Q) and then by a hydrogen peroxide stage reinforced with pressurized oxygen (PO), resulting to an overall LQPO sequence. The use of violuric acid was dictated by the results of a preliminary study, where the bleaching efficiency of various natural (syringaldehyde and *p*-coumaric acid) and synthetic (violuric acid and 1-hydroxybenzotriazole) mediators were assessed. The outstanding results obtained with laccase-violuric acid system fulfil most of the characteristics of commercial dissolving pulp, totally acceptable for viscose manufacturing or carboxymethylcellulose (CMC) derivatives, with the added advantage that the enzymatic treatment saved 2 h of reaction time and about 70% of hydrogen peroxide consumption, relative to a conventional hydrogen peroxide process (PO).

4.1 Introduction

Most wood pulp produced - 1.86×10^8 ton (FAO, 2012) - is used in the paper and packaging industry but a substantial part is employed to obtain cellulose-based products with important applications in industrial sectors such as pharmaceutical, textile, food and printing, among others. Cellulose-based products are classified into cellulose derivatives and regenerated cellulose (Köpcke et al., 2008) and the raw material typically used for the production of these products is low-yield chemical bleached pulp called dissolving-grade pulp. Dissolving pulp is defined by high content of cellulose (90–99%), small amounts of hemicellulose (2–4%) and only traces of residual lignin, extractives and minerals. Removing hemicelluloses from wood fibres is essential with a view to facilitating the processing of cellulose during the production of viscose. Hemicelluloses can affect the filterability of viscose, the xhantation of cellulose, and the strength of the end product (Christov and Prior, 1993). In addition, high brightness, low degree of polymerization and very uniform molecular weight distribution are also highly desirable qualities; in contrast fibre strength is not an issue.

According to FAO (2012), the current world production of dissolving-grade pulp is 4.22×10^6 ton; however, the demand for speciality cellulose has become apparent in recent years and prospective consumer markets indicate that this interest will continue in subsequent decades. This upturn in the dissolving pulp market is considered to be due to an increasing demand for textile materials in Asian countries, environmental and agricultural restrictions on cotton cultivation and the important point that dissolving pulp can be an environmentally friendly alternative to synthetic fibres.

Dissolving pulp has traditionally been obtained by the alkaline pre-hydrolysis kraft (PHK) process or acid sulfite process. However, these conventional pulping processes have some drawbacks relating to the quality of the final product and the pollution they generate. In view of these disadvantages, new industrial technologies such as organosolv pulping or the removal of hemicellulose by alkaline, nitren and cuen extraction have been developed and studied. In the same way, the enzymes have been used alone and in combination with alkaline extraction to develop effective alternatives to chemical processes (Wallis and Wearne, 1990; Jackson et al., 1998; Bajpai and

Bajpai, 2001; Puls et al., 2006; Ibarra et al., 2010b; Schild and Sixta, 2011). In addition, dissolving pulp is more expensive to obtain than paper-grade pulp in terms of chemical consumption, production rate, inventories and storage space (Hillman, 2006) and hence the interest to upgrade paper-grade pulps to dissolving quality pulps. Furthermore, the potential of a sulphite mill biorefinery, i.e. separation of lignocellulosic materials (LCM) with different properties into different streams and the further processing of these fractions to obtain high-value byproducts, has become desirable.

In terms of bleaching treatments, chlorine-based chemicals were typically used in order to control cellulose degradation and achieve a desired viscosity level, but in response to environmental concerns these technology were largely abandoned. The new bleaching processes such as the elemental chlorine free (ECF) and the totally chlorine free (TCF) are based on oxygen-derived compounds. A typical ECF sequence comprises multi-stages, oxygen delignification (O), chlorine dioxide (D), alkaline extraction (E) and hydrogen peroxide (P) stages in any combinations, while a conventional TCF sequence includes an oxygen delignification (O) stage, an acid (A) stage, an ozone (Z) stage and a hydrogen peroxide (P) stage in different multi-positions. Also, biotechnology has provided highly promising results in delignifying or bleaching paper-grade pulps. In particular, the laccases are known to catalyse the oxidation of phenolic substrates; however the feasibility of laccase application may be improved in the presence of a redox mediator (viz. as a laccase–mediator system, LMS). The mediator acts as an intermediate to allow the enzyme to indirectly oxidize large molecules or even non-phenolic substrates. Mediator oxidation by laccase produces a high redox potential which dramatically expands the range of laccase-oxidizable compounds. LMS have been widely applied in alkaline pulps (Bourbonnais et al., 1997; Chakar and Ragauskas, 2004; Andreu and Vidal, 2011; Aracri and Vidal, 2011; Valls et al., 2013). To our knowledge, however, sulfite pulp processed with laccase plus a natural or synthetic mediator has not so far been reported. Moreover, as demand for dissolving pulp is estimated to increase during the next decades, the potential of LMS can offer a good alternative for traditional bleaching processes. With this aim, a biobleaching sequence using a laccase-mediator system in combination with a chemical bleaching stage was developed for efficient bleaching of

softwood sulphite cellulose. In preliminary tests, two synthetic (violuric acid and 1-hydroxybenzotriazole) and two natural compounds (*p*-coumaric acid and syringaldehyde) were used as mediators and their bleaching responses assessed in terms of pulp and effluent properties. The mediator with the highest bleaching potential was selected for further use in an extended TCF biobleaching sequence.

4.2 Materials and Methods

4.2.1 Raw material

Unbleached sulfite cellulose obtained from a mixture of 60% spruce (*Picea abis*) and 40% pine (*Pinus sylvestris*) and cooked at Domsjö mill (Sweden), was used as raw material. Prior to bleaching treatments, fibre samples were conditioned at pH 4 adjusted with H_2SO_4, stirred at 2% pulp consistency for 30 min and washed with de-ionized water in a glass filter funnel. This step was needed to remove contaminants and metals, and also to bring the pulp to the pH required for enzymatic treatment. The main characteristics of the starting pulp were as follows: 4.2 ± 0.2 kappa number, 61.25 ± 0.6% ISO brightness, 511 ± 11 mL/g viscosity. Carbohydrate composition, as determined by high-performance liquid chromatography (HPLC), was as follows: 92.6 ± 0.04% glucan, 6.6 ± 0.1% mannan and xylan and 0.8 ± 0.02% glucuronic acid.

4.2.2 Enzyme and mediators

Commercial laccase from *Trametes villosa* was used in combination with the natural mediators syringaldehyde (SA) and *p*-coumaric acid (pCA), and the synthetic mediators 1-hydroxybenzotriazole (HBT) and violuric acid (VA). The enzyme was supplied by Novozymes® (Denmark) and the mediators were purchased from Sigma–Aldrich. Laccase activity was determined by monitoring the oxidation of ABTS 2,2′azino*bis*(3-ethylbenzthiazoline-6-sulphonate) in 0.1 M sodium acetate buffer at pH 5 at 25 ºC. One activity unit is defined as the amount of laccase required to convert 1 μmol/min of ABTS to its cation radical (ε_{436} = 29300 $M^{-1}cm^{-1}$).

4.2.3 Preliminary bleaching tests: selection of the mediator

Unbleached sulfite cellulose was treated with a laccase–mediator system (LMS) consisting of the enzyme and either a synthetic compound (HBT or VA)

or a natural compound (SA or pCA). All treatments, at 5% pulp consistency, were conducted in an oxygen pressurized reactor (0.6 MPa), at stirring rate of 30 rpm, using 50 mM sodium tartrate buffer at pH 4, a dose of 20 U/g odp (oven dried pulp) of laccase and one of 1.5% odp of each mediator at 50 ºC for 4 h. A few drops of the surfactant Tween 20 (0.05% w/v) were also added. The enzymatic conditions were similar to those previously used by Valls et al.,(2012) with eucalyptus pulp. The sequence was completed with a chemical bleaching stage involving alkaline peroxide bleaching procedure. Fibre samples, at 5% consistency, were treated with 2% odp H_2O_2, 1.5% odp NaOH, 1% odp DTPA (diethylenetriaminepentaacetic acid) and 0.2% odp $MgSO_4$ in a Datacolor Easydye AHIBA oscillating individual reactor at 90 ºC for 2 h (Aracri et al., 2009; Fillat et al., 2010; Andreu and Vidal, 2011). After each stage, residual liquors were collected for subsequent analysis, and pulp samples filtered and extensively washed for further processing. A laccase control treatment (K_L) was conducted in parallel under the same conditions but with no presence of mediator. A conventional hydrogen peroxide bleaching treatment, i.e. a P stage applied directly to the starting pulp, was also performed in order to compare the bleaching efficiency of the laccase-mediator system in combination with a hydrogen peroxide bleaching stage.

A xylanase stage (X) was described as a pre-trial test to assess the efficiency of the enzyme on sulfite cellulose. The enzyme used was a commercial xylanase (Pulpzyme HC) supplied by Novozymes®. The X stage used 3 U/g odp xylanase at 10 % consistency adjusted with Tris-HCl buffer at pH 7 at 50 ºC for 2 h. After treatment, liquors were recovered and the resulting pulp was extensively washed as reported elsewhere for eucalyptus pulp (Valls et al., 2010e).

4.2.4 Extended biobleaching sequence: influence of the H_2O_2 dose and reaction time reduction

An extended TCF biobleaching sequence including a laccase-mediator treatment was applied to the initial pulp. The sequence was designated LQPO/P, where L denotes the enzymatic treatment, Q a chelating stage and PO/P a hydrogen peroxide stage lasting 6 h —the first four reinforced with pressurized oxygen and then followed by a depressurization where oxygen is removed.

The enzymatic stage (L) was carried out with the laccase-violuric acid system (Lac-VA), using the same conditions as in the preliminary tests. The enzymatic treatment was followed by a Q stage involving the use of chelating agents to reduce the contents in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) capable of degrading the bleaching agents and cellulose during the subsequent peroxide bleaching treatment (Heijnesson et al., 1995). The Q stage was performed with 1% odp DTPA at 5% consistency at pH 5-6 (adjusted with H_2SO_4 1 N) in polyethylene bags at 85 ºC for 1 h. The biobleaching sequence was completed with a chemical bleaching stage involving alkaline hydrogen peroxide bleaching procedure (PO/P). The operating conditions of PO stage differed from those of the preliminary tests —emphasising the incorporation of a multiple sequential steps. Thus, PO was carried out at 5% consistency in oxygen pressurized (0.6 MPa) reactor, using a stirring rate of 30 rpm under the following conditions: 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$ at 90 ºC for 4 h. This stage was performed in three consecutive steps (PO_1 = 1 h reaction, PO_2 = 1 h reaction, PO_4 = 2 h reaction) each involving the addition of 10% odp H_2O_2 and no interstep washing. In the last P stage, pressure was released and the temperature was maintained at 90 ºC for 2 additional hours without addition of H_2O_2. These last two hours of treatment were performed in order to examine the potential influence of residual hydrogen peroxide on the extent of fibre bleaching. A small amount of pulp was removed after each PO step, filtered, residual liquors collected and then extensively washed with de-ionized water in a filter funnel for subsequent analysis. A control sequence (KQPO/P) treated identically as the enzymatic sequence but in the absence of laccase and mediator was also studied. A conventional chemical hydrogen peroxide sequence (PO/P) was also performed in order to examine in terms of industrial application the effect of an enzymatic on the overall bleaching process. Thus, the enzymatic stage was omitted and the PO/P treatment was applied directly to the initial pulp using the same conditions defined for the enzymatic sequence.

4.2.5 Pulp properties

The sugar composition of the initial pulp and after xylanase treatment (X) was determined by high performance liquid chromatography (HPLC). Samples were studied on a duplicate basis using a modified version of TAPPI 249 cm-09 test method. Because the column failed to resolve xylose, mannose and

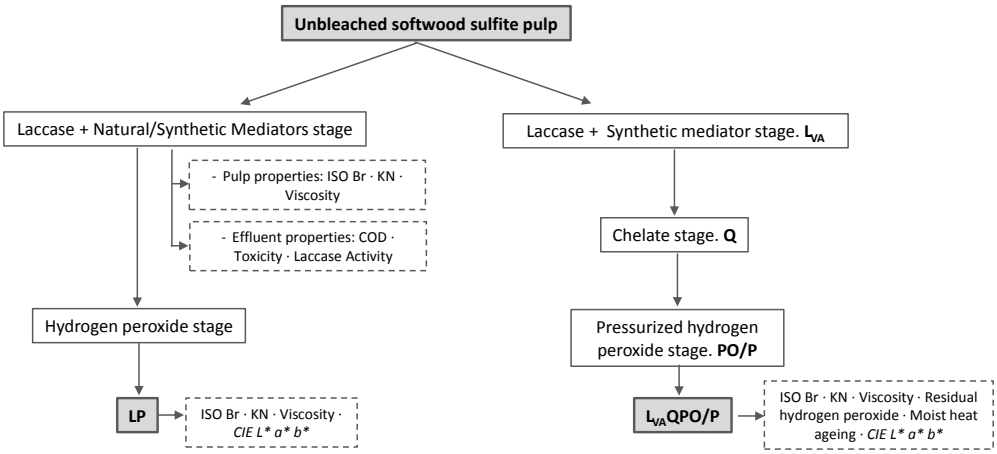
galactose, their combined content was expressed as xylose (Chapter 3, section 3.7.1).

Initial and treated pulp samples were characterized in terms of kappa number, ISO brightness and viscosity according to ISO 302:2004, ISO 2470:2009 and ISO 5351:2004, respectively. Fibre optical properties were measured in terms of the CIE $L^*a^*b^*$ color coordinates, namely: lightness (L^*), red–green (a^*) and yellow–blue (b^*). Chroma (C^*), which is the perpendicular distance of a point from the lightness axis [$C^* = (a^{*2} + b^{*2})^{1/2}$] and represents the amount of color "saturation" of a sample, was also used to characterize the bleaching process. Biobleached fibre samples were subjected to accelerated thermal ageing treatment by moist heating (at 80 ºC and 65% RH) in a HC 2020 Heraeus-Vötsch climatic chamber according to ISO 5630-3:1996 (Chapter 3, section 3.7.2).

4.2.6 Effluent properties

The effluents from the xylanase treatment were collected and analysed by thin-layer chromatography (TLC) as described elsewhere (Valls et al. 2010). The effluents from L stage in combination with different mediators were collected and analysed for chemical oxygen demand (COD), residual laccase activity, colour and toxicity (Chapter 3, section 3.7.4).

Schema 4-1 summarizes the experiments conducted and the properties analysed for each treatment.



Schema 4-1 Schema of work applied to the unbleached sulfite pulp and the respective studied properties

4.3 Results and discussion

4.3.1 Preliminary bleaching tests: influence of the mediators

Four different compounds were used in combination with commercial laccase in order to evaluate their bleaching efficiency in terms of pulp and effluent properties, as well as to compare their bleaching potential with a control sequence and a conventional hydrogen peroxide process. However, a xylanase stage (X) was contemplated as a pre-trial test in order to assess the efficiency of xylanase on sulphite cellulose. Thin Layer Chromatography (TLC) revealed that xylanase caused no release of sugars from the pulp despite its ability to remove xylans. Complementary to TLC, the HPLC analyses after the X stage showed no decrease in xylan and mannan fraction. This may have resulted from the initial low content in xylan and probably the residual xylan was in the form of lignin-carbohydrates complexes which are not readily attacked by xylanase (Gübitz et al., 1997). Indeed, no significant differences in delignification and brightness properties were detected in X-treated pulp relative to the starting pulp (Table 4-1 and Table 4-2). This led us to exclude the xylanase stage from the biobleaching sequence.

Table 4-1 Main properties of initial pulp and pulp resulted from a xylanase treatment

	Initial pulp	Xylanase treated pulp
Glucan (%)	92.6 ± 0.4	92.7 ± 0.02
Xylan + Mannan (%)	6.6 ± 0.05	6.6 ± 0.04
Glucuronic acid (%)	0.8 ± 0.02	0.6 ± 0.0
Acetic acid (%)	0.04 ± 0.01	0.1 ± 0.02
Viscosity (mL/g)	511 ± 26	496 ± 4

Table 4-2 summerizes the main properties of treated fibers after enzymatic stage (L) and after hydrogen peroxide stage (P).

Table 4-2 Main properties of biobleached pulps (results ± confidence interval)

	KN	ISO Brightness (%)	L^*	a^*	b^*	C^*	Effluent colour (kg Pt/ t pulp)
Initial	4.18 ± 0.09	61.3 ± 1.0	87.78	1.28	9.33	9.42	-
Xylanase	3.60 ± 0.38	60.8 ± 0.7	87.42	1.24	9.14	9.22	-
KL	4.04 ± 0.09	60.8 ± 0.2	87.62	1.25	9.45	9.53	2 ± 0
L_{HBT}	3.09 ± 0.15	55.6 ± 0.2	86.24	1.17	12.20	12.26	67 ± 0.5
L_{VA}	1.07 ± 0.17	56.7 ± 0.3	86.84	1.23	12.15	12.21	15 ± 0.1
L_{SA}	3.52 ± 0.03	52.1 ± 0.2	84.8	1.98	13.21	13.36	36 ± 0.6
L_{pCA}	3.88 ± 0.14	51.2 ± 0.5	84.2	2.04	13.10	13.26	61 ± 0.5
KLP	1.92 ± 0.04	68.0 ± 2.4	89.72	-0.22	6.71	6.71	-
$L_{HBT}P$	0.94 ± 0.28	70.2 ± 1.1	90.8	-0.47	6.65	6.67	-
$L_{VA}P$	0.56 ± 0.26	77.8 ± 0.2	94.15	-0.72	6.57	6.61	-
$L_{SA}P$	1.68 ± 0.04	69.6 ± 0.3	92.05	-0.13	9.43	9.43	-
$L_{pCA}P$	1.25 ± 0.31	66.5 ± 1.6	89.44	-0.57	7.50	7.52	-

The laccase–mediator treatments (L stage) were found to reduce ISO brightness (Figure 4-1a) and kappa number (Figure 4-1b) in all samples. This is suggestive of delignification, but also of coloration of the pulp as a consequence of the formation of chromophores groups due to the oxidative action of the enzymatic treatment. The greatest reduction in lignin content was obtained with VA, followed by HBT and then by the natural compounds, pCA and SA (Figure 4-1b). Interestingly, Aracri et al. (2010) and Andreu and Vidal (2011), previously observed an increase in kappa number after an LMS stage in the

presence of *p*-coumaric acid (pCA), syringaldehyde (SA), acetovanillone (AV) and vanillin (V) suggesting the occurrence of cross-linking or cross-coupling reactions of these natural compounds adsorbed onto sisal and kenaf fibres. Cadena et al. (2010c) found hexenuronic acids (HexA) could act as coupling partner of the mediator. The initial unbleached sulfite fibres had low content in xylan and no hexenuronic acids, which may have reduced the possibility of bonding between mediator and fibres, hence the probability of radical coupling reactions. The laccase control treatment (KL) caused no reduction in kappa number as well as no variation in ISO brightness, indicating that laccase alone was unable to modify pulp properties. Figure 4-1b confirms that the L stage caused delignification and boosted bleaching in the presence of a mediator. In fact, all mediators reduced kappa number with respect to the initial pulp after the P stage by: 2.5 points SA, 2.9 points pCA, 3.2 points HBT and 3.6 points VA. By contrast, on the conventional sequence (P) the kappa number was reduced by only 0.79 points despite the increased brightness (67% ISO). The greatest effect was that of LVAP, which caused an increase of 28% in ISO brightness and a decrease of 87% in kappa number; while, the control sequence (KLP) increased brightness by 12% ISO and reduced kappa number by 54% with respect to the initial pulp in both examples.

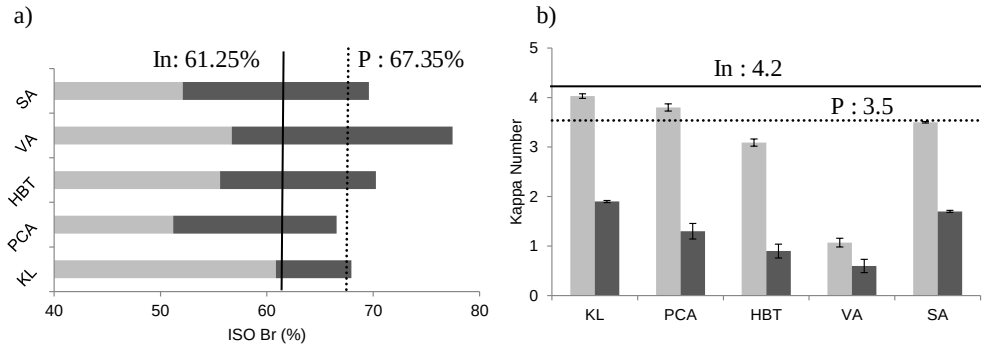


Figure 4-1 (a) Changes in ISO Brightness and (b) kappa number of pulp obtained from the enzymatic stage (grey bars/columns) and hydrogen peroxide stage (black bars/columns). The confidence intervals are included for the kappa number graph. Black line indicates the value of the initial pulp (In) and the dashed line indicates the value of the conventional hydrogen peroxide treatment (P). The references of each LMS treatment are detailed as follows: KL: control treatment without addition of mediator; PCA: p-coumaric acid; HBT: 1-hydroxybenzotriazole; VA: violuric acid; SA: syringaldehyde

Some authors such as Moldes et al. (2008) and Aracri et al. (2009) have hypothesized that the loss of brightness observed after the enzymatic treatment (Figure 4-1a, grey bars) may indicate the coexistence of lignin removal with condensation and oxidation of compounds from the mediator effluents or formation of chromophores in the pulp. When phenolic compounds (such as SA and pCA) are used as mediators for pulp bleaching, the delignification effect may be hindered by adverse reactions involving the phenoxy radicals generated by enzymatic oxidation of the mediators. These assumptions are consistent with the colour analyses of the effluents from the L stage and also with an unfavourable effect on optical properties of pulp. As can be seen from Figure 4-2a, all mediators exhibited higher colour values than did the laccase control treatment (K_L, no presence of mediator); however, this parameter was slightly better with VA, which gave the lowest colour value and the highest brightness result. In addition, in terms of optical properties (Figure 4-2b), paper handsheets exhibited marked colour after the enzymatic stage (L) with pCA and SA (natural phenolic compounds). Colour changes were mainly characterized by an increase in yellowness (b^*) and one in redness (a^*). On the opposite, the treatments with VA and HBT (synthetic compounds) led to essentially the same colour results as the

control sequence except for a slight increase in yellowness (a shift in b^* from blue to yellow).

Optical properties including chroma (C^*, a measure of colour saturation) and lightness (L^*, a measure of amount of colour) were also analysed. As can be seen from Figure 4-2c, C^* was increased and L^* slightly decreased by the L stage in all treatments; by contrast, C^* and L^* for the control pulp were close to the values for the initial pulp (In). The increase in C^*, which indicates coloration of the pulp, is consistent with the above-stated potential formation of quinones. However, the P stage decreased C^* and increased L^* coordinates with respect to L stage and the initial pulp. Similar results were previously obtained by other authors for different raw materials (Moldes et al., 2008; Moldes and Vidal, 2011; Martín-Sampedro et al., 2012). Based on the foregoing, LMS improves the optical properties of pulp, not only by increasing brightness and lightness, but also by reducing color in bleached pulp after applying a P stage.

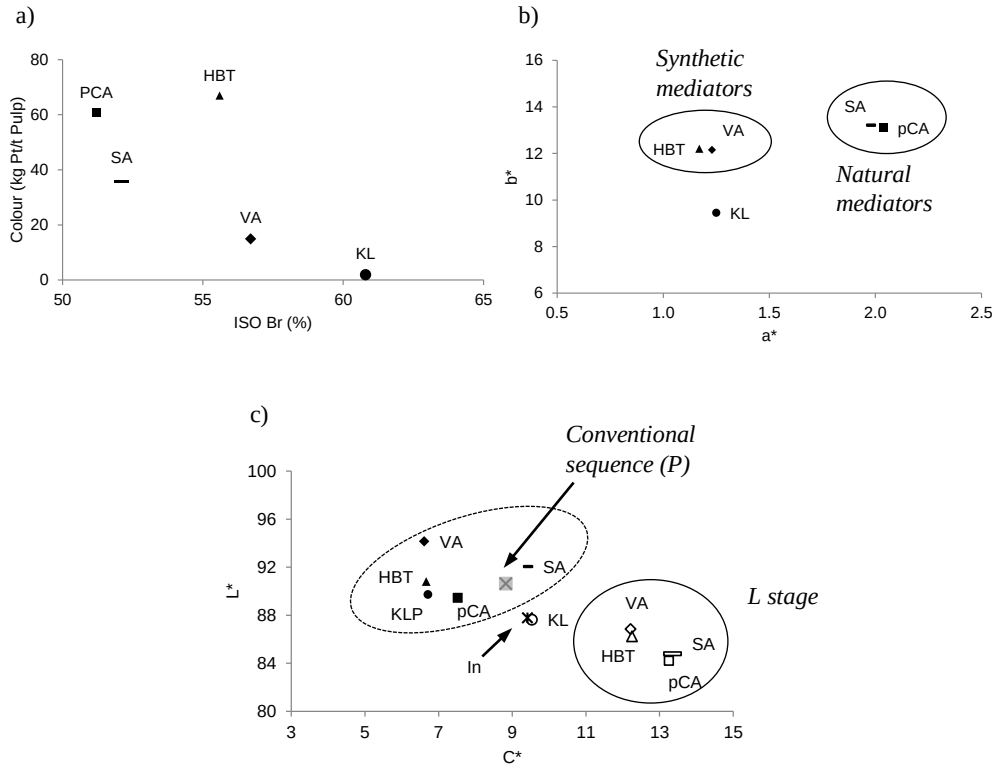


Figure 4-2 (a) Effluent color property as a function of ISO brightness of pulp resulted from the enzymatic stage (L). (b) Optical properties of the treated pulp resulted from the enzymatic stage (L): CIE a^* b^* color coordinates. (c) Optical properties of the treated pulp resulted from the enzymatic stage (white symbols) and from the hydrogen peroxide stage (black symbols). CIE L^* C^* color coordinates. The references of each LMS treatment are detailed as follows: KL: control treatment without addition of mediator; PCA: *p*-coumaric acid; HBT: 1-hydroxybenzotriazole; VA: violuric acid; SA: syringaldehyde

In general, the respective enzymatic treatments caused no significant change in viscosity, which was only 2.5% lower after L stage with respect to the control treatment (Table 4-3). This finding reflects that the incorporation of LMS treatment boosted biodelignification, i.e. reduction of kappa number without important degradation on carbohydrate chains. This cellulose preservation provided by LMS was previously observed by other authors (Martín-Sampedro et al., 2012) but using different raw material. However, cellulose loss was more pronounced after the P stage with all mediators except for pCA. These results are in agreement with the increased brightness and marked reduction in kappa number observed after the hydrogen peroxide stage. As known from the

literature, viscosity is an important parameter in the viscose process and obtaining quality rayon requires final viscosity values in the 200-300 mL/g range (Henriksson et al., 2005; Kvarnlöf et al., 2006; Köpcke et al., 2008). A lower viscosity is not acceptable as it gives low viscose fibre strength. However, a higher viscosity than that required for the xanthation step is desirable in order to have better control of the process and hence to the final product.

Table 4-3 Viscosity values (± confidence intervals) obtained from the enzymatic stage (L) and the hydrogen peroxide treatment (LP) for each mediator and the corresponding laccase control treatment.

	Viscosity (mL/g)	
Initial	511 ± 26	
Conventional sequence (P)	468 ± 10	
	L	LP
KL (Laccase control)	492 ± 8	464 ± 15
HBT	480 ± 3	430 ± 13
VA	509 ± 10	493 ± 0
SA	490 ± 27	472 ± 52
*p*CA	494 ± 25	502 ± 19

Table 4-4 illustrates the environmental impact of the process in terms of toxicity, chemical oxygen demand (COD) and laccase inhibition as determined in the effluents from the L stage (enzymatic treatment). The natural mediators, SA and pCA, exhibited a higher toxicity than the synthetic mediators (HBT and VA), with VA as the least toxic. Similar results were previously reported with different raw materials (Aracri et al., 2009; Fillat et al., 2010; Andreu and Vidal, 2011). The control treatment, which excluded the mediator, was scarcely toxic; this confirms the hypothesis that toxicity in this process may result from the generation of intermediate and degraded products from the oxidized mediators since mediator solution in its original form introduced no toxicity in the effluents (Aracri et al., 2010; Fillat et al., 2011). The control laccase treatment resulted in the lowest COD value although some authors (Aracri et al., 2010; Fillat et al., 2011) found buffer suspension to contribute in this result. The increased COD values obtained with respect to the control can be ascribed to the species dissolved during the treatment (lignin and carbohydrates) or due to the oxidized form of the mediator. Therefore, these high values of COD obtained

with the mediators treatments may indicate that this stage needs to be optimized in terms of mediator dose by means of statistic plan (Fillat and Roncero, 2009). Concerning residual laccase activity measured from the L-effluents, it must be said that the laccase inhibition increased with increasing efficiency of the treatment (i.e. reduction of kappa number with a brightness improvement), being VA as the most efficient mediator.

Table 4-4 Effluent properties: Toxicity, COD and laccase inhibition values (±standard deviation) obtained from the enzymatic stage (L) for each corresponding mediator and the corresponding laccase control treatment

	Toxicity (equitox/m^3)	Chemical Oxygen Demand (kg O_2/t_{pulp})	Laccase Inhibition (%)
KL	4 ± 2	5 ± 0	94.7 ± 0.3
HBT	12 ± 1	90 ± 4	98.8 ± 0.0
VA	3 ± 0.5	101 ± 1	99.6 ± 0
SA	170 ± 0	141 ± 5	91.0 ± 0.18
pCA	215 ± 25	105 ± 0	97.5 ± 0.3

Based on the above-described results, the laccase–violuric acid system exposed the best pulp and effluent properties. In fact, VA proved the most efficient mediator for the biobleaching sequence as it led to the smallest kappa number and highest brightness without an adverse effect on cellulose integrity. Also, the Lac–VA system gave the least toxic and coloured effluents. In addition, the proposed system provided even better results than a simple conventional alkaline hydrogen peroxide process; this testifies to the delignification-boosting effect of LMS on softwood sulfite cellulose. Moreover and consistent with the previous observations, a recent cyclic voltammetry study (Aracri et al., 2013), used to assess the efficiency of a laccase–enhancer system in oxidative processes, revealed that violuric acid was the most efficient choice for oxidizing lignin. This was most probably a result of a more favorable balance between reactivity and stability in the ensuing radicals. A part from the excellent efficiency of the Laccase-VA system, the present study allowed to test the natural mediators SA and pCA for the first time on this type of cellulose fiber and to obtain information of potential use for future experiments.

4.3.2 Extended biobleaching sequence: influence of the H_2O_2 dose and reaction time reduction

In view of the results obtained with violuric acid, an extended biobleaching sequence in order to more accurately assess the performance of the Lac–VA system and the influence of a hydrogen peroxide stage was explored. So, a biobleaching sequence designated LQPO/P was conducted; where: L denotes an enzymatic stage, Q a chelating stage and PO/P pressurized hydrogen peroxide bleaching followed by further bleaching at atmospheric pressure with no washing between steps. The results were compared with those for a control sequence without presence of laccase and mediator (KQPO/P) and also with those for a conventional sequence (PO/P).

Table 4-5 summarizes the main characteristics of biobleached sulfite fibers, control treated sulfite fibers and conventional bleached sulfite fibers.

Table 4-5 Kappa Number (KN), ISO brightness, viscosity and hydrogen peroxide consumption results (± confidence interval) for initial pulp and respective treated pulps (biobleached, control and conventional sequences).

	KN	ISO brightness (%)	Viscosity (mL/g)	Hydrogen peroxide consumption (%)
Initial	5.2 ± 0.2	58.7 ± 0.6	552 ± 8	-
L	2.3 ± 0.1	55.4 ± 0.4	522 ± 5	-
LQ	2.3 ± 0.1	56.9 ± 1.0	483 ± 18	-
$LQPO_1$	0.5 ± 0.3	84.2 ± 0.2	413 ± 1	86
$LQPO_2$	0.6 ± 0.4	87.5 ± 0.1	373 ± 3	76
$LQPO_4$	0.2 ± 0	89.1 ± 0.1	343 ± 13	86
K	4.7 ± 0.2	55.0 ± 1.0	480 ± 22	-
KQ	4.2 ± 0.2	57.9 ± 0.5	486 ± 13	-
$KQPO_1$	1.9 ± 0.2	78.0 ± 0.1	415 ± 1	91
$KQPO_2$	1.1 ± 0	83.1 ± 0.1	405 ± 5	86
$KQPO_4$	1.0 ± 0.2	84.4 ± 0.2	420 ± 32	78
PO_1	2.1 ± 0.35	76.1 ± 0.3	526 ± 32	67
PO_2	1.9 ± 0.04	81.8 ± 0.3	485 ± 5	80
PO_4	1.5 ± 0.01	84.0 ± 0.1	455 ± 15	88

Firstly, it is important to point out that, results from P_5 and P_6 steps (hydrogen peroxide treatment at atmospheric pressure) were excluded from the

discussion because any significant difference with respect to the previous Po_4 stage was detected. Therefore, the respective bleaching sequences were referred from now on as: LQPO, KQPO and PO.

Changes in residual lignin during the bleaching process were assessed in terms of kappa number. As can be seen from Figure 4-3, the laccase–mediator system efficiently reduced kappa number from 5.3 to 2.3 after the enzymatic stage. The kappa number reduction can be directly ascribed to the removal of residual lignin, since the presence of no hexenuronic acids could not contribute to this property. The enzymatic sequence (LQPO) caused 56% delignification after the L stage; however, the buffering stage of the control sequence (KQPO) also afforded 13% delignification with respect to the initial pulp. Therefore, the actual delignification contributed by the laccase–mediator stage in the LQPO sequence was about 43%.

The delignifying effect of PO_1 step with respect to the previous stage in the LQPO sequence was 79%. The marked decrease obtained after PO_1 step was directly due to the laccase–VA system since the control sequence (KQPO) only provided 56% delignification after $KQPO_1$. Hydrogen peroxide is known to be an effective bleaching agent capable of oxidizing residual lignin in pulp. The conventional hydrogen peroxide process (PO) caused 61% delignification at Po_1 relative to the initial pulp, but failed to reach the same level as the $LQPO_1$ sequence. This result further confirms the boosting effect of L stage and the alkaline pH used in the PO stage facilitated dissolution of degraded lignin. Overall, the greatest change was caused by Po_1 step, but the next multiple steps also contributed, to a lesser extent, to the final results. The kappa numbers obtained at the end of the bleaching sequences were <0.5 in LQPO, 1 in KQPO and 1.5 in PO.

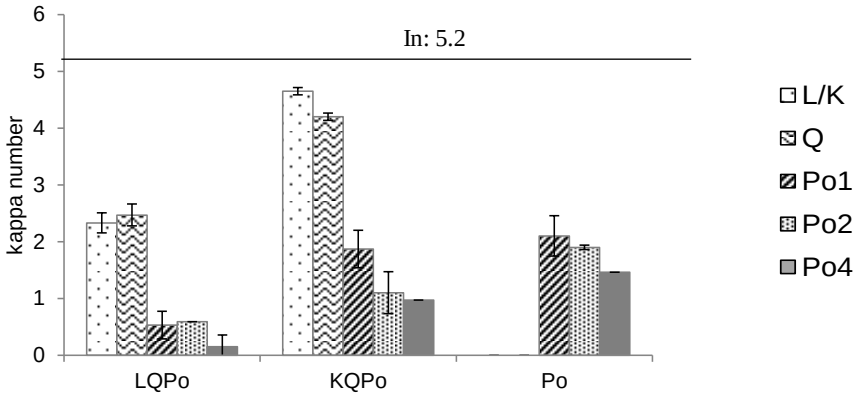


Figure 4-3 Kappa number (± confidence intervals) of treated pulps obtained from each stage of the sequences: LQPO (enzymatic treatment), KQPO (control treatment) and PO (conventional treatment). Black line indicates the kappa number initial pulp (In)

Figure 4-4 illustrates the brightness increase obtained after each stage in the enzymatic sequence (LQPO), the control sequence (KQPO) and the conventional hydrogen peroxide bleaching sequence (PO). Despite the drop in brightness observed after the enzymatic stage (L), the laccase-VA system was more efficient in raising pulp brightness at the end of the whole sequence than was the control sequence (KQPO) or even the conventional chemical bleaching sequence (PO). All treatments markedly increased brightness after PO stage, which is consistent with the increased reduction in kappa number observed. Limiting the bleaching process for only 4h with the aid of the enzyme–mediator system led to 89% ISO brightness, which is very close to the typical levels for commercial dissolving pulps.

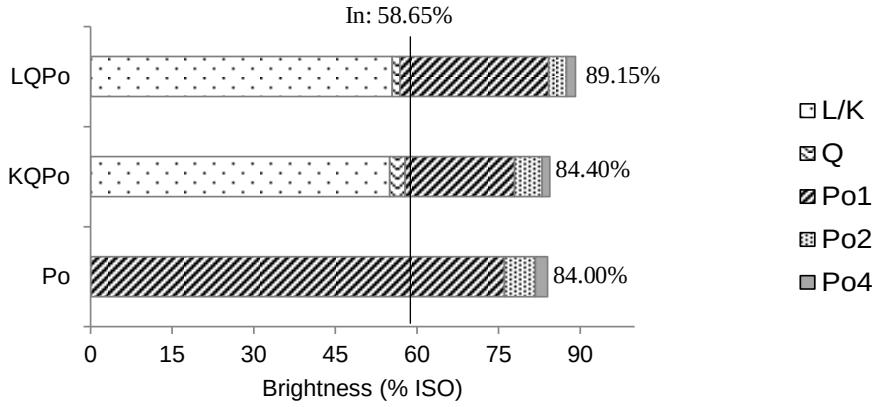


Figure 4-4 ISO brightness (± confidence intervals) of treated pulps obtained from each stage of the sequences: LQPO (enzymatic treatment), KQPO (control treatment) and PO (conventional treatment). Black line indicates the ISO brightness initial pulp (In)

Accelerated pulp ageing was conducted in order to assess the effect of an enzymatic treatment on the stability of optical properties. Pulp brightness was reduced by 10.1% with the L_{VA} treatment, but only by 0.3% with the control treatment (K) after moist heating ageing treatment (Table 4-6). Cadena et al. (2010) postulated that hexenuronic acids (HexA) contributed to 69% brightness reversion in eucalyptus TCF pulp during an accelerated ageing. However, the pulp used in this study contained no HexA, so the brightness loss observed after the L_{VA} stage suggests that the enzymatic treatment produces chromophores or oxidizable structures that boost reversion of optical properties. In fact, the chroma coordinate was increased 22% by L treatment while after the control treatment (K) was reduced by a 3.5%, with respect to initial chroma value and before the ageing treatment (

Figure 4-5). Other authors (Moldes and Vidal, 2008; Aracri et al., 2009) also related the changes in the CIE $L^* C^*$ color coordinates to the formation of chromophoric groups. In addition, Chakar and Ragauskas (2001) studied the relative amounts of quinones in residual lignins isolated from a softwood kraft pulp before and after LMS treatments; and the P NMR spectral analyses confirmed the formation of quinones from $LMS_{VA, NHA, HBT}$. However, the total brightness loss detected for $L_{VA}QPO_4$ and $KQPO_4$ samples by effect of accelerated

ageing was 13.0% and 12.2%, respectively (Table 4-6). This result indicates that chromophoric groups formed during the laccase treatment were removed or altered during the hydrogen peroxide stage. Interestingly, the complete biobleaching sequence led to the highest value of brightness without a detriment to brightness stability.

Table 4-6 ISO brightness before and after moist heating ageing treatment for initial and respective treated pulps

	ISO Brightness (%)				
Moist heating ageing treatment	Initial	L	K	$L_{VA}QPO_4$	$KQPO_4$
0h	58.70	55.40	57.85	89.20	81.80
144h	57.15	49.80	57.65	77.60	71.80

Figure 4-5 represents the CIE L^* C^* color coordinates before and after the accelerated ageing treatment. After 144h of ageing treatment, chroma (C^*) and lightness (L^*) coordinates of control-treated pulp (K) remained constant while the enzymatic-treated pulp (L_{VA}) suffered an increase in C^* and a diminution in L^* due to the quinones or carbonyl species generated during the L stage. As observed with brightness loss index, although the enzymatic sequence ($L_{VA}QPO_4$) led to lower L^* and higher C^* values than control sequence ($KQPO_4$), both sequences experimented similar changes. As a conclusion, it can be said that the introduction of an L stage had no negative impact on the final brightness result of the whole bleaching sequence.

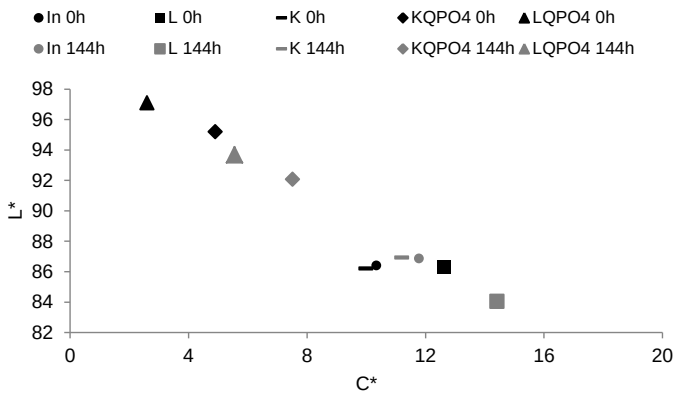


Figure 4-5 Effect of accelerated ageing by moist heating treatment on the CIE L* C* color coordinates for pulps obtained with the enzymatic (L), control (K), KQPO4 and LVAQPO4 treatments. Black and grey symbols represent values before and after a moist heating ageing treatment, respectively.

Figure 4-6 illustrates the hydrogen peroxide consumption at each hydrogen peroxide step. The hydrogen peroxide steps, PO_1, PO_2 and PO_4, indicate the different additions of H_2O_2 during the PO bleaching treatment. As can be seen from the first graph, the $LQPO_1$ treatment provided 84% ISO brightness with a single addition of H_2O_2 and only 1 h of treatment; by contrast $KQPO_1$ treatment required three additions of H_2O_2 and 4 h of reaction time to reach the same brightness level. The conventional treatment by its side, also needed the maximum conditions (3 additions of H_2O_2 and 4 h of treatment) to obtain approximately 84% ISO brightness. In other words, introducing an enzymatic stage in the bleaching process reduces the reaction time by 2 h and let to save about 70% H_2O_2, at a given brightness of 84% and with respect to a conventional bleaching sequence. In addition, in terms of effectivity (i.e. relation between hydrogen peroxide consumption and brightness levels), the $LQPO_1$ treatment had a bleaching effectivity of 32% versus 26% for PO_1 treatment (conventional bleaching sequence). This result further confirms that the laccase-VA system boosts the bleaching effect of hydrogen peroxide on sulfite cellulose and regarding the conventional bleaching sequence (PO), even using a high charge of hydrogen peroxide, more chemical stages or additional treatments are required to reach the highest brightness level (~90 % ISO) of the enzymatic sequence.

