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Flax fibre modification using enzyme systems to obtain high-value cellulose products

Doctoral Thesis

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La Doctora TERESA VIDAL LLUCIÀ, Catedrática de Universidad y la Doctora Mª BLANCA RONCERO VIVERO, Profesora Titular del Departamento de Ingeniería Textil y Papelera de la Universitat Politècnica de Catalunya

CERTIFICAN:

Que **Amanda Fillat Latorre**, Licenciada en Biología, ha realizado bajo su dirección el trabajo de investigación **"Flax fibre modification using enzyme systems to obtain high-value cellulose products"** que presenta para optar al grado de Doctor.

Y para que así conste expiden el presente certificado en Terrassa a 5 de mayo de 2011.

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A mi familia

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NOMENCLATURE

A	Absorbance
ABTS	2,2'-azinobis-(3-etilbenzotiazolina-6-sulfonat)
AQ	Anthraquinone
AS	Acetosyringone
BI	Bleaching index
C*	Chroma
CFU	Colony forming units
CLK	Klason lignin content
COD	Chemical oxygen demand
DRI	Dye removal index
DTPA	Diethylenetiamineoentaacetic acid
ECF	Elementary chlorine free
FTIR	Fourier transform infrared spectrocopy
G	Guaiacyl
H NMR	Hydrogen nuclear magnetic resonance
HBT	1-hydroxybenzotriazol
HexA	Hexenuronic acid
HPLC	High performance liquid chromatography
k/s	Chromophores groups content index
KN	Kappa number
Knlig	Kappa number due to lignin

L(l)	Length-weighted average length
L(n)	Numerical average length
L* a* b*	Chromatic coordinates
LiP	Lignin Peroxidase
LOI	Lateral order index
MnP	Manganase peroxidase
MS	Methyl syringate
MtL	Myceliophthora thermophila laccase
OD	Optical diffraction
Odp	Oven dried pulp
P	Hydrogen peroxide stage
PCA	p-coumaric acid
PcL	*Pycnoporus cinnabarinus* laccase
Po	Hydrogen peroxide pressurized stage
Py-GC/MS	Pyrolysis coupled with gas chromatography/mass spectrometry
Q	Chelating stage
Rpm	Revolutions per minute
S	Syringyl
SA	Syringaldehyde
TCF	Totally chlorine free
TCI	Total crystallinity index
TGA	Thermogravimetry analyses
TGE	Tryptone glucose extract

TLC	Thin layer chromatography
TMAH	Tetramethylammonium hydroxide
TU	Toxicity units
U	Enzyme activity units
VA	Violuric acid
VP	Versatil peroxidase
W(l)	Length-weighted average width
W(n)	Numerical average width
Wa	Acidic washing
W_aL	Long flax fibres (bast fibres)
W_aS	Short flax fibres (core fibres)
X	Xylanase stage
XRD	X-ray diffraction

INDEX

Abstract

The aim of this thesis is to modify flax pulp fibres (*Linum usitatissimum*) by more friendly environmental processes. Pulp and paper research is focussing through enzyme systems investigation for developing green chemistry technologies due to existing environmental concerns and to legal restrictions. Moreover, it exists also an increasing strategic interest in using flax fibres to obtain high-quality specialty papers. That is why we study the application of biotechnology as an efficient alternative to traditional industrial processes based on the use of chemical agents.

This work is framed by two of the main research topics of the Paper and Graphic Specialty Laboratory in the Textile and Paper Engineering Department of the Universitat Politècnica de Catalunya. One research line is based on pulp bleaching and is focused basically on the study of enzymatic systems as biobleaching agents; the other research topic that has been recently introduced in our investigation group is the use of enzymes as functionalisation agents by promoting the grafting of several compounds. Laccase is the main enzyme used in this thesis; it is an oxidoreductase that can assist reactions in an eco-friendly way since laccase uses air and produces water as the only by-product. Moreover, laccase can work under mill conditions and has wide application potential.

The first part of this thesis involved the use of enzymes to bleach flax pulp. The aim was to explore the potential of various natural mediators (lignin-derived compounds) for delignifying flax fibres in order to identify the most efficient and ecofriendly choice among them. Afterwards, we assessed the use of various enzyme delignification stages in an industrial bleaching sequence. The ensuing totally chlorine free (TCF) sequence comprised various laccase-mediator system treatments (L stage) followed by a chelating stage (Q stage) and a subsequent bleaching step with hydrogen peroxide (Po stage). A xylanase pretreatment was additionally carried out. Laccases used came from the fungi *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*; the performance of several natural mediators was compared with the obtained with the application of various synthetic mediators. In addition, the lack of studies on the properties of effluents from the treatment of non-wood pulp with laccase and natural mediators led

us to examine effluent properties upon biotreatments and after different bleaching stages. The results obtained warrant upscaling any of the biobleaching sequences for flax pulp as they provide sustainable flax fibre with a high cellulose content and brightness above 80% ISO. The use of xylanase pretreatment was found to efficiently remove HexA and enhance delignification by laccase.