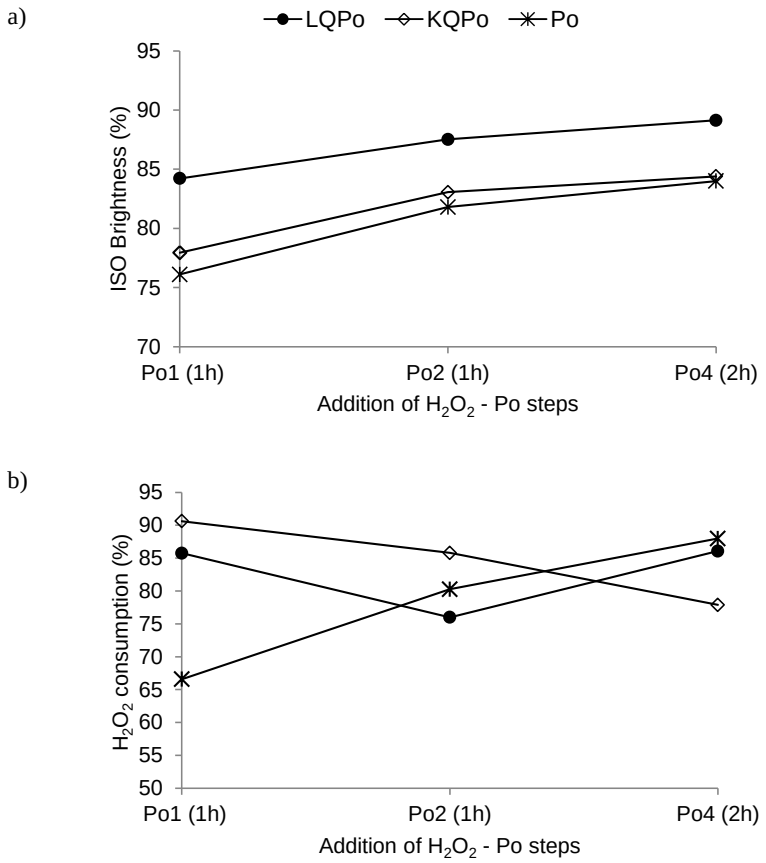


Figure 4-6 Evaluation of the multiple sequential hydrogen peroxide steps (PO_1, PO_2 and PO_4) in terms of ISO Brightness (a) and hydrogen peroxide consumption (b) for the different bleaching sequences: LQPO (enzymatic treatment), KQPO (control treatment) and PO (conventional treatment). Residual hydrogen peroxide was measured from the different bleaching effluents after each PO step and consumption values were calculated with respect to the charge of H_2O_2 for each PO step. The PO_1 step was 1h of reaction, the PO_2 was 1 h of treatment and PO_4 was 2h of reaction.

In the second graph (Figure 4-6b), during the first hour of treatment and first addition of H_2O_2, PO conventional bleaching sequence consumed 19% and 24% less H_2O_2 than did the LQPO and KQPO sequences, respectively. Although, after and L stage involving Lac-VA system there was less lignin moiety available for bleaching, these differences in H_2O_2 consumption may have been the result of the dissimilar nature of the samples or structural factors. Moldes et al. (2008) previously reported that in the case of laccase treatment in combination with HBT or VA, during the P stage, the hydrogen peroxide was consumed mainly to

oxidize chromophoric groups and brightness enhanced as a result. Unlike LQPO, the conventional sequence (PO) included no enzymatic treatment, so no chromophores groups from laccase treatment were present; this accounts for the response of the pulp to the PO_1 step. The enzymatic sequence (LQPO) consumed 86%, 76% and 86% of H_2O_2 in PO_1, PO_2 and PO_4, whereas conventional sequence (PO) consumed 67%, 80% and 88% in PO_1, PO_2 and PO_4, respectively. Importantly, the evaluation of residual hydrogen peroxide in the bleaching effluents confirmed that an excess of H_2O_2 dose was employed and therefore this stage needs to be optimized for any industrial application.

Finally, the pulp samples from the three bleaching sequences were subjected to viscosity measurements in order to assess the effect of each treatment on cellulose integrity. A gradual decrease in viscosity was observed as the bleaching process progressed. Degradation increased with increasing severity of the treatment and a decrease in cellulose chain length was detected for all samples at the end of the bleaching process. An L stage may have two different effects on cellulose, namely: (a) direct degradation of cellulose; and (b) alteration of functional groups in it facilitating its oxidation in the subsequent alkaline treatment (PO stage). It is important to clarify that because viscosity measurements are made in an alkaline medium, the measurements made after the L stage actually represented the extent of degradation caused by both effects (Fillat and Roncero, 2009). With respect to the initial viscosity value (552 ± 8 mL/g), the enzymatic sequence made a 13% yield loss while the control treatment caused only 5% yield loss after the L/K stage, respectively. Interestingly, after the Po_1 step (single addition of H_2O_2 and 1 h of treatment), the enzymatic sequence and the control sequence led to similar viscosity values ($LQPO_1$= 413 ± 1 and $KQPO_1$= 415 ± 1 mL/g, respectively), what means a 25% yield loss, but presented different final kappa number and ISO brightness values. Moreover, at the end of the bleaching process, the viscosity of the conventional bleaching sequence (PO_4 = 455 ± 15 mL/g) was higher than that presented for the enzymatic sequence ($LQPO_4$= 343 ± 13 mL/g) but the enzymatic sequence gained the highest value of brightness (~90%). So, as can be seen from Figure 4-7, similar values of viscosity were obtained for a target brightness value (e.g. ~84% ISO) but not under similar conditions. This observation indicates that the yield

loss is related to brightness value and there is a compromise between both properties.

According to literature, during the viscose process it is necessary to decrease viscosity around 200–300 mL/g with a pre-aging stage because too high viscosity affects the cellulose processability (Henriksson et al., 2005). The enzymatic sequence provided a final value (343 ± 13 mL/g) that can be considered acceptable for viscose manufacturing (rayon) and CMC derivatives rather than cellulose derivatives applications such as acetate and nitrate (Batalha et al., 2011). Control sequence and conventional sequence exhibited higher viscosities indicating that pre-aging step was required. The main issue related with viscosity was the limiting value (552 mL/g) of the starting pulp.

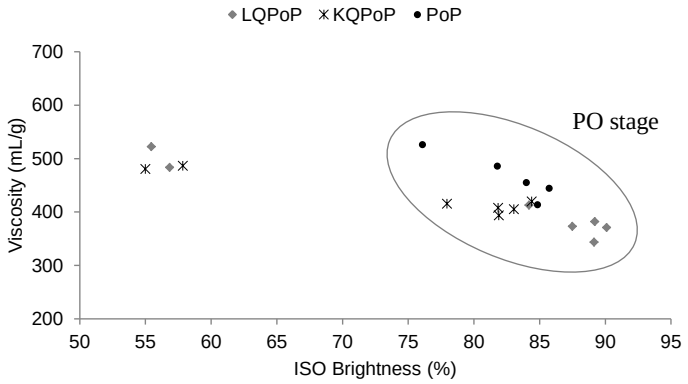


Figure 4-7 Viscosity values as a function of ISO brightness for the different bleaching sequences: LQPO (enzymatic treatment), KQPO (control treatment) and PO (conventional treatment)

4.4 Conclusions

Initially, a comparative study evaluating the efficiency of natural and synthetic compounds was conducted. From the tested mediators, the laccase-violuric acid system (LVA) yielded the best results. Then, an extended TCF biobleaching sequence (LQPO) using VA as mediator was implemented. The impact of L stage in the entire biobleaching sequence was assessed via the efficiency of PO stage in terms of H_2O_2 consumption and reaction time, with respect to a conventional treatment. Significantly, the incorporation of a laccase–mediator stage into a bleaching sequence for unbleached sulfite cellulose, led to final pulp with improved properties: high brightness level, low

lignin content and viscosity closely related to the value required for manufacturing cellulose derivatives.

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Chapter 5

Studying the effects of laccase bleaching treatment to meet dissolving pulp characteristics

Abstract

The enzymatic biobleaching sequence (L_{VA}QPO) using laccase from *Trametes villosa* in combination with violuric acid (VA) and followed by a pressurized hydrogen peroxide treatment was found to give good bleaching properties, i.e. low lignin content, high ISO brightness and brightness stability against moist heat ageing (Chapter 4). In addition, the bleached fibers met dissolving pulp requirements: high brightness, low content of hemicellulose, satisfactory pulp reactivity, no significant cellulose degradation detected by α-cellulose and HPLC. The incorporation of a laccase-mediator system to bleach sulfite pulps can be a good alternative to traditional bleaching processes since thermogravimetric analysis (TGA) showed that the laccase treatment prevented the adverse effect of hydrogen peroxide on fibre surface as observed during a conventional hydrogen peroxide bleaching treatment (PO). Although VA exhibited the best results in terms of bleaching properties, the performance of natural mediators, such as *p*-coumaric acid and syringaldehyde, was discussed in relation to changes in cellulose surface detected by TGA.

5.1 Introduction

Recently, laccase–mediator systems have been found to interact with fiber surfaces in various ways; thus, they can deposit onto fibers through condensation, promote grafting of the mediator on lignin surfaces or cause oxidative degradation of lignin (Barneto et al., 2012). Also, as shown by Saarinen et al. (2009) using a quartz crystal microbalance, and by Barneto et al. (2013) using X-ray photoelectron spectroscopy (XPS) and thermogravimetric analysis (TGA), laccase remains adsorbed on fiber surfaces after the enzymatic treatment. In fact, XPS revealed the presence of increased amounts of nitrogen on fiber surfaces after the enzyme treatment, and TGA large amounts of cellulose that underwent degradation at a low temperature. Thermal degradation of bleached pulp occurs in two stages, namely: degradation of amorphous cellulose at around 300 ºC and subsequent degradation of crystalline cellulose with rapid mass loss at a higher temperature level close to 350 ºC. The ensuing mass loss derivative curve (DTG) exhibits a typical sharp peak. A chemical or enzymatic treatment (e.g., a laccase bleaching treatment) increases the amount of cellulose that is degraded at a low temperature (i.e., amorphous cellulose) or even splits the peak for crystalline cellulose into two peaks. In this situation, crystalline cellulose in microfibrils loses its identity and behaves as amorphous or "paracrystalline" cellulose (Ioelovich et al., 2010), even though glucosyl units in microfibrils remain in their original location (Barneto et al., 2013). That is, laccase bleaching modifies the surface but has no effect on micrifibril core, which remains crystalline. The differential behaviour of surface and inner cellulose during thermal degradation make interesting the use of thermogravimetric analysis (TGA) in order to monitor changes in cellulose fibre surfaces during pulping and bleaching (Barneto et al., 2011b). In the previous chapter it was demonstrated that violuric acid (VA) in combination with *Trametes villosa* laccase and complemented with a multiple sequential hydrogen peroxide stage reinforced with oxygen, was able to bleach softwood sulfite pulp obtaining excellent bleaching properties.

In the present work, TGA is used to monitor changes in fibre surfaces of softwood sulfite pulp with high cellulose content resulted from the action of an enzymatic bleaching treatment. Besides studying cellulose surface modification, the main interest of this study was to examine the resulted biobleached sulfite

pulps (LVAQPO) in terms of dissolving pulp characteristics in order to satisfy the market-like requirements. Dissolving pulps consisting of wood derived celluloses are the main source for the manufacture of viscose rayon and cellulose derivatives, such as cellulose esters and cellulose ethers (Gehmayr et al., 2011). Dissolving pulps are defined by high cellulose content and small amounts of residual lignin, extractives and minerals, as well as high brightness and very uniform molecular weight distribution (Sixta, 2006; Ibarra et al., 2010a, 2010b). Besides the mentioned characteristics, reactivity is one of the most important quality parameters of dissolving pulps (Köpcke et al., 2008; Ibarra et al., 2010b; Schild and Sixta, 2011). Therefore, all the avobe mentioned properties were studied and measured in the present chapter.

5.2 Materials and methods

5.2.1 Raw material

Pulps obtained from the preliminary tests conducted in Chapter 4, section 4.2.3, were used for further characterization. All treatments were performed following the conditions decribed in 3.3.1 and 3.3.2 (*Hydrogen peroxide treatment (P stage)*).

Biobleached sulfite fibers resulted form LVAQPO sequence (Chapter 4, section 4.2.4) were used for further characterization. The operating conditions are described in detail elsewhere (3.3.1, 3.3.2, Chelating treatment (Q stage) and 3.3.2, *Oxygen-reinforced hydrogen peroxide treatment (PO/P stage)* sections).

5.2.2 Characterization of enzyme-treated pulp: surface and bleaching properties

The effects of the bleaching process produced on fibre surfaces were examined by thermogravimetric analysis carried out on a Mettler Toledo TGA/SDTA851e/LF1600 thermobalance. XRD measurements were performed on a SIEMENS D-500 BRAG-BRENTANO θ/2θ geometry X-ray diffractometer (Chapter 3, section 3.7.1).

5.2.3 Characterization of biobleached pulp: chemical properties

The cellulose reactivity of the pulp samples was determined according to slightly modified version of Fock's method (Fock, 1959; Ibarra et al., 2010b).

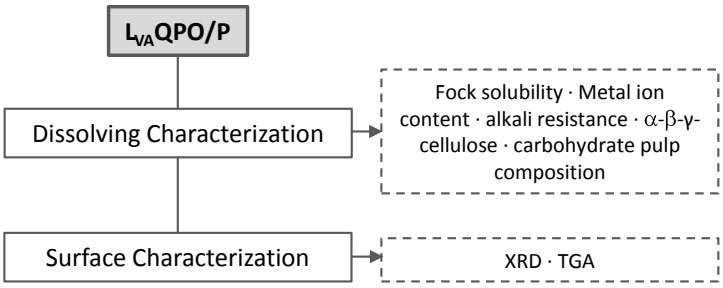
This is a micro-scale process simulating the industrial viscose process for manufacturing regenerated cellulose.

α-, β- and γ-celluloses were determined according to TAPPI method T 203 cm-09. Alkali resistance (R10 and R18) was determined according to TAPPI standard T 235 cm-09. All determinations were performed in duplicate.

The carbohydrate composition of the initial pulp and the respective treated samples was determined by high performance liquid chromatography (HPLC). Two replicates of the resulting samples were hydrolyzed using a modified version of the TAPPI test method T 249 cm-09.

The determination of calcium, iron and manganese in pulps was conducted by atomic absorption spectroscopy according to SCAN-CM 38:87 and was carried out in duplicate (Chapter 3, section 3.7.1).

Schema 5-1 summarizes the experiments conducted and the properties analysed for each treatment.



Schema 5-1 Schema of work applied to the biobleached sulfite pulp and the respective studied properties

5.3 Results and discussion

As concluded in Chapter 4, the enzymatic treatment presented satisfactory results in terms of bleaching-related pulp properties and the introduction of an L stage in the bleaching process provided a reduction in hydrogen peroxide dose with respect to conventional bleaching treatment. However, the results were not conclusive enough as regards dissolving pulp characteristics, so further study was needed. In the following part of this chapter, the quality of dissolving pulp

was assessed mainly via carbohydrate composition, viscosity, reactivity, alkali solubility and metal ion content. Therefore, $L_{VA}QPO_4$, $KQPO_4$ and PO_4 samples were selected and subjected to an extended study.

5.3.1 Characterization of biobleached dissolving pulp: chemical properties

As can be seen from Table 5-1, the carbohydrate composition of the bleached pulp samples was similar in all cases and essentially the same as in the initial pulp. This result was to be expected since, unlike cellulases or xylanases, a laccase–mediator system acts on the diminution of lignin content rather than on carbohydrate content of the pulp (Gübitz et al., 1997; Köpcke et al., 2008; Ibarra et al., 2010a). However, it is important to emphasize that the hemicellulose content fell in the acceptable range for commercial dissolving pulp (<10%) (Christov et al., 1998; Köpcke, 2010) in all cases.

Table 5-1 Mean values (± standard deviation) of carbohydrate composition, ISO brightness and kappa number for the different bleaching treatments.

	Initial	L_{VA}	$KQPO_4$	$L_{VA}QPO_4$	PO_4
Glucan (%)	92.6 ± 0.04	92.2 ± 0.4	91.9 ± 1.48	92.7 ± 0.2	92.7 ± 0.1
Xylan + mannan(%)	6.6 ± 0.05	6.9 ± 0.4	7.0 ± 1.1	6.5 ± 0.1	6.5 ± 0.2
Glucuronic acid (%)	0.8 ± 0.02	0.7 ± 0.2	0.6 ± 0.05	0.8 ± 0.3	0.8 ± 0.3
Acetic acid	0.04 ± 0.01	0.1 ± 0.0	0.3 ± 0.2	0.1 ± 0	0.1 ± 0
ISO brightness (%)	58.75 ± 0.6	55.43 ± 0.4	84.40 ± 0.2	89.14 ± 0.1	84.00 ± 0.1
Kappa number	5.3 ± 0.1	2.33 ± 0.1	0.97 ± 0.21	0.15 ± 0.0	1.46 ± 0.0
Fock solubility (%)	63 ± 6	-	65 ± 12	65 ± 11	67 ± 14
Viscosity (mL/g)	552 ± 8	-	420 ± 32	343 ± 13	455 ± 15

Another important property of dissolving pulp is reactivity, which has been previously quantified after the application of a cellulase or xylanase treatment by several authors (Köpcke et al., 2008; Ibarra et al., 2010a, 2010b; Köpcke, 2010). However, the Fock's solubility of laccase-bleached pulp was analyzed for the first time in this work. As can be seen from Figure 5-1, no substantial differences in the amount of reacted cellulose between bleaching treatments

were observed. According to Gehmayr and Sixta (2012), reactivity measured with Fock's test represents the amount of pulp that is dissolved in the viscose-like solution. Consequently, the results are strongly influenced by pulp viscosity and by the amount of alkali-soluble hemicellulose present, which might be misleading in terms of cellulose reactivity (Gehmayr and Sixta, 2012). Therefore, the little change observed can be explained in this way. Accurately comparing our bleaching sequences in this respect was made difficult by the fact that viscosity differed markedly although alkali solubility only moderately between bleaching sequences. In Figure 5-1, a moderate increase in Fock solubility can be associated to high values of viscosity, with the exception of initial pulp which had the highest viscosity value but also the lowest reactivity number. In particular, the enzymatic sequence experimented a 2.5% of reactivity increase, followed by the control sequence with a 3.0% and then the conventional hydrogen peroxide sequence with a 6.1% of reactivity improvement, with respect to initial pulp. However, this trend should be taken in caution since enzymatic sequence suffered the highest viscosity loss, 38%, while the conventional treatment only an 18%. Importantly, all the Fock's solubility values are similar to those obtained by Köpcke et al. (2008) for commercial dissolving pulp. In general, the conventional hydrogen peroxide bleached pulp (PO_4) had similar chemical properties (lignin content, brightness and viscosity) as control treated pulp ($KQPO_4$) and also a comparable Fock's solubility. The reactivity and viscosity results obtained with the enzymatic sequence ($L_{VA}QPO_4$) were slightly lower than PO_4 and $KQPO_4$, but under similar Fock solubility value the enzymatic treated pulp reached a higher final brightness value.

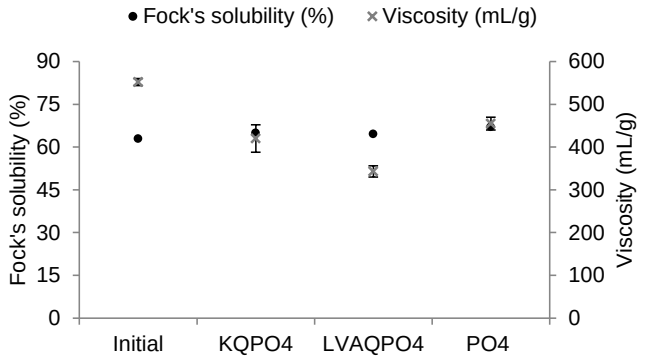


Figure 5-1 Viscosity (± standard deviation) and Fock's solubility obtained with the different bleaching treatments: initial (unbleached sulfite pulp), $KQPO_4$ (control sequence with no laccase or mediator), $L_{VA}QPO_4$ (enzymatic sequence) and PO_4 (conventional sequence). (The confidence interval of reactivity values was <8, with and alpha of 0.05).

Table 5-2 summarizes other interesting properties of dissolving pulp. Among chemical properties, alkali solubility is a measure of cellulose degradation, and also a loss or retention of hemicellulose during pulping and bleaching processes. In general, in 18% NaOH (S18) the hemicelluloses are soluble, whereas in 10% NaOH (S10) low-molecular weight cellulose (degraded cellulose) and the hemicellulose are dissolved (Tappi T-235 cm-00). In other words, alkali resistance can be defined as the fraction of pulp that is insoluble in sodium hydroxide at different concentrations (R18 = 100–S18 and R10 = 100–S10). On the other hand, α-cellulose is defined as the residual portion which is insoluble in 17.5% NaOH. This α-cellulose has a high molecular weight. Likewise, β-cellulose is the alkali-soluble fraction of pulp, which consists mainly of hemicelluloses that are insoluble under slightly acidic conditions (pH 2.5). Based on the foregoing, a relationship between α-cellulose and alkali resistance (R18) can be established; similarly, alkali solubility (S18) can be related to hemicellulose and β-cellulose fraction. However, as can be seen from Table 5-1 and Table 5-2, the concentration of hemicellulose (xylan and arabinan) failed to match pulp solubility in 18% NaOH, and nor did the β-cellulose fraction. Therefore, solubility of the pulps was mainly influenced by hemicellulose and cellulose of short chain. In addition, the small differences in β-cellulose fraction, and hence in S18 and R18, during the bleaching process, can be ascribed to differences in carboxylic acid content. By contrast, α-cellulose contents were

similar with all bleaching treatments and consistent with carbohydrate composition (particularly with glucan fraction).

Table 5-2 Characteristic parameters used to describe market-like dissolving pulp. Initial (unbleached) sulphite pulp, $L_{VA}QPO_4$ (enzymatic sequence), $KQPO_4$ (control sequence) and PO_4 (conventional sequence). The standard deviations were below ± 0.7 in all cases.

		Initial	$L_{VA}QPO_4$	PO_4
R10	%	88.7	86.1	85.2
R18	%	90.3	86.6	90.2
S18	%	9.7	13.4	9.8

		Initial	$KQPO_4$	$L_{VA}QPO_4$
α-cellulose	%	88.3	88.1	87.6
β-cellulose	%	7.5	7.0	5.8
λ-cellulose	%	4.2	5.4	6.1

The X-ray diffraction (XRD) technique measures the proportion of crystalline material in cellulose. Deconvoluting X-ray signals and isolating the contribution of amorphous cellulose led to crystallinity values of 64 ± 2% for all treated pulp samples. A high value of crystallinity was obtained since crystallinity in bleached pulp typically ranges from 52% to 65 % (Andersson et al., 2004; Leppänen et al., 2009). The X-ray results confirmed that an enzymatic bleaching treatment was essentially a surface process (Roncero et al., 2000) that causes virtually no change in fiber chemical composition or crystalline cellulose integrity. In other words, there was no alteration of the planes of anhydroglucose units inspected by the X-ray technique, so pulp crystallinity remained unchanged during the bleaching treatment.

The content in metal ions of dissolving pulp is also very important because a high concentration can affect the processability of cellulose derivatives (Table 5-3). The fact that the calcium and iron contents of the control and enzymatically treated pulp samples were similar suggests that the L stage does not alter pulp composition. Also, none of the samples was found to contain manganese at any stage during the bleaching process.

Table 5-3 Contents in metal ions (± standard deviation) of pulp subjected to different bleaching treatments. Nd: not detected. $KQPO_4$ (control sequence); $L_{VA}QPO_4$ (enzymatic sequence)

		$KQPO_4$	$L_{VA}QPO_4$
[Ca]	mg/kg pulp odp	177 ± 110	189 ± 0
[Fe]	mg/kg pulp odp	479 ± 186	519 ± 0
[Mn]	mg/kg pulp odp	Nd	Nd

5.3.2 Effect of the biobleaching sequence on fiber surface

Pulps biobleached with the laccase–VA system and complemented with a hydrogen peroxide treatment resulted in good dissolving pulp characteristics meeting market-like requirements. The surface changes of the pulp during the biobleaching treatment were assessed using thermogravimetric analysis.

TGA is sensitive to chemical changes in fiber surfaces because such changes can alter the thermal degradation path of pulp. Consequently, if a bleaching process results in enzyme adsorption or surface oxidation, then the hydrogen bond network which protected the surface of crystalline cellulose will be partially broken. In order to understand the effect of laccase adsorption on the thermal degradation of pulp requires considering that fibers contain both amorphous and crystalline cellulose. Cellulose is organized as long bundles of cellulose polymer chains called microfibrils, which includes crystalline and amorphous zones (Tsuji et al., 1992). In the crystalline cellulose region, cellulose chains form planes where equatorial hydroxyl groups in pyranose rings are hydrogen-bonded. The amorphous regions, which consist of non-ordered cellulose chains, are located mainly between cellulose crystallites (Nishiyama et al., 2003).

As can be seen from Figure 5-2a and b, the pulp underwent surface changes in the course of biobleaching process. A comparison of the initial and PO_4 curves in Figure 5-2a reveals that the hydrogen peroxide treatment damaged fiber surfaces considerably. In fact, this bleaching step caused a substantial decrease in the crystalline cellulose peak, which suggested alteration of fiber crystalline surfaces. In addition, the region from 300 to 325 ºC in the PO_4 curve fell above the curve for the initial pulp, thus indicating that the hydrogen peroxide treatment increased the amount of cellulose volatilizing at low temperatures (i.e., amorphous and paracrystalline cellulose). As a reference, commercial TCF

bleached dissolving pulp was also anlysed, showing similar pattern as PO_4 sample. These alterations of pulp may have resulted from alkaline depolymerization by effect of β-elimination reactions breaking bonds between adjacent glucosyl units (Barneto et al., 2011b). These results differed from with others obtained for treated eucalyptus and kenaf pulp, where a hydrogen peroxide treatment had a substantial cleaning effect (viz., removing deposits on the cellulose surface) and led to pulp giving sharper peaks than the initial or LMS-treated pulp (Andreu et al., 2013; Barneto et al., 2013). The absence of residual lignin and low content of hemicellulose detected by KN and HPLC, respectively, may account for such an important difference. As viscosity values showed, hydrogen peroxide caused a marked degradation of cellulose chains and TGA detected the actual surface changes since no deposits were removed. However, although an alkaline hydrogen peroxide treatment damages fiber surfaces, when pulps were previously treated with a laccase–mediator system or buffer solution resulted in some peculiarities. Contrasting the $KQPO_4$ and $L_{VA}QPO_4$ curves with the PO_4 curve revealed that the former two fell below the latter at low temperatures but above it at high temperatures (Figure 5-2b). These results mean that the reduction in crystalline cellulose by effect of the hydrogen peroxide was less marked when the pulp was previously treated with laccase–VA–sodium tartrate or sodium tartrate alone. As noted earlier, the buffer seemingly has a surface protective effect on this type of pulp that is strong enough to prevent a subsequent adverse effect of hydrogen peroxide.

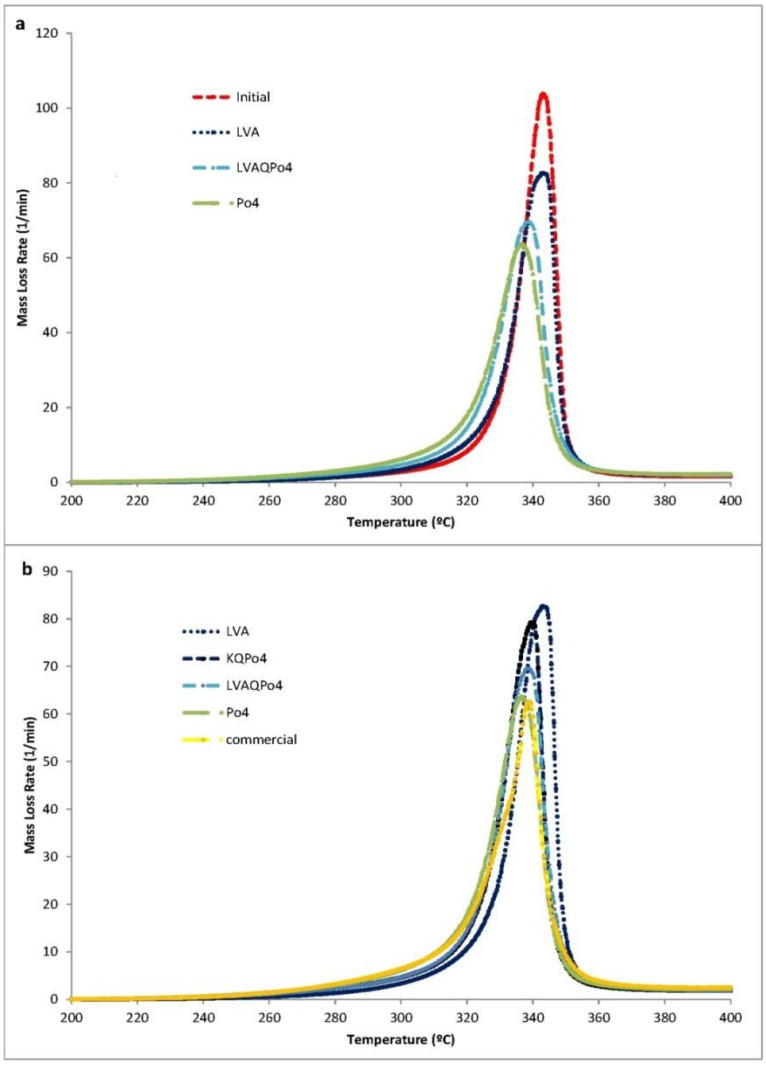


Figure 5-2 DTG curves for pulp samples at different stages of the biobleaching process. Heating rate, 20 ºC/min and the environment was in air. Initial (unbleached sulphite pulp), L_{VA} (enzymatic treatment), $L_{VA}QPO_4$ (enzymatic sequence), PO_4 (conventional sequence) and commercial TCF bleached dissolving pulp. The mass loss rate was normalized to the initial mass of sample.

Putting together TGA results and the above-described chemical properties, the initial pulp exhibited the highest viscosity value but also the lowest reactivity result. These observations can be explained by the fact that initial pulp contained more amount of crystalline cellulose than treated pulps, as TGA curves demonstrated. Although the PO_4 and $KQPO_4$ treatments led to pulp with similar chemical properties, $KQPO_4$ fibers had a "cleaner" surface judging by the

TGA results. On the other hand, treated biobleached pulp ($L_{VA}QPO_4$) exhibited a good reactivity value, an acceptable viscosity result and, even more important, better surface properties than PO_4-treated pulp and bleached commercial dissolving pulp.

5.3.3 Influence of the mediator on fibre surfaces

As demonstrated in Chapter 4, the bleaching efficiency of *Trametes villosa* laccase in combination with the natural mediators, *p*-coumaric acid (*p*CA) and syringaldehyde (SA), on unbleached sulfite pulp was very low and did not present potential capacity for meeting dissolving pulp characteristics. The differences in bleaching properties between natural and synthetic mediators can be explained by particular changes on fibre surface. Therefore, this part of the work intends to elucidate the changes that cellulose underwent due to the action of LMS during a bleaching process, using a thermogravimetric technique.

As can be seen from Figure 5-3, the initial pulp exhibited a sharp peak around 300–350 ºC indicating a high content in crystalline cellulose. The peak was sharper than the typical peaks for unbleached kenaf (Andreu et al., 2013) and unbleached eucalyptus pulps (Barneto et al., 2013), and suggestive of dissolving pulp composition. Clean and crystalline cellulose are thermally degraded at high temperatures spanning a narrow range because ordered cellulose chains yield crystallites that are protected from external attack by a surface hydrogen bond network (Barneto et al., 2011b). The control treatments, which included (KL) or excluded laccase (K), caused no change in chroma value as observed in Table 5-4. Also, based on the TGA results, fibers resulted from control treatment appeared more “crystalline” since the mass loss rate was higher at high temperatures. This fact contrasts with that obtained with kenaf pulp and eucalyptus pulp (Andreu et al., 2013; Barneto et al., 2013), where laccase or buffer were adsorbed onto fiber surfaces and increased the amount of “paracrystalline” cellulose. Seemingly thus, tartrate buffer can be adsorbed on pulp surfaces and alter their thermal degradation path by effect of the formation of new hydrogen bonds between lone electron pairs in oxygen atoms from the reagents (carboxyl group) and hydrogen atoms in hydroxyl groups from cellulose molecules (Barneto et al., 2013). Based on our results for biobleached softwood sulfite pulp, the buffer solution seemed to produce a cleaning and protective effect on fiber surfaces rather than being adsorbed onto them.

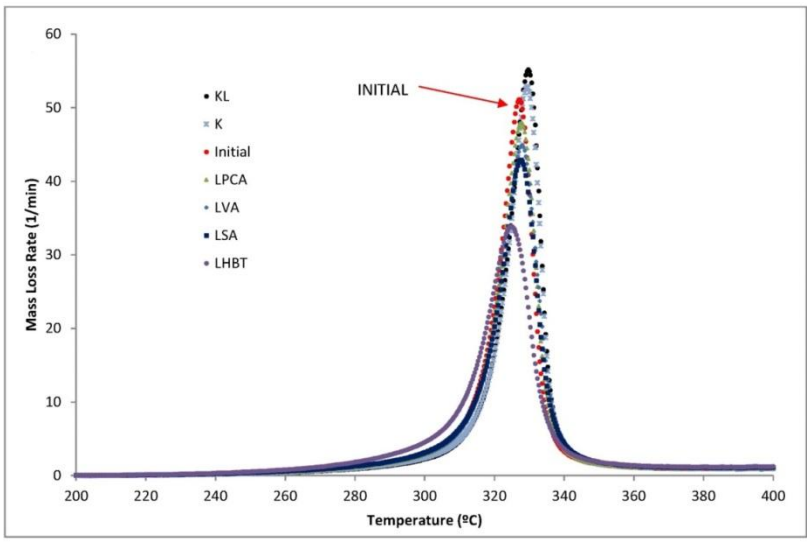


Figure 5-3 Changes in the thermal degradation pathway for the initial pulp during biobleaching at 10 ºC/min in an air atmosphere. The mass loss rate was normalized to the initial mass of sample.

As shown in Figure 5-3, the amount of cellulose degrading at a low temperature increased by effect of treatment with laccase in combination with a mediator in the following order: *p*-coumaric acid < violuric acid < syringaldehyde < hidroxybenzotriazole. In the same way, the DTG curves of pulp samples treated with the mediators SA, PCA and VA were similar to that for the initial pulp, but the mass loss rate resulting from the laccase–HBT treatment was considerably shifted to lower temperatures and its maximum much lower.

Combining TGA results and pulp optical properties revealed that dissolving pulps subjected to a LMS treatment presented a particular behavior. In the case of natural mediators such as syringaldehyde (SA), laccase is known to promote condensation of mediator molecules onto fiber surfaces in eucalyptus (Valls et al., 2014), flax (Fillat et al., 2011) and kenaf pulp (Barneto et al., 2012); however, only non-wood pulp (flax and kenaf) has been found to undergo substantial grafting — as reflected in an increased kappa number. As noted earlier, the TGA curves excluded condensation and grafting reactions for the pulp samples treated with the natural mediators because the thermal degradation paths were similar to that for the initial pulp. In addition, the kappa number of treated sulfite pulp

was slightly diminished. However, the increased chroma observed after the L stage suggested the formation of chromophores (quinones) by oxidation of residual lignin in the pulp (Aracri et al., 2009). Delignification after L was higher with the synthetic mediators than with their natural ones (Table 5-4). In fact, the reactivity and stability of N–O· radicals (VA) are seemingly better balanced than in phenoxy radicals (SA, pCA), which may account for the better performance of the former radicals as mediators for laccase-based delignification (Andreu et al., 2013). Despite the reduction in lignin content observed with LVA and LHBT, these enzyme-treated pulp samples exhibited higher colour saturation (C^*) than the initial pulp but lower than that obtained with the natural mediators. Specifically, VA had the highest delignification and lowest gain in chroma (C^*) after the enzymatic stage. According to Barneto et al. (2011a), the laccase–HBT system partially oxidizes the outer cellulose layer, changing hydroxyl groups to carbonyl groups, disordering the crystalline surface and increasing the paracrystalline cellulose content as a result. Figure 5-3 testifies to these effects: the amount of cellulose that volatilizes at a low temperature increased with HBT. Moreover, these results were confirmed with the viscosity values where a minimum cellulose preservation of 90% was obtained with LHBTP vs. the 97% obtained with LVAP.

Table 5-4 Delignification and colour (as defined in terms of chroma) of pulp samples from the L and P stages. K, control treatment with no laccase or mediator; KL, laccase control treatment; P, hydrogen peroxide stage directly applied to the initial pulp and initial pulp. (–) The stage was not performed.

	L stage		P stage	
	Delignification (%)	C^*	Delignification (%)	C^*
K	13	10.70	-	-
KL	4	9.53	55	6.71
*p*CA	9	13.26	69	7.52
SA	16	13.36	60	9.43
HBT	26	12.26	79	6.67
VA	74	12.21	86	6.61
P	-	-	17	8.83
Initial		9.42		

5.4 Conclusions

In Chapter 4, it was shown that the enzymatic sequence LVAQPO provided biobleached sulfite pulps with a reduction in hydrogen peroxide dose about 70% and reaction time of 2h in comparison to simple hydrogen peroxide treatment (PO). In the present study, the resulted biobleached sulfite pulps were thoroughly investigated since presented a potential use as dissolving pulp and its applications. The basic requirements that define dissolving pulps were successfully satisfied: low content of hemicellulose, satisfactory values of Fock's solubility, no significant cellulose degradation as shown by α-cellulose and HPLC results, high brightness value and good optical properties (C^* and L^* coordinates). Furthermore, XRD and TGA showed that crystallinity and fiber surface were not altered by the application of LMS. In the second part, the biobleaching performance of natural mediators (SA and PCA) in combination with *Trametes villosa* laccase was discussed in relation to their fiber surface modification since treated pulps exhibited low brightness results and poor delignification in comparison to the synthetic mediators, VA and HBT. TGA confirmed that no grafting or condensation reactions onto sulfite fibers occurred in the presence of natural mediators and no significant changes in fiber surface in comparison to control treatment were observed.

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Chapter 6

Improving pulp quality of biobleached sulfite pulp by the action of different endoglucanases

Abstract

A TCF sulfite pulp, bleached at the laboratory scale with a laccase–violuric acid system and complemented with a pressurized hydrogen peroxide stage, was treated with two endoglucanases, one obtained from *Paenibacillus barcinonensis* (B) and the other one produced from *Cerrena unicolor* (F) to improve cellulose reactivity. The treated pulps were evaluated in terms of brightness, viscosity, α-cellulose, water retention value (WRV), fibre morphology, Fock solubility, NMR and carbohydrate composition of pulps and liquors. Results revealed that both endoglucanases improved cellulose reactivity, albeit in a different way; thus, B caused no scissions in the cellulose chain and no significant reduction in fibre length, whereas F decreased viscosity and shortened fibre length, leading to lower reactivity value. The liquor composition of soluble carbohydrates released by the enzymatic treatments revealed the B had a processive mode of action since short oligosaccharides, cellobiose and glucose, were obtained. F hydrolysed, from high to low concentration, cellobiose, glucose and cellotriose. Importantly, environmentally friendly dissolving pulp with 90% Fock solubility was obtained, combining two enzymatic treatments: a laccase–mediator system (LMS) and then a cellulase from *Paenibacillus barcinonensis* (B). In order to improve the quality of final dissolving pulp, a pulp purification step was introduced before the B endoglucanase treatment. The cold caustic extraction lead to reduce the amount of hemicelluloses by 42% with respect to biobleached

pulp, but Fock solubility was also reduced. However, complementing the purification step with F treatment, improved Fock solubility by 17%, although some presence of cellulose II was detected by NMR.

6.1 Introduction

The introduction of new technologies to bleach dissolving pulps using enzymatic treatments and complemented with treatments based on oxygen-derived compound can be an alternative to traditional bleaching processes, such as elemental chlorine free (ECF) and totally chlorine free (TCF) processes. In Chapter 4 and Chapter 5, a laccase–mediator system (LMS) proved to be effective to bleach never-dried softwood sulfite pulp. The proposed LVAQPO sequence provided acceptable results in terms of dissolving pulp requirements and had a positive contribution from an environmentally point of view. However, in the direction of improving pulp reactivity and the quality of the final product (i.e. reduce the amount of hemicelluloses) further treatments were required.

The bioconversion process uses enzymes, such as cellulase, to break down cellulose into sugars that can be later fermented into ethanol. Therefore, following the biorefinery concept, endoglucanases are also used for upgrading paper-grade pulps to dissolving-grade pulp, and obtain hemicellulose free dissolving pulp as a primary product, but also lignin, bioethanol and a number of complementary products. The removal of hemicelluloses from paper kraft pulp is the main challenge to convert it into dissolving pulp. Cold caustic extraction (CCE), commonly used as post treatment to pre-hydrolysis kraft process (PHK) pulp for acetate grade dissolving pulp production can dissolve selectively hemicelluloses (Sixta, 2006; Arnoul-Jarriault et al., 2014). The dissolution of hemicelluloses during CCE is related to structural change of cellulose pulps when they are subjected to concentrated sodium hydroxide solution (> 8% w/v).

Most cellulolytic microorganisms produce a battery of cellulases which act synergistically to solubilize crystalline cellulose (Lynd et al., 2002; Chiriac et al., 2010, 2013). Hydrolysis of cellulose (i.e. cleavage of the (1-β-4) glycosidic bonds) depends on three classes of enzymes: cellobiohydrolases (CBHs; EC 3.2.1.91), endoglucanases (EGs, EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). Cellobiohydrolases remove cellobiose from either the non-reducing or the

reducing ends of chain with higher apparent activity on crystalline cellulose, and as a result, a rapid production of soluble sugars but slow decrease of the degree of polymerization (DP) is observed. Endoglucanases are typical multi-domain enzymes consisting of a catalytic domain that randomly cleaves β-1,4-glycosidic bonds in the middle of the polysaccharide chains and typically act on more irregular physical structures of the substrates, giving a rapid reduction of the DP but slow release of soluble sugars from crystalline cellulose (Ek et al., 2009). The degradation of amorphous cellulose located on the fibre surface and in between microfibrils, resulted in increasing exposed crystalline surfaces, and improving the swelling ability and reactivity of pulp (Henriksson et al., 2005; Gehmayr et al., 2011). Beta-glucosidases (β-D-glucopyranoside glucohydrolases) are enzymes that hydrolyze glycosidic bonds to release nonreducing terminal glucosyl residues from glycosides and oligosaccharides (Cairns and Esen, 2010).

Interestingly, EG treatments have become more and more popular in recent years to improve pulp reactivity and, as a side effect, precisely adjust pulp viscosity (Henriksson et al., 2005; Engström et al., 2006; Köpcke et al., 2008; Ibarra et al., 2010a). Strong alkaline treatment (> 8% wt NaOH) transforms cellulose I to cellulose II. Cellulose II is unfavorable for the production of cellulose derivatives or regenerated cellulose such as viscose for textile fibers because it has an antiparallel orientation relative to cellulose I and a more entangled and complex hydrogen-bonding network than the natural form (Ibarra et al., 2010a); as a result, the presence of cellulose II considerably affects the accessibility of cellulose. However, it is known that cellulose II is attacked by endoglucanases (Rahkamo et al., 1998), which was speculated to play a role in reactivity increase of the pulp after EG treatment (Henriksson et al., 2005; Engström et al., 2006; Kvarnlöf et al., 2006; Gehmayr and Sixta, 2012).

Therefore, in this chapter, pulp quality and Fock solubility of our biobleached fibers was improved by two endoglucanase enzymes one obtained from *Paenibacillus barcinonensis* (B) and the other from *Cerrena unicolor* (F). Cellulase action was evaluated in terms of Fock solubility, ^{13}C-CP/MAS NMR, water retention value (WRV), fibre morphology, cellulose degradation and carbohydrate composition of pulp. Another important contribution of the present study was to examine the composition of dissolved carbohydrate present in the liquors. Understanding the mode of action of the respective cellulases, in

terms of pulp activation and cellulose modification, provided interesting information in view of a potential industrial application. The work was completed with a pulp purification step, with 9% w/v NaOH, at 25 ºC, before the endoglucanase treatments. The combination of a cold caustic extraction followed by a hydrolytic treatment was evaluated in terms of Fock solubility, hemicellulose content and ^{13}C-CP/MAS NMR.

6.2 Materials and Methods

6.2.1 Pulp and enzymes

Unbleached sulfite cellulose obtained from a mixture of 60% Norway spruce (*Picea abies*) and 40% Scots pine (*Pinus sylvestris*) was used as a starting pulp. The pulp was cooked at Domsjö Fabriker mill (Sweden) and the characteristics were as follows: 4.2 ± 0.2 kappa number, 61.25 ± 0.6% ISO brightness and 511 ± 11 mL/g viscosity, and a carbohydrate content as determined by HPLC of 88.5 ± 0.3% glucan, 6.0 ± 1.3% mannan, 3.0 ± 0.3% galactan, 2.4 ± 0.4% xylan, and 0.3 ± 0.2% rhamnan. Then, starting pulp was biobleached using a laccase from *Trametes villosa* and violuric acid (VA) as a mediator.

Two different endoglucanases were used in the hydrolytic treatments. B (EC. 3.2.1.4) was produced from the newly identified species *Paenibacillus barcinonensis* by Universitat de Barcelona (Spain) (Chiriac et al., 2010). F endoglucanase was supplied by Fungal Bioproducts® (Spain) and was produced from *Cerrena unicolor*. The activity as U/g dry enzyme powder of the cellulase preparation was: 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the cellulase, respectively (see section 3.2.3).

6.2.2 Biobleaching sequence (L) for unbleached sulfite pulp

Prior to the bleaching treatment, unbleached sulfite fibers were conditioned at pH 4 adjusted with H_2SO_4, stirred at 2% pulp consistency for 30 min and washed with de-ionized water in a glass filter funnel. This step was needed to remove contaminants and metals, and also to bring the pulp to the pH required for the enzymatic treatment.

The bleaching process was conducted at the laboratory scale. A TCF biobleaching sequence including a laccase–mediator treatment was applied to unbleached sulfite cellulose. The sequence was designated L_{VA}PO, where L

denotes the enzymatic treatment and PO a hydrogen peroxide stage reinforced with oxygen. Treatment conditions are described in Chapter 3, section 3.3.1 (Laccase-mediator treatments) and 3.3.2 (Bleaching treatment). Subsequently, the treated pulps were extensively washed with de-ionized water and stored until use.

6.2.3 Enzymatic hydrolytic treatments and cold caustic extraction

The resulting biobleached pulp (LVAPO) was subjected to enzymatic hydrolysis with the two endoglucanases, B and F. The former enzyme was used at 120 U/g odp (L_B120 treatment) and the latter at 12 U/g odp (L_F12 treatment). The enzymatic treatments were performed in polyethylene bags that were placed in a laboratory water bath and following the conditions detailed in Chapter 3, section 3.4. A control treatment (L_K) was also performed under the same conditions but in the absence of enzyme. In parallel, the biobleached pulp (LVAPO) was also treated with a cold caustic extraction in an Easydye Ahiba oscillating individual reactor from Datacolor. The treatment was performed at 10% (w/w) consistency adjusted with 9% (w/v) NaOH at 25 ºC for 1h. Then the hydrolytic treatments with B or F enzymes were conducted, following the same conditions described earlier but using an Easydye Ahiba oscillating individual reactor.

6.2.4 Analysis of pulp properties

The starting and treated pulp samples were characterized in terms of kappa number, ISO brightness, viscosity, α-cellulose and water retention value (WRV) according to ISO 302:2004, ISO 2470:2009, ISO 5351:2004, TAPPI method T-203 cm-99 and ISO 23714, respectively. The bleaching process was also characterized in terms of chroma (C^*), which is the perpendicular distance of a point from the lightness axis [$C^* = (a^{*2} + b^{*2})^{1/2}$]. The degree of polymerization (DP) was calculated from intrinsic viscosity values, using the equation of Evans & Wallis (1987) (SCAN-CM 15:88). The number of scissions in the cellulose chain (CS) was calculated according to Bouchard et al., (2000).

Cellulose reactivity of the pulp samples was determined according to slightly modified version of Fock's method (Fock, 1959; Ibarra et al., 2010b). This is a micro-scale method simulating the industrial viscose process for manufacturing

regenerated cellulose. Prior to analysis, the samples were dried and conditioned in a climate room at 23 ºC and 50% RH overnight.

Carbohydrate composition of initial and treated pulps was determined using high performance liquid chromatography (HPLC). Samples were studied on a duplicate basis using a modified version of TAPPI 249 cm-09 test method.

^{13}C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ^{13}C.

The morphological properties of the fibres (viz., length, width and curl), and the content in fines of the pulp samples were determined in accordance with TAPPI T 271 on a Metso kajaaniFS300 fibre analyser. Surface SEM images of the handsheets were taken on a JEOL JSM-6400 microscope.

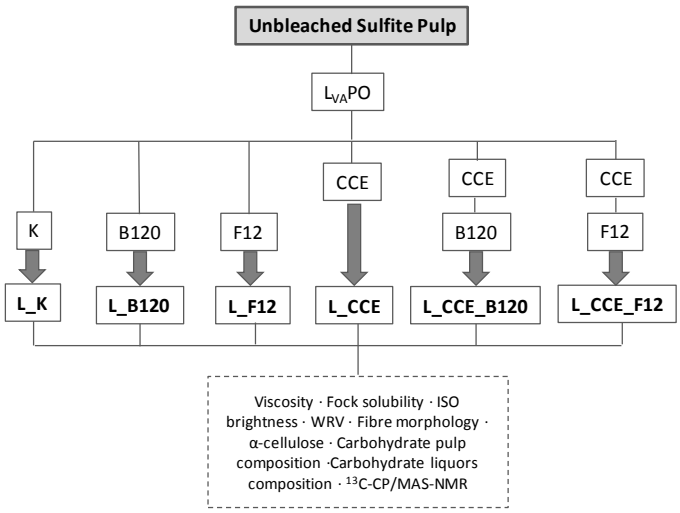
All the procedures mentioned above are described in Chapter 3, sections 3.7.1 and 3.7.2.

6.2.5 Effluent properties

Dissolved carbohydrates present in the liquors released from the cellulase treatments of biobleached dissolving pulps were quantified by HPLC (Agilent 1200 HPLC instrument), using a BIO-RAD Aminex HPX-87H ion-exchange resin column that affords the separation of glucose, xylose and arabinose (Chapter 3, section 3.7.4).

The concentration of oligosaccharides present in the treatment liquors was also determined by HPLC, using a Biorad Aminex HPX-42A ion-exchange column (Garcia-Ubasart et al., 2013a) (Chapter 3, section 3.7.4).

Schema 6-1 summarizes the experiments conducted and the properties analysed for each treatment.



Schema 6-1 Outline of the plan of unbleached sulfie pulps and the respective studied properties

6.3 Results and Discussion

In previous chapters (Chapter 4 and Chapter 5), sulfite pulp was satisfactorily biobleached by a combination of a laccase-violuric acid treatment, chelant stage and a pressurized hydrogen peroxide treatment. These biobleached pulps (LVAQPO) were characterized in terms of Fock solubility, viscosity, ISO brightness, alkali resistance, α-cellulose, metal ion content and thermal degradation by TGA. The pulps exhibited acceptable market dissolving pulp characteristics but in the direction of improving pulp reactivity and the quality of the final product (i.e. lower amount of hemicelluloses) further treatments were required. Therefore, this work wants to elucidate the effect of two endoglucanases, one from *Paenibacillus barcinonensis* (B) and the other one from *Cerrena unicolor* (F), on biobleached sulfite pulps (LVAPO). The cellulase treatments were compared with those where a cold caustic extraction with 9% (w/v) of NaOH was introduced before the hydrolytic treatment. As a novelty, the carbohydrate composition of liquors was analyzed and leads to understand the mechanism of action of each enzyme.

6.3.1 Fock Solubility

Table 6-1 shows the Fock solubility results. The initial pulp had a Fock solubility value of 76.6%, while L_F12 treatment had an 88% and the L_B120 treatment reached a Fock solubility value of 91%.

Table 6-1 Fock solubility values obtained for each sample

	Fock solubility (%)	Chain scission (CS)
LVAPO (=L)	76.6 ± 3.7	0.39
L_K	76.3 ± 5.5	0
L_B120	90.9 ± 1.9	0.03
L_F12	88.1 ± 1.1	0.16
L_CCE	69.7 ± 3.6	-
L_CCE_B120	70.8 ± 3.7	-
L_CCE_F12	82.4 ± 2.4	-

In Figure 6-1 is shown the relation of Fock solubility increase and chain scission values (CS), calculated from the viscosity results. As expected, the control cellulase treatment did not undergo Fock solubility improvement, although the viscosity value was slightly different from biobleached pulp. With respect to biobleached pulp, the L_B120 improved Fock solubility by 18.7% with no variation on viscosity. Therefore, this change was due to the action of the own endoglucanase treatment. The L_F12 treatment also suffered a Fock solubility increase but a viscosity loss with respect to initial pulp was found. Therefore, the improvement on Fock solubility cannot only be related to the enzyme action.

The relationship between pulp solubility according to Fock method and chain scission values provided useful information to understand the action that each enzyme caused on biobleached cellulose fibres (Figure 6-1). Special attention had the B endoglucanase which provided the highest increase in Fock solubility (18.7 % respect to LVAPO) even though CS was virtually zero, suggesting that this enzyme produced a fibrillated effect rather than cutting the fibres, as previously demonstrated by some authors (Cadena et al., 2010c; Garcia-Ubasart et al., 2013a). L_F12 treatment improved Fock solubility by 13.5 % and exhibited slightly greater CS number of 0.16 with respect to starting pulp, which is in agreement with its slightly greater viscosity loss. As can be seen from Table

6-1 and Figure 6-1, the buffer solution used in the control treatment (L_K) did not contribute to improve Fock solubility or shorten fibres and CS value remained 0.

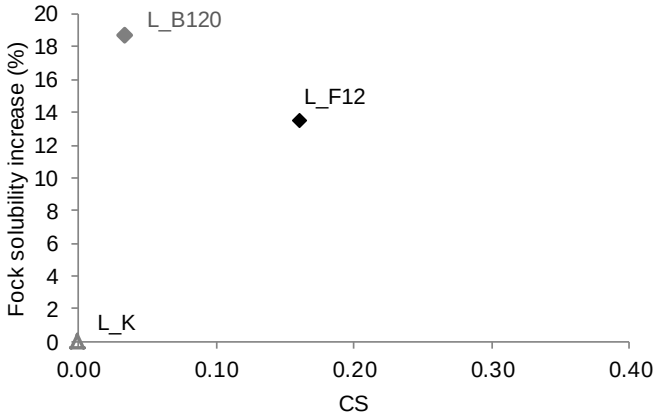
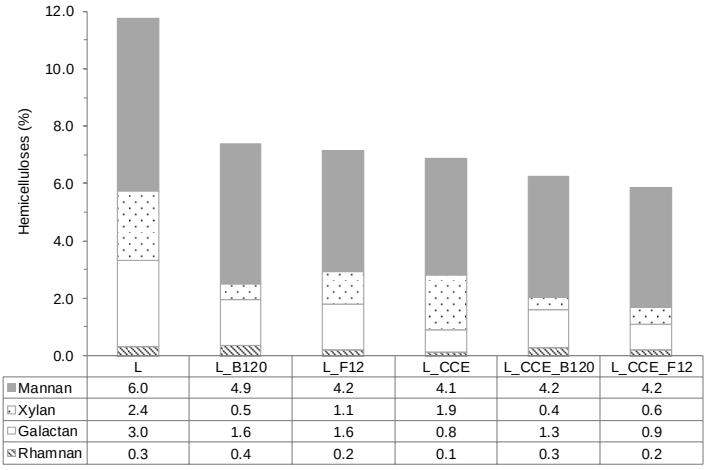


Figure 6-1 Fock solubility increase (calculated with respect to the previous treatment) and number of chain scission for each treatment.

Carbohydrate composition was determined by HPLC, with special interest on the hemicelluloses content (Figure 6-2). The hemicellulose composition of biobleached pulp (LVAPO) was as followed: 6.0% mannan, 3.0% galactan, 2.4% of xylan and 0.3% rhamnan. With B endoglucanase treatment, the hemicellulose content was diminished by 37% and the final amount of hemicelluloses was 7.4%. As can be seen, the highest reduction was observed with xylan fraction (77%), then galactan (47%) and finally mannan (19%), with respect to biobleached pulp. F endoglucanase decreased the amount of hemicelluloses by 39% which corresponds to a 7.1% of final hemicelluloses. The xylan fraction suffered a reduction of 53%, the galactan a 47% and the mannan a 30%. From results, it is observed that B and F endoglucanases exhibited the same pattern of action for galactan (i.e. similar removal) but acted differently on mannan and xylan fraction. Interestingly, the xylan removal was superior to mannan for both endoglucanase treatments, being B endoglucanase more efficient on xylan removal than F. On the contrary, F was slightly more effective on mannan fraction than B. Finally, the amount of rhamnan was not modified by the respective enzymatic treatments.

In terms of pulp purification step, the strong alkaline treatment before the hydrolytic treatment also contributed to reduce the total amount of hemicelluloses by 42% with respect to biobleached pulp (L), reaching a final content of 6.8% (Figure 6-2). Interestingly, the higher effect was produced on galactan fraction and was greater than the one observed for endoglucanase treatments alone. In contrast, xylan removal was lower than the ones achieved by endoglucanase treatments and no changes on mannan fraction were detected.

Combining cold caustic extraction followed by the respective cellulase treatments, hemicelluloses removal was further extended, with respect to L_CCE sample. The final amount of hemicelluloses for L_CCE_B120 and L_CCE_F12 was 6.2% and 5.8%, respectively. To be precise, only xylan fraction was affected while galactan and mannan carbohydrates remained unaltered. As previously observed, the B endoglucanase had slightly higher action on xylan fraction than F. In particular, B reduced the amount of xylans by 79% while the F a 70%. To conclude, a synergy effect between CCE and endoglucanase led to further diminished xylan content, but a limiting mannan and galactan fractions resulted resitant to both treatments.



	L	L_B120	L_F12	L_CCE	L_CCE_B120	L_CCE_F12
Mannan	6.0	4.9	4.2	4.1	4.2	4.2
Xylan	2.4	0.5	1.1	1.9	0.4	0.6
Galactan	3.0	1.6	1.6	0.8	1.3	0.9
Rhamnan	0.3	0.4	0.2	0.1	0.3	0.2

Figure 6-2 Hemicellulose composition of treated pulps determined by HPLC. Biobleached pulp and the respective endoglucanase treatments, biobleached pulp followed by cold caustic extraction and then the respective endoglucanase treatments. The fraction of each carbohydrate is indicated in the table.

6.3.2 Pulp properties

It can be appreciated in Table 6-2 that the brightness, viscosity, the α-cellulose and the contents in fines, of the treated control pulp was practically identical with those of the starting pulp. By contrast, B and F treatments caused a brightness loss with respect to biobleached sulfite pulp. Viscosity results manifested that the enzymatic treatments had no effect on cellulose integrity, although L_F12 seemed to present a slightly lower value. On the other hand, α-cellulose was adversely affected by the enzymatic treatments, particularly with B. It should be noted that viscosity represents the average molecular weight of all cellulose chains, whereas α-cellulose indicates the content of undegraded, higher molecular weight cellulose in pulp. Therefore, although no damage of cellulose chains was perceived, the content in high-molecular weight cellulose (α-cellulose) was considerably altered by B enzyme. Regarding the proportion of fines, enzymatic treatments increased the amount of fines by up to 24% with L_B120 and 53% with L_F12, relative to LVAPO sample. Likewise, fibre length was reduced by 19% and 41%, respectively. The latter results suggest that both endoglucanases act on cellulose chains but have a different effect on fibre length, which is also suggestive of a different mechanism of action.

Table 6-2 Results (± confidence intervals) of brightness, chroma coordinate (C*), viscosity and α-cellulose. The content of fines and fibre length were determined according to TAPPI standard and calculated as an arithmetic mean for the different treated pulps.

	ISO Brightness (%)	*C**	Viscosity (mL/g)	α-cellulose	Fines (%)	L (n) (mm)	WRV
LVAPO	88.45 ± 0.11	3.07	395 ± 6	87.3 ± 0.39	30.98 ± 1.04	0.85 ± 0.02	1.1 ± 0.13
L_K	88.95 ± 0.11	3.00	453 ± 28	89.5 ± 0.71	30.96 ± 1.04	0.86 ± 0.01	1.1 ± 0.04
L_B120	82.90 ± 0.27	4.14	384 ± 88	80.4 ± 0.02	38.32 ± 1.12	0.69 ± 0.02	1.26 ± 0.11
L_F12	85.75 ± 0.60	3.20	348 ± 43	83.4 ± 0.61	47.30 ± 0.52	0.50 ± 0.01	1.12 ± 0.03

The biobleached pulp treated with B increased the WRV by 17% but fibre length was only reduced by 19% and the content of fines increased by 24%, with respect to LVAPO pulp (Figure 6-3). By contrast, L_F12 treatment did not modify the WRV (4% increase) but raised the content in fines by 53% and reduced fibre length by 42%, regarding biobleached pulp. The respective results suggest that B enzyme might alter fibre surface via external fibrillation (Cadena

et al., 2010c; Garcia-Ubasart et al., 2013a) while F enzyme showed a preferable action to cut the fibres.

The differences found between fibre length, fine content, WRV and CS pointed out that B and F endoglucanases had different performing mechanism.

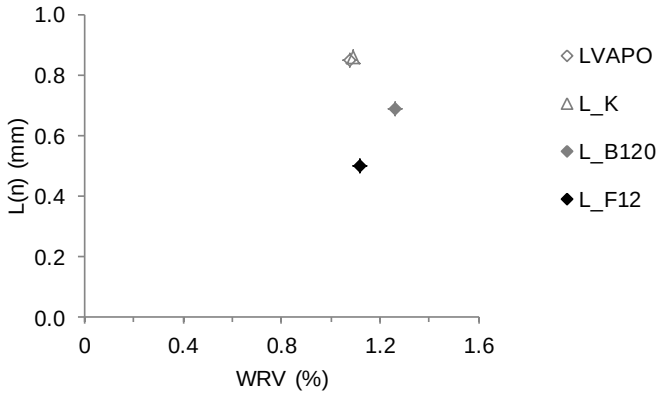


Figure 6-3 Relation between fibre lengths determined according to TAPPI standard and calculated as an arithmetic mean and water retention value (WRV) for biobleached sulphite pulps treated with B and F enzyme.

6.3.3 ^{13}C-CP/MAS NMR

The solid state ^{13}C-NMR spectra of biobleached pulp, the enzymatic treated pulps and 9% (w/v) NaOH extracted (CCE) pulps followed by endoglucanase treatments are illustrated in Figure 6-4. The spectra of L_CCE_B120 and L_CCE_F12 samples exhibited different polymorphic form than samples submitted to an endoglucanase treatment alone. The introduction of a 9% (w/v) NaOH extraction converted cellulose I to cellulose II, since the C-6 signal at 65 ppm increased, obtaining two peaks with nearly identical heights at 65 ppm and 61 ppm. Nevertheless, the C-1 signal did not exhibit a shoulder at 108 ppm, which is characteristic of cellulose II (Janzon et al., 2008a). The greater proportion of cellulose II of L_CCE_B120 and L_CCE_F12 samples can be related to the low Fock solubility values, in comparison to L_B120 and L_F12 (Krässig, 1993; Janzon et al., 2008a).

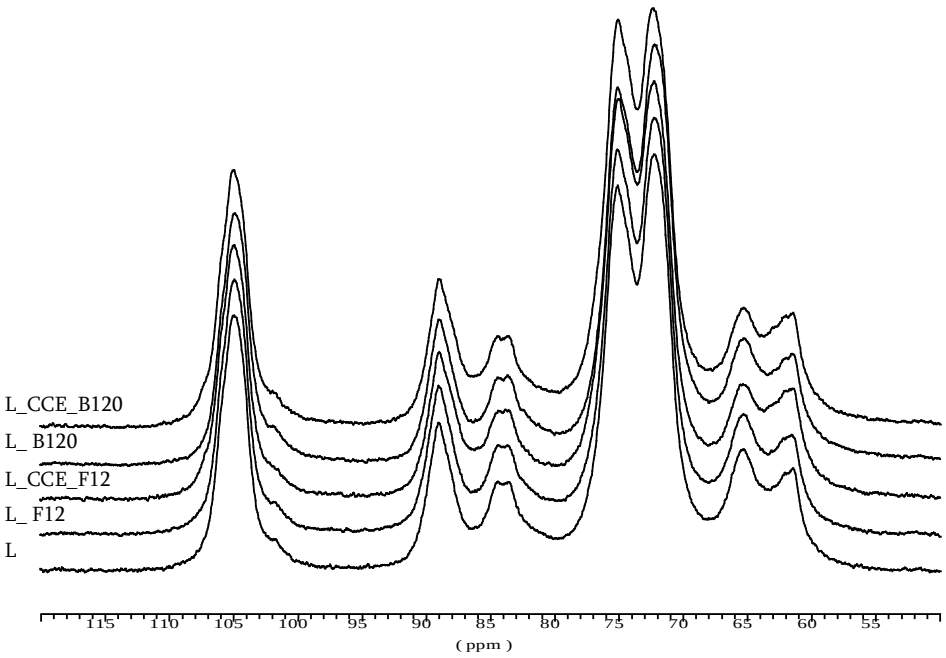


Figure 6-4 ^{13}C-CP/MAS NMR spectra of biobleached pulp (L) compared to samples treated with B and F endoglucanases alone or after an extraction with 9% (w/v) NaOH.

Figure 6-5 shows SEM images of handsheets made from LVAPO, L_B120, L_F12 and L_K treated pulps. Interestingly, the action of F enzyme was apparently observed in increased amount of free particles and disruption of the cellulose wall structure. With the L_B120 treatment, differences in appearance with respect to control and starting pulp were inappreciable; however, the treated pulp was 19% more reactive with less percentage of hemicelluloses, but similar in viscosity and fiber length to the starting pulp.

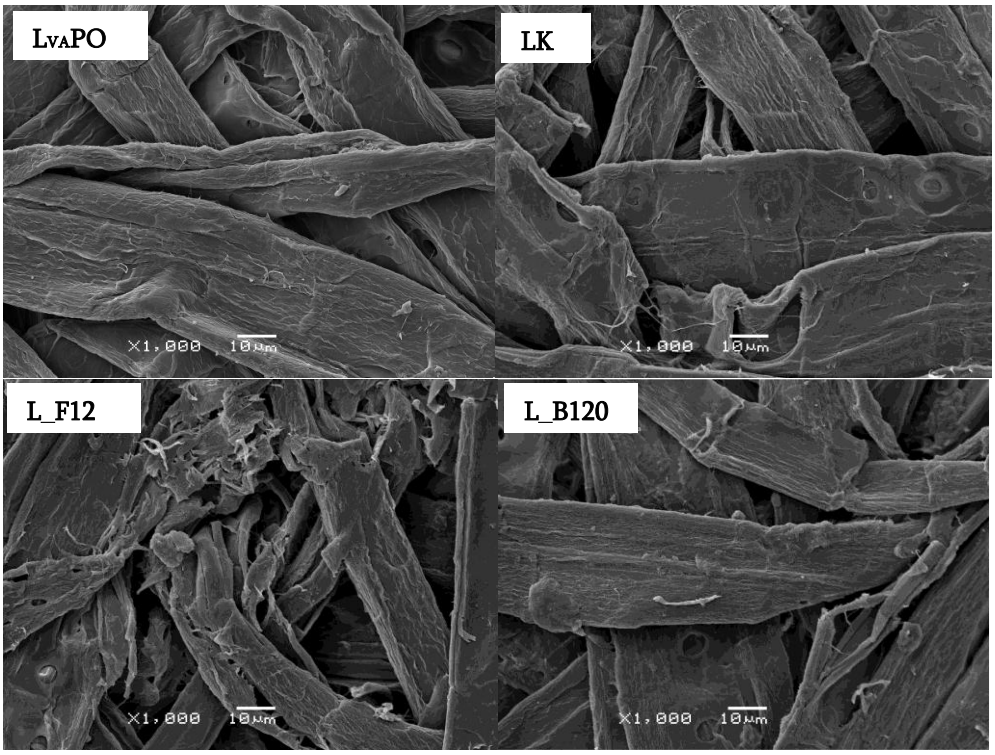
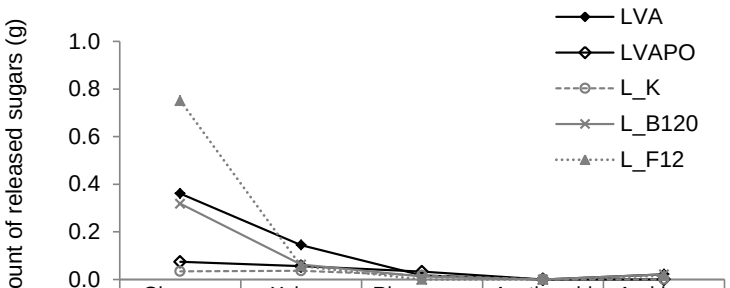


Figure 6-5 SEM images of the biobleached treated pulps and the respective hydrolytic treatments

6.3.4 Mode of action of B and F endoglucanases

In order to further explain the results, the concentration and composition of dissolved sugars present in the liquors released from the enzymatic treatments were determined by HPLC. Comparing the results for the different treatments required expressing the composition of dissolved monosaccharides relative to the effluent volume resulted from each treatment. As can be seen from Figure 6-6, the Lva treatment produced a greater release of glucose and xylose than did LvaPO. As also observed with Fock solubility and fiber morphology, the results for the control treatment (L_K) revealed that the buffer solution by itself had no effect on carbohydrate composition, while L_F12 treatment caused the greatest release of glucose, followed by L_B120, but taking into consideration that enzyme dosage differed between cellulases. Specifically, L_B120 treatment presented a 77% of glucose with respect to the total amount of carbohydrates and L_F12 a 91%. These results are consistent with the number of chain scissions caused by each enzyme and with the extent cellulose degradation,

expressed as viscosity. The amount of xylose in the liquors was smaller than that of glucose in all treatments with an exception of control treatment (L_K), which led to very similar amounts of the two monomers. As observed for carbohydrate pulp composition, L_B120 treatment was more efficient to remove xylose than F_12, although 30% of F activity corresponds to a xylanase activity. In other words, L_B120 effluent contained 15% of xylose, while L_F12 only 7%.



	Glucose	Xylose	Rhamnose	Acetic acid	Arabinose
LVA	0.361	0.144	0.017	0.000	0.022
LVAPO	0.074	0.055	0.033	0.000	0.000
L_K	0.034	0.036	0.016	0.000	0.000
L_B120	0.318	0.063	0.012	0.000	0.021
L_F12	0.751	0.056	0.000	0.000	0.016

Figure 6-6 Amount of dissolved monosaccharides (g) found in the liquors of each treatment

In addition, the low amount of oligosaccharides detected in L_B120 treatment is consistent with the low decrease of pulp viscosity. Table 6-3 shows the concentration and composition of oligosaccharides in the effluents as determined by HPLC.

Table 6-3 Concentration of oligosaccharides (mg/mL) in enzymatic treatment liquors

mg/mL	L_B120	L_F12	L_K
Glucose	0.270	0.829	0.000
Cellobiose	0.592	1.486	0.000
Cellotriose	0.000	0.05	0.000

Glucose standard oligomers were used as references. The pattern of products released by the L_F12 treatment decreased in the following sequence: cellobiose > glucose > cellotriose. The fact that oligosaccharides of different size and

glucose monomer are found is consistent with the mode of action of F, which randomly cleaves glycosidic bonds. With L_B120 treatment, cellobiose in major proportion and glucose in lower were identified in the liquors but no presence of cellotriose or larger oligosaccharides. The contents in hydrolysis products suggest that B is an endoglucanase with an exo type mode of action (Chiriac et al., 2010). According to Chiriac et al. (2010), the absence of long oligosaccharides among the hydrolysis products from crystalline cellulose would indicate a processive mode of action of B, as previously reported for some endoglucanases of family 9 (Irwin et al., 1998; Zverlov, 2003). In addition, the low amount of oligosaccharides detected in L_B120 treatment is consistent with the low decrease of pulp viscosity.

6.4 Conclusions

Two different cellulases, B and F, were studied in biobleached sulfite pulp ($L_{VA}PO$) with the intention to increase Fock solubility and bring a satisfactory advantage to the viscose process. The enzymes, however, exhibited a different mode of action. Thus, while B increased Fock solubility strongly but had little effect on fiber length, F increased Fock solubility to a lesser extent but produced a strong reduction in fiber length and increased notably the amount of fines. In terms of the number of chain scission, the F endoglucanase produced higher values than B endoglucanase. The soluble carbohydrate composition present in the liquors as determined by HPLC confirmed that B endoglucanase had an exo type mode of action since cellobiose and glucose in different proportion were detected. On the other hand, the F liquors contained cellobiose, glucose and traces of cellotriose soluble oligomers.

The most salient contribution of this study is that the introduction of a biobleaching sequence involving a laccase-mediator system (LMS) provides an environmentally friendly process without detracting the final characteristics of a dissolving pulp, and the subsequent endoglucanase treatment (L_B120) reached a 91% of Fock solubility. On the other hand, the cold caustic extraction introduced before the hydrolytic treatment could reduce the percentage of hemicellulose although the final Fock solubility was lower due to the formation of cellulose II as observed by ^{13}C-CP/MAS NMR. However, complementing the purification step with F12 treatment, lead to improve Fock solubility and reach a final value of 82.4% and obtain the lowest amount of hemicelluloses (5.8%).

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Chapter 7

Evaluation of the action of different endoglucanases on a bleached commercial dissolving pulp

Abstract

A fully bleached commercial acid dissolving pulp was treated with two endoglucanases, one obtained from *Paenibacillus barcinonensis* (B) and the other one produced from *Cerrena unicolor* (F) with the intention to improve cellulose reactivity and processability in the viscose process or during the manufacturing of cellulose derivatives. B cellulase was tested under 120 U/g odp (oven dry pulp) and the F cellulase under two conditions, 12 U/g odp and 60 U/g odp. In addition, a purification stage, consisting in a cold caustic extraction (CCE) of 9% w/v NaOH, was applied before or after the enzymatic treatment in order to reduce the amount of hemicelluloses and improve the action of enzymes. The treated pulps were evaluated in terms of brightness, viscosity, water retention value (WRV), fibre morphology, carbohydrate composition, Fock solubility and NMR. In general, results revealed that both endoglucanases improved cellulose reactivity, albeit in a different way; thus, B caused no scissions in the cellulose chain and no significant reduction in fibre length, whereas F strongly decreased viscosity, shortened fibre length and increased considerably the amount of fines. The result of applying two different doses of F cellulase was reflected on Fock solubility and fibre morphology. F60 treatment was found to give the highest value of Fock solubility and the biggest reduction of fibre length. The effect of both endoglucanases on Fock solubility was increased by introducing an earlier CCE stage. Finally, a CCE_B120 pulp with 3% of hemicellulose and 69% of Fock solubility was obtained.

7.1 Introduction

Cellulose presents an extremely compact structure due to its supramolecular structure (viz., its intermolecular and intramolecular hydrogen bonds). The structure of cellulose consists of crystalline and non-crystalline domains (Krässig, 1993; Köpcke et al., 2008). This non uniform structure is responsible for the low accessibility and reactivity of cellulose, and hence for its limited solubility in some special solvents. The crystalline domain of cellulose is highly ordered and exhibits extensive hydrogen bonding between molecular chains. As a result, cellulose can crystallize into various polymorphs. Natural cellulose (cellulose I) is the form found in nature and occurs in two allomorphs: I_α and I_β (Nishiyama et al., 2002, 2003; Ciolacu et al., 2011). Cellulose II is the crystalline form that emerges after regeneration from different media or mercerization with aqueous sodium hydroxide (Langan et al., 2001, 2005; Nishiyama et al., 2003). Cellulose III_I and III_{II} are obtained by treating cellulose I and II, respectively, with liquid ammonia or an organic amine (Wada et al., 2004).

Strongly alkaline pre-treatments are used in the production of carboxymethyl cellulose and viscose for textile fibres (Mozdyniewicz et al., 2013). Such treatments cause native cellulose (cellulose I) to swell and after washing shrink back to another allomorphs, cellulose II, through Na-cellulose (Klemm et al., 1998; Van de Weyenberg et al., 2006; Pönni et al., 2014). The conversion of cellulose I into Na-cellulose is fast. Cellulose II is unfavourable for the production of cellulose derivatives or regenerated cellulose such as viscose for textile fibres because it has an antiparallel orientation relative to cellulose I and a more entangled and complex hydrogen-bonding network than the natural form (Ibarra et al., 2010a); as a result, cellulose II has higher difficulty of dissolution than cellulose I (Krässig, 1993).

The presence of cellulose II considerably affects the accessibility of cellulose. Thus, the reactivity of pulp is defined as the accessibility of hydroxyl groups at C6 and C2/C3 of the glucose monomer units to reactants, and dictates the processability of dissolving pulps for the viscose process (Sixta, 2006; Gehmayr and Sixta, 2012).

Endoglucanase (EG) treatments have become increasingly popular in recent years to improve pulp reactivity and, as a side effect, precisely adjust pulp

viscosity (Henriksson et al., 2005; Engström et al., 2006; Köpcke et al., 2008; Ibarra et al., 2010a). Endoglucanases (EG) are typical multi-domain enzymes consisting of a catalytic domain that randomly cleaves β-1,4-glycosidic bonds in polysaccharide chains. These enzymes preferentially degrade amorphous cellulose located on the fiber surface and in between microfibrils, thereby increasing exposed crystalline surfaces, and the swelling ability and reactivity of pulp (Henriksson et al., 2005; Gehmayr et al., 2011). Additionally, cellulose II is attacked by endoglucanases (Rahkamo et al., 1998), which was speculated to play a role in reactivity increase of the pulp after EG treatment (Henriksson et al., 2005; Engström et al., 2006; Kvarnlöf et al., 2006; Gehmayr and Sixta, 2012). However, accessibility is indeed a structural parameter that depends on the nature of the interactions between reactants and the cellulose substrate, and such interactions are influenced by the availability of the inner surface and the fibril architecture. The properties of cellulose depend directly on the moisture regain of the particular fibrous material and on environmental moisture (Ciolacu et al., 2011). One other key concept in fully understanding enzymatic degradation of cellulose is the influence of parameters such as accessibility, crystallinity and supramolecular structure of the substrate.

The outstanding results obtained from a biobleached sulfite pulp (LVAPO) combined with cold caustic extraction and the respective endoglucanase treatments (Chapter 6), supported the idea to conduct a similar investigation but with TCF bleached commercial pulp. The different hemicelluloses content between biobleached sulfite pulp (LVAPO) and commercial bleached dissolving pulp may account for different results. This work focused on providing more knowledge about the effect of CCE treatment on cellulose activation. Therefore, further treatments, such as CCE before or after endoglucanase treatment, were studied. In addition, cellulase action was evaluated in terms of Fock solubility, ^{13}C-CP/MAS NMR, water retention value (WRV), fiber morphology, carbohydrate composition and cellulose degradation.

7.2 Materials and Methods

7.2.1 Pulp and enzymes

A commercial dried totally chlorine-free (TCF) bleached sulfite dissolving-grade pulp obtained from a mixture of 60% Norway spruce (*Picea abies*) and

40% Scots pine (*Pinus sylvestris*) was used as a raw material. The pulp was cooked and bleached at Domsjö Fabriker mill (Sweden) and presented the following characteristics: 91.70 ± 0.15% ISO brightness and 474 ± 0.7 mL/g viscosity with a carbohydrate composition as determined by high-performance liquid chromatography (HPLC) of 95.6 ± 0.3% glucan, 2.8 ± 0.2% mannan, 0.8 ± 0.0% xylan, 0.6 ± 0.3 galactan and 0.2 ± 0.2% rhamnan.

Two different endoglucanases were used in the hydrolytic treatments. B (EC. 3.2.1.4) was produced from the newly identified species *Paenibacillus barcinonensis* by Universitat de Barcelona (Spain) (Chiriac et al., 2010, 2013). The cellulase activity was 60 CMCase U/mL. F endoglucanase was supplied by Fungal Bioproducts® (Spain) and was produced from *Cerrena unicolor*. The activity as U/g dry enzyme powder of the cellulase preparation was: 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the cellulase, respectively. In both cases, the cellulase activity was determined by measuring the amount of reducing sugars released from carboxymethyl cellulose (CMC, Sigma) according to Somogyi-Nelson method (Spiro, 1966). An activity unit (U) is the amount of enzyme capable of converting 1 μmol of substrate per second.

7.2.2 Enzymatic hydrolytic treatments and cold caustic extraction (CCE)

Prior to the hydrolytic treatments, the pulp was disintegrated according to ISO 5263-1:2004 and stored at 4 ºC until use. Commercial dried TCF-bleached dissolving pulp was subjected to an enzymatic hydrolysis treatment followed by a cold caustic extraction (CCE) treatment or vice versa. Two different endoglucanases, cellulase B and cellulase F, were used at different doses, namely: 120, 12 and 60 U/g (odp), corresponding to samples B120, F12 and F60, respectively (Chapter 3, section 3.4). The treated pulps were extensively washed with de-ionized water. A control treatment (K) under the same conditions as the enzymatic treatment but in the absence of enzyme was also developed. A cold caustic extraction (CCE) treatment was conducted in an Easydye Ahiba oscillating individual reactor from Datacolor. The treatment was performed at 10% (w/w) consistency adjusted with 9% (w/v) NaOH at 25 ºC for 1 h. Treated pulps were washed with de-ionized water until the filtrate pH was neutral.

The treatments were named as follows: CCE, K, K_CCE, B120, CCE_B120, B120_CCE, F12, CCE_F12 and F60. After each enzymatic or chemical treatment, an amount of pulp sample was withdrawn for subsequent characterization.

A summary of the work conducted in this chapter is shown in Schema 7-1. Techniques and methods used for treated-pulps characterization are also detailed.

7.2.3 Analysis of pulp properties

The starting and treated pulp samples were characterized in terms of kappa number, ISO brightness, viscosity and water retention value (WRV) according to ISO 302:2004, ISO 2470:2009, ISO 5351:2004 and ISO 23714, respectively. The degree of polymerization (DP) was calculated from intrinsic viscosity values, using the equation of Evans & Wallis (1987) (SCAN-CM 15:88) and pulp degradation was assessed via the number of scissions in the cellulose chain (CS) (Bouchard et al., 2000)

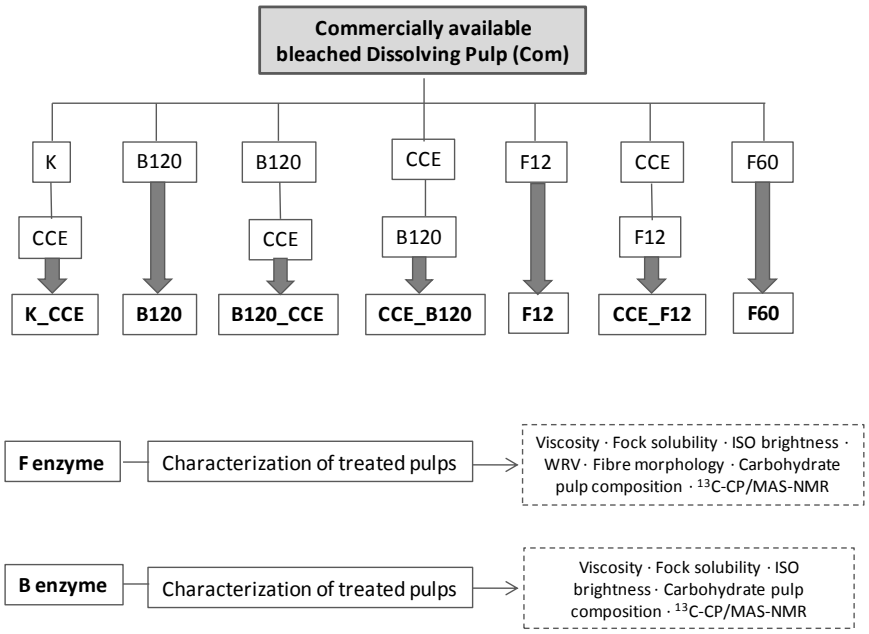
The cellulose reactivity of the pulp samples was determined according to slightly modified version of Fock's method (Fock, 1959; Ibarra et al., 2010b).

Carbohydrate composition of TCF-bleached commercial dissolving pulp and the resulted treated pulps were determined using high performance liquid chromatography (HPLC) using a modified version of TAPPI 249 cm-09 test method.

^{13}C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ^{13}C.

The morphological properties of the fibres (viz., length, width and curl), and the content in fines of the pulp samples were determined in accordance with TAPPI T 271 on a Metso kajaaniFS300 fibre analyser. Fibers were analysed by light microscopy according to ISO 9184-3:1990.

More detailed information about each technique and method is provided in Chapter 3, 3.7.1and 3.7.2 sections.



Schema 7-1 Schema of work applied to dried bleached commercial dissolving pulp and the respective studied properties.

7.3 Results and Discussion

During the manufacturing of cellulose derivatives and regenerated cellulose, the accessibility of cellulose is a key parameter to control, since a no homogeneous substitution of the hydroxyl groups of the cellulose chain might lead to the production of low-quality derivatives (Köpcke, 2010). In the production of regenerated cellulose, the ideal achievement is to obtain a complete dissolution of the cellulose structure. However, due to its supramolecular structure is difficult to achieve. In addition, the non-uniform and compact structure of cellulose limits its dissolution to some special solvents. On the other hand, the solvents used to dissolve cellulose are usually expensive, highly toxic and have harmful effects on the environment. Hence, the great interest to improve cellulose accessibility and as a result reduce the consumption of solvents.