In a second part of the thesis, the study was based on the ability of laccases to graft phenolic compounds onto the flax fibres. We demonstrated the capacity of the laccase from *P. cinnabarinus* to couple covalently several lignin-derived compounds and to functionalise the treated fibres. In this way, the optical properties of treated pulps were measured showing and increase in coloration caused by the laccase induced grafting of the phenols; furthermore, it was verified that antimicrobial properties were conferred to the paper products obtained by this functionalisation method.

Our knowledge of the oxidized phenolic compounds activity on the fibres that form unbleached pulp had many gaps. In order to fill the lack in acquaintance of the different performance of this by-laccase active compounds, several analytical techniques were used namely, pyrolysis coupled to gas chromatography/mass spectrometry, X-Ray diffraction, ATR-FTIR, thermogravimetry, pulp fibre fractionation or size exclusion chromatography among other techniques. These techniques have allowed a better understanding of the processes that happen in the different treatments with laccase and phenolic compounds.

Resum

Aquesta tesi neix del interès de modificar fibres de lli (*Linum usitatissimum*) mitjançant processos més respectuosos amb el medi ambient amb el propòsit d'obtenir productes d'alt valor afegit. De fet, la recerca en el sector industrial de fabricació de pasta i paper està orientada cap al desenvolupament de tecnologies "netes" degut a la gran preocupació ambiental i a les restriccions legals existent. D'altra banda, també existeix un interès estratègic creixent en l'ús de fibres no provinents de la fusta, tals com les de lli, per a obtenir papers especials d'alta qualitat. Per aquesta raó, s'estudia l'ús de la biotecnologia com a alternativa eficient als processos industrials tradicionals basats en la utilització d'agents químics.

Aquest treball s'emmarca dins d'una de les dues línies de recerca principals del laboratori de l'Especialitat Paperera i Gràfica del Departament d'Enginyeria Tèxtil i Paperera de la Universitat Politècnica de Catalunya. Una de les línies es basa en el blanqueig de pasta i tracta bàsicament de l'aplicació de sistemes enzimàtics com a agents bioblanquejants. L'altra línia de recerca, que ha sigut recentment introduïda en el nostre grup, es centra en l'ús d'enzims com a agents funcionalitzadors per a promoure l'acoblament de diferents compostos en les fibres. Entre aquests enzims estan les lacases, que son oxidoreductases que poden participar en diferents reaccions de forma respectuosa amb el medi ambient, ja que utilitzen oxigen com a donador d'electrons i produeixen aigua com a únic subproducte. A més, les lacases son actives sota condicions industrials i tenen una amplia aplicació biotecnològica.

Així doncs, la primera part d'aquesta tesi es fonamenta en l'ús d'enzims per a blanquejar pasta de lli. L'objectiu és explorar el potencial de diferents mediadors naturals (compostos derivats de la lignina) per a deslignificar fibres de lli, amb l'objectiu d'identificar els més eficients i respectuosos amb el medi ambient. Seguidament, s'estudia l'aplicació de vàries etapes enzimàtiques en una seqüència típica de blanqueig industrial.

Les seqüències TCF estudiades consten de diferents tractaments amb el sistema lacasa-mediador (etapa L) seguits d'una etapa quelant (etapa Q) i una etapa posterior amb peròxid d'hidrogen (etapa Po). També s'investiga la idoneïtat d'introduir un

pretractament enzimàtic amb xilanasa. Les lacases utilitzades en l'etapa L provenen dels fongs *Pycnoporus cinnabarinus* i *Myceliophthora thermophila,* les quals s'apliquen amb alguns mediadors naturals i es comparen amb altres de sintètics. A més, motivats per la falta d'estudis sobre la caracterització dels lleixius resultants dels biotractaments de pastes no fusteres, s'han avaluat les propietats dels efluents després de les diferents etapes de blanqueig. Els bons resultats obtinguts després de realitzar aquestes experiències justifiquen l'escalat de qualsevol de les seqüències de bioblanqueig amb pasta de lli estudiades en aquest treball. Al final de les seqüències, s'obtenen fibres de lli amb un alt contingut en cel·lulosa i amb una blancor per sobre de 80% ISO. L'ús d'un pretractament amb xilanasa dona lloc a una eliminació eficient de grups hexenurònics i a una millora en la deslignificació causada per la lacasa.