With the previous observations in mind, the present work deals with the intention to improve pulp reactivity. Therefore, cellulase treatments in

combination with strong alkaline treatments were performed. Fock solubility, carbohydrate composition, fibre morphology and WRV of treated pulps were analysed.

7.3.1 Fock solubility

The reactivity of pulp was assessed using Fock's method, which measures the amount of pulp that is dissolved in a viscose-like solution (Fock, 1959; Ibarra et al., 2010a; Gehmayr and Sixta, 2012). As can be seen from Table 7-1, both enzymes increased Fock solubility: from 67.3% (starting pulp, Com) to 76.1% with the F12 treatment and 95.8% with the F60 treatment. The B120 treatment also increased Fock solubility up to 78.7%. The high reactivity obtained with F60 is comparable to previously reported values of Henriksson et al., 2005; Ibarra et al., 2010a for similar softwood TCF bleached dissolving pulp. According to Henriksson et al. (2005), endoglucanase attacks the less ordered cellulose regions between and on the surface of the fibrils, leading to fibre wall swelling and therefore an increase in the accessibility to solvents. A strong alkaline treatment (CCE) caused an important Fock solubility loss (26%) with respect to commercial dissolving pulp. The same negative effect was observed for K_CCE and B120_CCE samples, the latter giving a Fock solubility reduction of 31%. Interestingly, when the CCE treatment was introduced firstly and then followed by endoglucanase treatment a Fock solubility improvement was found, although final values were lower than the ones presented by endoglucanase treatments alone.

Table 7-1 Fock solubility value of each treatment

	Fock solubility (%)
Com	67.3 ± 2.1
F12	76.1 ± 3.2
F60	95.8 ± 0.2
B120	78.7 ± 1.5
K	61.2 ± 6.0
CCE	49.8 ± 4.4
K_CCE	48.9 ± 5.0
CCE_F12	69.1 ± 3.7
B120_CCE	54.0 ± 0
CCE_B120	69.2 ± 4.2

The Fock solubility increase was calculated with respect to the value obtained from the previous stage of each treatment. As can be seen from Figure 7-1, a cold caustic extraction by itself or the combination of a cold caustic extraction after an enzymatic treatment (B120_CCE) diminished the Fock solubility of the final pulp due to the hornification phenomena, which is an aggregation of microfibrils. Gehmayr et al. (2011) previously found drying or strong dewatering of pulp after a treatment at high alkalinity induce the formation of molecular aggregates via inter- and intra fibrillar hydrogen bonding of the cellulose microfibrils.

The high values of Fock solubility obtained by F60 and F12 treatments can be expressed as a 42% and 13% of reactivity increase with respect to the starting pulp. Fock solubility was also considerably improved by alkaline extraction and then followed by enzymatic treatment. Although the increase in reactivity was similar for CCE_F12 and F60, the final Fock solubility level obtained was 69% with the former and 96% with the latter. In the case of B120, the hydrolytic treatment caused a 17% reactivity increase with respect to commercial pulp, and led to a reactivity value of 79%. Using a subsequent alkaline stage had an adverse effect, but inserting it prior to the hydrolytic treatment increased Fock solubility by a further 39% reaching a 69% value. These results can be explained by the fact that the cold caustic treatment (concentration > 8%wt) transforms cellulose I into cellulose II and endoglucanases have greater affinity for the latter form (Engström et al., 2006; Köpcke et al., 2008; Gehmayr et al., 2011).

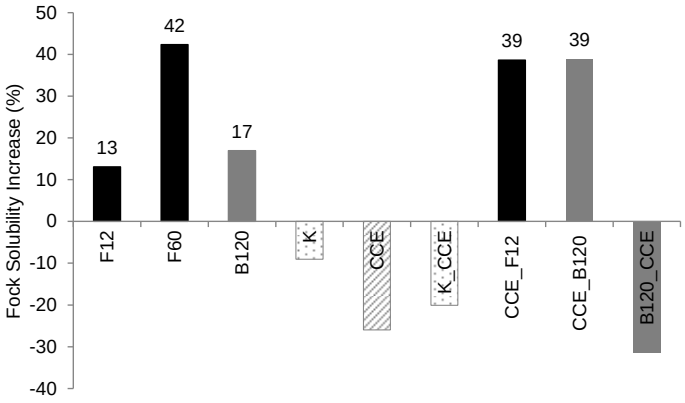


Figure 7-1 Reactivity increase according to Fock method calculated with respect to the reactivity value of the previous stage for each enzymatic and control treatment.

In Figure 7-2 is shown Fock solubility and viscosity results. Interestingly, K, CCE and K_CCE showed slightly higher viscosities than original pulp (Com). This result can be explained by the dissolution of hemicelluloses of low molecular weight which effect the determination of viscosity. However, in terms of Fock solubility, a significant loss was detected with CCE treatment. Specifically, a decrease of 26 and 20% was found for CCE and K_CCE, respectively. The strong alkaline concentration is responsible for the transformation of cellulose I to cellulose II. The cellulose II polymorph has a closer and denser structure and as a result present lower reactivity (Sixta, 2006). On the other hand B120 and CCE_B120 did not suffer cellulose degradation with respect to Com sample, but B120 treatment experimented a 17% of Fock solubility improvement. As observed earlier, the combination of a B120 followed by CCE treatment resulted in a lower Fock solubility.

F12, F60, CCE_B120 and CCE_F12 samples presented lower viscosities than commercial dissolving pulp, but similar range value between them. In terms of Fock solubility, interesting differences were observed. Therefore, the greater Fock solubility of F12 and F60 samples than CCE_B120 and CCE_F12 samples was due to the enzyme action itself. Significantly, comparing F60 and F12 treatments led to affirm that higher dose of endoglucanase had positive influence on Fock solubility. By its side, CCE_B120 and CCE_F12 presented same Fock solubility values but much higher than CCE sample. Therefore, the

endoglucanase treatment was responsible for the Fock solubility improvement. It is known that endoglucanases have greater affinity to cellulose II.

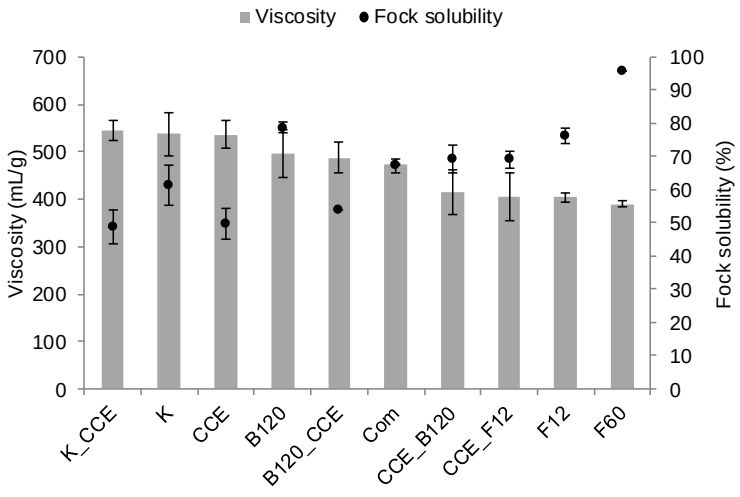


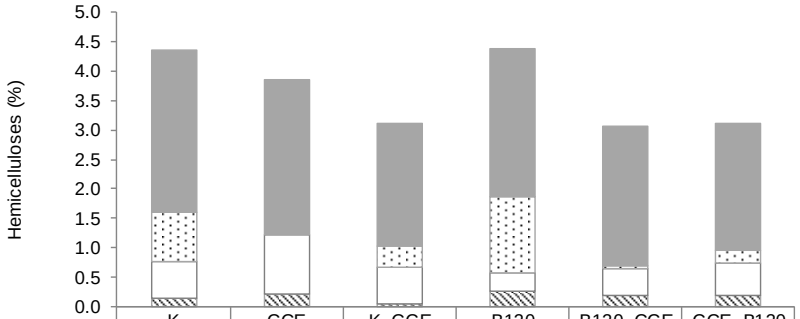
Figure 7-2 Fock solubility increase, calculated with respect to the previous treatment, and viscosity values for each treatment. Non-filled symbols indicates endoglucanase treatments alone and filled symbols corresponds to combined treatment (endoglucanase and CCE)

7.3.2 Carbohydrate composition of treated pulps

Carbohydrate composition was determined by HPLC, with special attention on the hemicelluloses content (Figure 7-3). The control treatment presented the following carbohydrate composition: 2.8% mannan, 0.8% xylan, 0.6% galactan and 0.2% rhamnan. All treatments reduced the amount of xylans with exception of B120 treatment, with respect to control treatment. A comparison of control treatment (K) and control treatment followed by strong alkaline treatment (K_CCE) let to distinguish the action resulted from the own alkali treatment. With K_CCE treatment the amount of xylan and mannan was reduced by 57% and 25%, respectively. The B endoglucanase treatment did not diminish the global amount of hemicelluloses, however, with the introduction of a cold caustic extraction a variation was found. Interestingly, no differences were detected with the combination of an alkaline extraction before (CCE_B120) or after the enzymatic treatment (B120_CCE) and the final amount of hemicelluloses was 3.1% in both cases. The action of F endoglucanase on mannan fraction was higher than the one produced by B. The CCE_F12 treatment reduced the amount of hemicelluloses to a final content of 3.4%,

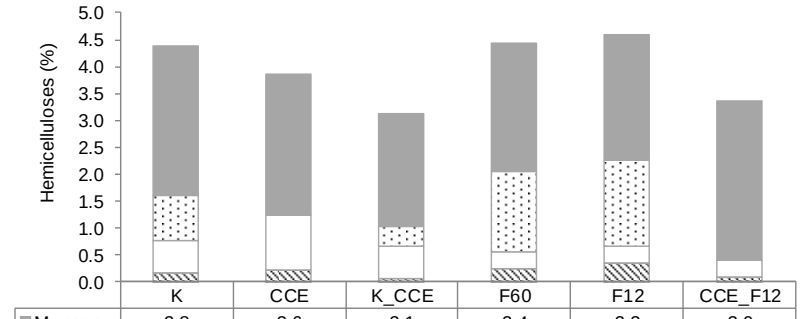
affecting principally the xylan fraction. Working at higher enzyme dose (F60 treatment) did not result in higher reduction of hemicelluloses; in fact, the final amount of hemicelluloses was the same as the control treatment.

a)



	K	CCE	K_CCE	B120	B120_CCE	CCE_B120
Mannan	2.8	2.6	2.1	2.5	2.4	2.1
Xylan	0.8	0.0	0.4	1.3	0.1	0.2
Galactan	0.6	1.0	0.6	0.3	0.4	0.6
Rhamnan	0.2	0.2	0.1	0.3	0.2	0.2

b)



	K	CCE	K_CCE	F60	F12	CCE_F12
Mannan	2.8	2.6	2.1	2.4	2.3	3.0
Xylan	0.8	0.0	0.4	1.5	1.6	0.0
Galactan	0.6	1.0	0.6	0.3	0.3	0.3
Rhamnan	0.2	0.2	0.1	0.2	0.3	0.1

Figure 7-3 Hemicellulose composition of treated pulps determined by HPLC. (a) B endoglucanase treatments and (b) F endoglucanase treatments.

7.3.3 Cellulase effects

Once already detected that Fock solubility was significantly increased by the endoglucanases treatments, and a diminution of the hemicellulose content was observed by CCE treatment; the ISO brightness and viscosity properties were also measured after each enzymatic and chemical treatment (Table 7-2).

The results showed that both enzymes lead to substantially reduced brightness at the end of the process. Thus, the B120 and F60 treatments decreased brightness by 9.21% and 3.32%, respectively, from the initial pulp (Com); by contrast, the control treatment (K) decreased it by only 1.1%. Interestingly, the increase of the enzyme dose from F12 to F60 resulted in no significantly greater brightness reduction. The brightness loss observed is thought to be caused by the enzyme solution (i.e., the mixture where the enzymes were prepared) since inserting a treatment at high sodium hydroxide concentration after the enzymatic treatment (B120_CCE) raised brightness to virtually the same level as in the starting pulp. Also, B and F cellulases caused a marked increase in colour saturation (C^*); however, removal of adsorbed enzyme products by subsequent cold caustic treatment decreased chroma, which suggests that cellulose chains were not damaged or modified. In order to clarify these results, the enzymes were denatured at a high temperature (> 90 ºC) before use and then the enzymatic treatments were performed following the same conditions. The results revealed a significant brightness loss and hence that the enzymes itself did not contribute to the adverse effect on brightness.

In general, F cellulases degraded cellulose chains and decreased viscosity, but using a high enzyme dose of F (F60 sample) resulted in no further viscosity loss with respect to F12. According to Gurnagul et al. (1992), a heterogeneous attack of cellulase on fibre wall can weaken fibres without significantly diminishing cellulose degree of polymerization or viscosity, which is consistent with our results at high enzyme doses. The B120 treatment did not cause an adverse effect on viscosity (Cadena et al., 2010c; Garcia-Ubasart et al., 2013a), even with the combination of a cold caustic extraction and a posterior enzymatic treatment. The treatment with buffer solution alone (K), and the cold caustic extraction applied after the buffer treatment (K_CCE), resulted in no cellulose degradation with respect to starting pulp. The gain of viscosity observed after a CCE with respect to initial pulp can be explained by the alkaline pH used, which is responsible for dissolving degraded cellulose and low molecular weight hemicelluloses.

Table 7-2 Results of ISO brightness (± confidence intervals), Chroma coordinate (C*), viscosity (± confidence intervals) and chain scission (CS) for treated pulps. CCE: cold caustic extraction, K: control treatment with no presence of enzyme, B and F cellulases.

	ISO Brightness (%)	C*	Viscosity (mL/g)	Chain scission (CS)
Com	91.70 ± 0.2	1.62	476 ± 1	-
CCE	91.90 ± 0.2	1.75	536 ± 29	0
B120	83.25 ± 1.6	4.76	495 ± 50	0
B120_CCE	91.10 ± 0.5	1.94	488 ± 32	0.02
CCE_B120	84.00 ± 0.9	5.16	415 ± 47	0.35
F60	88.65 ± 0.9	1.89	390 ± 6	0.26
F12	88.40 ± 1.7	2.32	404 ± 11	0.21
CCE_F12	89.10 ± 0.7	2.15	406 ± 50	0.38
K	90.60 ± 0.7	2.30	538 ± 45	0
K_CCE	90.85 ± 0.3	1.59	544 ± 21	0.16
Inactivated B120 enzyme	74.80 ± 10	7.94	Not measured	-
Inactivated F60 enzyme	87.80 ± 0.1	3.13	Not measured	-

Intrinsic viscosity data allowed us to calculate the number of cellulose chain scissions (CS) caused by the enzymatic treatments and cold caustic extraction. CS represents the number of chain cleavage steps per initial cellulose chain. As can be seen from Figure 7-4a, B cellulase-treated pulp exhibited none chain scission value while F cellulase had a chain scission value around 0.2. These results indicated that B and F endoglucanases must have acted differently on cellulose chains. Cold caustic extraction (CCE) gave a negative CS value that was assumed to be zero; however, a subsequent enzymatic treatment (CCE_B120) increased CS by 35%. This behaviour was not observed when the caustic treatment was applied after the enzymatic treatment, B120_CCE, which failed to raise the number of scissions of CCE_B120. Also, in the case of CCE_F12 a higher CS was obtained compared to the simple F12 enzymatic treatment. From these results can be concluded, that the presence of cellulose II promoted during the cold caustic extraction facilitated the action of the enzymes. On the other hand, the combined treatment CCE_F12 led to more scissions than F60 at a high enzyme dose, which further underlines the favourable effect of cold caustic extraction. Janzon et al. (2008a) reported that the conversion of cellulose I into cellulose II starts at a NaOH concentration about 6–7 wt% in wood pulps and above 10 wt% in cotton linters. Also, cellulose II has proved especially

susceptible to hydrolysis by endoglucanases (Atalla, 1979; Rahkamo et al., 1998). Similarly to us, Gehmayr and Sixta (2012) stated that pulps with an increasing proportion of cellulose II experienced an increasing extent of maximal chain scission and this effect may be attributed to enhanced accessibility of the cellulose II modification.

Plotting the proportion of fines as a function of the number of chain scissions with F treatments (Figure 7-4b) afforded some interesting conclusions. F cellulase reduced fiber size and as a result the amount of fines (viz., fibers with a particle size between 0.0–0.2 mm) relative to the starting pulp increased significantly, whereas the control treatment (K) did not. The higher the cellulase dose used was, the higher was the content in fines, which suggests increased degradation of cellulose chains. The F60 treatment increased the amount of fines by 42% and 18% relative to initial and F12 treatment, respectively. Again, introducing a cold caustic extraction before the enzymatic treatment increased the content in fines by up to 9% and the number of chain scissions by 46% in CCE_F12 treated pulps relative to F12. These results suggest that the high sodium hydroxide concentration contributed to fiber swollen and opened-up the cellulose structure, thereby facilitating the action of the posterior enzyme. However, a cold caustic extraction (CCE) by itself did not modify the number of chain scissions. In addition, the application of a strong alkaline treatment after the buffer treatment neither caused greater scissions than control treatment alone. In terms of fiber morphology, the fiber length and the proportion of fines of K_CCE were identical to the values for K. However, the content in fines of control treatment was increased by 13.5% with respect to the starting pulp although no degradation of cellulose fibers was apparent from viscosity results. This result can be explained by the oscillating agitation used during the treatment that helped to release small particles.

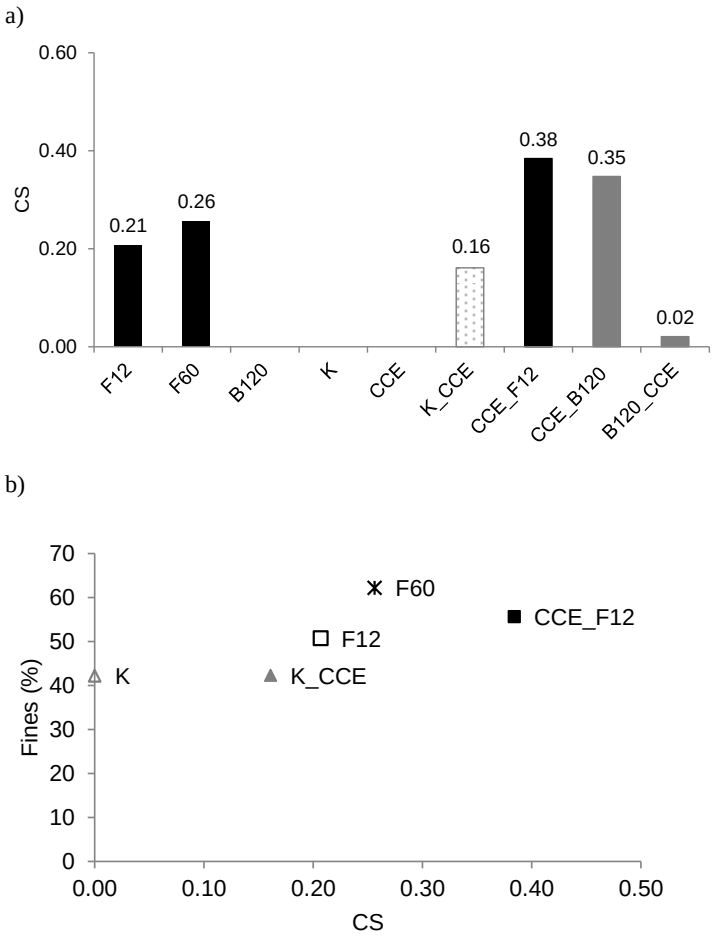


Figure 7-4 (a) Chain scission number for the different studied samples. (b) Proportion of fines obtained from Metso Kajaani FS300 against the number of chain scission for F treatments and their respective control treatments.

7.3.4 ^{13}C-CP/MAS NMR

The solid state ^{13}C-NMR spectra of commercial dissolving pulp, the enzymatic treated pulps and the combination of 9% NaOH extracted (CCE) pulps before or after endoglucanase treatments are shown in Figure 7-5. The C-6 signal of commercial dissolving pulp consisted of two peaks nearly identical heights at 64 and 66 ppm. This spectrum suggests a small presence of cellulose II and this cellulose polymorph is more susceptible to enzymatic hydrolysis by endoglucanases (Krässig, 1993; Teleman, 2001). Therefore, B endoglucanase treatment was dominated by cellulose I signal at 66 ppm while the F12

treatment increased the signal intensity but in less extent. The 9% NaOH extraction presented different polymorphic form from commercial dissolving pulp, but no signals of cellulose II. The most pronounced structural changes were detected with CCE_F12 sample. A high presence of cellulose II was apparent by a perceptible shoulder at 108 ppm of C-1 signal and an increased C-6 signal intensity at 64 ppm.

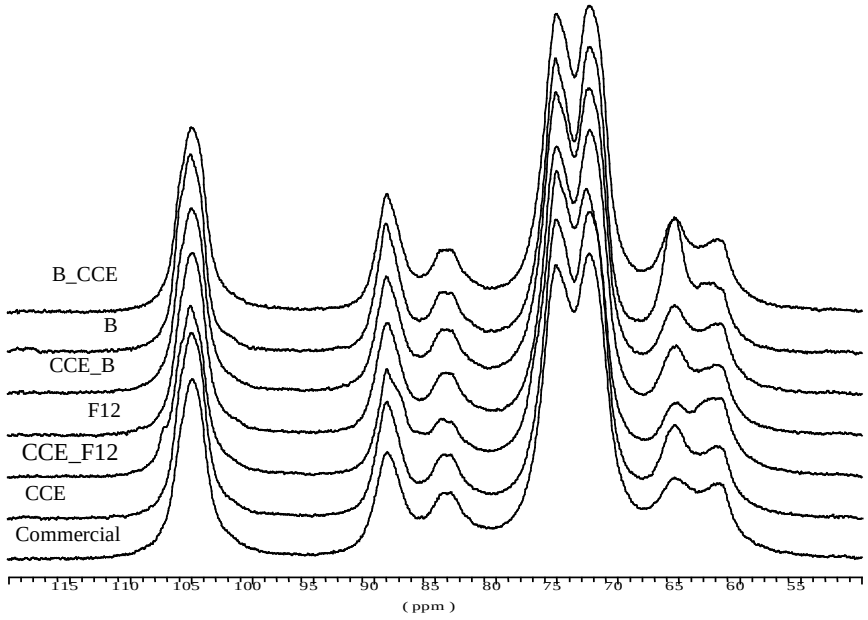


Figure 7-5 ^{13}C-CP/MAS NMR spectra of commercial dissolving pulp compared to samples treated with 9% NaOH before or after a hydrolytic treatment.

7.3.5 Fiber morphology

The outcomes of the F cellulase treatments were examined in greater detail (Table 7-3). The water retention value (WRV) provides information about the integral pore volume of a pulp and the content of hydrophilic groups (Gehmayr et al., 2011). Fines are thought to increase WRV improvement since these particles have more accessible –OH groups and plays a major role in dewatering. Treating commercial dissolving pulp with the lowest dose of F cellulase (F12 treatment), reduced fiber length and substantially increased the proportion of fines, and as a result of that the WRV rose to 1.1%. By contrast, a high enzyme

dose (F60 treatment) had virtually no effect on WRV even though fibre length was shortened by 57%, and the amount of fines increased to 41%, with respect to commercial dissolving pulp. The conditions used in the control treatment, i.e. oscillating agitation and the buffer solution had slight effect on fibre morphology. Introducing a cold caustic extraction (K_CCE) stage resulted in no change in fibre morphology or WRV relative to the control treatment (K). The greatest change in WRV was produced by CCE_F12, which suggests increased hydrogen bonding; fibres were shortened by 43% and the content in fines grew by up to 34%, with respect to starting pulp. In terms of fibre width, a slight increase was observed with F12 treatment, but a substantial greater size was found with F60 treatment, with respect to commercial pulp. Interestingly, K_CCE, CCE_F12 and F60 presented the same value of fibre width. A comparison of K with K_CCE on the one hand, and F12 with CCE_F12 on the other hand, revealed that the incorporation of an alkaline step into the process enhanced the fibre width.

Table 7-3 Water retention value (WRV) (± confidence interval) and fiber length and fiber width (± confidence intervals) determined according to TAPPI standard and calculated as an arithmetic mean for the F treatments.

	WRV	Fiber length, Ln (mm)	Fiber width, Wn (μm)	Fines (%)
Com	0.79 ± 0.04	0.65 ± 0.01	21.3 ± 0.1	36.48 ± 0.43
K	1.00 ± 0.02	0.60 ± 0.0	22.2 ± 0.0	42.18 ± 0.60
K_CCE	0.99 ± 0.07	0.57 ± 0.0	23.6 ± 0.0	42.30 ± 0.98
F60	0.88 ± 0.18	0.28 ± 0.01	23.5 ± 0.7	62.18 ± 0.49
F12	1.11 ± 0.21	0.47 ± 0.02	22.6 ± 0.2	50.71 ± 0.39
CCE_F12	1.27 ± 0.31	0.37 ± 0.02	23.5 ± 0.1	55.59 ± 1.60

Combining fiber length results with WRV results (Figure 7-6) some conclusions can be drawn. In general, the lower the fiber length resulted, the higher the WRV became, with the exception of F60 treatment. Focusing the attention on the control treatment followed by a caustic extraction (K_CCE), it failed to improve WRV and to alter cellulose fibre size relative to control treatment (K). The CCE_F12 suffered a slight improvement in WRV but a 10% reduction in fibre length, with respect to F12 treatment; although these differences were more significant with respect to starting pulp.

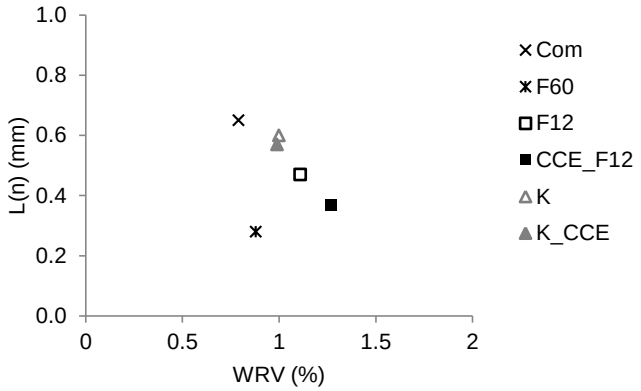


Figure 7-6 Relation between fibre lengths determined according to TAPPI standard and calculated as an arithmetic mean and water retention value (WRV) for biobleached sulphite pulps treated with B and F enzyme.

Figure 7-7 represents the population fractions determined according to TAPPI standard. As can be seen, the proportion of fibres in the length range of 0.5–3.2 mm was considerably diminished when the F60 treatment was performed, and thereby the content of fines increased by up to 70% (length range of 0.0-0.2 mm) with respect to starting pulp. However, the effect caused by the enzyme was not detected in the length range of 0.2-0.5 mm since the same population as starting pulp was obtained. After F60 treatment, the increase of fines was followed by CCE_F12 and then F12. The CCE_F12 treatment increased the amount of fines by up to 35% and the F12 treatment by up to 39%. F12 and CCE_F12 treatments presented similar proportion of fibres in the length range of 0.2-0.5 mm, but the differences in the long length fraction became more accentuated. As observed earlier, the control treatment had similar population fractions as starting pulp, except for slight difference in the content of fines and therefore different proportion of fibres in the range of 0.2-1.2 mm. These results can be explained due to the oscillating agitation used in the treatments which facilitated the release of small particles.

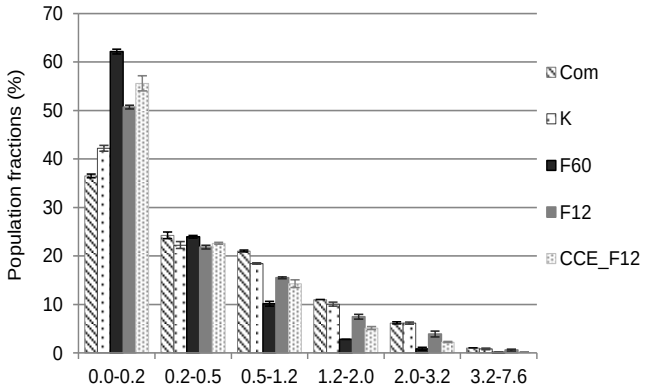


Figure 7-7 Population fractions (± confidence intervals) for the FB treatments determined according to TAPPI standard

The microscopy images (Figure 7-8) provided visual confirmation of the previous results. Thus, the F60 sample exhibited a high content in fines but also in long fibers. In addition, slight difference between F12 and CCE_F12 were detected.

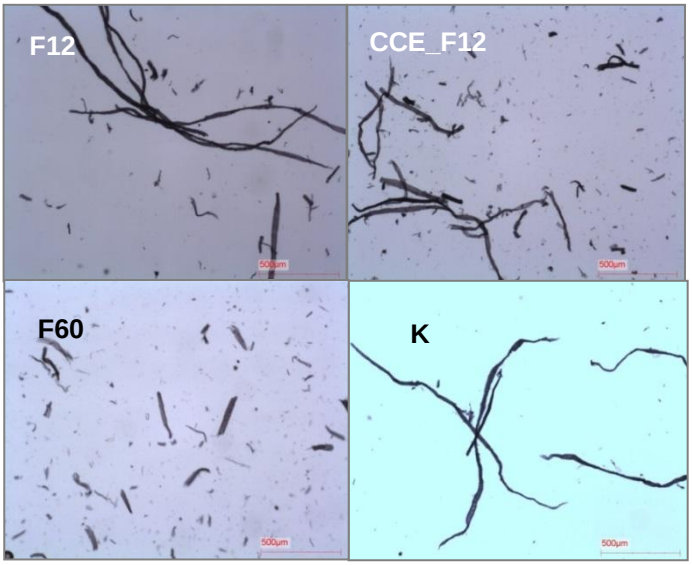


Figure 7-8 Optic Microscopy images for the F treatments. The pictures were taken with a magnification of 40.

7.3.6 Comparison of fiber source and respective endoglucanases

Finally, a comparison between biobleached treated sulfite pulp Chapter 6 and bleached commercial treated dissolving pulp Chapter 7 in terms of Fock solubility and carbohydrate composition, provided more information about the mechanism of action of endoglucanase and cold caustic extraction treatments.

Concerning Fock solubility results, the application of B or F endoglucanase resulted more positive on **biobleached dissolving pulp rather than bleached commercial dissolving pulp**, even when a cold caustic extraction was previously applied. By its side, a cold caustic extraction (CCE) treatment had different effect on respective studied pulps. A much higher Fock solubility loss was found for bleached commercial dissolving sample (-26%) in comparison to biobleached sample (-9%). Actually, commercial dissolving pulp had lower content of hemicelluloses than biobleached sulfite pulp, but lower hemicellulose removal was achieved. Specifically, **CCE treatment decreased hemicelluloses by 12% for Com_CCE and about 40% for L_CCE samples, with respect to each original pulp**. The action of CCE treatment was limited to the accessibility of residual hemicelluloses. Therefore, from these results two effects were suggested by CCE treatment: on the one hand, CCE helped to reduce mainly galactan fraction and then mannan and xylan fractions of biobleached sulfite pulp, and on the other hand, was responsible for the formation of cellulose II in commercial dissolving pulp leading to lower Fock solubility.

Moreover, the presence of cellulose II polymorph detected by NMR and the low Fock solubility value of Com_CCE sample were in agreement with the proposed explanation. Regarding to the combined treatments (CCE + endoglucanases) different Fock solubility improvements were found: commercial dissolving samples (CCE_B120 and CCE_F12) exhibited a 39% of gain, while L_CCE_B120 and L_CCE_F12 biobleached samples only provided a 2% and 18%, with respect to CCE treatment. Once more, the different improvement can be explained by the different effect of CCE treatment: conversion of cellulose I to cellulose II or hemicellulose removal, respectively.

To conclude, **same enzymatic treatment conditions, biobleached sulfite upgraded pulp presented higher Fock solubility value than bleached commercial treated dissolving pulp**.

Concerning carbohydrate composition B and F endoglucanases performed the same mechanism of action on biobleached sulfite pulp and commercial dissolving pulp. Specifically, both enzymes reduced galactan and mannan fractions by about 50% and 15%, respectively, of commercial dissolving pulp. By contrast, both endoglucanase attacked mainly xylan, followed by galactan and mannan fractions of biobleached sulfite pulp. Interestingly, xylan removal was more effective with B endoglucanase (79%) in comparison with F (54%), but similar percentage of reduction was found for galactan and mannan.

7.4 Conclusions

Two different cellulases, named as B and F, were used to increase the reactivity according to Fock method of dried bleached commercial dissolving pulp with the intention to bring a satisfactory advantage to the viscose process. Working at high enzyme dose, F60 treatment, provided a reactivity increase of 42%, which belongs to a 96% Fock solubility. In terms of fiber morphology, the amount of fines increased significantly after F60 treatment, which is in connection with somewhat viscosity loss. The F12 treatment provided a 13% Fock solubility improvement. Interestingly, applying a cold caustic extraction (CCE) treatment before the hydrolytic treatment (CCE_F12) resulted in further increased Fock solubility than an enzymatic hydrolysis alone (F12), although the relative value was much lower. In terms of WRV, this property was also improved with the combination of a cold caustic extraction and hydrolytic treatment (CCE_F12). The NMR results revealed that the starting pulp had already an important proportion of cellulose II, but after the CCE_F12 treatment this amount of cellulose II became higher. The B enzyme did not cause viscosity loss, although the Fock solubility was improved by 17% with respect to commercial pulp, which corresponds to a 79% of Fock solubility.

7.5 References

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Chapter 8

Modification of bleached commercial dissolving pulp by Laccase-TEMPO treatments

Abstract

In this work, laccase-TEMPO (Lac-T) treatments were applied to commercial bleached dissolving pulp in order to modify cellulose structure by introducing new functional groups such as carbonyl and carboxyl groups. As a result, dry and wet strength improvements were assessed as well as the solubility behavior towards a xanthate reaction. The effect of a refining step (R) before the oxidative treatment, the presence of oxygen pressure, TEMPO dose (2 or 8% oven dried pulp, odp) and reaction time (8 or 20h) were thoroughly examined. The most salient results were those obtained in the presence of oxygen pressure. Results revealed that Lac-T 8% (O_2) introduced an amount of aldehyde and carboxyl groups of 182.15 and 242.70 μmol/g, respectively, but resulted in poor wet strength, whereas the treatment R+Lac-T 8% (O_2) presented similar values of aldehyde and carboxyl groups but higher wet strength was achieved. Specifically, Lac-T (8%, O_2) had a wet-to-dry strength ratio of 18% compared to R+Lac-T (8%, O_2) which accounted for 26%, indicating that the refining pretreatment improved strength-related properties. In addition, for similar pulp viscosity, R+Lac-T 8% presented higher wet zero-span tensile strength, which suggest the formation of hemiacetal linkages between the new introduced aldehyde groups and free available hydroxyl groups resulting from the fibrillation. The increase in wet-to-dry strength was accompanied by a diminution in Fock solubility, although an 80% of Fock solubility was obtained.

This diminution was explained by a lower accessibility, closer and more compact structure.

8.1 Introduction

Cellulose is a linear homopolymer consisting of anhydro-β-D-glucopyranose units (AGU) that are linked together by (1-β-4) glycosidic bonds. Every AGU contains three hydroxyl groups in the position C2, C3 and C6. The ability of these hydroxyl groups to form hydrogen bonds plays a major role in the formation of fibrillar and semicrystalline packing, which governs the important physical properties of this highly cohesive material (Klemm et al., 1998). Importantly, the presence of these reactive hydroxyl groups allows cellulose to be modified and functionalized, by introducing new functional groups to improve its nano-dispersibility or even confer it an added-value for specific applications such as medical (scaffolding material), agricultural, cosmetic and pharmaceutical (Dias et al., 2003; Kumar and Banker, 2008; Isogai et al., 2010; Coseri et al., 2013; Jaušovec et al., 2014). In the paper industry, carbonyl and carboxyl functionalities are known to play a decisive role in the pulping process and hence in the final paper properties. For example, sheets made from the partially oxidized fibers experienced higher wet and dry tensile index, presumably due to an increased opportunity of electrostatic interactions between anionic pulp and cationic polyamideamine-epichlorohydrin, which was added as a wet-strength agent (Kitaoka et al., 1999).

Cellulose oxidation can be induced by very different processes, e.g., radiation, energy impact or the application of oxidizing reagents (Potthast et al., 2007). These chemical oxidants can be divided into non-selective (nitrogen oxides (Butrim et al., 2007), alkali metal nitrites and nitrates (Painter, 1977), ozone (Johansson and Lind, 2005) and permanganates (Manhas et al., 2007); and selective, such as periodates (Fras et al., 2005; Calvini et al., 2006; Potthast et al., 2009) and nitroxyl radicals (de Nooy et al., 1996; Isogai and Kato, 1998; Vignon and Tahiri, 2000; Coseri et al., 2009, 2013; Biliuta et al., 2010). Periodates are specific oxidants capable of oxidizing the vicinal hydroxyl groups at carbon atoms 2 and 3 in an anhydroglucose unit (AGU) of cellulose, to form two aldehyde groups. As a result, the carbon carbon bond between the carbon atoms 2 and 3 is broken (Coseri et al., 2013). Nitroxyl radicals have been widely studied for catalytic and selective oxidation of primary hydroxyl groups of

polysaccharides under aqueous conditions (Saito and Isogai, 2004). In particular, TEMPO-mediated oxidation is an efficient method for introducing carboxyl and carbonyl functional groups into cellulose in aqueous suspension (de Nooy et al., 1996; Saito and Isogai, 2004). Two TEMPO-mediated oxidation systems have been reported so far: TEMPO/NaBr/NaClO system at pH 10-11 (de Nooy et al., 1996; Isogai and Kato, 1998; Saito and Isogai, 2006, 2007) and TEMPO/NaClO/$NaClO_2$ system at pH 4-7 (Saito et al., 2009, 2010). NaClO and $NaClO_2$ are used as the primary oxidants in each system. Applying TEMPO/NaBr/NaClO to native cellulose significant amounts of sodium carboxylate groups and small amounts of aldehyde groups are formed, which is usually accompanied by important depolymerization. On the other hand, the TEMPO/NaClO/$NaClO_2$ system avoided the depolymerization of the oxidized cellulose, although the efficiency for the formation of carboxylate groups was somewhat lower (Isogai et al., 2011a). The catalytic conversion of the primary hydroxyl groups to carboxyl via aldehydes has been widely studied over the last two decades, but the mechanism is still under discussion.

The presence of carboxylic groups can promote re-wetting and re-swelling of fibers, and contribute to the negative charge of fibers, which favorably influences fiber flexibility, pulp refining, and dry-strength of papers (Hubbe et al., 2007). Carbonyls in cellulose are “hot spots” along the carbohydrate chain acting as localized sites of increased chemical instability where cleavage will primarily occur. Oxidized groups in cellulose are chiefly responsible for strength loss and decreased performance parameters in pulp, paper, textiles and other cellulosic materials. They also account for general ageing of cellulosic (Lewin, 1997), as well as thermal and light-induced yellowing process (Potthast et al., 2005). However, works using TEMPO-oxidized mechanism have been found to considerably improve wet tensile strength. Development of this property was ascribed to the formation of substantial amounts of aldehyde groups on the surfaces of cellulose fibers as intermediate structures during the course of TEMPO-mediated oxidation. Once formed, the aldehyde groups seemingly established covalent interfiber bonds through hemiacetal linkages with sterically close hydroxyl groups in cellulose (de Nooy et al., 1996; Saito and Isogai, 2006, 2007; Isogai et al., 2010, 2011b; Okita et al., 2010). As a considering point, the carbonyl or carboxyl groups introduced may also be contemplated as reactive

"chemical hooks" for further chemical modification or graft co-polymerization (Potthast et al., 2007).

In this line, but in view of introducing biotechnology concept, some recent works demonstrated the use of laccase as a cocatalyst in stoichiometric oxidation. Laccase, having a redox potential in the range of 0.7–0.9 V, can easily oxidize the stable oxyl- radical form of TEMPO to oxoammonium ion (E° 0.2 V). This ion is the actual oxidant, while laccase would regenerate TEMPO from the generated hydroxyl-amine. Then, either acid-induced disproportionation of TEMPO, or further oxidation of it by laccase, would form the oxoammonium ion once again (Fabbrini et al., 2001). Some authors demonstrated the good performance of a chemoenzymatic modification using laccase as biocatalyst and TEMPO as a enhancer (Viikari et al., 1999; Bragd et al., 2001; Aracri et al., 2011, 2012; Patel et al., 2011; Aracri and Vidal, 2012; Xu et al., 2013; Jaušovec et al., 2014). The interest of cellulosic modification and functionalization have been explored using various methods including hydrophobization with laccase-lauryl gallate (Garcia-Ubasart et al., 2012, 2013b; Cusola et al., 2013, 2014a, 2014b) or antimicrobial properties with laccase-natural phenols (Fillat et al., 2012) and antioxidant capacity (Cusola et al., 2015).

Based on the foregoing, the purpose of this work was to study laccase-TEMPO oxidation of a commercial dissolving pulp, to evaluate the dissolution behavior towards Fock reactivity, and the degree of functionalization towards wet strength improvement. Therefore, different experimental conditions, such as TEMPO dose (2% or 8% oven dried pulp (odp)), reaction time (8h or 20h), presence or absence of oxygen pressure and a refining pretreatment were studied.

8.2 Materials and Methods

8.2.1 Pulp, enzymes and reagents

A totally chlorine-free (TCF), bleached dried commercial dissolving-grade pulp was used as a starting pulp and presented a 91.70 ± 0.15% ISO brightness and 522 ± 4 mL/g viscosity. The carbohydrate composition, as determined by high-performance liquid chromatography (HPLC), was as follows: 95.6 ± 0.3%

glucan, 2.8 ± 0.2% mannan, 0.8 ± 0.0 xylan, 0.6 ± 0.3% galactan and 0.2 ± 0.2% rhamnan.

A laccase from *Trametes villosa* (TvL) with an activity of 746 U/mL supplied by Novozymes® (Denmark), was used for the oxidative treatments. The enzyme activity was measured as the oxidation of 5 mM 2,20-azinobis(3-thylbenzothiazoline-6-sulfonic acid) (ABTS) to the cation radical (ε_{436} =29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 24 °C. One activity unit (U) was defined as the amount of enzyme transforming 1 μmol of ABTS per min. 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) purchased from Sigma–Aldrich was used as mediator for the oxidative treatments.

8.2.2 Pulp refining and oxidative treatments

Prior to oxidative treatments, a portion of the starting pulp was refined (R) at 5000 rev in a PFI mill according to ISO 5264, and the remained was used as received with no refining step.

Laccase–TEMPO oxidation treatments were performed at room temperature, at 5% consistency in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 and 20 U/g odp laccase (Lac). Different experimental conditions such as, TEMPO (T) dose, reaction time and presence or absence of oxygen pressure (O_2) were tested in order to provide the greatest increases in carboxyl and aldehyde groups, and hence improve wet and dry tensile strength. The specific conditions used for each treatment are described in Table 8-1 and were taken from the study conducted by Aracri et al., (2012) and considering the highest formation of aldehyde and carboxyl groups. Pulp samples treated under identical conditions as in the chemoenzymatic treatment but in the absence of TEMPO (Cont-Lac), laccase and TEMPO (Cont-Buffer) were used as controls. After treatment, each pulp was filtered and washed with de-ionized water until a colourless, neutral filtrate was obtained.

Table 8-1 Detailed conditions of the performed treatments

Sample ID	Pulp Refined at 5000 rev	TEMPO dose (% odp)	Applied O_2 pressure (MPa)	Time (h)
Lac-T 2%, no O_2, 8h	-	2	-	8
Lac-T 8%, no O_2, 8h	-	8	-	8
Lac-T 8%, 8h	-	8	0.6	8
R+Lac-T 8%, 8h	✓	8	0.6	8
R+Lac-T 8%, 20h	✓	8	0.6	20
R+Cont-Lac, 20h	✓	-	0.6	20
R+Cont-Buffer, 20h	✓	-	0.6	20

8.2.3 Analysis of pulp properties

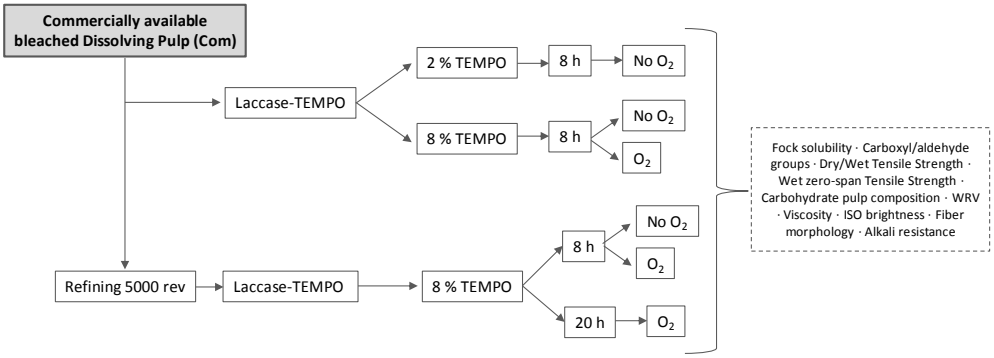
Pulp brightness, and Fock solubility were assessed according to ISO 3688 and Fock (1959), respectively. Pulp viscosity (as intrinsic viscosity for a sample of cellulose dissolved in a dilute solution of cupriethylenediamine) was determined in accordance with ISO 5351:2010. Because viscosity measurement is conducted using alkaline Cuen solution, carbonyl groups derived from chemoenzymatic treatment can suffer β-elimination reaction and final viscosity results are misunderstood (Roncero et al., 2003). This problem was avoided by treating the TEMPO-oxidized samples with 2% $NaBH_4$ at 5% consistency at room temperature for 30 min in order to reduce carbonyl groups to hydroxyl groups (Roncero et al. 2003). After this post-treatment, viscosity was determined again using Cuen solution. The degree of polymerization (DP) and the number of scissions in the cellulose chain (CS) were also calculated. The respective equations are detailed in 3.7.1. The bulk acid group content and aldehyde groups were determined by conductimetric titration as described in section 3.7.1.

Fiber length and width, and the content in fines (%) of samples were measured using Kajaani fiber analyzer (FS300, Metso automation, Finland) according to TAPPI T 271. Water retention value (WRV) was determined according to ISO 23714 and surface SEM images of the handsheets were taken on a JEOL JSM-6400 microscope. Zeta potential of the fibers was determined according to Cadena et al. (2009), using Mütek zeta potential equipment (SZP-06, Mütek, Germany).

Once treated, the samples were used to prepare handsheets on a Rapid-Köthen laboratory former according to ISO 5269-2. Mechanical properties were

assessed in accordance with the standards in brackets as follows: dry tensile strength (ISO 1924-3:2005), wet tensile strength (ISO 3781), tearing resistance (ISO 1974:1990), air permeance (ISO 5636-5:2003) and bulk density. The wet zero span tensile strength (WZSTS) was measured according to T-273 pm-95 and ISO 15361:2000, respectively, on a Zero-Span 1000 Pulmac tester, using strips previously soaked in de-ionized water for 5 s. A wet-to-dry (W/D) strength ratio of more than 15% should be considered wet-strength paper (Scott, 1996).

Schema 8-1 summarizes the experiments conducted and the properties analysed for each treatment.



Schema 8-1 Outline of the experiments conducted in commercial dissolving pulp and the properties analysed

8.3 Results and Discussion

In Chapter 7, bleached commercial dissolving pulp was fully characterized in terms of carbohydrate composition, ^{13}C-CP/MAS NMR, Fock solubility, ISO brightness, fiber morphology, viscosity and WRV. Then commercial dissolving pulp was further purified with chemoenzymatic treatments (specifically with combinations of cold caustic extraction (CCE) and endoglucanase treatments). That cellulose modification led to Fock solubility improvement and the formation of cellulose II. In the same line of chemoenzymatic modification of cellulosic pulps, oxidative reactions in presence of Laccase-TEMPO system were conducted in this work.

8.3.1 Relating functional groups to dry and wet strength properties

Good strength-related properties is not an important requirement for dissolving pulp, but testing dry and wet strength (DTI and WTI) is a way to evaluate the amount of new functional groups introduced in cellulose fibers. Therefore, mechanical properties were in depth analyzed. As shown in Table 8-1, two control treatments (R+Cont-Lac, 20 h and R+Cont-Buffer, 20 h both with oxygen pressure) were performed in order to observe any contribution from laccase and buffer parameters. No changes in carboxyl/aldehyde content, fiber morphology, viscosity, Fock solubility and WRV were found, with respect to starting and refined pulp. ISO brightness and α-cellulose reults were slightly lower than the ones of refined sample. Consequently, the results of these treatments are not further discussed from here on.

Table 8-2 Properties measured for commercial dissolving pulp, refined pulp, Lac-T treated and respective control treatments

	Commercial dissolving pulp	Refined commercial dissolving pulp	R +Lac-T 8%, 20h, O_2	R+Cont-Lac, 20h, O_2	R+Cont-Buffer, 20h, O_2
Fock solubility (%)	67.3 ± 2.1	35 ± 4	80 ± 10	41 ± 5	37 ± 4
Brightness (%)	90.3 ± 0	91.68 ± 0.15	61 ± 2	83.96 ± 0	83.54 ± 2.0
Viscosity (mL/g)	476 ± 0.7	474 ± 1	151 ± 4	554 ± 38	533 ± 27
Viscosity after $NaBH_4$ treatment (mL/g)	522 ± 4.0	505 ± 5	127 ± 6	527 ± 40	516 ± 6
Carboxylic acids (μmol/g)	35.6 ± 2.4	47.6 ± 8	310 ± 15	28.1 ± 6.3	49.3 ± 3.7
Aldehyde groups (μmol/g)	0	0	0	0	0
α-cellulose (%)	92 ± 0	92.4	91 ± 12	88 ± 0.2	88 ± 0.42
WRV	0.79 ± 0.01	0.6 ± 0.03	4.3 ± 0.1	1.27 ± 0	1.37 ± 0.09
Fines (%)	38.77 ± 0.01	49.72 ± 0	46 ± 0	55.39 ± 0	53.24 ± 0
L(l) (mm)	1.53 ± 0.01	0.82 ± 0.02	0.85 ± 0.02	0.77 ± 0.03	0.79 ± 0.01

In Figure 8-2 is shown the content of carboxyl (COOH) and aldehyde functional groups (CHO); and dry/wet strength. As can be seen from Figure 8-2a, the original pulp presented 35.6 μmol/g of carboxyl group and no presence of aldehyde groups. In general, Laccase-TEMPO treatments with no oxygen pressure and no refining step, increased carboxyl group moderately with respect to the starting pulp. Two doses of TEMPO (2 and 8% odp) were studied, failing to show significant differences in terms of carboxylic group content. Specifically, a 32% and 46% of improvement was detected for Lac-T 2% and Lac-T 8%, with respect to original pulp. Carboxyl groups are known to contribute to dry strength in paper (Hubbe et al., 2007); but results showed that the low content in carboxylic acids led to low dry tensile strength values with no differences between samples (Figure 8-2b). On the other side, the use of 2% odp TEMPO provided an amount of 55.6 μmol/g of aldehyde groups, whereas the use of 8% odp TEMPO only 6.9. It is known that, aldehyde groups once formed in the native cellulose fibers are able to form hemiacetals with hydroxyl groups of cellulose sterically close to each other. However, as suggested by some authors (Saito and Isogai, 2006), not all aldehydes groups formed in the TEMPO-oxidized cellulose fibers necessarily contribute to the development of wet strength of the sheets. Pulp samples treated with Lac-T 2% and Lac-T 8% exhibited a wet tensile index of 0.7 and 0.8 Nm/g, which correspond to a 32 and 60% of improvement, with respect to original pulp. In other words, the low amount of aldehyde groups introduced was not enough to develop good wet strength property since an only wet-to-dry (W/D) strength ratio of 11 and 14% was achieved, respectively.

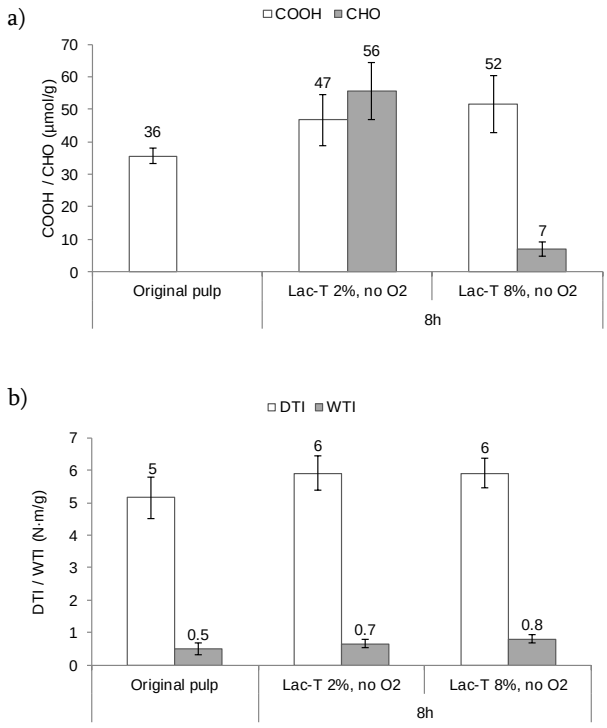


Figure 8-1 Carboxylic (COOH)/ aldehyde (CHO) group content (± standard deviation), dry / wet tensile index (± standard deviation) for original pulp and respective enzymatic oxidative treatments.

On the other hand, applying oxygen pressure in the system (Lac-T 8%, O_2), let to introduce a greater amount of aldehyde and carboxyl groups, 182 and 243 µmol/g, respectively; as compared to only 35.5 µmol/g of carboxyl group in the original pulp (Figure 8-2a). It is known that dissolving pulps exhibit poor strength properties and low bonding availability due to low content in hemicelluloses. However, the laccase-TEMPO treatment using oxygen pressure (Lac-T 8%, O_2) increased dry and wet strength to 9.3 and 1.7 N·m/g (Figure 8-2b), from 5.2 and 0.5 N·m/g, respectively, in the original pulp. The Lac-T 8% (O_2) sample had a W/D ratio of 18% indicating that wet strength was well developed.

The effect of a refining treatment was also studied (Figure 8-2). As observed for original pulp, the refined pulp presented carboxylic groups (47.6 µmol/g) but no aldehyde groups. The fibrillation effect resulted from the refining treatment provided in turn a positive effect in mechanical properties. Thus, the greater

interfibre bonds allowed to improve dry and wet strength with respect to the original pulp. The combined treatment, R+Lac-T 8% (O_2), provided an amount of carboxyl and aldehyde groups of 290.7 and 210.9 μmol/g, respectively. The content of aldehyde functional groups was slightly higher than Lac-T 8% (O_2) and the carboxylic content was the same since refined pulp already had 48 μmol/g. However, the combined treatment, R+Lac-T 8% (O_2), showed a markedly improvement in dry and wet strength, with a W/D strength ratio of 26%. These results demonstrated that the refining treatment did not contribute to introduce much greater functional groups but bettered strength-related properties due to fibrillation effect. The marked improvement observed was due to the combined effect of the large amounts of functional (carboxyl and aldehyde) groups and new free hydroxyl groups resulting from the fibrillation. The presence of both functional groups led to the formation of hemiacetal linkages. It was observed that a near-unity aldehyde/carboxyl ratio ensured good wet strength property. Therefore, the poor bonding capability of dissolving pulp due to low content in hemicelluloses justified the refining pretreatment in terms of strength developed property.

In addition, maintaining same conditions (8% TEMPO and oxygen pressure) but extending the reaction time to 20h provided a 309.5 μmol/g of carboxylic acids but no presence of aldehyde groups (Table 8-2). Patel et al. (2011) observed that in Lac-T system carboxyls were mainly formed in later phases of the reaction when the carbonyl generation became slower or even leveled off. However, the mechanism behind the formation of carboxyl is still under study. Some authors proposed the formation of carboxyls mainly as a result of aldehyde subsequent autoxidation under oxygen-saturated medium or a direct participation of the oxoammonium ion in the reaction (Patel et al., 2011). It is described in the literature that Lac-T system is able to modify cellulose by introducing predominantly carbonyl groups and, to a much lesser extent carboxyl groups (Aracri et al., 2011; Patel et al., 2011; Aracri and Vidal, 2012; Jaušovec et al., 2014). However, this trend was only observed when no oxygen pressure was used, since higher content of carboxyl than aldehyde groups was found for the rest of samples, suggesting that cellulose composition of fibers influences the reaction pattern of TEMPO-oxidation.

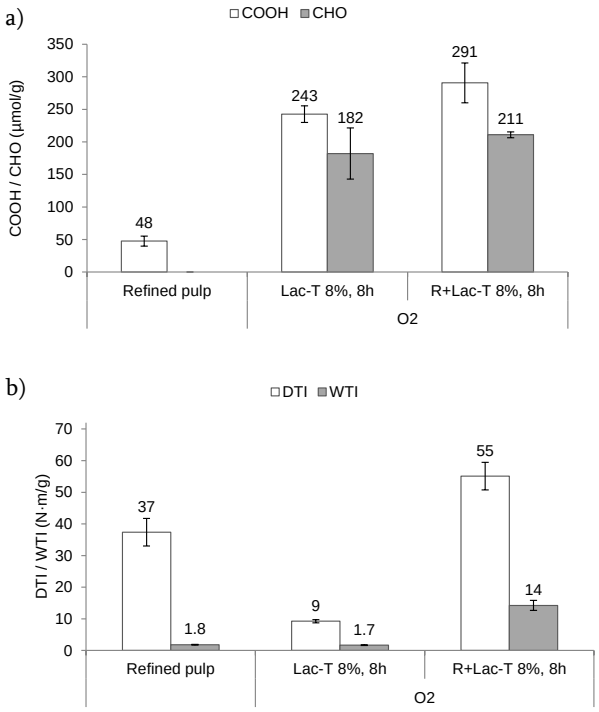


Figure 8-2 Carboxylic (COOH)/ aldehyde (CHO) group content (± standard deviation), dry / wet tensile index (± standard deviation) for refined pulp at 5000 rev and respective enzymatic oxidative treatments.

The water retention value (WRV) is a measure of the water absorption capacity of fibers and provides information about the degree of swelling of TEMPO-oxidized samples (Gehmayr et al., 2011). WRV is influenced by the content and distribution of hydrophilic carboxyl groups introduced and specific surface areas or degrees of fibrillation of the cellulose (Saito et al., 2007). Moreover, it is known that carboxyl groups increased hydrophilic character compared to hydroxyl groups. Therefore, a good correlation between WRV and carboxylic acids was found, with exception of Lac-T 8% treatment performed under oxygen pressure (Figure 8-3). Since Lac-T 8% (O_2) and R+Lac-T 8% (O_2) presented nearly the same carboxyl content, it can be hypothesize that the respective carboxyl groups were not distributed in the same region, and as a result the hydrophilic character of carboxyl groups was altered.

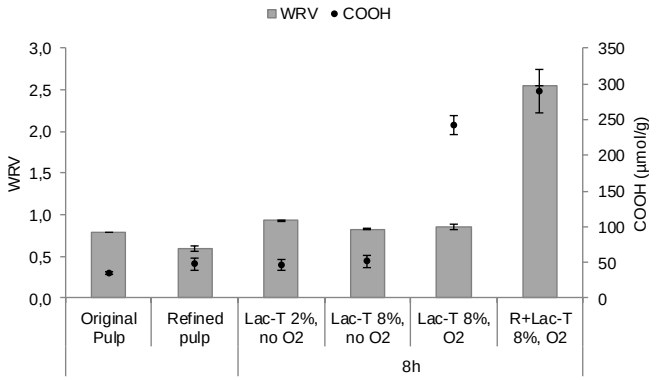


Figure 8-3 Water Retention Value (WRV) (± standard deviation) and content of carboxylic acids (± standard deviation).

Table 8-3 summarizes the morphologycal and mechanical properties of treated pulp samples. In terms of fiber morphology, the refining treatment caused an important reduction of fiber length, from ~1.5mm to ~0.8mm, and increased the content of fines, with respect to the original pulp. Interestingly, the presence or absence of oxygen during the Lac-T treatment led to a different yield in functional groups but had no effect on fiber morphology. The oxidation of primary hydroxyl groups occurs only on the surfaces of fibrils in cellulose fibers, and this regioselective oxidation probably resulted in such specific water-absorption behavior without any significant changes in the fibrous morphology (Saito et al., 2007). Introducing a refining step before the enzymatic treatment decreased fiber length to the same extent as the refining treatment alone. The smaller fiber length was directly associated to the refining treatment rather than to oxidative modification.

Regarding mechanical properties, the refining step provided an important improvement in tear strength with respect to the original pulp. The Lac-T treatments also increased tear strength, which suggests that higher strength was required to tear the fiber network. This improvement was due to increased inter-fiber hydrogen bonding provided by new introduced carboxyl groups (Barzyk et al., 1997). However, combining a refining treatment with a Lac-T treatment led to a loss of tear strength that can be ascribed to one of fiber strength during oxidation which prevailed at a high bonding level.

The refining treatment markedly reduced the air permeance and was accompanied by a density increase. This effect was not observed when oxidative treatments were performed alone and same air permeance as original pulp was found. By its side, R+Lac-T 8% (O_2) sample offered higher air permeance relative to refined pulp (R), although exhibited same bulk density.

The surface charge can also be measured by zeta potential of fibers. Due to the presence of carboxylic groups (weak acid group) on the surface, the zeta potential values were negative. Original pulp exhibited a zeta potential of -342 mV, and a notable charge decrease was obtained for all Lac-T oxidized pulp samples. Consistent with the presence of ionized carboxylic acids at the surface of fibers, similar zeta potential values were obtained for Lac-T 2%, Lac-T 8% and Lac-T 8% (O_2). The substantial difference in zeta potential of R+Lac-T 8% sample was thought to be caused by a modification of ionic distribution and repulsive forces between fibers and fines. Fiber hydrophilicity and swelling, which are highly relevant on fiber-fiber bonding, are also affected by fiber charge.

Table 8-3 Physical and chemical properties of Lac-T treated pulps

	Air Permeance (μm/Pa·s)	Density (g/cm^3)	L (l) (mm)	Fines (%)	Tear strength Index ($mN \cdot m^2/g$)	Z potential (mV)
Original pulp	639 ± 10	0.42 ± 0.01	1.53 ± 0.01	38.77± 0.01	4.01 ± 0.52	-342 ± 18
Refined pulp	40 ± 2	0.72 ± 0.01	0.82 ± 0.02	49.72 ± 0.0	6.71 ± 0.23	-
Lac-T 2%, no O_2, 8h	621 ± 8	0.41 ± 0.01	1.6 ± 0.02	35.3 ± 1.3	5.94 ± 0.83	-133 ± 0
Lac-T 8%, no O_2. 8h	593 ± 40	0.4 ± 0.01	1.6 ± 0.03	40.2 ± 1.1	5.51± 0.66	-144 ± 29
Lac-T 8%, O_2, 8h	622 ± 7	0.41 ± 0.01	1.6 ± 0.02	39.4 ± 1.6	5.72 ± 0.77	-148 ± 3
R+Lac-T 8%, O_2, 8h	292 ± 37	0.7 ± 0.0	0.8 ± 0.0	57.7 ± 0.2	3.23 ± 0.22	-42.15 ± 3

8.3.2 Influence of the treatment conditions on fiber properties

Figure 8-4 illustrates degree of polymerization after $NaBH_4$ treatment and the Fock solubility results. The Lac-TEMPO pulp samples were subjected to a post-reduced treatment with $NaBH_4$ in order to avoid further degradation during the viscosity measurements since Cuen-solution (cupriethylenediamine) is used. It is known that under alkaline conditions aldehyde groups at C-6 and keto groups at C-2 undergo β-elimination reactions that cause the cleavage of the cellulose chain (Patel et al., 2011). As can be seen from Figure 8-4, all oxidative treatments caused a considerably degradation of pulp and this depolymerization was more pronounced when the oxidative treatment was performed under oxygen pressure, showing about 85% of degree of polymerization loss, with respect to initial pulp. Patel et al. (2011) also suggested that degradation processes of the enzymatic approach are most probably homolytic (radical) and can also account for cellulose degradation.

In contrast to some authors (Aracri et al., 2011, 2012; Aracri and Vidal, 2012), no depolymerization due to carbonyl groups was observed during viscosity measurements since same viscosity values determined directly after Lac-T treatment or after a post-reduced treatment with $NaBH_4$ were found (Table 8-4). According to Potthast et al. (2005), the formation of carbonyl hydrates and/or hemiketals protects pulp from all reactions attributable to the reactive carbonyl moieties. In addition, double-bonded carbonyl groups are base-sensitive but relatively stable in acidic media, whereas hydrates and hemiacetals are stable in basic media, but are very labile under acidic conditions. These postulations are consistent with viscosity results. Moreover, these results suggested that reducing end groups are mostly present as hemiacetals and only to small extent as aldehydes or aldehyde hydrates. Therefore, as other authors described (Jaušovec et al., 2014), chemical oxidation of carbonyl to carboxyl form by $NaClO_2$ was inefficient due to the precedent formation of highly-stable covalent hemiacetal linkages, between primarily C-6 aldehyde groups and surrounding hydroxyls groups in cellulose.

Table 8-4 Viscosity values (± standard deviation) determined directly with Cuen solution or after applying a reduced treatment with $NaBH_4$, Fock solubility (± standard deviation) and chain scission values.

	Original pulp	Refined pulp	Lac-T 2%, no O_2, 8h	Lac-T 8%, no O_2, 8h	Lac-T 8%, O_2, 8h	R+Lac-T 8%, O_2, 8h
Viscosity (mL/g)	476 ± 1	474 ± 1	189 ± 16	197 ± 2	87 ± 13	99 ± 13
$NaBH_4$ viscosity (mL/g)	522 ± 4	505 ± 5	185 ± 10	192 ± 11	110 ± 27	96 ± 19
Fock solubility (%)	67 ± 4.3	35 ± 2.1	89 ± 0.7	91 ± 3.7	96 ± 3.8	77 ± 3.1
Chain scission (CS)	-	0	2.4	2.2	5.2	6.1

Fock solubility increased with decreasing pulp viscosity (Figure 8-4). However, because Fock solubility is highly dependent on the degree of polymerization, deep analysis must be conducted in order to draw accurate conclusions. The treatments Lac-T 2% and Lac-T 8% treatment, which used no oxygen pressure, exhibited an 89 and 91% Fock solubility, respectively. An identical increase in Fock solubility can be assumed since both pulp samples had a similar viscosity. The presence of oxygen pressure increased the amounts of carboxyl and aldehyde groups, and as a result higher value of Fock solubility was obtained. The new carboxyl groups in the glucose monomer shows increased hydrophilic character compared to the hydroxyl group and may act as a kind of spacer in-between the cellulose microfibrils, reducing the aggregation tendency (Gehmayr et al., 2012). It is interesting to remark that refined pulp (R) suffered a notably Fock solubility loss, but the treatment R+Lac-T let to retrieve Fock solubility value. Importantly, after the refining treatment the effect of Lac-T treatment was more marked than the ones observed for the others treatments. As noted earlier, the refining effect helped to improve strength properties, but the treatment R+Lac-T 8% (O_2) led to a lower final Fock solubility value than Lac-T 8% (O_2). The difference in Fock solubility can be explained by the presence of hemiacetal linkages that hindered cellulose dissolution. In addition, Gehmayr et al. (2012) suggested that low Fock solubility performance can be

attributed to the partially oxidized C6 carbons that are not available for xanthation reactions with CS_2.

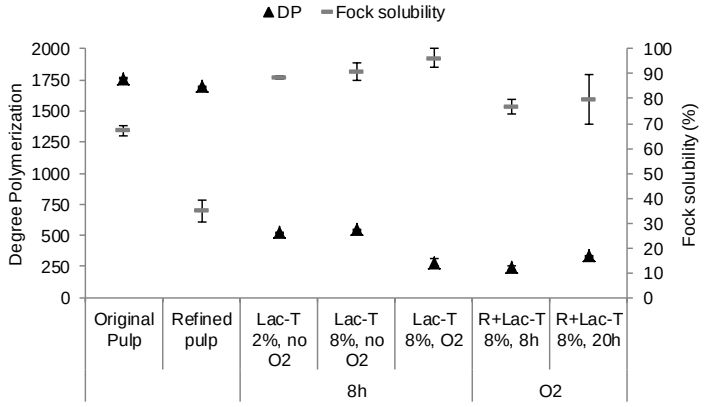


Figure 8-4 Degree of polymerization (DP) determined after subjecting pulps to a reduced treatment with $NaBH_4$ (±standard deviation) and Fock solubility, as a % of reacted cellulose, (± standard deviation) for original pulp, refined pulp at 5000 rev and respective enzymatic oxidative treatments.

As can be seen in Figure 8-5, a decreased viscosity was associated to an increased number of scissions in the cellulose chain. As mentioned earlier, cellulose depolymerization was due to the chemoenzymatic oxidative treatment itself since no degradation due to carbonyl groups was detected. The treatments conducted with no oxygen pressure exhibited about 2.4 chain scissions (CS), whereas the addition of oxygen pressure caused a greater viscosity loss and gave a CS value of 5.2. The increased CS values obtained in the presence of oxygen pressure, suggested that due to the cleavage of the connecting carbon-carbon bond additional aldehydes might be present at C-6, or at C-2 or/and C-3. These new aldehydes groups were not the result of the Lac-T oxidation of primary hydroxyl groups. The refining treatment followed by the oxidative treatment (R+Lac-T 8%) increased the CS up to 6.1 and also showed the biggest Fock solubility improvement. This is consistent with the results of other authors (Beltramino et al., 2015) who also observed that higher chain scissions corresponded to higher pulp solubility.

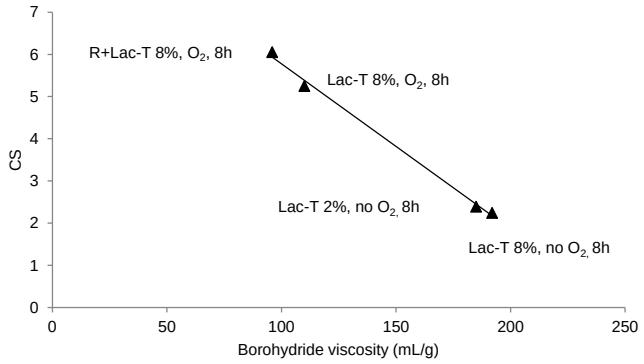


Figure 8-5 Chain scission (CS) against viscosity, all values determined after subjecting pulps to a reduced treatment with $NaBH_4$.

Figure 8-6 shows wet zero-span tensile index (WZSTI) against viscosity values. Wet-zero span test measures the intrinsic strength of a single fiber (in independence of fiber network) (Hagglund, 2004) and indirectly providing information about the effect caused by the treatment to the fibre structure. As observed earlier, cellulose underwent considerable degradation after oxidative treatments but fiber strength was not affected. The treatments conducted with no presence of oxygen pressure led to no differences between the resulting pulps, which however exhibited higher wet zero-span strength than the original pulp. The addition of oxygen pressure to the system increased cellulose degradation but no variation in fiber strength was observed, with respect to the original pulp. The combination of a refining step followed by the oxidative treatment (R+Lac-T 8%, O_2) resulted in no further degradation with respect to Lac-T 8% (O_2) but in much higher intrinsic fiber strength. This result can be ascribed to the formation of hemiacetal linkages which difficulty fiber individualization. In addition, the R+Lac-T 8% (O_2) sample was found to develop good wet strength property which may have influenced on fiber strength.

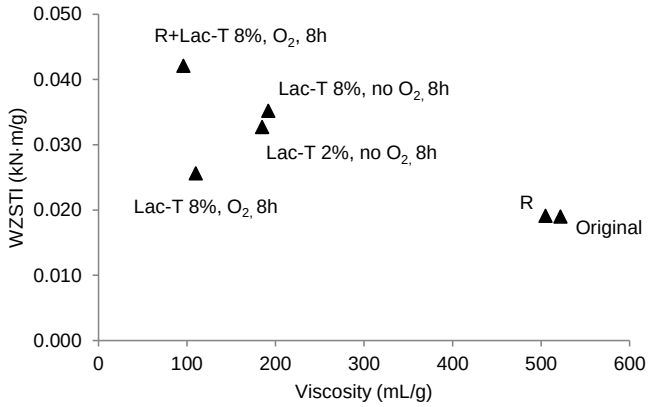


Figure 8-6 Wet Zero-Span Tensile Index (WZSTI) against viscosity results determined from pulps subjected to a post-reduced treatment with $NaBH_4$.

Figure 8-7 shows a cross sectional view of handsheets made from Lac-T 2% (no O_2, 8 h), Lac-T 8% (no O_2, 8 h), Lac-T 8% (O_2, 8 h), refined pulp (R) and R+Lac-T 8% (O_2, 8h). In agreement with fiber length and bulk density results, no differences between non-refined chemoenzymatic oxidative treatments were found. The refining treatment increased the bonding ability and as a result a highly compacted handsheet was observed. Interestingly, the combined treatment (R+Lac-T 8%) revealed lower adhesion and sheet compaction relative to the refined sample. Both samples had the same fiber length and bulk density, but differed in air permeance. Thus, the R+Lac-T sample showed more pore structures, which explain the higher air permeance value.

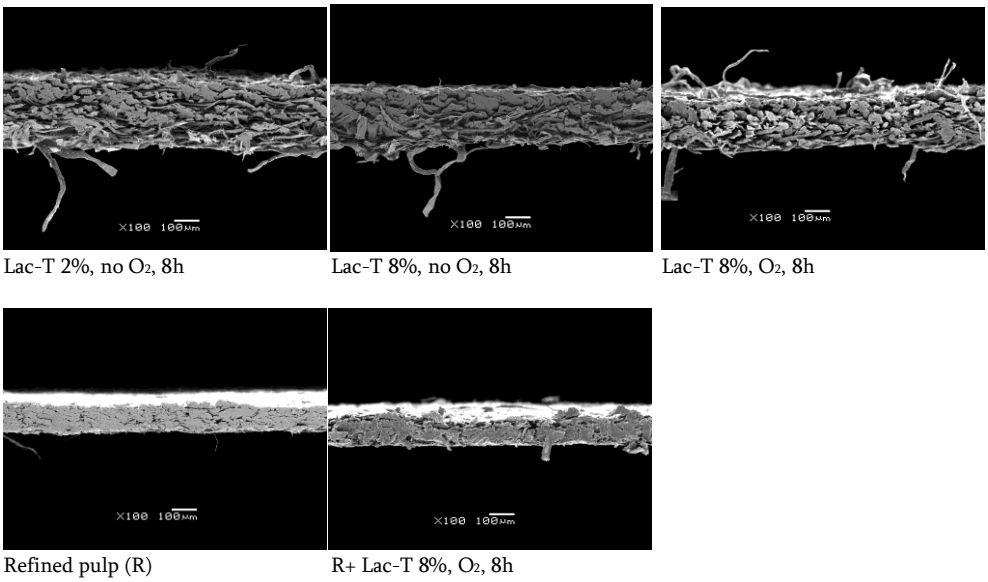


Figure 8-7 Cross section of laccase.TEMPO oxidized samples by Scanning Electron Microscope (SEM).

8.4 Conclusions

In the present work, chemo-enzymatic modification of commercial bleached dissolving pulp using laccase as biocatalyst and TEMPO as enhancer was investigated. Different treatment conditions, such as the presence or absence of oxygen pressure, TEMPO dose, reaction time and the introduction of a refining step before the oxidative treatment, were studied in order to obtain the greatest content of carboxyl and aldehyde groups. Due to the presence of these functional groups, dry and wet strength, and Fock solubility behavior were measured. The treatment Lac-T which used no oxygen pressure provided low carboxyl and aldehyde contents and therefore no wet strength was developed, even though it increased Fock solubility by 35% with respect to the original pulp. Importantly, using oxygen pressure brought higher amount of carboxyl and aldehyde groups, strength-related properties were improved but further cellulose degradation was observed. The treatment Lac-T 8% (O_2) improved Fock solubility by 43%, regarding original pulp, and led to an acceptable wet strength ratio (W/D=18%). On the other hand, R+Lac-T 8% (O_2) caused slightly increase in carboxyl and aldehyde groups with respect to Lac-T 8% (O_2) sample, but improved the wet strength property significantly, with a W/D strength ratio

of 26%. These results demonstrated that the fibrillation effect resulted from the refining treatment let to form hemiacetal covalent linkages with aldehyde groups derived from Lac-TEMPO treatment and available intra- hydroxyl groups. Specifically, the synergy between fibrillation and a favorable aldehyde/carboxyl ratio provided a positive effect on wet strength property. As a relevant conclusion, the refining treatment exhibited a Fock solubility value similar to that of non-refined treated pulp, but improved bonding capability and as a consequence strength-related properties.

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Chapter 9

Study of the acetylation conditions on dissolving pulps upgraded by enzyme-assisted treatments

Abstract

Biobleached sulfite fibers were subjected to new chemo-enzymatic treatments to investigate their suitability for heterogeneous- and homogeneous-phase acetylation. The results were compared with those from a commercially-available dissolving fiber grade, obtained by traditional chemical methods, which was used as reference. A range of surface acetylation degrees, as assessed by FTIR and titration, was achieved by varying the concentration of acetic anhydride in the nonpolar solvent used in the heterogeneous phase. The degree of substitution was affected extensively by relatively small changes in reactant concentration. As expected, the acetyl group content correlated with acetic anhydride activity in the system. The precursor chemo-enzymatic and reference fibers differed in terms of hemicellulose concentration and fiber morphology (length and fine content). As a consequence, different extents of acetylation were achieved for these samples. At the highest acetic anhydride reactant concentration, acetyl groups content of up to 24 and 8% were measured, respectively. Under this condition, the fiber length of the chemo-enzymatic and reference fibers was reduced by 68 and 87% (with corresponding fine fraction content of 83 and 93%). These changes were accompanied by a remarkable gain in fiber coarseness. The substitution of hydroxyl groups by acetyl moieties resulted in a lower hydrophilicity (highest water contact angles of 55° and 64° for the acetylated chemo-enzymatic and reference fibers, respectively). This is a

remarkable increase compared with those of the non-acetylated fibers, between 28 and 31°, depending on the sample. In addition, in both cases a higher wet tensile strength was observed. Homogeneous phase acetylation of chemo-enzymatic and reference fibers resulted in relatively higher acetyl groups content (up to 36 and 33%, respectively). Also, negligible differences were observed in the fiber mechanical and morphological properties measured at a given degree of acetylation. Overall, it is concluded that chemo-enzymatic treatment is as feasible alternative towards the production of dissolving fiber grades for efficient acetylation.

9.1 Introduction

Acetylation is a commonly used chemical modification in which acetyl groups (CH_3CO^-) react with the surface hydroxyl groups (OH) of cellulose, making its surface less hydrophilic. The acetylation process depends on the accessibility of cellulose fibers and the susceptibility of OH groups in the crystalline and less crystalline domains of the cellulose fibers (Kalia et al., 2014). The greater the accessibility, the easier it is for the reactants to diffuse into the interior of the cellulose. There are two methods for acetylation, namely, heterogeneous (in fiber dispersions) and homogeneous (in solution). The heterogeneous acetylation process is performed in the presence of a non-solvent, such as toluene, benzene or carbon tetrachloride. The reaction product (cellulose acetate) is insoluble and thereby, this process preserves the morphological structure of the fiber. In contrast, cellulose acetate is dissolved during the homogeneous acetylation and, therefore, it demands solvents capable of deconstructing the crystalline network and interacting with the anhydro-glucose units of cellulose. This is usually done by reducing or eliminating inter and intra- molecular hydrogen interactions. In the homogenous phase, sulfuric acid is used as a catalyst and it combines with the cellulose, forming sulfate linkages (cellulose sulfate), but, most of these are removed during acetylation via an exchange with acetyl groups. In the next step, cellulose is transesterified from sulfate to acetate in the presence of acetic anhydride. The initial reaction occurs mainly in the amorphous regions of cellulose. It is important that the final cellulose acetate contains only a very small amount of sulfate groups because they affect the properties adversely, especially the color. When acetylation is virtually complete, the reaction mixture is viscous and clear. The excess of acetic

anhydride is then neutralized by adding aqueous acetic acid, which helps to desulfate the residual sulfate linkages. The presence of water diminishes cellulose chain degradation.

The extent to which the three available hydroxyl groups are substituted, the degree of substitution (DS), does not quite reach the maximum of three units per anhydroglucose unit (as in cellulose triacetate). Cellulose triacetate (DS > 2.8) displays a limited solubility in acetone and is reported for use in a relatively narrower number of commercial applications (Cao et al., 2007). Diacetates with a DS from 2.2 to 2.7 (also named secondary acetates) are the most commonly reported cellulose esters. They are soluble in acetone and other organic solvents (Steinmeier, 2004; Fischer et al., 2008; Wan Daud and Djuned, 2015), and can be used in applications such as coatings, films, textiles, synthetic polymeric membranes, among others.

Following a heterogeneous phase, it is possible to obtain more crystalline and less biodegradable cellulose acetates (CA) than those produced through homogeneous routes (Barud et al., 2008). On the other hand, the advantages of acetylation in homogeneous phase include the excellent control of the degree of substitution (DS) and the possibility of a uniform distribution of the functional groups along the polymer chain (Ass et al., 2004).

Importantly, CA is usually produced from high quality cellulose fibers, namely, dissolving grades derived from cotton or wood (α-cellulose content of > 95%) (Saka and Matsumura, 2004; Roselli et al., 2014; Wan Daud and Djuned, 2015). In the case of cotton sources, issues related to the large land area required for farming and water required for irrigation, result in high economic costs. Further, the so-called “cotton gap” motivates a need for more extensive utilization of dissolving-grade fibers derived from wood. According to FAO (2012), dissolving-grade fibers constitute a small share of the global pulp production, but prospective consumer markets indicate that this share will increase in the coming decades. Based on this scenario, new technologies are being suggested as alternative to traditional dissolving pulp production processes. In Chapter 4 and Chapter 6 (Quintana et al., 2013, 2015), laccase-mediator system was used to bleach sulfite pulp and the conversion to dissolving-grade was achieved by cellulase treatment. According to the results,

the obtained chemo-enzymatic dissolving-grade fibers exhibited suitable characteristics for use in the synthesis of cellulose derivatives.

In the present chapter, fibers obtained via chemo-enzymatic treatments of biobleached fibers (termed herein as *L_F* and *L_CCE_F*) and fibers further modified by Laccase-mediated oxidation (termed herein as *L__CCE_F+Lac-T)* were investigated as far as their suitability to synthesize acetylated cellulose. A bleached commercial dissolving-grade fiber, used as a reference and termed "*Com*", was used for comparison. This study focuses on the surface acetylation reactions, typical of heterogeneous acetylation, while homogeneous acetylation was also carried out for comparison purposes. In terms of surface acetylation, given doses of acetic anhydride (Ac_2O) were tested and the degree of acetylation was evaluated by FTIR spectroscopy. Paper handsheets were produced from fibers that were acetylated on the surface (heterogeneous phase) and characterized in terms of contact angle, mechanical strength and surface morphology. Samples obtained by homogeneous acetylation phase were used to prepare transparent films via solvent casting and characterized in terms of the tensile strength. This work, therefore, aims at determining if chemo-enzymatic treatment is a suitable alternative for the synthesis of materials with low hydrophilicity via heterogeneous and homogeneous acetylation.

9.2 Materials and Methods

9.2.1 Raw material

Precursor fibers. As starting fiber material, **unbleached** sulfite cellulose fibers were used and obtained as a mixture of 60% Norway spruce (*Picea abies*) and 40% Scots pine (*Pinus sylvestris*) (Dömsjo Fabriker mill, Sweden). Fiber characteristics included a kappa number of 4.2 ± 0.2, ISO brightness of 61.25 ± 0.6% and viscosity of 511 ± 11 mL/g. The carbohydrate content, as determined by high-performance liquid chromatography (HPLC), was 88.5 ± 0.3% glucan, 6.0 ± 1.3% mannan, 3.0 ± 0.3% galactan, 2.4 ± 0.4% xylan, and 0.3 ± 0.2% rhamnan. As a reference fiber source, a totally chlorine-free (TCF) **bleached** commercial dissolving-grade pulp was employed. This pulp was obtained from the unbleached fibers (as indicated above). It has an ISO brightness of 91.70 ± 0.15% and viscosity of 474 ± 0.7 mL/g. The carbohydrate composition, also determined by HPLC, included 95.6 ± 0.3% glucan, 2.8 ± 0.2% mannan, 0.8 ±

0.0% xylan, 0.6 ± 0.3% galactant and 0.2 ± 0.2% rhamnan. The bleached fibers were obtained by sulfite digestion followed by chemical bleaching at the Dömsjo Fabriker mill (Sweden). These fibers, which are used commercially, are thereafter referred to as *Com*.

9.2.2 Enzyme treatments

A laccase, *Trametes villosa* (TvL), used for *biobleaching* the fibers was supplied by Novozymes® (Denmark), with an activity of 746 U/mL. Violuric acid (VA), the mediator used for this enzymatic treatment, was purchased from Sigma-Aldrich and used as received. A *hydrolytic* treatment was also applied involving an endoglucanase produced from *Cerrena unicolor* (supplied by Fungal Bioproducts®, Spain). The activity as U/g dry enzyme powder of the cellulase preparation was 1700 CMCase U/g and 680 U/g for the cellulase and xylanase activity on the cellulase, respectively.

For biobleaching, unbleached sulfite fibers were first conditioned at pH 4 adjusted with H_2SO_4, stirred at 2% solids content for 30 min and washed with de-ionized water in a glass filter funnel. This step was needed to remove contaminants and metals, and also to bring the fiber dispersion to the pH required for the enzymatic treatment. The biobleaching process was conducted at the laboratory scale and included a sequence denoted as $L_{VA}(PO)(PO)$, where L_{VA} denotes an enzymatic (lacasse) treatment and PO the hydrogen peroxide stage assisted with oxygen. The conditions used are detailed in 3.3 Bleaching assays (*Laccase-mediator treatments* and Oxygen-reinforced hydrogen peroxide treatment (PO/P stage))

The resulting *biobleached* fibers ($L_{VA}(PO)(PO)$), denoted here as *L*, for simplicity, were used in two different additional treatments to produce the chemo-enzymatic samples used later for acetylation reactions. One was subjected to enzymatic hydrolysis with an endoglucanase (resulting in fibers that are denoted thereafter as *L_F*) and the other with application of cold caustic extraction before endoglucanase treatment (resulting in fibers that are denoted thereafter as *L_CCE_F*). The purpose of introducing an endoglucanase treatment was to improve fiber reactivity. By its side, cold caustic extraction was a purification stage where hemicelluloses were removed and as a result fiber quality was improved. Both enzymatic treatments employed 12 U/g odp enzyme,

and the conditions followed are described in section 3.4 (Endoglucanase assays). The cold caustic extraction was conducted in a polyethylene bag. The treatment was performed at 10% (w/ w) solids adjusted with 9% (w/v) NaOH at 25 °C for 1 h. Treated fibers were washed with de-ionized water until the filtrate pH was neutral (section 3.4 Endoglucanase assays). Additionally, *L_CCE_F* fibers were further modified by TEMPO-mediated oxidation (resulting in fibers that are denoted thereafter as *L_CCE_F+Lac-T)*. Laccase–TEMPO oxidation treatments were performed at room temperature, at 5% consistency in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5, 20 U/g odp laccase (Lac), 8% odp TEMPO and 8h of reaction.

9.2.3 Analysis of pulp properties

L_F, L_CCE_F, and *Com Fiber Analysis.* The commercial dissolving grade and chemo-enzymatic fiber samples (*Com, L_F* and *L_CCE_F*) were characterized in terms of kappa number, brightness and viscosity according to ISO 302:2004, ISO 2470:2009, ISO 5351:2004, respectively. The cellulose reactivity of the fiber samples was determined according to slightly modified version of Fock's method (Fock, 1959; Köpcke et al., 2010). Carbohydrate composition of treated fibers was determined using high performance liquid chromatography (HPLC).

^{13}C-CP/MAS NMR spectra were recorded in a Bruker AMX-300 instrument operating at 7.05 T and at 75.5 MHz for ^{13}C. Samples were immersed in deionized water for at least 2h. All measurements were performed at 290 ± 1K. The magic angle spinning (MAS) rate was 4 kHz. The cross-polarization contact time was 1 ms and the recycle delay time 2.5 s. Acquisition time was 98.3 ms and sweep-width was 31.2 kHz. The number of scans was 5100.

All methods and techniques are described in more detail in section 3.7.1 and 3.7.2.

Heterogeneous (surface) and homogenous acetylation. In the heterogeneous phase acetylation, 2.0 g oven dried pulp (odp) of each type (*Com, L_F, L_CCE_F*) was disintegrated and then filtered using a filter paper (Whatman 1) for water removal. The samples were then placed in a glass beaker containing a mixture of 20 mL of acetic acid (99.7% w/w) and 35 mL toluene. The dispersion was stirred for 5 min and 0.2 mL sulfuric acid (95% w/w) was added. Then, a desired

amount of acetic anhydride (Ac_2O) was added and the mixture was stirred for 1 h at room temperature. The specific conditions for acetylation reactions were as follows: 0.53, 2.67, 5.35 and 10.7 g Ac_2O per weight (g) of dried fiber sample (*Com, L_F, L_CCE_F*) (Table 9-1). The reaction was quenched by adding 6 mL of distilled water and ethanol, 3:7 v/v. The mixture was allowed to stand for 20 min and then washed 3 times with methanol and finally with water until neutral pH (Figure 9-1).

The heterogeneous acetylated fibers were analyzed by Fourier transform infrared spectroscopy (FTIR) by using a Nicolet Avatar 360 spectrophotometer (Nicolet Instrument Corporation). The samples were prepared by mixing 1 mg of the sample in a matrix of 300 mg of KBr followed by pressing. The spectrum was recorded in the range of 400–4000 cm^{-1} and 32 scans were run at 4 cm^{-1} resolution.

Table 9-1 Conditions used for heterogeneous acetylation reactions depending on the activity or concentration of acetic anhydride (Ac_2O)

g Ac_2O/ g of dried sample	Ranking of Ac_2O activity (nomenclature used)	*L_F*	*L_CCE_F*	*Com*
0.53 (~1mL Ac_2O)	Lowest	✓	X	✓
2.67 (~5 mL Ac_2O)	Low	✓	✓	✓
5.35 (~10 mL Ac_2O)	Medium	✓	✓	✓
10.7 (~20 mL Ac_2O)	High	✓	✓	✓

Homogeneous acetylation was performed as reference. For this purpose, 2.5 g odp of respective fiber type (*Com*, *L_F, L_CCE_F, L_CCE_F+Lac-T*) was disintegrated and then filtered using a filter paper for water removal. Then, 50 mL of acetic acid was added to the sample, stirred 5 min and then filtered. This step was done by duplicate. After filtration, 45 mL of acetic acid (99.7% w/w) and 0.25 mL sulfuric acid (95% w/w) was dropped into the sample and stirred for 1 min. Then, 5.35 g Ac_2O/g dried fiber (~12.5 mL Ac_2O) was added and continuously stirred for 30 min at room temperature. The reaction was quenched with the addition of 6.25 mL of distilled water and acetic acid at a ratio of 3:7 v/v, respectively. Finally, cellulose acetate (CA) was obtained by pouring the viscous reaction mixture into distilled water obtaining a continuous

droplet and with constant stirring. With precipitation, cellulose acetate was regenerated. The obtained product was washed with distilled water until neutrality and subsequently dried using a freeze-drying (Figure 9-1).

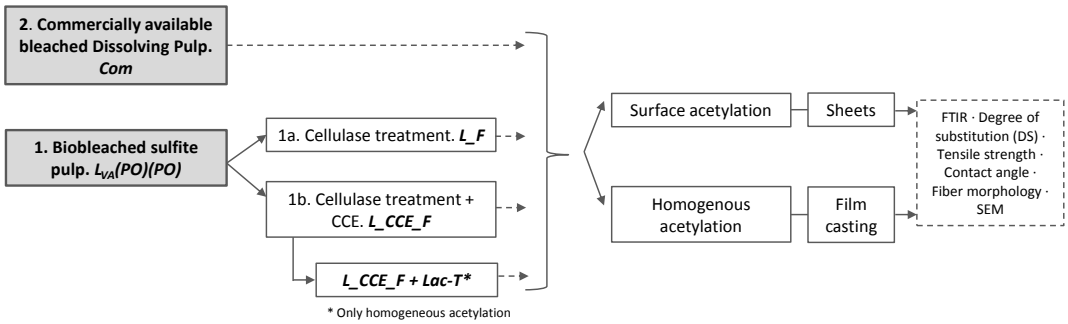


Figure 9-1 Outline of experimental plan and samples studied. Biobleached (L or LVA(PO)(PO)) sulfite fibers were subjected to endoglucanase treatment (1a, *L_F*) or cellulase treatment after cold caustic extraction (1b, *L_CCE_F*). Heterogeneous (surface) or homogeneous acetylation reactions were applied to *L_F*, *L_CCE_F*. Fiber handsheets or films were prepared and characterized. Bleached commercial dissolving fibers (Com) were used as a reference, and same heterogeneous and homogeneous acetylation reactions were performed.

Acetyl group content. Firstly, the respective acetylated sample was ground and 100 mg (oven dried) were weighed accurately and placed into 20 mL of 75% v/v of ethanol in an Erlenmeyer flask. The bottle, loosely stoppered, was heated to 50-60 °C for 30 min for better swelling of the material. Then, 20 mL of 0.5 N NaOH solution was added to the sample and the mixture was heated to 50-60 °C for 15 min. A blank was also conducted but in absence of fiber sample. The flasks were stoppered tightly and allowed to stand at room temperature for 72h. The excess alkali was then titrated with 0.5 N HCl using phenolphthalein as indicator. An excess of about 1 mL of 0.5 N HCl was added and allowed the NaOH to diffuse from the regenerated cellulose overnight. The small excess of HCl was titrated with 0.5 N NaOH to a phenolphthalein end point. The percentage of acetyl groups was calculated as follows:

$$Acetyl\ groups, \% = \quad D - C\ Na + \ A - B\ Nb\ \cdot (F/W)$$

where:

A = NaOH solution required for titration of the sample, mL

B = NaOH solution required for titration of the blank, mL

Nb = Normality of the NaOH solution

C = HCl required for titration of the sample, mL

D = HCl required for titration of the blank, mL

Na = Normality of the HCl solution

F = 4.305 for acetyl

W = sample mass used, g

Handsheets from surface acetylated fibers and cellulose acetate films. Fibers obtained by surface acetylation were used for preparing handsheets. For sheet manufacture 1g of each sample at 1% solids was disintegrated and poured into an over-pressurized device (< 1 bar pressure difference) allowing few minutes drainage to obtain a web or handsheet of the acetylated fibers. The device was equipped with open mesh fabric screen (Sefar Nitex 03-10/2, mesh opening of 10 μm with open area of 2%) to remove the excess water and retain the fibrils. The webs were pressed between two blotting papers using a metal roller (10 kg) and then dried at 80 °C, for 1 h in a tumble drier. The obtained sheets were then stored in a conditioned room (23 °C and 50% relative humidity) until further use.

Cellulose acetate (CA) obtained from homogeneous acetylation reaction was used for preparing transparent films by means of a casting technique. Dried cellulose acetate was dissolved in given amounts of acetone in order to obtain a concentration of 8 wt%. The solutions for film casting were firstly centrifuged at 6000 rpm for 10 minutes. The supernatant was carefully transferred and centrifuged again at 2000 rpm for 5 minutes. The films were cast by pouring the transparent solution on a glass plates, well distributed and followed by drying in a vacuum desiccator for at least 2h. The film samples were finally kept in a desiccator.

Surface acetylated handsheets and cellulose acetate films characterization. Surface acetylated handsheets were used to determine different properties. The morphological characteristics of fibers (viz., length, width and curl), and fine content were determined in accordance with TAPPI T 271 on a Metso

kajaaniFS300 fiber analyzer. High-resolution imaging of surfaces (handsheets were taken on a JEOL JSM- 6400 scanning electron microscope (SEM). Samples were placed on the SEM sample holding stub with the aid of conductive double side sticky carbon film and coated with Au/Pd alloy prior to analysis. The wetting characteristics of the acetylated handsheets was determined by the initial water contact angle (WCA) using a Dataphysics OCA15EC contact angle goniophotometer (Dataphysics, USA). A 4 μL water drop was dropped to the sample surface, and an image capture ratio of 25 frames/s was used to calculate the initial contact angle. A minimum of ten readings were taken on every sample to reduce possible influence of the heterogeneity of the surface. Also, changes in contact angle were monitored until complete absorption of each water drop. Wet and dry tensile strength of the surface acetylated sheets were measured on a MTS 400/M Vertical Tensile Tester equipped with a 50 N load cell, in accordance with ISO 1924-3:2005.

The surface of transparent films were directly analyzed on a Perkin Elmer Spectrum 100 FT-IR spectrometer equipped with universal ATR sampling accessory by performing 32 scans at 1 cm^{-1} intervals over the wavenumber range 4000−400 cm^{-1}. The measuring cell was washed with de-ionized water and ethanol between measurements. Tensile strength tests for CA films resulted from homogeneous acetylation reactions were performed on a MTS 400/M vertical Tensile Tester, with a cross-head speed of 40 mm/min. Specimen strips presented 10 mm width and 40 cm length. Dry zero-span strength, initial water contact angle and water drop test were also measured (Chapter 3, 3.7.3).

9.3 Results and discussion

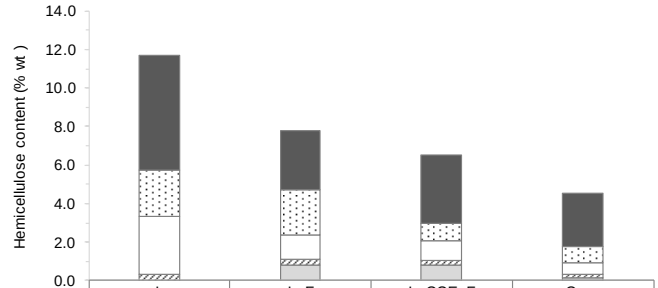
Precursor fiber characterization. The effect of acetylation on the quality of the systems obtained from *L_F, L_CCE_F* or *Com* was evaluated. The characteristics of respective treated fibers are indicated in Table 9-2. All sequences applied resulted in similar lignin content, as assessed by the kappa number, ISO brightness and viscosity. However, the commercial dissolving fibers (*Com*) exhibited the highest ISO brightness. Similar values of Fock solubility were found for all the fibers; however, compared to *L_F, L_CCE_F* solubility was higher. This result can be related to the transformation of cellulose I into cellulose II during the cold caustic extraction (concentration > 8

wt) and the fact that endoglucanases have a greater affinity for the latter allomorph (Engström et al., 2006; Köpcke et al., 2008; Gehmayr and Sixta, 2011). It is also known that lower viscosity can influence cellulose solubility; however, in general all fibers presented comparable viscosity.

Table 9-2 Main characteristics (mean ± standard deviation) of L_F, L_CCE_F fibers as well as Com reference.

	L_F	L_CCE_F	Com
Kappa Number	< 0.5 ± 0	< 0.5 ± 0	<0.5
ISO Brightness (%)	84.6 ± 0.9	83.7 ± 1.5	90.3 ± 0.1
Viscosity (mL/g)	473 ± 55.4	447 ± 18.5	476 ± 0.7
Fock solubility (%)	66.9 ± 2.9	71.5 ± 2.3	67.3 ± 2.1

The carbohydrate composition was determined by HPLC, with special attention to the hemicelluloses content (Figure 9-2). The endoglucanase treatment applied to the biobleached fibers (*L*) to obtain *L_F* reduced the amount of hemicelluloses by 35%, especially the mannan and galactan fractions. The introduction of a cold caustic extraction followed by hydrolytic treatment (*L_CCE_F*) further decreased the amount of hemicelluloses by 46.2%. To be precise, compared to *L_F, L_CCE_F* treatment contribution amounted to 11.2%, resulting in a smaller xylan fraction and similar mannan and galactan content. The lowest hemicellulose content was measured in *Com*.



	L	L_F	L_CCE_F	Com
Mannan	6	3.1	3.6	2.8
Xylan	2.4	2.3	0.9	0.8
Galactan	3	1.3	1.0	0.6
Rhamnan	0.3	0.3	0.3	0.2
others	0.0	0.8	0.8	0.2

Figure 9-2 Hemicellulose composition of biobleached pulp (L), samples after chemo-enzymatic treatment (L_F and L_CCE_F) and commercial dissolving pulp (Com). The total content of hemicelluloses is indicated above each column.

Solid state ^{13}C-NMR spectra of biobleached fibers (*L*), biobleached fibers followed by endoglucanase treatment (*L_F*), and biobleached fibers submitted to cold caustic extraction (9 % (w/v) NaOH) followed by endoglucanase treatment (*L_CCE_F*) are included in Figure 9-3. Caustic extraction converted cellulose I to cellulose II: the C-6 signal at 64 ppm increased, obtaining two peaks with nearly identical heights at 66 and 64 ppm. The C-1 signal did not exhibit a shoulder at 108 ppm, which is characteristic of cellulose II (Janzon et al., 2008a). Note that the greater proportion of cellulose II in *L_CCE_F* is associated with the low Fock solubility value (Krässig, 1993; Janzon et al., 2008b).

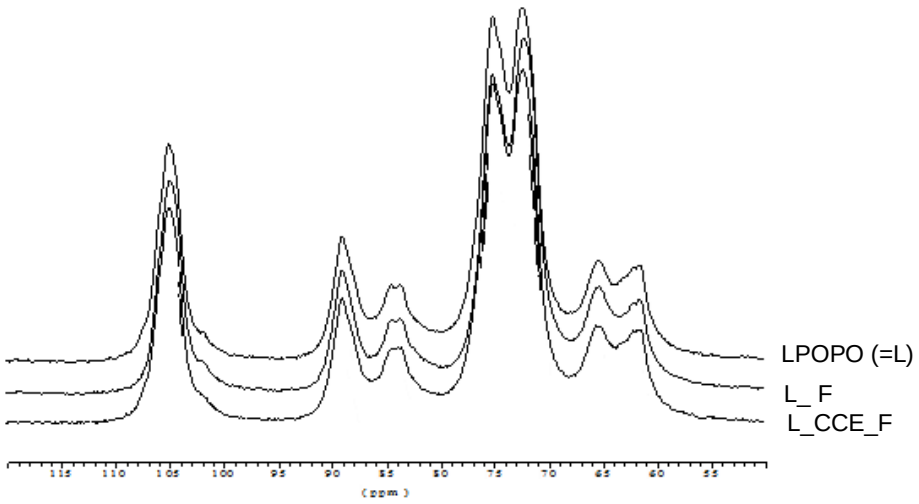
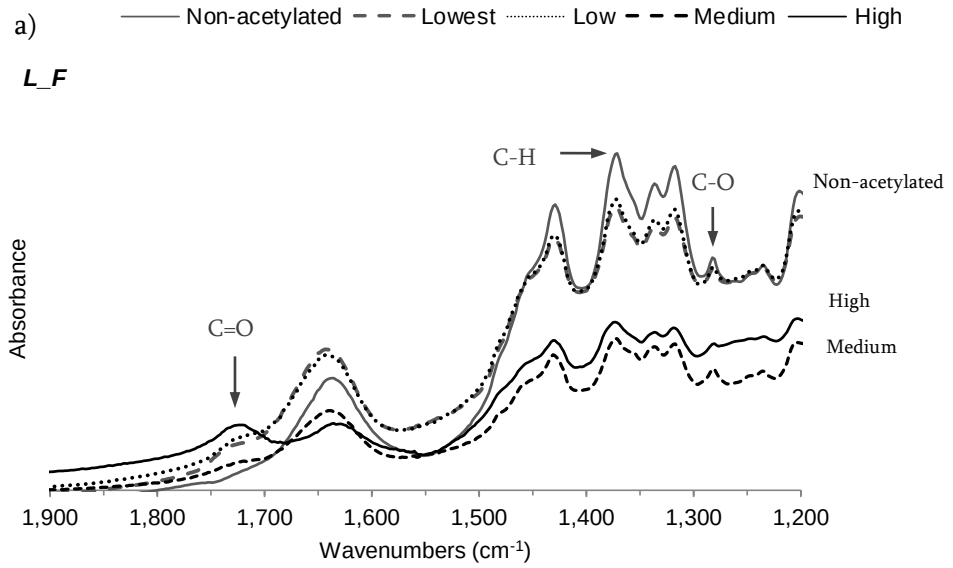


Figure 9-3 ^{13}C-CP/MAS NMR spectra of biobleached pulp (LPOPO, enzymatic stage followed by two pressurized hydrogen peroxide stages) compared to biobleached fibers followed by an endoglucanases treatment (*L_F*), and biobleached fibers submitted to cold caustic extraction with 9 % (w/v) NaOH and then an endoglucanase treatment (*L_CCE_F*)

Heterogenous acetylation phase. Different degrees of surface acetylation were achieved by varying the concentration of acetic anhydride (Ac_2O) in the nonpolar solvent used. The Ac_2O loading indicated in Table 9-1 correspond to relative activities denoted thereafter as "lowest", "low", "medium" and "high" (Table 9-1). The effect of acetylation on *L_F*, *L_CCE_F* and *Com* samples was assesed via FTIR spectroscopy (Figure 9-4). Changes in non-acetylated and acetylated samples were identified. Specifically, the structural changes of acetylated fibers were confirmed by the appereance of three new bands characteristic of the acetyl group vibration at about 1735-1740, 1368-1375 and 1259-1277 cm^{-1}. The peaks located at 1735-1740 cm^{-1} were attributed to the C=O stretching of carbonyl in the ester bonds. The peaks located at 1368-1375 cm^{-1} were assigned to C-H symmetrical deformation in methyl group. The vibration peaks between 1259 and 1277 cm^{-1} corresponded to C-O stretching of the acetyl group. The absence of peaks in the 1840-1760 cm^{-1} region demonstrated that there was no residual, unreacted acetic anhydride in the acetylated fibers. Also, the absence of absorption in 1700 cm^{-1} for carboxylic group implied that the

products are also free of the byproduct of acetic acid (Rodionova et al., 2011; Cunha et al., 2014; Muhammad Djuned et al., 2014; Mashkour et al., 2015).

In the case of *Com* fibers, it is noted that by increasing the amount of Ac_2O used for acetylation resulted in a higher intensity of the C=O band at 1735 cm^{-1}; at the same time, a decrease in the C-O band at 1235 cm^{-1} was clear. Although the C-H band at 1375 cm^{-1} is charactersitic of acetylated fibers, no variation in absorption was evident for the different acetylated conditions. A similar observation applies to *L_F* and L_CCE_F fibers but differences in the intensity peak at 1735 cm^{-1} with respect to the acetylation conditions were less pronounced. For both, *L_F* and *L_CCE_F*, a high intensity peak at 1735 cm^{-1} was observed when a high (10.7 g) Ac_2O level was introduced.



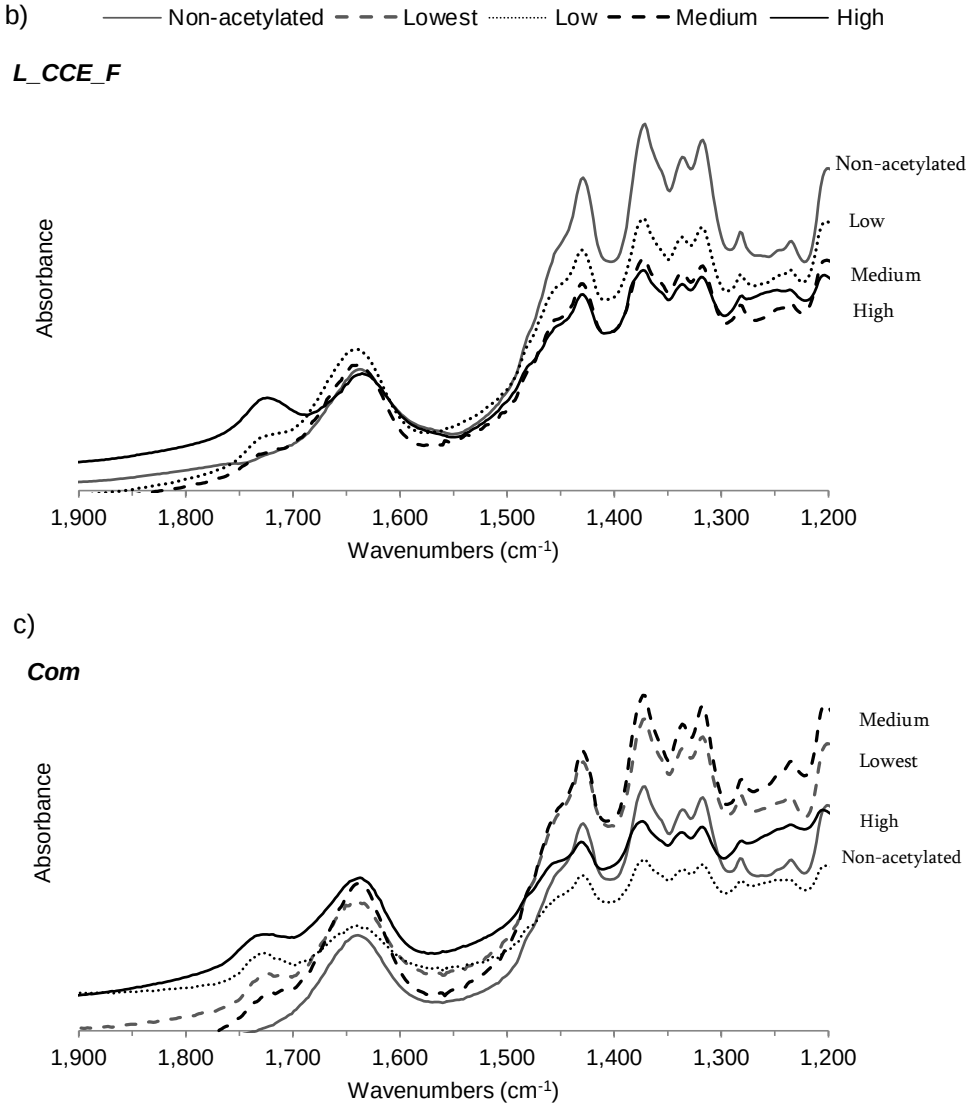


Figure 9-4 FTIR spectra for L_F (a), L_CCE_F (b) and Com (commercial dissolving pulp) (c) samples at different acetylated levels (minimal, low, medium and high).

The degree of acetylation (i.e. acetyl group content) was determined by titration with NaOH and HCl (Figure 9-5). *L_F* sample did not show differences in terms of acetyl content after reaction with lowest and medium Ac_2O levels, but a significant gain in acetyl group content was observed for the high dose (10.7 g). In fact, from all studied fibers, the highest acetyl group content (~24%) was determined for the *L_F* sample. In general, compared to *L_F*, acetylation of

L_CCE_F fibers yielded a smaller amount of acetyl groups. The application of "low" Ac_2O levels was not effective in incorporating enough acetyl groups. Only by using two or four-fold the Ac_2O dosage level introduced a suitable amount of acetyl groups. Specifically, 13% and 15% of acetyl group content were measured for medium and high Ac_2O dosages. In the case of *Com* sample, a gradual improvement in acetyl group content was observed from the lowest (0.53 g Ac_2O) to the medium (5.35 g Ac_2O) Ac_2O addition. Interestingly, a high Ac_2O addition produced a relatively small acetylation degree. *Com* subjected to medium conditions resulted in 15% acetyl group substitution, while only 7.9% was measured at high Ac_2O levels (a 47.3% reduction). Actually, same acetyl content was found using low and high amounts of Ac_2O.

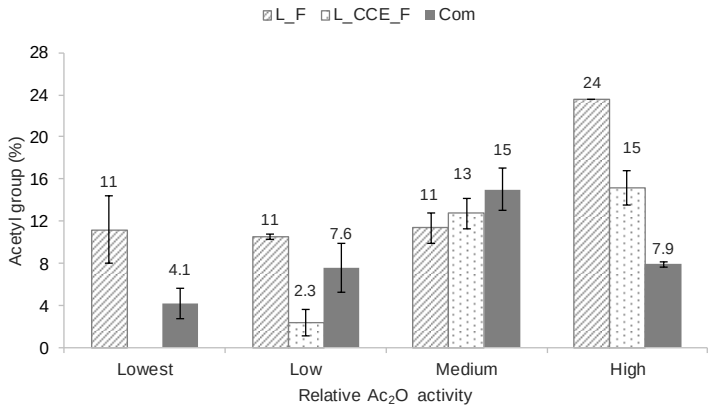


Figure 9-5 Content of acetyl group (%) as a function of acetic anhydride used (g) in the respective acetylation reactions for *L_F*, *L_CCE_F* and *Com* samples.

Changes in fiber morphology upon chemo-enzymatic treatment and acetylation. Endoglucanase treatment (*L_F*) of the biobleached fibers (*L*) caused a significant reduction (~65%) in fiber length and increase of the fines content. The fiber length decreased from 1.8 mm (L) to 0.68 and 0.62 mm for *L_F* and *L_CCE_F* samples, respectively. As can be seen in Table 9-3, a further length reduction was observed for *L_F, L_CCE_F* and *Com* samples upon acetylation (from lowest to high Ac_2O reaction levels). Specifically, *Com* sample consisted at first of longer fibers than those in *L_F* and *L_CCE_F*, but at high acetylation conditions (5.35 and 10.7 g Ac_2O) the fiber length reduction (and fines generation) was more severe for the *Com* sample. A high acetylation degree was

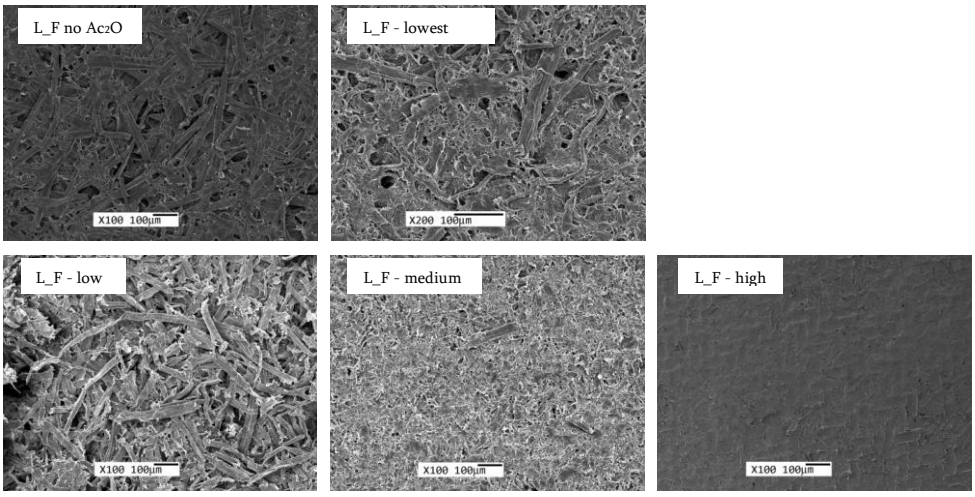
achieved in *Com* by using medium level conditions (5.35g Ac_2O). The greatest reduction in fiber length (87%) and amount of fines generated (93%) took place when high Ac_2O levels were used (10.7 g). Importantly, the acetyl groups incorporated on the cellulose surface are associated with an increase of mass (coarseness results) and fiber width. *L_F* and *L_CCE_F* also suffered a reduction in fiber length with increasing acetylation degree. In particular, at the highest acetylation level (23.6% of acetyl group) of *L_F*, a mass gain (coarseness) of about 158% was observed compared to the initial value. Meanwhile, equivalent values for *L_CCE_F* at the highest acetylation level (15.2% of acetyl groups) were measured (178%, Table 9-3).

Table 9-3 Fiber morphology according to TAPPI standard for the respective acetylated pulps (L_F, L_CCE_F and Com) at different acetylated levels.

Sample and acetylation conditions (Table 9-1)	L (mm)	Fines (%)	Coarseness (mg/m)	Fiber curl (%)	Fiber width (μm)
L_F non-acetylated	0.68 ± 0.02	57.5 ± 0.7	0.19 ± 0.01	12.3 ± 0.2	27.5 ± 0.2
L_F lowest	0.34 ± 0.0	71.9 ± 0.3	0.37 ± 0.02	9.9 ± 0.5	30.4 ± 0.4
L_F low	0.37 ± 0.0	69.3 ± 0.1	0.31 ± 0.0	9.7 ± 0.1	28.4 ± 0.4
L_F medium	0.23 ± 0.01	82.1 ± 0.4	0.49 ± 0.0	7.1 ± 0.0	31.1 ± 0.04
L_F high	0.22 ± 0.01	82.2 ± 0.2	0.50 ± 0.0	6.1 ± 0.1	31.8 ± 0.3
L_CCE_F non-acetylated	0.62 ± 0.01	58.2 ± 0.1	0.19 ± 0.01	12.9 ± 0.4	27.3 ± 0.04
L_CCE_F low	0.27 ± 0.01	79.2 ± 0.5	0.34 ± 0.02	8.8 ± 0.1	27.9 ± 0.2
L_CCE_F medium	0.26 ± 0.01	80.8 ± 0.2	0.31 ± 0.0	8.1 ± 0.1	27.9 ± 0.1
L_CCE_F high	0.20 ± 0.0	84.5 ± 0.5	0.53 ± 0.01	5.9 ± 0.1	31.7 ± 0.03
Com non-acetylated	1.33 ± 0.02	43.0 ± 0.7	0.19 ± 0.0	20.2 ± 0.1	24.0 ± 0.1
Com lowest	1.25 ± 0.01	45.0 ± 0.5	0.19 ± 0.0	17.5 ± 0.1	22.8 ± 0.1
Com low	1.19 ± 0.03	46.4 ± 0.2	0.16 ± 0.0	15.85 ± 0.1	23.5 ± 0.1
Com medium	0.30 ± 0.0	65.0 ± 0.6	0.26 ± 0.0	8.4 ± 0.1	22.2 ± 0.2
Com high	0.18 ± 0.01	92.5 ± 0.1	0.92 ± 0.04	6.9 ± 0.2	29.2 ± 0.2

Surface changes in handsheets of acetylated fibers. The change in the surface morphology of the acetylated fibers was evaluated by scanning electron microscopy (SEM). Since acetylation mainly involves the substitution of hydroxyl groups by acetyl units, an increase in paper porosity was expected due

to a decrease in bonding area. However, such effect was not evident. A clear fiber degradation due to acetylation reactions was confirmed by fiber morphology and also by SEM analyses. As can be seen for all samples treated at the medium Ac_2O level, fiber length was reduced; in addition, the greater amount of fines produced yielded a more entangled structure, with smaller pore size. The greatest changes were observed for fibers subjected to more severe acetylation conditions. In this case, in fact, whole fibers were not observed at the given SEM magnification and the pattern of the mesh used for web preparation was observed (Figure 9-6).



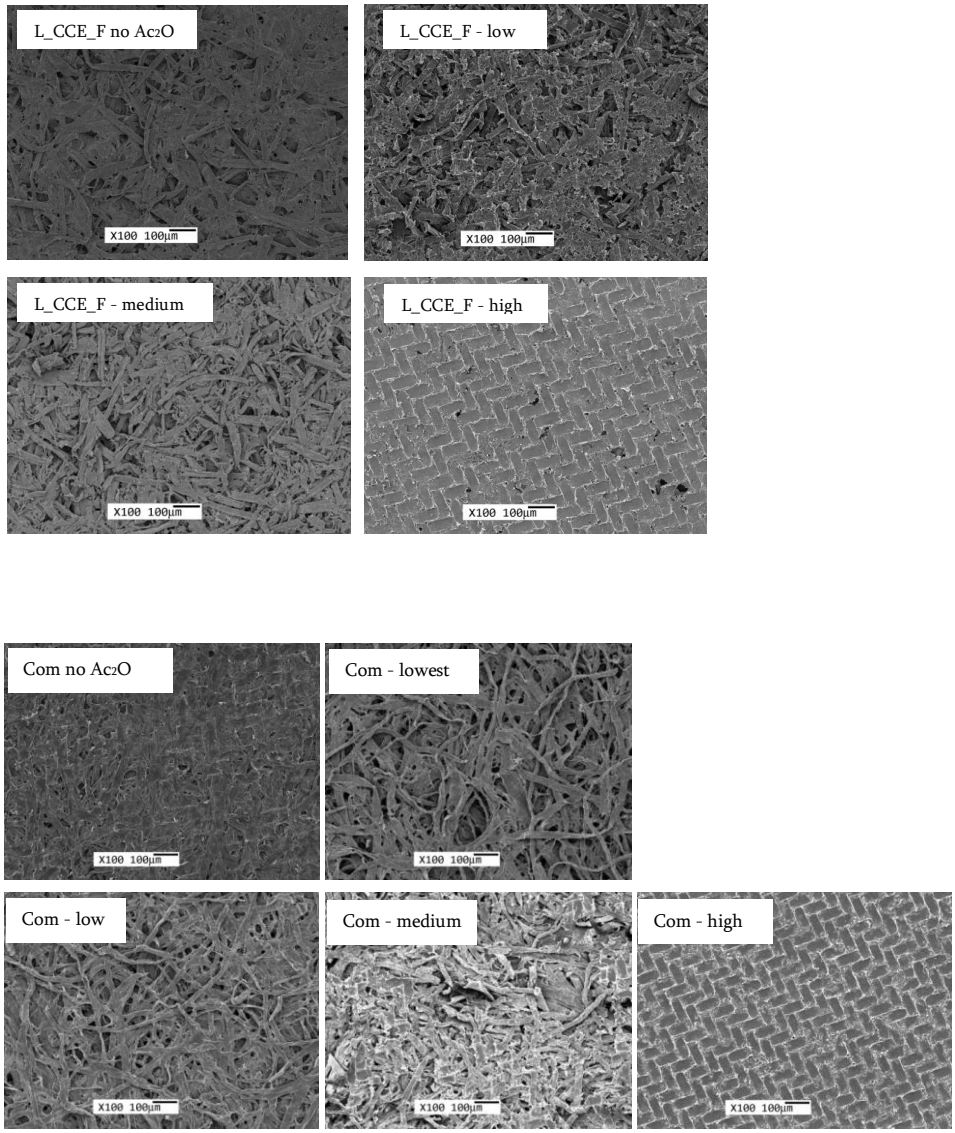


Figure 9-6 SEM images at different acetylated levels for biobleached pulp followed by an endoglucanase treatment (*L_F*), biobleached pulp submitted to cold caustic extraction and then an endoglucanase treatment (*L_CCE_F*) and commercial dissolving pulp (*Com*) as a reference

Mechanical properties of the fibers webs. The effect of fiber morphology and acetylation degree was assessed as far as the mechanical properties of the corresponding handsheets. The increased acetylation level of *Com* fibers resulted in higher dry tensile strength, with important strength improvement from lowest (0.53g) to low (2.67g) Ac_2O reactant levels. This was somewhat

surprising since a high acetylation level is expected to limit hydrogen bonding capacity since acetyl groups substitute –OH groups available in the cellulose network. Therefore, the strength enhancement may be explained by the generation of fines and, as a result, additional –OH groups were available for bonding. On the other hand, using fibers obtained at high Ac_2O reaction levels did not result in higher acetyl groups. A negative effect in tensile strength was noted. In fact, fibers acetylated at extreme (low and high dosages) of Ac_2O yielded similar acetyl content (~7.9%) but different strength values. *Com* subjected to high Ac_2O reaction level suffered a strength loss of about 93% with respect to the sample with fibers at low Ac_2O level. These results can be attributed to changes in fiber morphology: samples obtained from "high" acetylation conditions presented considerably higher fines content and much shorter fibers, giving weaker bonding. Therefore, a compromise between fiber strength and free hydroxyl groups determines the strength of the handsheets. An opposite trend in strength was observed for handsheets made with the acetylated *L_F* and *L_CCE_F* samples. As the content of acetyl groups increased the tensile strength decreased, with the exception of the samples obtained at the "high" Ac_2O level, which displayed a slight strength improvement. The gain in strength can be related to the higher fines content. In addition, the strength loss of *L_F* and *L_CCE_F* samples observed as the acetylation degree increased was in agreement with a diminishing bulk density. For the highest *L_CCE_F* Ac_2O level a denser and more compact structure was observed (Table 9-4). Significantly, *L_F* sample with the highest acetylation degree exhibited same strength as the non-acetylated *L_F* sample.

Table 9-4 Bulk density of respective acetylated samples

Sample and acetylation conditions (Table 9-1)	Bulk density (g/cm^3)
L_F non-acetylated	0.565
L_F lowest	0.426
L_F low	0.380
L_F medium	0.458
L_F high	0.542
L_CCE_F non-acetylated	0.497
L_CCE_F low	0.308
L_CCE_F medium	0.355
L_CCE_F high	0.606
Com non-acetylated	0.398
Com lowest	0.271
Com low	0.277
Com medium	-
Com high	0.847

The wet strength of fibers carrying the highest amount of acetyl groups produced slightly higher wet strength than that of fibers of lower degrees of acetylation. The wet-to-dry (W/D) strength ratio provides information about the wet-strength development. It is generally agreed that a web with a W/D > 15% should be considered as having wet-strength (Scott, 1996). Only *L_CCE_F* and *Com* fibers (high Ac_2O level) developed such feature upon acetylation. Specifically, *L_CCE_F* and *Com* fibers with 15.2 and 7.9 % acetyl group content produced webs with a W/D strength ratio of 22 and 48%, respectively.

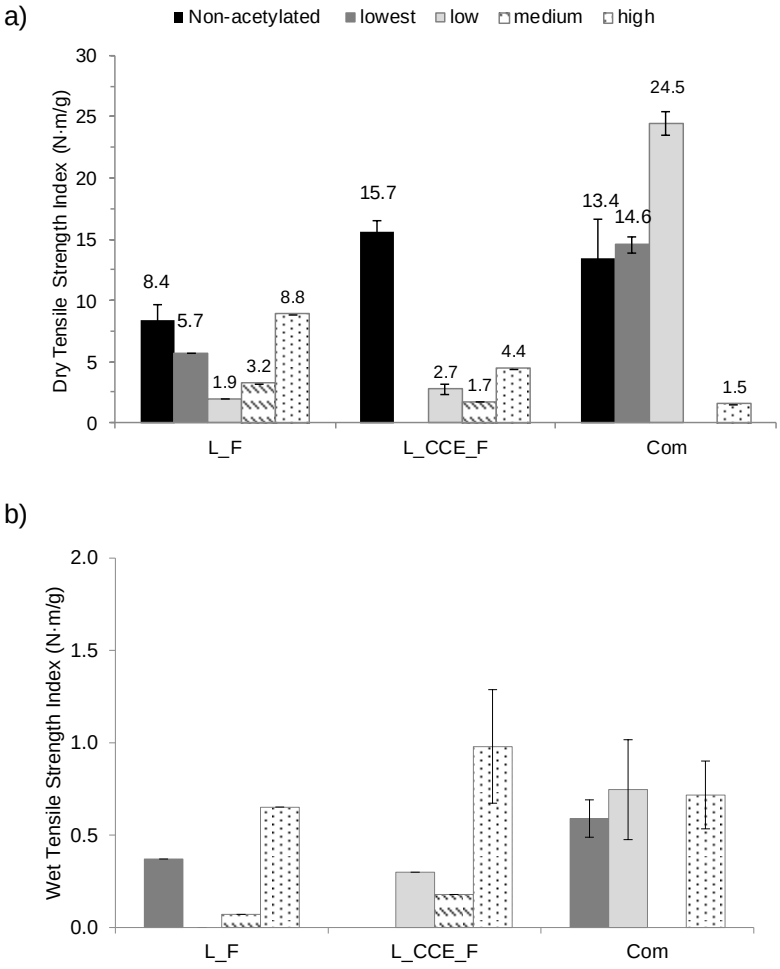


Figure 9-7 Wet and dry Tensile strength of respective pulps at different acetylated levels

Wetting properties of acetylated fibers. The effect of acetylation treatment on the hydrophilicity of the fibers was examined by means of initial water contact angle (WCA) of the respective handsheets (Figure 9-8). In general, the water absorption of paper depends on the porous structure of the sheet and the nature of the interactions that occur between fibers and the fluid (Mashkour et al., 2015). Acetyl groups were expected to reduce the hydrophilicity of fibers and lower the interfiber bonding. The different WCA observed between non-acetylated and acetylated samples confirm the effect of chemical surface modification. Samples with the highest degree of acetylation presented twice the WCA value compared to non-acetylated ones. To be precise, a WCA of 58 and

52° were obtained for acetylated *L_F* and *L_CCE_F* samples, confirming that the acetylation reactions reduced the hydrophilicity of fibers. Interestingly, *Com* sample showed different pattern: using high dose of Ac_2O did not provide the maximum acetyl content, although it gave the highest WCA (64°). In fact, using medium dose of Ac_2O resulted in 12% of acetyl group (largest amount) but showed same WCA as non-acetylated Com sample.

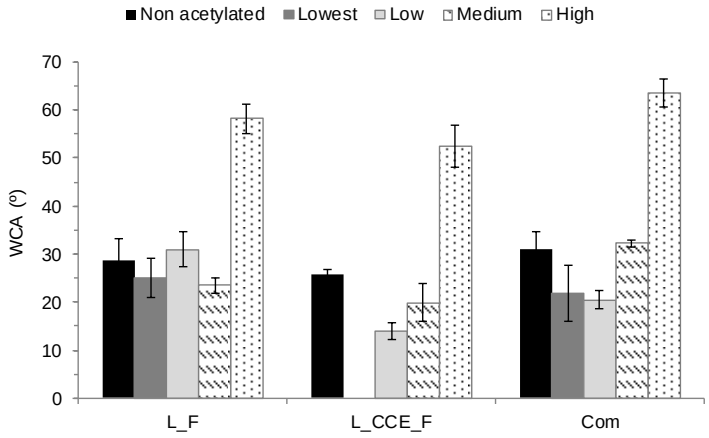


Figure 9-8 Initial water contact angle as a function of acetylation conditions for handsheets produced with *Com*, *L_F* and *L_CCE_F* fibers, as indicated.

Changes in WCA are mainly due to absorption in the sheet structure and to evaporation—the latter, however, is only relevant for relative long absorption times (Cusola et al., 2013). As shown in Figure 9-9a, water drops were absorbed rapidly for non-acetylated samples, giving an equilibrium WCA close to 0°. The greatest acetylated L_F and L_CCE_F samples also showed fast drop absorption, 2.4 and 56 s respectively (Figure 9-9b). In contrast, Com acetylated sample indicated no change in water drop during 2 min and a WCA of 45° was recorded after 20 min. Finally, after about 36 min the water drop was fully absorbed.

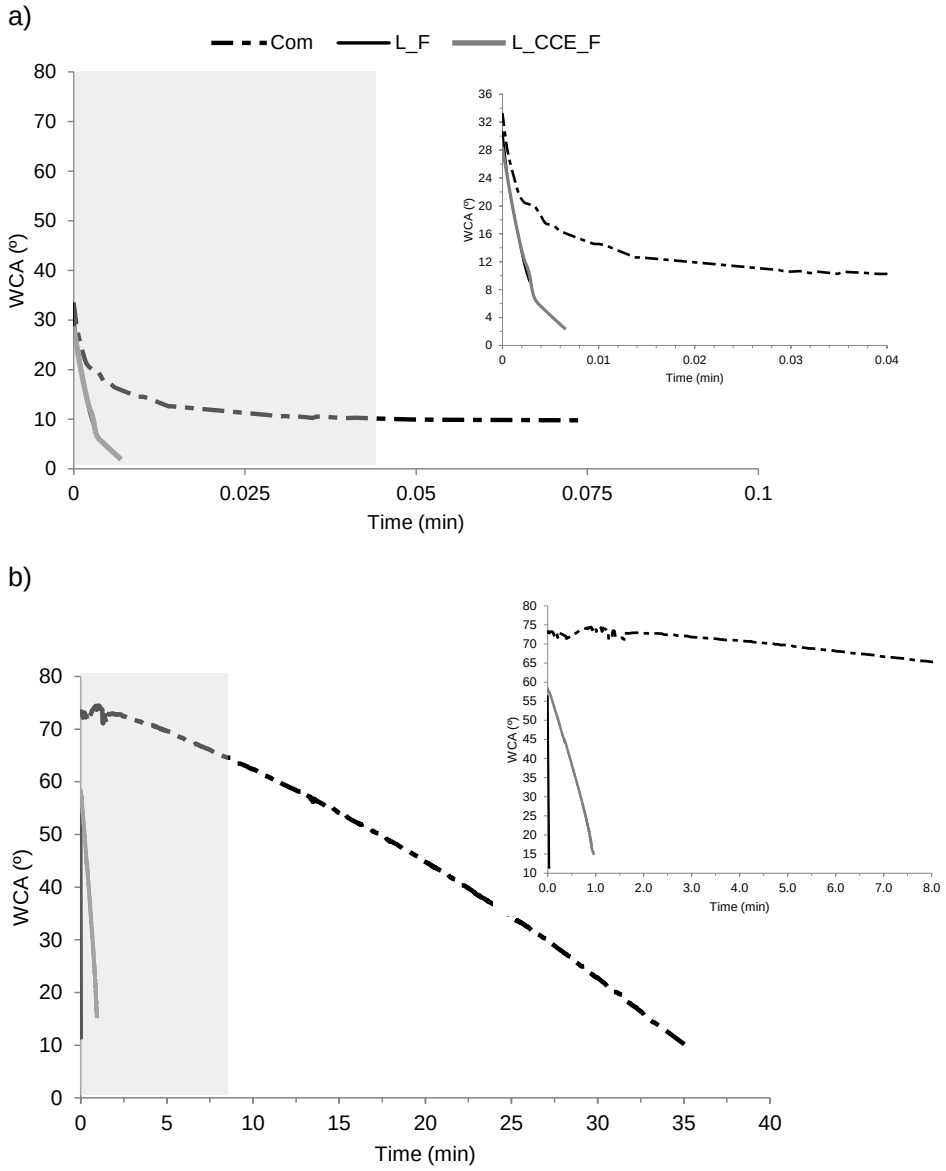


Figure 9-9 Temporal variation of contact angle as a function of water absorption for the respective non-acetylated (a) and the highest acetylated (b) *Com*, *L_F* and *L_CCE_F* samples. Inset graphs show in more detail the absorption behavior of different samples

Homogenous acetylation phase. Homogeneous acetylation was conducted in order to evaluate the dissolution behavior of fibers treated chemo-enzymatically (*L_F* and *L_CCE_F*) and fibers further modified by TEMPO-mediated oxidation (termed herein as *L_CCE_F+Lac-T).* The results were compared to *Com*

reference fibers. In the absence of toluene in the acetylation medium (homogeneous acetylation), a higher percentage of substituted acetyl groups are determined relative to the results from heterogeneous acetylation. FTIR spectroscopy confirmed that acetylation reactions were substantial, as indicated by the fingerprint peak at 1730 cm^{-1} (Figure 9-10). Importantly, the low intensity of absorbance peaks in the mentioned regions indicates that *L_CCE_F + Lac-T* fibers have a low degree of acetylation. One explanation for the clearly poor acetylation could be low presence of free available hydroxyl groups, due to the previous oxidation by Lac-T treatment to aldehyde or carboxylic groups. Quantification of the degree of substitution (DS) by titration showed similar acetylation degrees for all studied fibers. Values between 33 to 36% of acetyl substituted groups were found (Table 9-5), indicating a high level of acetylation compared to available commercial cellulose acetate (from Sigma-Aldrich ~39%). The amount of acetyl groups is in agreement with the peak intensity in the characteristic regions showed by chemoenzymatic and Com samples, with excpetion of *L_CCE_F+Lac-T* fibers. From high to low acetylation achievement: L_CCE_F > L_F > Com and *L_CCE_F+Lac-T*. It is suggested that the presence of aldehyde and carboxylic groups generated during TEMPO-mediated oxidation may mislead the amount of acetyl groups determined by titration.

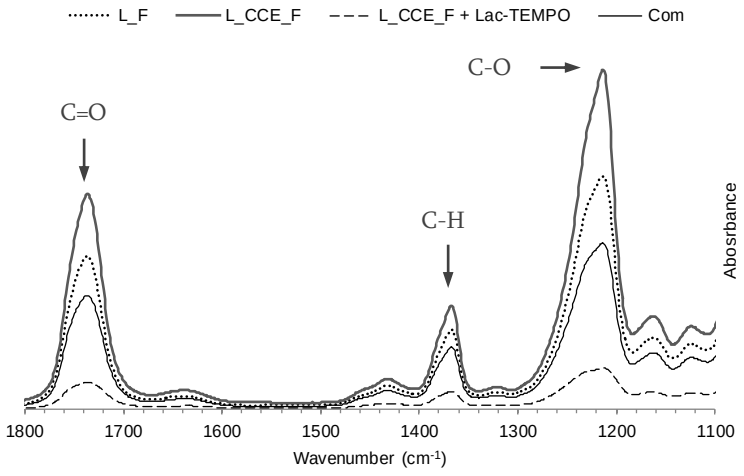


Figure 9-10 FTIR spectroscopy for cellulose acetate from *L_F*, *L_CCE_F*, *L_CCE_F+Lac-T* and *Com* samples

Fibers obtained after acetylation were freeze-dried, then dissolved in acetone and the resulting viscous solution was used to prepare films via solvent casting. Films made from the chemo-enzymatic samples presented notably greater strength values than those of heterogeneous acetylation reaction at the highest acetylation level. However, despite the fact that similar acetyl content was measured for all samples, *Com* films presented a tensile strength three times higher than those measured for the samples acetylated after chemo-enzymatic treatment. In terms of dry zero-span tensile strength, Com and chemoenzymatic acetylated fibers showed values in the same range, but *L_CCE_F+Lac-T* significantly lower. This result can be related to the severe oxidation and degradation caused by TEMPO-mediated reaction. As observed with heterogeneous acetylation reactions, the presence of acetyl groups reduced the hydrophilic character, giving a contact angle between 66 and 76°. Although high hydrophobicity was not achieved (contact angle < 90°), water drops remained long time on the surface until complete absorption for all acetylated samples. Overall, cellulose acetate fibers with new functional groups and high strength-related properties were achieved, highlighting the poor efficiency for *L_CCE_F+Lac-T* fibers.

Table 9-5 Acetyl groups determined by titration method , dry tensile strength Index, dry zero-span tensile strength, WDT and contact angle after subjecting *L_F, L_CCE_F, L_CCE_F+Lac-T* and Com samples to homogenous acetylation reaction

	Acetyl Groups (%)	Dry Tensile Strength Index (N·m/g)	Dry zero-span tensile strength (kN/cm)	Water drop test (s)	Contact Angle (°)
L_F	36.2 ± 4.9	19 ± 3	0.06 ± 0.01	5810 ± 117	76 ± 3
L_CCE_F	35.5 ± 3.9	22 ± 11	0.07 ± 0.009	5435 ± 293	67 ± 4
L_CCE_F + Lac-T	42 ± 1	-	0.011 ± 0.005	7634 ± 1804	66 ± 5
Com	33.3 ± 4.4	67 ± 28	0.05 ± 0.006	5445 ± 507	67 ± 7

9.4 Conclusions

Various surface acetylation conditions were studied from dissolving fiber grade (*Com*) and from a set of newly introduced fibers obtained by chemo-enzymatic treatment of biobleached sulfite fibers (*L_F*, *L_CCE_F*). The respective fibers presented different hemicellulose content and fiber

morphology. As a result, upon given heterogeneous reaction conditions, different acetylation degrees were achieved. FTIR and acetyl content titrations confirmed the fact that much higher acetyl group content was developed for the more severe acetylation conditions. Morphological studies revealed that acetyl groups were introduced via heterogeneous reactions on the surface of the fibers, as indicated by the gain in coarseness that was observed. In addition, the fiber length decreased with the acetylation degree and a larger amount of fines were produced. Fiber degradation was observed upon acetylation reactions, which increased the amount of hydroxyl groups available on the surface of the fibers. Handsheets from fibers with the highest acetylation level exhibited similar dry tensile strength than non-acetylated ones, with the exception of *Com* fibers that suffered a remarkable loss in strength. Importantly, these acetylated fibers offered wet tensile strength and lower hydrophilicity compared to the non-acetylated grades. Compared to the heterogeneous acetylation, homogeneous reactions led to higher acetyl group degree of substitution. These samples exhibited good solubility in acetone and produced transparent films (solvent casting) with enhanced dry strength. In conclusion, the results of this study indicate that it is possible to improve water resistance by enzymatic treatment of fibers, and yield a fiber quality that is similar to that of commercial dissolving-grade fibers.

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Chapter 10

General discussion

The present chapter presents a general summary of the results and findings obtained in this thesis.

The manufacture of dissolving pulp has increased markedly in recent years principally due to a higher demand for textile fibers in Asian countries. A growth of cotton production is difficult to achieve because of the large land area required for farming and the amount of water required for irrigation, which results in higher economic costs. Therefore, the problem of "cotton gap" connected with the interest to shift from petroleum-based products to bio-renewable carbon feedstock, makes dissolving-grade pulp a topic of great interest. Then, the framework of the present thesis is justified by the continuous demand of dissolving pulp that market studies are forecasting for the following years. Currently, most of the studies are focused on the conversion of paper-grade pulps to dissolving-grade pulps. There are a huge number of publications that put forward new technologies such as organosolv pulping or the removal of hemicelluloses by alkaline, Nitren and Cuen extractions. Enzymatic treatments or new solvents such as ionic liquids (ILs) are also suggested as a replacement of the conventional processes. Based on this scenario, the work developed in this thesis also considers the introduction of new technologies (i.e. based on enzymatic procedures) in order to purify softwood sulfite pulps and achieve pulps with dissolving quality. The process starts from the bleaching stage, continues with the purification stages and ends with the obtainment of a cellulose acetate derivative that supports the value of the new process.

10.1 Biobleaching treatments (Chapter 4)

Biobleaching interest

Based on the interest that dissolving pulps have been gained, the first part of the thesis draws the attention to bleaching treatments. The introduction of new technologies to bleach dissolving pulps using an enzymatic-aided process and complemented with treatments based on oxygen-derived compound can be an alternative to traditional bleaching processes, such as elemental chlorine free (ECF) and totally chlorine free (TCF). Dissolving pulp has traditionally been obtained by the alkaline pre-hydrolysis kraft (PHK) process or acid sulfite process. To our knowledge, the laccase–mediator system (LMS) has been widely applied in alkaline pulps (Bourbonnais et al., 1997; Chakar and Ragauskas, 2004; Andreu and Vidal, 2011; Aracri and Vidal, 2011; Valls et al., 2013) but has not attracted widespread attention on sulfite pulps. Therefore, a biobleaching sequence using a laccase-mediator system (LMS) in combination with a chemical bleaching stage was proposed for efficient bleaching of softwood sulfite cellulose.

Selecting the most suitable mediator

Initially, preliminary tests were conducted in order to assay the bleaching potential of different mediators. Two synthetic compounds, **violuric acid (VA)** and **1-hydroxybenzotriazole (HBT)**; and two natural compounds, ***p*-coumaric acid (*p*CA)** and **syringaldehyde (SA)** were used and their bleaching responses assessed in terms of pulp and effluent properties. After the enzymatic treatment, pulps were submitted to a hydrogen peroxide treatment (P) in order to further improve pulp quality. In short, a LP sequence was performed. Furthermore, a comparative study between chemoenzymatic sequence with respect to control sequence (no presence of enzyme) and conventional sequence (a hydrogen peroxide stage alone) was conducted in order to highlight the action of the enzyme alone.

In terms of delignification, after L stage all mediators reduced the lignin content but to different extent. The greatest reduction was obtained by synthetic mediators. Specifically, VA and HBT provided a 74 and 26% of delignification with respect to initial pulp; while SA and pCA only accounted for 16 and 9%. Laccase alone resulted in 4% of delignification indicating that enzyme was

unable to modify cellulose composition. After hydrogen peroxide stage (P), all properties were bettered, i.e. lignin content was further reduced and brightness was successfully improved. LVAP sample exhibited 77.75% ISO brightness and kappa number <0.5, far from the results exhibited for other mediators. Significantly, conventional sequence (P) by its side only provided 67.35% ISO brightness and kappa number of 3.5. These results led to the conclusion that **L stage boosted the delignification** effect and **improved final brightness level** (Figure 10-1).

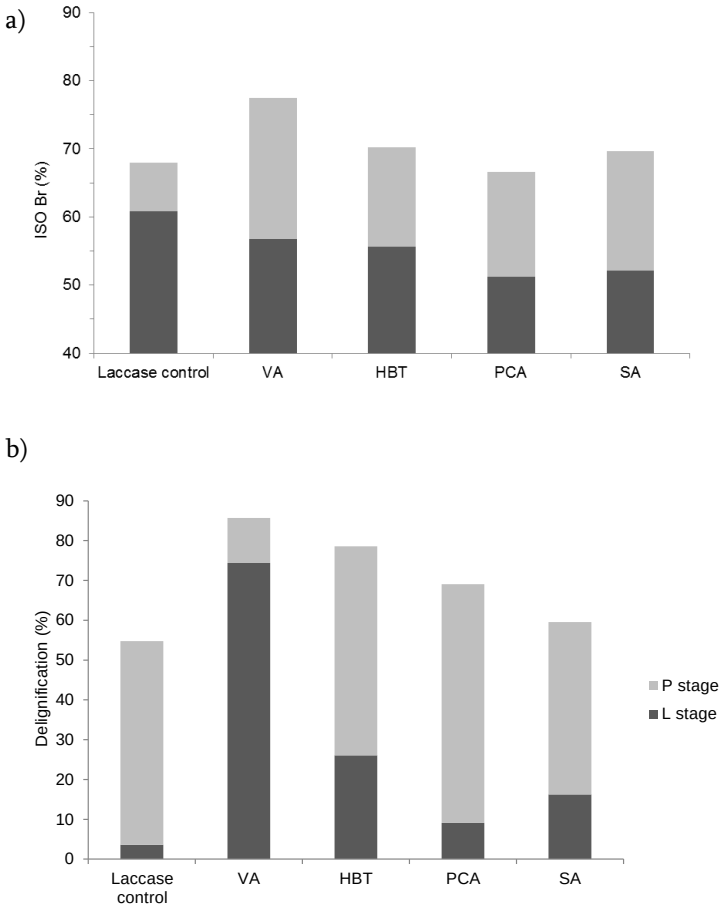

Figure 10-1 (a) ISO brightness and (b) delignification after L and P stage for each mediator

The lignin removal observed after L stage was accompanied by a brightness loss as a consequence of the formation of chromophores groups due to the oxidative action of LMS. Treated pulps exhibited higher color values (CIE *L*a*b**,

C^*) than did the laccase control treatment (no presence of mediator). Color changes were mainly characterized by an increase in yellowness (b^*) and in redness (a^*). A gain in chroma (C^*) value was also observed and justified by the formation of quinones. From all mediators, VA was the one which suffered the lowest brightness reduction (6.8%), which corresponded to a 56.7% ISO brightness. Some authors postulated that natural mediators in combination with laccase were found to increase kappa number, suggesting the occurrence of cross-linking or cross-coupling reactions of these natural mediators to fibers. Other works described that hexenuronic acids (HexA) could act as a coupling partner of the mediator. However, since no kappa increase was found and no hexenuronic acids were present in the starting pulp, no radical coupling reactions were considered to explain the brightness loss. Importantly, the subsequent P stage could overcome the negative effect brought by L stage on optical properties. C^* and L^* coordinates were decreased and increased, respectively reaching similar values as control treatment and conventional sequence.

As other authors observed, no significant degradation on carbohydrate chains was perceived after L stage. Besides, cellulose loss was more pronounce after the P stage for all mediators, except for *p*CA. Importantly, obtaining quality rayon requires values in the 200-300 mL/g range. However, a higher viscosity than that required for the xhantation step is desirable in order to have better control of the process and hence to the final product. In particular, LVAP sample only suffered a 3.5% viscosity loss, giving a value of 493 mL/g.

Therefore, **the introduction of an L stage before a chemical stage was absolutely justified** since better brightness values and higher delignification percentage were obtained with respect to control and conventional sequences, without affecting viscosity results. From all studied mediators, **VA** proved to be the **most efficient** for biobleaching sulfite pulp, as it led to the smallest kappa number and highest brightness without an adverse effect on cellulose integrity.

Fiber surface changes of preliminary bleaching tests: TGA

The differences in bleaching properties between natural and synthetic mediators can be explained by particular changes on fiber surface. Thermogravimetric analysis (TGA) is sensitive to chemical changes in fiber

surface because such changes can alter the thermal degradation path of pulp. Consequently, if a bleaching process results in enzyme adsorption or surface oxidation, then the hydrogen bond network which protected the surface of crystalline cellulose will be partially broken. Cellulose is organized as long bundles of cellulose polymer chains called microfibrils, which include crystalline and amorphous zones (Tsuji et al., 1992). In the crystalline cellulose region, cellulose chains form planes where equatorial hydroxyl groups in pyranose rings are hydrogen-bonded. Clean and crystalline cellulose are thermally degraded at high temperatures spanning a narrow range because ordered cellulose chains yield crystallites that are protected from external attack by a surface hydrogen bond network (Barneto et al., 2011a).The amorphous regions, which consist of non-ordered cellulose chains, are located mainly between cellulose crystallites (Nishiyama et al., 2003). Initial pulp exhibited a sharp peak around 300–350 ℃ indicating a high content in crystalline cellulose. Fibers resulted from control treatments, which included (KL) or excluded (K) laccase, appeared to be more "crystalline" since the mass loss rate was higher at high temperatures. In other words, buffer solution seemed to produce a cleaning effect on fiber surfaces. This fact contrasts with results found by other authors were laccase or buffer solution were adsorbed onto kenaf or eucalyptus fibers and increased the amount of "paracrystalline" cellulose. The amount of cellulose degrading at a low temperature increased in the following order: p-coumaric acid < violuric acid < syringaldehyde < hidroxybenzotriazole. Specifically, L_{HBT} exhibited the biggest difference with respect to initial pulp, while others L-mediator treated samples presented similar pattern as the initial sample. It is known, that laccase–HBT system partially oxidizes the outer cellulose layer, changing hydroxyl groups to carbonyl groups, disordering the crystalline surface and increasing the paracrystalline cellulose content as a result. In addition, the reactivity and stability of N–O· radicals are seemingly better balanced than in phenoxy radicals, which may account for the better performance of HBT or VA radicals as mediators for laccase-based delignification. Lastly, condensation and grafting reactions for the pulp samples treated with natural mediators were excluded since DTG curves were similar to initial pulp and no kappa increase was detected.

Environmental impact of LMS treatment

The environmental impact of effluents resulted from the LMS treatment was evaluated in terms of **toxicity**, **chemical oxygen demand (COD)** and **laccase inhibition**. According to the method used for quantifying LMS toxicity (microbe *Vibrio fischeri*), showed violuric acid (VA) as the least toxic compound. Mediator solution in its original form (Aracri et al. 2011) and control treatment (no presence of mediator) showed low values of toxicity. Therefore, these results demonstrated that toxicity may result from the generation of intermediate and degraded products of oxidized mediators. The increased COD values obtained with respect to the control can be ascribed to the species dissolved during the treatment (lignin and carbohydrates) or due to oxidized form of the mediator. Residual laccase activity was measured from L-effluents and all results revealed an important laccase inhibition (> 90%) suggesting that laccase treatment worked satisfactorily.

10.2 Extended biobleaching sequence (Chapter 4)

Based on the preliminary biobleaching results, an extended biobleaching sequence was explored in order to more accurately assess the performance of the Lac-VA system and the influence of a hydrogen peroxide stage. The novelty, with respect to preliminary tests, was to introduce a **chelating stage (Q)** and a **pressurized hydrogen peroxide treatment (PO)**. So, a biobleaching sequence designated **LQPO** was conducted. The results were compared with those for a control sequence without presence of laccase and mediator (**KQPO**) and also with those for a conventional sequence (**PO**).

Delignification and brightness properties

The enzymatic sequence (LQPO) caused 56% delignification after the L stage; however, the buffering stage of the control sequence (KQPO) also afforded 13% delignification with respect to the initial pulp. Therefore, the actual delignification contributed by the laccase–mediator stage in the LQPO sequence was about 43%. The delignifying effect of Po_1 step with respect to the Q stage in the LQPO sequence was 79%. This important decrease was directly due to the laccase–VA system since the control sequence (KQPO) only provided 56% delignification after the same PO_1 step and the conventional hydrogen peroxide process (PO) caused 61% at PO_1 relative to initial pulp. The noticeable delignification undergone by conventional sequence was not enough to reach

the same level as LQPO$_1$ sequence, and the contribution of the next multiple Po steps failed to achieve a kappa number lower than 0.5.

As expected, the observed delignification was accompanied by a brightness increase for all sequences. Important to remark that despite the drop in brightness observed after the enzymatic stage (L), the enzymatic sequence was more efficient in raising pulp brightness at the end of the whole sequence than was the control sequence (KQPO) or even the conventional chemical bleaching sequence (PO). Enzymatic sequence led to 89% ISO brightness, whereas control and conventional sequence only provided 84% ISO brightness (Figure 10-2).

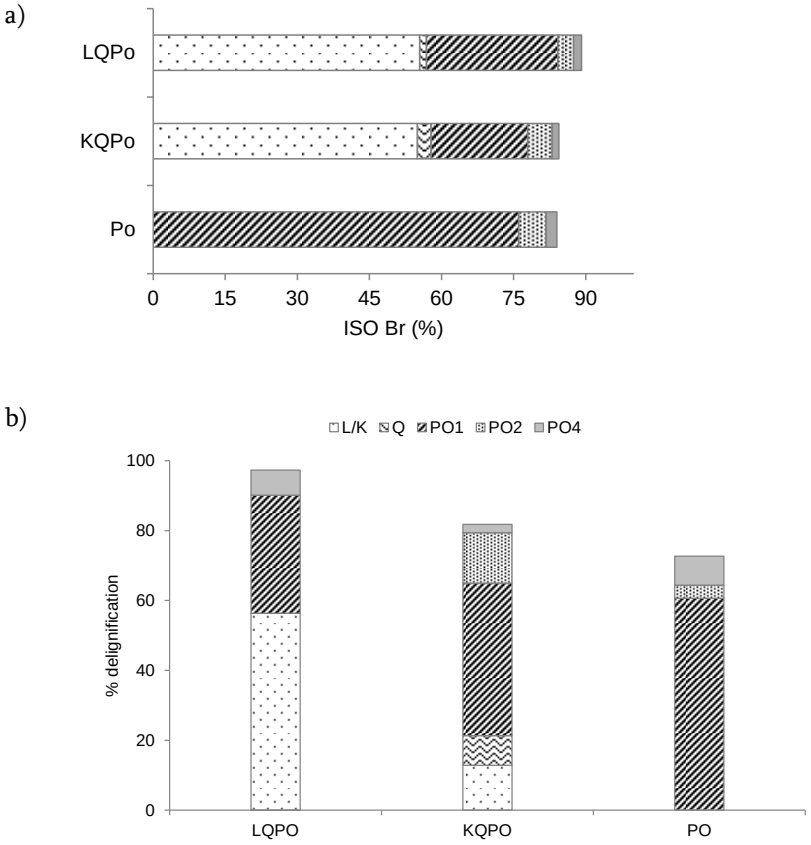

Figure 10-2 (a) ISO Brightness and (b) delignification of each stage for the respective sequences

Could the effect of H_2O_2 in brightness property be boosted?

The pressurized hydrogen peroxide stage was conducted in three consecutive steps (PO$_1$ = 1 h reaction, PO$_2$ = 1 h reaction, PO$_4$ = 2 h reaction) each involving

the addition of 10% odp H_2O_2 and no interstep washing. With the purpose to boost the effective bleaching capability of hydrogen peroxide, the extension of hydrogen peroxide at atmospheric pressure with no washing between steps was examined. Pressure was released and the temperature was maintained at 90 °C for 2 additional hours without addition of H_2O_2. Results revealed that residual hydrogen peroxide did not influence the extent of fiber bleaching, since ISO brightness results were not further improved with respect to the previous pressurized hydrogen peroxide stage. All studied sequences showed same behavior, therefore the bleaching sequence was limited to 3 additions of 10% H_2O_2 and 4h of reaction.

Could H_2O_2 be saved with a previous LMS stage?

As mentioned earlier, $LQPO_1$ treatment provided 84% ISO brightness with a single addition of H_2O_2 and only 1 h of reaction; by contrast $KQPO_1$ treatment and Po_1 conventional treatment required three additions of H_2O_2 and 4 h of reaction to reach the same brightness level. In other words, **L_{VA}QPO sequence saved 2h of reaction and about 70% H_2O_2 to meet the same brightness value (~84% ISO) as control and conventional sequences**. These results confirmed that the laccase–VA system enhanced the bleaching effect of hydrogen peroxide on sulfite cellulose, leading to obtain a brightness level of 89% ISO at the end of the full sequence. The bleaching effectivity (i.e. relationship between hydrogen peroxide consumption and pulp brightness) also assured the boosted effect of L_{VA} stage since $LQPO_1$ treatment exhibited 32% of effectivity versus 26% for PO_1 treatment of conventional sequence.

ISO brightness results were closely related to hydrogen peroxide consumption. Therefore, monitoring the actual consumption of hydrogen peroxide at each addition point let to optimize the operating conditions. The enzymatic sequence (LQPO) consumed 86%, 76% and 86% of H_2O_2 in PO_1, PO_2 and Po_4 steps, whereas conventional sequence (PO) consumed 67%, 80% and 88% of H_2O_2, respectively. The difference in H_2O_2 consumption may have been the result of the dissimilar nature of the samples or structural factors. It was found that enzymatic-treated pulp presented less lignin moiety available when hydrogen peroxide treatment was applied in comparison to pulp submitted to conventional sequence. However, (Moldes et al., 2008) previously reported that in the case of laccase treatment in combination with HBT or VA, the hydrogen

peroxide was consumed mainly to oxidize chromophoric groups. The conventional sequence (PO) included no enzymatic treatment, so no chromophores groups from laccase treatment were present; this accounts for the response of the pulp to the Po_1 step. Importantly, the evaluation of residual hydrogen peroxide in the bleaching effluents confirmed that an excess of H_2O_2 dose was employed and therefore this stage needs to be optimized for any industrial application.

Cellulose degradation

Cellulose integrity was evaluated in terms of viscosity determined in Cuen solution. With respect to initial viscosity value (552 ± 8 mL/g), the enzymatic sequence produced a 13% yield loss while the control sequence caused only 5% yield loss after L or K stages with respect to original pulp. Degradation was more pronounced with hydrogen peroxide treatment. Interestingly, after the Po_1 step (single addition of H_2O_2 and 1 h of treatment) the enzymatic sequence and the control sequence led to similar viscosity values ($LQPO_1$ = 413 ± 1 and $KQPO_1$ = 415 ± 1 mL/g, respectively), what means a 25% yield loss, but samples differed in final kappa number and ISO brightness values. Precisely, $LQPO_1$ exhibited an 84% ISO brightness and 0.53 KN, while $KQPO_1$ showed a 78% and 1.87, respectively. Therefore, giving a target brightness value of ~84% ISO, samples resulted in similar viscosity values but different operating conditions (reaction time and H_2O_2 dose) were required for each sequence.

Lastly, since enzymatic sequence obtained the highest brightness value (~90%), a lower viscosity value was found with respect to control and conventional sequences. Nevertheless, the final value can be considered acceptable for viscose manufacturing (rayon) and CMC derivatives rather than cellulose derivatives applications such as acetate and nitrate (Batalha et al., 2011).

Is brightness stability affected by LMS?

As mentioned earlier, an 89% ISO brightness value was obtained for $L_{VA}QPO$ sequence. However, the enzymatic stage (L_{VA}) generated chromophores and oxidizable structures since chroma value was increased with respect to starting pulp, but this modification was not observed for buffer stage (no presence of laccase neither mediator). Therefore, in order to assess the effect of an

enzymatic treatment on the stability of optical properties, an accelerated ageing treatment was conducted. Both samples were subjected to accelerated thermal ageing treatment by moist heating at 80 °C and 65% RH, for 144h. **The presence of chromophoric groups boosted brightness reversion since LVA treatment suffered a brightness loss of 10%, while buffer treatment only 0.3%, after accelerated ageing treatment**. Importantly, hydrogen peroxide treatment removed or altered those chromophoric groups since a similar brightness loss was observed for LVAQPO (-13%) and KQPO (-12%) sequences, although enzymatic sequence exhibited higher brightness value (78% ISO versus 72% ISO, respectively).

10.3 Dissolving pulp characterization (Chapter 5)

As discussed earlier, LVAQPO sequence was able to bleach softwood sulfite pulp obtaining excellent bleaching properties. Results suggested the potential of enzymatic bleaching as an alternative to traditional bleaching processes. Therefore, a fully study of dissolving pulp characteristics was needed in order to satisfy the market-like requirements. Having knowledge of hemicellulose content, cellulose reactivity and accessibility provided interesting information for likely dissolving pulp applications. Moreover, the differential behavior of surface and inner cellulose during thermal degradation make attractive the use of thermogravimetric analysis (TGA) in order to monitor changes of surface and inner cellulose during bleaching.

Effect of xylanase treatment

The application of a xylanase (X) stage as a previous step to the bleaching treatment (Chapter 4) did not contribute to reduce the xylan content and neither boosted the delignification effect in the following bleaching stages. Following reports from other authors, the dose of xylanase used was high enough to provide any significant variation on treated pulps. However, it is hypothesized that the residual xylan and manna (6.6% w/w) would be in the form of lignin-carbohydrate complexes and HC Pulpzyme xylanase was not effective to remove them. Based on these results, xylanase stage was excluded from the bleaching sequence.

Are hemicelluloses affected with the biobleaching sequence?

Carbohydrate composition was measured by high-performance liquid chromatography (HPLC). At the end of the biobleaching sequence (LVAQPO), **no reduction in hemicellulose content with respect to starting pulp was found**. This result was to be expected since, unlike cellulases or xylanases, laccase–mediator system acts on lignin moieties rather than on cellulose and hemicellulose fractions of fiber structure. However, the total hemicellulose content fell in the acceptable range for commercial dissolving pulp (<10%), giving a **glucan content of about ~93%**.

The extent of dissolved carbohydrate was determined by alkali solubility, which is a measure of cellulose degradation and also a loss or retention of hemicellulose during a bleaching process. In general, in 18% NaOH (S18) the hemicellulose is soluble, whereas in 10% NaOH (S10) low-molecular weight cellulose (degraded cellulose) and hemicelluloses are dissolved. With reference to starting pulp, biobleaching sequence increased S18, indicating that higher amount of hemicelluloses were soluble in 18% NaOH. Consistent with these results, alpha, beta and gamma celluloses were also analyzed. Consequently, enzymatic treated-samples exhibited higher λ-cellulose fraction and lower β-cellulose fraction than initial pulp. Since λ-cellulose corresponds to hemicelluloses and β-cellulose to degraded cellulose, the lower viscosity values observed for LVAQPo samples justified the above-stated results. Finally, as observed for glucan content (quantified by HPLC), α-cellulose fraction remained unchanged with same value as starting pulp.

Relevant dissolving pulp characteristics

Cellulose **reactivity** was quantified according to **Fock's method** that simulates, at a micro-scale, the industrial viscose process for manufacturing regenerated cellulose. The cellulose reactivity result represents the amount of pulp that is dissolved in the viscose-like solution. Consequently, the results are strongly influenced by pulp viscosity and by the amount of alkali-soluble hemicellulose present. Similar Fock solubility results were found for enzymatic sequence (LVAQPO), control sequence (KQPO) and conventional sequence (PO); but enzymatic sequence presented lower viscosity results in comparison to control and conventional sequences. As a relevant conclusion, the Fock

solubility value obtained by laccase treatment (67%) was comparable to those obtained by other authors for commercial dissolving pulp.

The content in **metal ions** of dissolving pulp is also very important because a high concentration can affect the processability of cellulose derivatives. Calcium, iron and manganese ions were analyzed, obtaining the highest concentration for iron, followed by calcium and no presence of manganese. Both sequences, enzymatically and control treated pulps exhibited same content suggesting that L stage does not alter metal composition. Moreover, the chelate stage (Q) did not provide a noteworthy effect on metal ions. In fact, the initial metal ion content was on average compared with commercial dissolving pulp. Therefore, the following studied biobleached sequences omitted the chelate stage, referred as $L_{VA}Po$.

In terms of **crystallinity**, X-ray results confirmed that an enzymatic bleaching treatment was essentially a surface process that causes virtually no change in fiber chemical composition or crystalline cellulose integrity. In other words, there was no alteration of the planes of anhydroglucose units inspected by the X-ray technique, so pulp crystallinity remained unaffected during the bleaching treatment. A crystallinity value of 64 ± 2% was found for initial and biobleached pulps.

Fiber surface changes of extended biobleaching sequence: TGA

The surface changes of pulps during the biobleaching treatment were assessed using thermogravimetric analysis. As mentioned earlier in Chapter 4, TGA is sensitive to chemical changes in fiber surfaces because such changes can alter the thermal degradation path of pulp.

A comparison of the initial and PO_4 (conventional sequence) DTG curves allowed to see that the hydrogen peroxide treatment damaged fiber surfaces considerably. In fact, PO stage caused a substantial decrease in the crystalline cellulose peak, which suggested alteration of fiber crystalline surfaces. In addition, PO_4 sample curve was above initial pulp in the region from 300 to 325 °C, indicating that the hydrogen peroxide treatment increased the amount of cellulose volatilizing at low temperatures (i.e. amorphous and paracrystalline cellulose). Consistent with the viscosity loss observed for PO_4 with respect to

initial sample, the alteration of PO_4 sample may have resulted from alkaline depolymerization by effect of β-elimination reactions breaking bonds between adjacent glucosyl units (Barneto et al., 2011b). In addition, low lignin and low hemicelluloses content proved by kappa number and carbohydrate composition (determined by HPLC) suggested that no deposits were removed with hydrogen peroxide treatment and therefore, DTG curves detected the actual cellulose surface degradation. Importantly, when pulps were previously treated with a laccase–mediator system or buffer solution, a less marked reduction of crystalline cellulose was detected after PO stage. **Therefore, the introduction of LMS was justified by the protective effect that gave on cellulose fibers when a posterior chemical stage was applied.**

10.4 Up-grading biobleached sulfite pulp to dissolving grade (Chapter 6)

As concluded in Chapter 4, the use of laccase-mediator system (LMS) proved to be effective to bleach never dried softwood sulfite pulp. The proposed $L_{VA}QPO_4$ sequence provided acceptable results in terms of dissolving pulp requirements and had a positive contribution from an environmentally point of view. However, in the direction of improving pulp reactivity and the quality of the final product (i.e. reduce the amount of hemicelluloses) further treatments were required. Therefore, two different **endoglucanases** were proposed as potential treatments to achieve cellulose activation. **Cold caustic extraction (CCE)** applied before the endoglucanase treatment was also studied in order to aid the conversion of biobleached sulfite pulp to dissolving-grade pulp. Just to put one in mind, one endoglucanase was obtained from *Paenibacillus barcinonensis* (B) and the other from *Cerrena unicolor* (F). The treatments conducted were named as follows: L_F12, L_B120, L_CCE, L_CCE_F12 and L_CCE_B120.

Optimizing biobleaching sequence

Firstly, referring to the bleaching treatment, an important modification was conducted in order to approach industrial conditions. Hence, the hydrogen peroxide stage was limited to only one step (1 h reaction), but still using 10% odp of reagent. As a result, a satisfactorily 88.45% ISO brightness was obtained with a similar viscosity value (395 ± 6) as the former studied biobleached

sequence (343 ± 13), where three multiple PO steps were performed. The brightness result correlates well with the residual hydrogen peroxide determined after each Po step, where it was concluded that hydrogen peroxide treatment required further optimization (Chapter 4). Moreover, the chelate stage (Q) did not provide a noteworthy effect on metal ions (Chapter 4). In fact, the initial metal ion content was on average compared with commercial dissolving pulp, and the slightly acidic pH used during the L stage was sufficient for metals removal. Therefore, Q stage was omitted and the biobleached sequence was referred from here on as LVAPO.

Removal of hemicelluloses

Biobleached pulp (LVAPO) presented an 11.7% (w/w) of hemicelluloses, with the following composition: 6.0% mannan, 3.0% galactan, 2.4% of xylan and 0.3% of rhamnan. **L_B endoglucanase** treatment diminished the hemicellulose content by 37% and the final amount of hemicelluloses was **7.4%** (w/w) (Figure 10-3). The highest reduction was observed with xylan fraction (77%), followed by galactan (47%) and finally mannan (19%), with respect to biobleached sulfite pulp. The amount of rhamnan was not modified with enzymatic treatment. **L_F endoglucanase** treatment decreased the amount of hemicelluloses by 39% which corresponded to a **7.1%** (w/w) of final hemicelluloses. The xylan fraction suffered a reduction of 53%, the galactan a 47% and the mannan a 30%. In both treatments, the xylan removal was superior to mannan, but the action caused on mannan fraction was higher by F than by B endoglucanase. As determined by Somogyi-Nelson method, F had also a xylanase activity, but this effect was not reflected on modifying pulp composition.

The **strong alkaline treatment (L_CCE)** contributed to reduce the total amount of hemicelluloses by 42% with respect to biobleached sulfite pulp, giving a final amount of **6.8%**. Significantly, hemicelluloses removal was further extended with the following respective cellulase treatments. The final amount of hemicelluloses for **L_CCE_B** and **L_CCE_F** was **6.2%** (w/w) and **5.8%** (w/w), respectively. Looking into carbohydrate composition (Figure 10-3), it was observed that L_CCE reduced the xylan content with respect to biobleached sample, and the use of a posterior cellulase treatment (L_CCE_B) helped to further minimize the xylan fraction. Interestingly, comparing L_B and L_CCE_B same xylan content was accomplished, indicating that CCE treatment did not

boost the xylan removal, although global hemicellulose content was different. From these results it is concluded that **a limit of chemoenzymatically accessible xylan removal was found,** even if combined treatments were used. In terms of F endoglucanase, L_F and L_CCE treatments presented same amount of xylan fraction, but **combining L_CCE_F produced a synergy effect and xylan fraction was further diminished.** Lastly, L_CCE_B and L_CCE_F pulps reached nearly the same xylan content. Enzymatic treatments alone almost halved the galactan content with respect to biobleached pulp, but results showed that galactan fraction was clearly susceptible to solubility under strong alkali solution (L_CCE) since much lower content than the ones exhibited by L_B and L_F samples was found. In fact, L_CCE_F showed the same galactan content as L_CCE sample. On the contrary observed by xylan and galactan fractions, mannan content was reduced to same value by presence of strong alkali solution or by cellulase action.

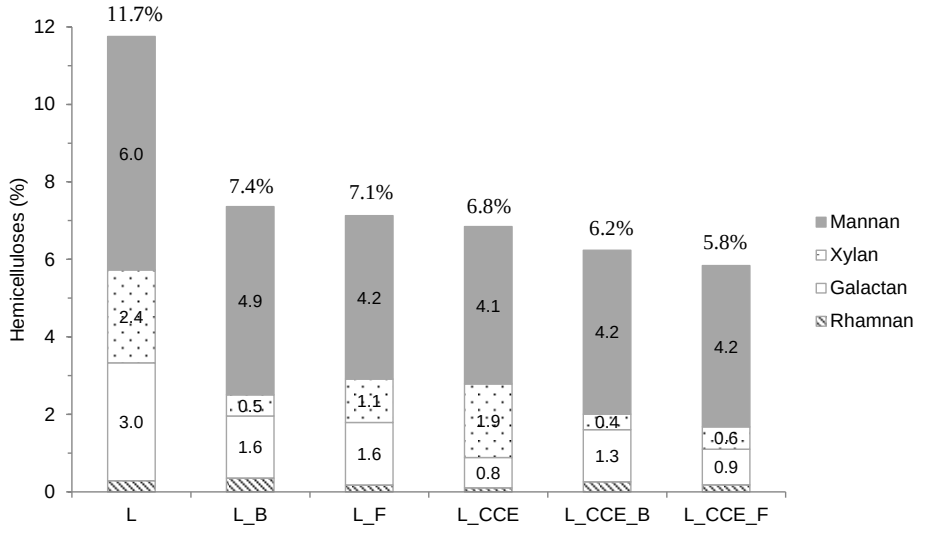

Figure 10-3 Evolution of hemicellulose composition along the treatments studied

Which information could be obtained from enzymatic reaction effluents?

The concentration and composition of dissolved sugars present in the liquors determined by HPLC provided knowledge about the mode of action of B and F endoglucanases, which we know from the literature that is specific for each

enzyme. Contrary to what was expected, a good correlation between pulp composition and effluent composition was difficult to establish. L_F treatment caused the greatest release of glucose, followed by L_B and then by L_K (Figure 10-4). These results are consistent with the number of chain scissions caused by each enzyme and with cellulose degradation, expressed as viscosity results. F enzyme contained xylanase activity and was capable of reducing hemicelluloses, but low concentration of xylose monomer was detected. It can be hypothesized that the xylanase activity released preferentially oligosaccharides of 2-3 xylose units rather than its monomer as some authors described (Beltramino et al., 2015). Glucose oligosaccharides in the effluents were also analyzed by HPLC. The pattern of products released by the L_F treatment decreased in the following sequence: cellobiose>glucose>cellotriose. The fact that oligosaccharides of different size and glucose monomer were found is consistent with the reported mode of action of F, which randomly cleaves glycosidic bonds. L_B treatment showed cellobiose in major proportion and glucose in lower, but no presence of cellotriose or larger oligosaccharides were identified in the liquors. The contents in hydrolysis products suggested that B is an endoglucanase with an exo type mode of action.

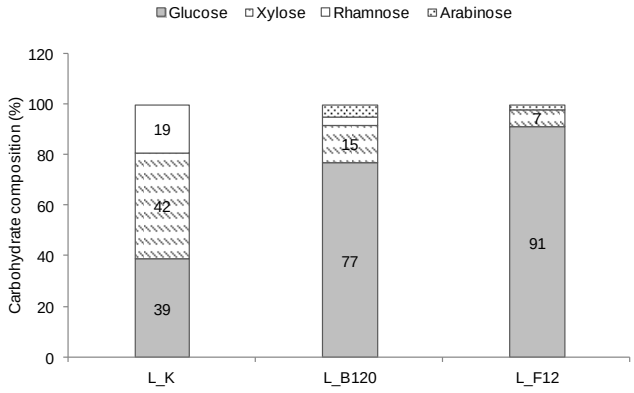

Figure 10-4 Percentage of each hemicelluloses fractions for control sequence and respective endoglucanase treatments

Studying changes of cellulose structure and ultrastructure

It is known that endoglucanase treatments hydrolyze glucose and cold caustic extraction dissolves hemicelluloses, however no important viscosity loss was observed after all treatments. In terms of cellulose chain scission (CS),

L_F12 caused slightly greater CS number than L_B120, and this small difference resulted in different Fock solubility improvement. Enzymatic biobleached pulp had a Fock solubility value of 77%, but after both endoglucanase treatments a gain in Fock solubility was observed. Specifically, L_B120 had a 91% of Fock solubility and L_F12 exhibited an 88%. Importantly, when a cold caustic extraction was applied after biobleached pulp (L_CCE), Fock solubility decreased moderately. However, this negative result was slightly improved by posterior endoglucanase treatment in the case of L_CCE_F12, while L_CCE_B120 sample remained constant. So, L_CCE_F exhibited a Fock solubility value slightly lower than L_F (82 vs. 88%), but the hemicellulose content was smaller. Endoglucanase action also contributed to modify fiber morphology. Specifically, fiber length was strongly reduced by L_F and to less extent by L_B treatments. These results suggested that B enzyme might alter fiber surface via external fibrillation while F enzyme showed preferable action to cut the fibers.

In conclusion, the combination of a purification step (CCE) with F endoglucanase treatment resulted in dissolving pulp charactersitics. **L_CCE_F sequence led to a final Fock solubility value of ~82% and to obtain the lowest amount of hemicelluloses (5.8% w/w)**. **L_CCE_B sequence could reduce the amount of hemicelluloses (6.2% w/w) but Fock solubility was negatively affected, obtaining a value of ~71%.**

Analyzing the effects of endoglucanase treatments on cellulose polymorph

The solid state ^{13}C-NMR spectra of treated pulps let to clarify the relationship between the effect of enzymatic and chemical stages with changes of cellulose structure. The spectra of L_CCE_B and L_CCE_F samples exhibited different polymorphic forms from samples submitted to an endoglucanase treatment alone. The introduction of a cold caustic extraction with 9% (w/v) NaOH converted cellulose I to cellulose II, since the C-6 signal at 64 ppm increased, obtaining two peaks with nearly identical heights at 66 and 64 ppm. Nevertheless, the C-1 signal did not exhibit a shoulder at 108 ppm, which is characteristic of cellulose II. The greater proportion of cellulose II of L_CCE_B120 and L_CCE_F12 samples can be related to the low Fock solubility values, in comparison to L_B120 and L_F12.

10.5 Effect of two different endoglucanases on commercial dissolving pulp (Chapter 7)

In view of the interesting results obtained for biobleached sulfite pulp with endoglucanase treatments (Chapter 6), approved the idea to conduct a similar study but on commercial dissolving pulp. Therefore, cellulose activation was also conducted by two different endoglucanases; one obtained from *Paenibacillus barcinonensis* (B) and the other from *Cerrena unicolor* (F). With respect to Chapter 6, this study went into more depth since additional treatments were conducted. Therefore, B endoglucanase was used at 120 U/g odp, while F endoglucanase at 12 and also at 60 U/g odp. Moreover, CCE treatment was studied before and after the respective enzymatic hydrolytic treatments. The treatments were named as follows: CCE, B120, CCE_B120, B120_CCE, F12, CCE_F12 and F60.

Removal of hemicelluloses

The evolution of carbohydrate composition of treated pulps is shown in Figure 10-5. With regard to the treatment using B120 enzyme, no hemicellulose reduction was found, but introducing a CCE before or after the hydrolytic treatment resulted in lower hemicellulose content (3.1%). In the same line, F treatment did not affect the hemicellulose content and only the presence of a CCE was able to decrease hemicelluloses. CCE treatment affected mainly xylan fraction and to less extend galactan composition. As a salient conclusion, B120_CCE, CCE_B120 and CCE_F12 presented same final amount of hemicelluloses.

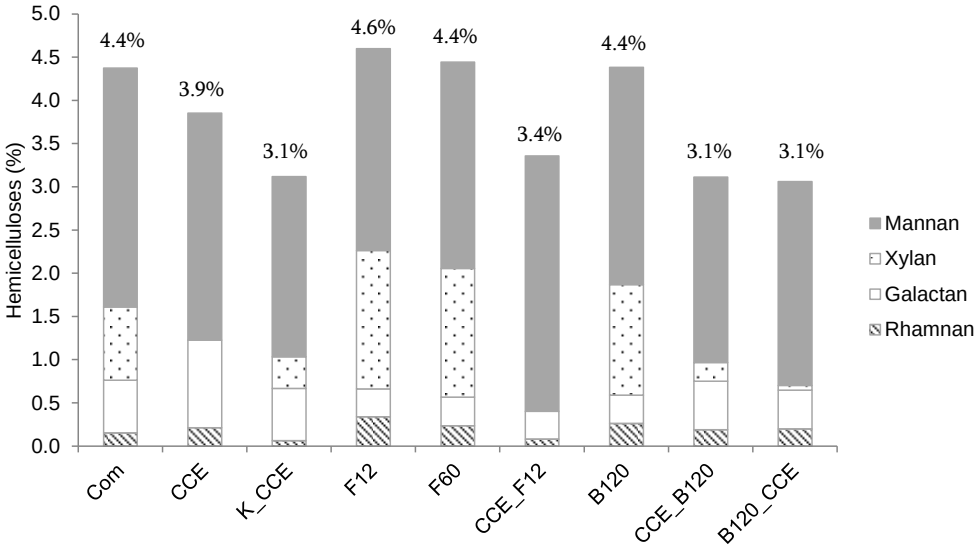

Figure 10-5 Evolution of hemicelluloses composition along the treatments studied

What is the best combination of treatments for improving Fock solubility?

All endoglucanase treatments increased Fock solubility: from 67.3% commercial dissolving pulp to 76.1% with F12 treatment and 95.8% with F60 treatment. The B120 also extended Fock solubility up to 78.7%. Some authors proposed that endoglucanase attacks the less ordered cellulose regions between and on the surface of the fibrils, leading to fiber wall swelling and therefore an increase in the accessibility to solvents. As happened with biobleached pulps, a CCE treatment by itself caused a strongly reduction of Fock solubility, with respect to commercial dissolving pulp leading to a value of 69.7%. Moreover, a similar Fock solubility loss was also observed by K_CCE treatment, with respect to control sample (K). This decrease can be explained due to the hornification phenomena which is an aggregation of microfibrils via inter- and intra fibrillar hydrogen bonding. It is described in the literature that a cold caustic extraction (NaOH > 8% w/v) transforms cellulose I into cellulose II, and the later polymorph is more susceptible to endoglucanase attack. This statement was confirmed by NMR spectra which showed presence of cellulose II for CCE_F12 sample. Therefore, the significant Fock solubility improvement detected by CCE_B120 and CCE_F12 with respect to CCE sample was in accordance with

the previous explanation. Importantly, this effect was not observed for B120_CCE which suffered an intense solubility loss with respect to B120 sample, but the Fock solubility value was higher than the one obtained by CCE sample, highlighting the positive effect brought by the previous B120 treatment. Lastly, the **combination of a CCE treatment before endoglucanase treatment appeared as a very promising both removing hemicelluloses and improving Fock solubility**.

Cellulose degradation

Fock solubility was closely related to viscosity and therefore a detailed analysis was conducted. In general, F cellulase degraded cellulose chains and decreased viscosity moderately, but the difference between low enzyme dose (F12) and high enzyme dose (F60), did not result in further viscosity loss. These results are consistent with the explanation that a heterogeneous attack of cellulase on fiber wall can weaken fibers without significantly diminishing cellulose degree of polymerization or viscosity. As other authors reported, the B120 treatment did not cause an adverse effect on viscosity, even with a posterior cold caustic extraction treatment. However, when the combination was CCE_B120, slightly lower viscosity value was found with respect to all B120-combined treatments. The gain of viscosity observed after a CCE with reference to initial pulp can be explained by the dissolution of degraded cellulose and low molecular weight hemicelluloses due to the alkaline pH. Intrinsic viscosity data allowed us to calculate the number of cellulose chain scission (CS). B cellulase-treated pulp had none chain cleavage while F cellulase had a chain scission value around 0.2. These results indicated that B and F endoglucanases must have acted differently on cellulose chains. As observed earlier Chapter 5), CCE did not cause any cleavage, but the subsequent enzymatic treatments increased CS number especially for CCE_F12 and not as much for CCE_B120. From these results can be concluded that **the presence of cellulose II formed during CCE treatment boosted the action of following cellulases**.

Relation between Fock solubility and viscosity

Regarding viscosity values two groups were distinguished. First group was B120 and B120_CCE which showed same viscosity values as starting pulp, but the former was accompanied by a Fock solubility gain while the latter suffered

an important reduction attributed to the presence of cellulose II polymorph as NMR confirmed. The second group was formed by F60, F12, CCE_F12 and CCE_B120 samples. Hence, under similar viscosity values, F12 sample exhibited lower Fock solubility improvement (+13%) than F60 (+42%), with respect to starting pulp. In other words, F60 showed a 26% of improvement with respect to F12 sample, confirming that the effect on Fock solubility was due to the enzyme action itself. In addition, from CCE_B120 treatment an interesting conclusion can be drawn since practically the same Fock solubility improvement as F60 treatment was achieved. Again, this result confirms the favorable action of enzyme because the preceded CCE treatment caused an important Fock solubility decrease.

Fiber morphology after F endoglucanase treatment

The outcomes of F cellulase treatments were examined in greater detail in terms of fiber morphology. F treatment shortened fiber length and this effect was more pronounced at high enzyme doses. So, F60 and F12 samples had a final fiber length of 0.28 and 0.47 mm, corresponding to a reduction of about 60 and 30%, with respect to commercial dissolving pulp. Although in smaller degree, the presence of a CCE also contributed to fiber length reduction since K_CCE and CCE_F12 presented shorter fibers than K and F12 samples. As a consequence, the fine content was increased, especially for F60, CCE_F12 and F12 samples. Examining the changes in fiber length according to population fractions would have knowledge about enzyme action. F60 treatment mainly affected fibers of long length (3.2-0.5 mm), while the proportion of shorter fibers was unaffected (0.2-0.5 mm). The same trend was observed by F12 and CCE_F12 but the variation was less accentuated than F60 treatment. In terms of fiber width, a greater size was found by F60, CCE_F12 and K_CCE samples in relation to starting pulp. Therefore, a comparison between CCE_F12 and K_CCE revealed that the strong alkaline step was responsible for fiber wall enhancement. Endoglucanase and CCE treatments also modified other properties: the hydrophilic character, measured according to WRV, was increased due to higher presence of free hydroxyl groups (-OH). Hence, WRV was increased as larger amount of fines were present, with the exception of F60 sample.

10.6 Biobleached sulfite pulp versus commercially available dissolving-grade pulp

Finally, a comparison between biobleached treated sulfite pulp (Chapter 6) and bleached commercial treated dissolving pulp (Chapter 7) in terms of Fock solubility, provided more information about the mechanism of action of endoglucanase and cold caustic extraction treatments (Figure 10-6).

Concerning Fock solubility results, the application of B or F endoglucanase resulted more positive on **biobleached dissolving pulp rather than bleached commercial dissolving pulp**, even when a cold caustic extraction was previously applied. By its side, a cold caustic extraction (CCE) treatment had different effect on respective studied pulps. A much higher Fock solubility loss was found for bleached commercial dissolving sample (-26%) in comparison to biobleached sample (-9%). Actually, commercial dissolving pulp had lower content of hemicelluloses than biobleached sulfite pulp, but lower hemicellulose removal was achieved. Specifically, **CCE treatment decreased hemicelluloses by about 12% for Com_CCE and about 40% for L_CCE samples, with respect to each original pulp**. The action of CCE treatment was limited to the accessibility of residual hemicelluloses. Therefore, from these results two effects were suggested by CCE treatment: on the one hand, CCE helped to reduce mainly galactan fraction and then mannan and xylan fractions of biobleached sulfite pulp, and on the other hand, was responsible for the formation of cellulose II in commercial dissolving pulp leading to lower Fock solubility.

Moreover, the presence of cellulose II polymorph detected by NMR and the low Fock solubility value of Com_CCE sample were in agreement with the proposed explanation. Regarding to the combined treatments (CCE + endoglucanases) different Fock solubility improvements were found: commercial dissolving samples (CCE_B120 and CCE_F12) exhibited a 39% of gain, while L_CCE_B120 and L_CCE_F12 biobleached samples only provided a 2% and 18%, with respect to CCE treatment. Once more, the different improvement can be explained by the different effect of CCE treatment: conversion of cellulose I to cellulose II or hemicellulose removal, respectively.

To conclude, **using same enzymatic and chemical treatment conditions, biobleached sulfite up-graded pulps presented higher Fock solubility values than bleached commercial dissolving pulps**.

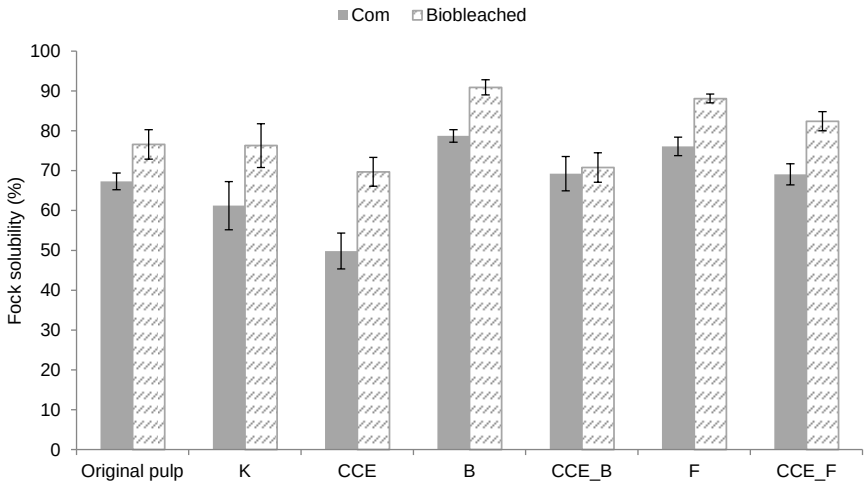

Figure 10-6 Fock solubility results for commercially available dissolving pulp (Com) and biobleached sulfite pulp.

10.7 Comparison between B and F endoglucanases

From effluent analysis and morphology data it was noticed that B and F endoglucanase presented different mode of action: F showed preferential action to cut the fibers and glucose oligosaccharides of up to 3 units were released, while B endoglucanase might alter fiber surface via external fibrillation and glucose and cellobiose constituted the most abundant oligosaccharides in the effluents. Nevertheless, using a different source of raw material (i.e. biobleached sulfite or commercial dissolving pulp) showed same trend and behavior.

Regarding Fock solubility, **B endoglucanase brought higher improvement than F enzyme** in both studied pulps**.** In fact, B endoglucanase contributed a gain of about 3.3% with respect to F enzyme and for both pulps. However, when the **combined treatments were studied**, **F enzyme provided superior results than B enzyme in the case of biobleached samples**. By contrast, applying a combined treatment on Com samples showed no differences between endoglucanases.

Therefore, the selected sequence for upgrading biobleached sulfite pulp was L_CCE_F in terms of good Fock solubility and low content of hemicelluloses.

10.8 Cellulose oxidation by laccase-TEMPO treatments (Chapter 8)

At this point, the discussion has been focused on the conversion of unbleached sulfite pulp to biobleached dissolving pulp by means of enzymatic (laccase and cellulase) and chemical treatments (hydrogen peroxide and cold caustic extraction). Specifically, laccase-VA system followed by a posterior pressurized hydrogen peroxide stage was used for biobleaching softwood sulfite pulp Chapter 4 and Chapter 5). Then, a cold caustic extraction treatment was introduced to upgrade biobleached sulfite pulp to dissolving pulp by means of hemicellulose removal, and finally pulp quality improvement was achieved by endoglucanase treatments (Chapter 6). In contrast to the work performed in Chapter 4, Chapter 5 and Chapter 6, this part of the study was based on the use of commercial bleached dissolving pulp in order to find out new uses or specific applications. In agreement with the research line performed in Chapter 7 (i.e. biomodification of commercial dissolving pulp by presence of cellulase and cold caustic extraction treatments); the use of standard dissolving pulp was justified by the increasing demand that market studies indicate for the next years. Therefore, in view of the characteristics that define dissolving pulps (i.e. high cellulose content), would be interesting to use TEMPO for cellulose oxidation. As a novelty and with the aim to reduce the use of environmentally harmful substances, the well-known traditional TEMPO-system was replaced by laccase-TEMPO system.

Studying the influence of different variables on cellulose oxidation

Cellulose fibers submitted to laccase-TEMPO oxidation resulted in new functional groups such as aldehyde and carboxylic acids. Therefore, the content of aldehydes or carboxylic acids is an indicator of the extent of cellulose modification. Moreover, these functional groups play a significant role in the final pulp and paper properties. So, evaluating dry and wet tensile strength also provides information about the degree of modification; although good strength-related properties are not required for dissolving pulps.

In order to obtain the highest degree of modification, different experimental conditions were studied. On the one hand, the contribution of laccase was studied by presence or absence of pressurized oxygen in the system. On the other hand, the development of wet strength was studied by the introduction of a pre-refining step, and the amount of new substituted functional groups as a function of TEMPO dose and reaction time.

In general, **laccase-TEMPO treatments with no oxygen pressure** increased moderately carboxylic acid content, with respect to starting pulp. Two doses of TEMPO, 2% and 8% odp, were studied failing to show significant differences in terms of carboxylic group content. Carboxylic acids are known to contribute to dry strength of paper. However, this effect was not observed since Lac-T 2% and Lac-T 8% showed low content of carboxylic acids, and supposed low dry tensile strength, with no differences between samples. New aldehyde groups were for the first time introduced, although in poor level. It is known that, aldehyde groups once formed in the native cellulose fibers are able to form hemiacetals with hydroxyl groups of cellulose sterically close to each other, but not all aldehydes groups formed in the TEMPO-oxidized cellulose fibers do not necessary contribute to the development of wet strength of the sheets. In fact, both samples exhibited a wet strength improvement with respect to commercial dissolving pulp, but the **wet-to-dry (W/D) strength ratio was lower than 15%**, indicating that wet strength paper was not achieved.

Significantly, applying oxygen pressure in the system (**Lac-T 8%, O_2**) let to introduce greater amount of carboxylic and aldehyde groups. Specifically, carboxylic acid multiplied five times the initial value. Aldehyde groups by their side, increased twenty-seven times compared to Lac-T 8%. As a consequence, dry and wet strength properties were markedly increased, providing a **W/D strength ratio of 18%**. The effect of a refining pre-treatment did not result in much higher functional groups but contributed positively to improve strength-related properties. **R+Lac-T 8% (O_2)** had nearly the same carboxylic and aldehyde content as Lac-T 8% (O_2), but the former exhibited **a W/D strength ratio of 26%**. Therefore, this significant improvement can be explained by the fibrillation effect, since the presence of higher hydroxyl groups on fiber surface led to develop hemiacetal linkages with the introduced aldehyde groups. Importantly, the poor bonding capability of dissolving pulp due to low content

of hemicelluloses justified the introduction of a refining pretreatment in order to develop good strength-related property.

From this study, we observed that a pre-refining step, laccase treatment conducted with presence of oxygen and a TEMPO dose of 8% were the best conditions for achieving high level of cellulose oxidation.

Could long time reaction maximize cellulose oxidation?

Then, using the best treatment conditions (i.e. R+Lac-TEMPO 8%, O_2) but extending reaction time to 20h provided higher amount of carboxylic acids but no presence of aldehyde groups. This result differed from the one obtained by laccase-TEMPO treated sisal pulp (Aracri and Vidal, 2012) under same treatment conditions since both functional groups were found. These results suggested that cellulose composition influenced on the efficiency of Lac-TEMPO oxidation, but the mechanism of carboxyl formation is still under study. Some authors proposed the formation of carboxyl mainly as a result of aldehyde subsequent autoxidation under oxygen-saturated medium or a direct participation of the oxoammonium ion in the reaction. Interestingly, the large amount of carboxyl groups made resulted pulp suitable for obtaining nanofibrillated cellulose. However, these results were not further investigated since NFC field was not the basis of this work.

Hemiacetal linkages

As discussed earlier, the increase of wet strength property with Lac-T treatment was related to the development of hemiacetal linkages. Results from wet-zero span tensile index (WZSTI), which measures the intrinsic strength of a single fiber (in independence of fiber network), also confirmed the development of hemiacetal linkages. All oxidative treatments exhibited higher values of WZSTI compared to original and refined pulps, while important depolymerization was occurred. The highest improvement was found for R+Lac-T 8% (O_2) which doubled intrinsic fiber strength value, followed by Lac-T 8% (O_2), then Lac-T 2% and finally Lac-T 8%. Therefore, the gain in WZSTI was related to the presence of new covalent interfibre bonds.

Changes in cellulose structure

The carboxylic acids have a direct influence on WRV which is a measure of the water absorption capacity and provides information about the degree of

swelling. Besides the hydrophilic groups, WRV is also influenced by the specific surface area or degrees of fibrillation of the cellulose. In general, the higher carboxyl groups, the higher WRV was obtained.

In terms of fiber morphology, the presence or absence of oxygen during Lac-TEMPO treatment did not have an effect on fiber morphology. In fact, Lac-TEMPO treated samples exhibited same fiber length and fine content as original pulp. But, as expected, a refining pre-treatment at 5000 rev reduced fiber length by 46% and increased the fine content up to 28%, with respect to original pulp. Combining the refining pre-treatment with Lac-TEMPO led to the same fiber length decrease as the refining treatment alone, but fine content was slightly superior. Therefore, the changes in fiber morphology were associated to the refining effect rather than Lac-TEMPO oxidative action. In addition, an important air permeance reduction accompanied by a density increase was found after the refining pre-treatment. The refined sample followed by Lac-TEMPO oxidation, did not reach the same air permeance as refined pulp alone, but presented closer structure than all Lac-TEMPO oxidative treatments.

Cellulose degradation and Fock solubility

The strong oxidative action of TEMPO system prompted an important degradation of pulp and this depolymerization was more pronounced when treatments were performed under oxygen pressure. Precisely, the highest viscosity loss was found for Lac-T 8% (O_2), followed by R+Lac-T 8% (O_2) sample. The cellulose degradation was quantified by viscosity measurements in Cuen solution. It is known that under alkaline conditions aldehyde groups at C-6 and keto groups at C-2 undergo β-elimination reactions that cause the cleavage of the cellulose chain. Therefore, the alkaline pH of Cuen solution can further degrade cellulose polymer during viscosity measurement and give a misleading viscosity result. In order to avoid this problem, pulps were subjected to a reduced treatment with $NaBH_4$ and then viscosity measurements were conducted. Interestingly, same viscosity values determined directly after Lac-TEMPO treatment or after a post-reduced treatment with $NaBH_4$ were found. These results suggested that reducing end groups were mostly present as hemiacetals and only to small extent as aldehyde or aldehyde hydrates. In addition, it is believed that the highly-stable covalent hemiacetal linkages,

between primarily C-6 aldehydes and cellulose surrounding hydroxyls protected the pulp from all reactions attributable to the reactive carbonyl moieties.

From viscosity results, it was calculated the number of chain scission (CS) due to the oxidative treatment itself. It was observed that the cleavage of the connecting carbon-carbon bond might provide additional aldehydes, but these aldehydes did not come from the oxidation of primary hydroxyl groups. Results also revealed that higher chain scission (CS) corresponded to higher values of Fock solubility, with exception of R+Lac-T 8% (O_2) sample. Fock solubility was improved as treatment conditions were more effective (i.e. TEMPO dose and presence of oxygen pressure). The improvement from low to high Fock solubility was as follows: Lac-T 2% < Lac-T 8% < Lac-T 8% (O_2). On the contrary, refined pulp (R) reduced markedly Fock solubility with respect to original pulp. Then, R+Lac-T 8% (O_2) treatment doubled Fock solubility value with respect to refined pulp, but did not succeed to reach same value as Lac-T 8% (O_2) sample. The lower Fock solubility value can be explained by the presence of hemiacetal linkages that hindered cellulose dissolution and also the partially oxidized C6 carbon were not available for xhantation reaction with CS_2.

To conclude, chemoenzymatic modification of commercial bleached dissolving pulp using laccase as biocatalysts and TEMPO as enhancer was satisfactorily achieved. The new functional groups resulted from Lac-T oxidation improved dry strength and provided new properties such as wet-strength.

10.9 Acetylation reactions on dissolving-grade pulps obtained from different chemoenzymatic paths (Chapter 9)

Following the same research line (i.e. cellulose modification) as Chapter 8, the last part of the thesis studied acetylation reactions on dissolving-grade pulps obtained from two different chemoenzymatic paths. In this part, cellulose modification was achieved by a chemical reaction with the introduction of acetyl groups. The biobleaching sequence studied in Chapter 4 and Chapter 5, and the chemoenzymatic conversion of sulfite pulp to dissolving-grade explored in Chapter 6, let to design two optimized procedures for obtaining dissolving-grade pulps, named: L_F and L_CCE_F. Then, acetylation reactions in heterogeneous and homogeneous phase were conducted and compared with

reference system from commercially available dissolving-grade pulp (Com). The objective of this work was to demonstrate the feasibility of using chemoenzymatic treated-pulps for the production of cellulose acetate films as alternative to traditional dissolving fiber grades. Moreover, the dissolution behavior of chemoenzymatic treated fibers (L_F and L_CCE_F) was evaluated by means of homogeneous acetylation reaction.

Acetylation in heterogeneous phase

Acetylation in heterogeneous phase was conducted in presence of acetic acid, toluene as a non-solvent and sulfuric acid as a catalyst. Then, specific amounts of acetic anhydride (Ac_2O) were studied and mixtures were stirred for 1h. The presence of a non-solvent made the product (cellulose acetate) insoluble and therefore fibers preserved the morphological structure. Reaction was quenched by adding a mixture of ethanol and water. The acetylated fibers were firstly washed with methanol in order to remove the non-solvent reagent, then washed with distilled water until neutralization and dried in a freeze-drying.

In the heterogeneous reaction, different degrees of surface acetylation were obtained by varying the concentration of acetic anhydride in the nonpolar solvent used. The specific conditions for acetylation reactions were as follows: 0.53, 2.67, 5.35 and 10.7 g Ac_2O per weight (g) of dried fiber sample. The nomenclature used, for simplicity, were as follows: “lowest”, “low”, “medium” and “high”. The structural changes of acetylated fibers were analyzed by FTIR technique, which confirmed the appereance of three new bands characteristic to the acetyl group vibration.

In the case of commercial pulp (Com), it was noted that by increasing the amount of Ac_2O used for acetylation resulted in a higher intensity of the C=O band at 1735 cm^{-1}. A similar observation applied to L_F and L_CCE_F pulps, but differences in the intensity peak at 1735 cm^{-1} (C=O) as a function to the acetylation conditions were less pronounced. For both L_F and L_CCE_F, a high intensity peak at 1735 cm^{-1} was observed when high (10.7g) Ac_2O was introduced.

Acetyl group content

FTIR showed qualitative changes between non-acetylated and acetylated samples but the quantification of acetyl groups was accomplished by titration,

where acetyl groups were neutralized with NaOH and the residual NaOH was treated against HCl. L_F sample did not exhibit differences in terms of acetyl content with lowest and medium dose of reagent, but a significant gain in acetyl group was observed when high dose of Ac_2O was used. Specifically, a 108% of improvement with respect to samples treated under medium conditions (5.35g Ac_2O) was observed. In fact, from all studied pulps the highest acetyl group content (24%) was determined by L_F sample using intense conditions. By its side, L_CCE_F acetylated sample presented lower amount of acetyl groups than L_F counterparts. Using low dosage of Ac_2O did not provide sufficient acetyl groups but, doubling and quadrupling Ac_2O dose let to introduce greater content of acetyl groups. Specifically, 13% and 15% of acetyl group were obtained for medium and high dose of Ac_2O. Com sample showed that by increasing Ac_2O dose resulted in a gradual improvement in acetyl group content with the exception of the highest Ac_2O dose, which underwent a decrease in acetylation level. Actually, same acetyl content was found using low and high dose of Ac_2O.

Changes in cellulose structure

As observed in Chapter 6 and Chapter 7, endoglucanase treatment caused a significant reduction (~65%) in fiber length and increased fine content, with respect to biobleached pulp (L_{VA}POPO). An additional length decrease was observed by intensifying the acetylation conditions (from lowest to high dose of Ac_2O) for L_F, L_CCE_F and Com samples. Specifically, Com sample exhibited at first longer fibers than L_F and L_CCE_F samples, but at medium and high acetylation conditions (5.35 and 10.7 g Ac_2O) the length reduction was more severe for Com sample. The maximum acetylation level of Com was achieved using medium conditions (5.35g Ac_2O), but the greatest loss of fiber length (87%) and the largest generation of fines (93%) where obtained when high-level of Ac_2O (10.7 g) was applied. Importantly, acetyl groups incorporated on the cellulose surface were associated to the mass increase (coarseness value) and higher fiber width. In particular, L_F sample at highest acetylation level (23.6% of acetyl group) showed a mass gain (coarseness value) of about 158%, while L_CCE_F sample at highest acetylation level (15.2% of acetyl groups) accounted for 178%, with respect to non-acetylated samples.

The effect of fiber morphology and acetylation degree was assessed as far as the mechanical properties of respective handsheets. The increased acetylation

level of Com fibers resulted in higher dry tensile strength, with important strength improvement from lowest (0.53 g) to low (2.67 g) Ac_2O dose. Importantly, acetyl groups are expected to limit hydrogen bonding since acetyl groups substitute –OH groups available in cellulose network. Therefore, this strength enhancement can be explained due to the generation of fines (i.e. additional available –OH groups). On the other hand, using the highest Ac_2O dose did not result in higher acetyl groups and a negative effect in tensile strength was noted. In fact, using low and high dose of Ac_2O gave similar acetyl content but different strength values. These results can be attributed to different fiber morphology: samples obtained from "high" acetylation conditions presented considerably higher fine content and much shorter fiber length, giving weaker fiber. Therefore, a compromise between fiber strength and free hydroxyl groups is needed in order to obtain strength-related handsheets. An opposite trend in strength was observed for handsheets made with acetylated L_F and L_CCE_F samples. As the content of acetyl groups increased, the tensile strength decreased, but at the high Ac_2O level a slight strength improvement was dispalyed. The gain in strength can be related to the higher amount of fines, and as a result new hydroxyl groups were available for hydrogen bonding. In addition, the strength loss of L_F and L_CCE_F samples observed as the acetylation degree increased was in agreement with a diminishing bulk density. For the highest L_CCE_F Ac_2O level a denser and more compact structure was found and the presence of fines contributed to generate a more entangle structure. Interestingly, L_F sample with the highest acetylation degree exhibited same strength value as non-acetylated L_F sample but the former had new functional groups.

The main consideration in terms of wet strength is that the highest amount of acetyl groups produced slightly higher wet strength values than pulps with lower acetylated levels. Importantly, the highest wet strength correlated with the highest dry strength values. Interestingly, a wet-to-dry (W/D) strength ratio provides information about the wet-strength development. It is generally agreed that a W/D > 15% should be considered as having wet-strength. Specifically, only L_CCE_F and Com fibers with 15.2 and 7.9% acetyl group content produced webs with a W/D strength ration of 22 and 48%, respectively.

Hydrophilicity behavior

The effect of acetylation treatment on hydrophilicity of the fibers was examined by means of initial water contact angle (WCA). Acetyl groups reduced the hydrophilicity of fibers since lower interfibre bonds were found. The different WCA observed between non-acetylated and acetylated samples confirmed that chemical surface modification occurred. In fact, samples with the highest degree of acetylation doubled the WCA with respect to non-acetylated samples. In particular, a WCA of 64, 58 and 52° was obtained for Com, L_F and L_CCE_F acetylated samples, respectively. Besides initial contact angle values, monitoring changes in WCA with time provided information about water absorption behavior. This is mainly due to absorption in the sheet structure and evaporation—the latter, however, is only relevant for relative long absorption times. For non-acetylated samples water drops were absorbed rapidly giving a WCA close to 0°. L_F and L_CCE_F acetylated samples also showed quick drop absorption. In contrast, Com acetylated sample kept same initial water contact angle for 2 min, but after 20 min WCA was halved.

Acetylation in homogeneous phase

Acetylation in the homogenous phase excluded the presence of a non-solvent and specific amount of acetic anhydride and sulfuric acid was used. In this case, the reaction latest only 30 min. Acetic anhydride is capable of deconstructing the crystalline network and interacts with anhydroglucose units of cellulose. Reaction was quenched by adding a mixture of ethanol and water. In the later route, a hydrolysis (i.e. release of acetic acid) was required in order to obtain cellulose acetate fibers. Therefore, viscous solution was droplet in a water bath and regeneration occurred.

Finally, homogeneous acetylation was conducted in order to evaluate the dissolution behavior of chemo-enzymatic treated fibers (L_F, L_CCE_F and L_CCE_F+Lac-T). The results were compared to the Com sample used as a reference. In the absence of toluene in the acetylation medium a higher percentage of substituted acetyl groups were found compared to the results from heterogeneous acetylation. FTIR spectroscopy confirmed that acetylation reactions were satisfactorily achieved since a fingerprint peak at 1730 cm^{-1} was observed. Importantly, noticeably lower intensity was found by L_CCE_F+Lac-T sample. The poor acetylation efficiency can be related to the low presence of

free hydroxyl groups, since Lac-T treatment oxidized hydroxyl groups to aldehyde or carboxylic form. Quantification the degree of substitution by titration showed that similar percentages were acquired for all studied fibers. Values between 33 to 36% of acetyl substituted groups were found. Fibers obtained after acetylation were freeze-dried, then dissolved in acetone solvent and the resulted viscous solution was used to prepare films by a casting technique. Although same acetyl content was described for all samples, Com increased three times the strength value with respect to both chemo-enzymatic samples. However, chemo-enzymatic samples still presented notably greater strength values than the ones exhibited for heterogeneous acetylation reaction at the highest acetylation level. To conclude, cellulose acetate fibers with new functional groups and high strength-related properties were achieved.

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Chapter 11

General conclusions

The high textile demand expected for the following years, has resulted in a number of new dissolving pulp mills to produce high purity cellulose from wood. This fact urges the purpose to find new bleaching and purification technologies alternative to dominant industrialized TCF sequences.

The novel and most salient points of this thesis are summarized as follows:

- Biobleaching considerations:

Softwood sulfite pulp was submitted to LMS treatment. Natural and synthetic mediators were assayed providing a valuable input into bleaching field since little bibliography is reported for this type of cellulose pulp.

The bleaching potential of *synthetic mediators* (HBT, VA) was superior to the *natural mediators* (SA, pCA).

SA and pCA accounted for low delignification and did not boost the bleaching effect of posterior hydrogen peroxide stage, providing a final ISO brightness close to the one obtained by a *conventional sequence* (i.e. a hydrogen peroxide treatment alone).

Condensation and grafting reaction between sulfite pulp and natural mediators were excluded since no KN increase was observed after L stage. Moreover, TGA curves were similar to initial pulp.

In reference to synthetic mediators, VA exposed the best results in terms of high brightness and low lignin content, with a minimal impact on effluent properties.

After *LMS stage* a significant brightness loss was found for all mediators, but hydrogen peroxide stage was able to overcome the negative effect on optical properties.

Lac-VA was used for exploring an extended biobleaching sequence where a chelating stage (Q) and a pressurized hydrogen peroxide stage (PO) were introduced. The proposed LVAQPO sequence enabled efficient bleaching without an adverse effect on cellulose integrity.

For a given ISO brightness value, LVAQPO sequence required lower H_2O_2 dose and reaction time in comparison to control and conventional sequences.

TGA curve of *conventional sequence* showed that PO stage caused a substantial decrease in the crystalline cellulose peak. In contrast, pulps previously treated with a Lac-VA resulted in less marked reduction of crystalline cellulose after PO stage. Therefore, the introduction of enzymatic stage was justified by a better final pulp quality and saving of chemical reagents.

- Considerations of cellulose modification:

a) Chemoenzymatic treatments for up-grading biobleached sulfite pulp and further purification of commercially available dissolving grade pulp.

Activation of *biobleached sulfite* samples was achieved by posterior endoglucanase treatments. Two endoglucanases, *Paenibacillus barcinonensis* (B) and *Cerrena unicolor* (F), were studied. In general, the endoglucanase treatment was found to improve Fock solubility.

Cellulose purification (i.e. hemicellulose removal) was obtained by means of a cold caustic extraction (CCE) with 9% w/v NaOH. Xylan fraction was the most affected followed by mannan fraction.

^{13}C-NMR spectra revealed that CCE converted cellulose I to cellulose II polymorphic form, diminishing Fock solubility values due to the hornification phenomena.

The combination of CCE followed by an endoglucanase treatment led to a synergic effect boosting hemicellulose removal and improving Fock solubility, with respect to CCE treatment alone. However, Fock solubility values were slightly lower in comparison to endoglucanase (B or F) treatments performed alone.

The studied endoglucanases exhibited different mode of action: B altered fiber surface via external fibrillation while F enzyme showed preferable action to cut the fibers. Hence, slightly higher chain scissions (CS) were found by F enzyme, although no significant viscosity loss was observed for both enzymes.

Composition of dissolved carbohydrates present in liquors provided knowledge about enzyme pattern degradation. L_F treatment released oligosaccharides of larger molecular weight (G2>G1>G3) than L_B treatment (G2>G1).

L_CCE_F12 sequence led to a final Fock solubility value of ~82% and to obtain the lowest amount of hemicelluloses (5.8% w/w). *L_CCE_B120* sequence could reduce the amount of hemicelluloses (6.2% w/w) but Fock solubility was negatively affected, obtaining a ~71%.

Commercially available dissolving grade pulp (Com) was submitted to further purification treatments.

A CCE treatment applied on commercial dissolving pulp caused a much higher Fock solubility loss than the one applied on biobleached sulfite cellulose.

A CCE treatment was studied before or after endoglucanase treatment. The latter combination caused a negative effect on Fock solubility since ^{13}C-NMR confirmed the presence of cellulose II.

High enzyme dose (F60) resulted in higher Fock solubility. However, no differences in cellulose degradation between low (F12) and high (F60) doses

were found. Therefore, that indicates that the improvement in Fock solubility was due to action of enzyme.

In terms of Fock solubility, the application of B or F endoglucanase was slightly more positive on *biobleached sulfite pulp* than *commercially available bleached dissolving grade pulp (Com)*. The action of hemicellulose removal by CCE treatment was more effective in *biobleached sulfite* samples than *Com* samples.

b) Cellulose oxidation by Laccase-TEMPO treatments.

Fibre modification based on the oxidation of cellulose using laccase-TEMPO system was assessed on sulfite pulp. Modification efficiency was enhanced by applying a refining stage prior to the enzyme treatment and performing laccase oxidation under pressurized oxygen.

New functional groups such as carboxylic acids and aldehydes were introduced, contributing to increase dry strength and develop wet strength property, respectively.

Wet strength is known to be developed by the new formed aldehyde groups that are able to form hemiacetals with hydroxyl groups of cellulose chains sterically close to each other. It is observed that these linkages hindered cellulose dissolution towards CS_2 reagent, providing low Fock solubility.

The strong oxidative action of TEMPO system prompted a significant degradation of cellulose. However, the alkaline pH used for viscosity measurements cause no further cellulose degradation suggesting that reducing end groups were mostly present as hemiacetals form.

- Considerations of acetylation reactions:

Fiber modification based on the introduction of acetyl groups was studied via heterogeneous and homogenous phase.

In the heterogeneous reaction, different degrees of surface acetylation were obtained by varying the concentration of acetic anhydride in the non-polar

solvent used. Results were confirmed by means of FTIR, quantification of acetyl groups and gain of lineal mass (coarseness) determined by cross polarized light.

The highest amount of acetyl groups produced slightly higher wet strength values than non-acetylated pulps. Moreover, acetyl groups reduced the hydrophilicity character of cellulose because lower interfibre bonds were found.

Homogeneous phase acetylation of chemo-enzymatic (L_CCE_F and L_F) and reference fibers resulted in relatively higher acetyl groups up to 36 and 33, respectively. By contrast, L_CCE_F+Lac-T sample presented low degree of acetylation as FTIR spectra revealed. This poor efficiency can be explained by the low presence of free hydroxyl groups, due to the previous oxidation by Lac-T treatment to aldehyde or carboxylic form.

From all acetylated samples, cellulose acetate films were obtained demonstrating the feasibility to use this new source as alternative to traditional dissolving fiber grades.

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