La segona part de la tesi es centra en desenvolupar l'habilitat de les lacases per a acoblar compostos fenòlics en les fibres. Es demostra la capacitat de la lacasa de *P. cinnabarinus* per acoblar covalentment alguns compostos naturals derivats de la lignina i per a funcionalitzar fibres de lli. A més, com a resultat amb elevada novetat, es verifica que s'han aconseguit funcionalitzar fibres a través d'un sistema enzimàtic, obtenint papers amb propietats antimicrobianes.

Amb el interès de raonar i entendre el mecanisme d'acció dels compostos fenòlics oxidats per la lacasa, s'utilitzen diferents tècniques analítiques, com per exemple: piròlisis acoblada a cromatografia de gasos/espectrometria de masses, difracció per raigs-X, ATR-FTIR, termogravimetria i fraccionament de fibres, entre d'altres. Aquestes tècniques, a més de corroborar l'acoblament dels fenols per acció de la lacasa, permeten una millor comprensió i coneixement dels processos que tenen lloc durant els tractaments amb lacasa i aquests compostos fenòlics en fibres de lli.

Resumen

La presente tesis surge del interés de modificar fibras de lino (*Linum usitatissimum*) mediante procesos más respetuosos con el medio ambiente con el propósito de obtener productos de alto valor añadido. De hecho, la investigación en el sector industrial de fabricación de pasta y papel está orientada hacia el desarrollo de tecnologías "limpias" debido a la gran preocupación ambiental y a las restricciones legales existentes. Por otro lado, también existe un creciente interés estratégico en el uso de fibras no madereras, tales como las de lino, para obtener papeles especiales de alta calidad. Es por ello que se recure al uso de la biotecnología como alternativa eficiente a los procesos industriales tradicionales basados en el uso de agentes químicos.

Este trabajo se enmarca dentro de dos de las principales líneas de investigación del laboratorio de la Especialidad Papelera y Gráfica del Departamento de Ingeniería Textil y Papelera de la Universitat Politècnica de Catalunya. Una de la líneas se centra en el blanqueo de pasta y trata básicamente de la aplicación de sistemas enzimáticos como agentes bioblanqueantes. La otra línea de investigación, que ha sido recientemente introducida en nuestro grupo, se basa en el uso de enzimas como agentes funcionalizadores para promover el acoplamiento de diferentes compuestos en las fibras. Entre estas enzimas están las lacasas, que son oxidoreductasas que pueden participar en diferentes reacciones de forma respetuosa con el medio ambiente, ya que utilizan oxígeno como donante de electrones y producen agua como único subproducto. Además, las lacasas son activas bajo condiciones industriales y tienen una amplia aplicación biotecnológica.

Así pues, la primera parte de esta tesis se fundamenta en el uso de enzimas para blanquear pasta de lino. El objetivo es explorar el potencial de varios mediadores naturales (compuestos derivados de la lignina) para deslignificar fibras de lino, con el objetivo de identificar los más eficientes y amigables con el medio ambiente. Seguidamente, se estudia la aplicación de varias etapas enzimáticas en una secuencia de blanqueo industrial típica.

Las secuencias TCF estudiadas constan de varios tratamientos con el sistema lacasa-mediador (etapa L) seguidos de una etapa quelante (etapa Q) y una etapa posterior con

peróxido de hidrógeno (etapa Po). También se investiga la idoneidad de introducir un pretratamiento enzimático con xilanasa. Las lacasas utilizadas en la etapa L provienen de los hongos *Pycnoporus cinnabarinus* y *Myceliophthora thermophila* y se aplican con varios mediadores naturales que se comparan con otros sintéticos. Además, motivados precisamente por la falta de estudios sobre la caracterización de efluentes provenientes de biotratamientos de pastas no madereras, se han evaluado las propiedades de los efluentes de las diferentes etapas de blanqueo. Los buenos resultados obtenidos tras realizar estas experiencias justifican el escalado de cualquiera de las secuencias de bioblanqueo de pasta de lino estudiadas en el presente trabajo. Al final de las secuencias, se obtienen fibras de lino con un alto contenido en celulosa y con valores de blancura por encima de 80% ISO. El uso de un pretratamiento con xilanasa da lugar a una eliminación eficiente de grupos hexenurónicos además de a una mejora de la deslignificación causada por la lacasa.

La segunda parte de esta tesis se centra en desarrollar la habilidad de las lacasas para acoplar compuestos fenólicos en las fibras. Se demuestra la capacidad de la lacasa de *P. cinnabarinus* para acoplar covalentemente varios compuestos naturales derivados de la lignina y para funcionalizar fibras de lino. Además, como resultado altamente novedoso, se verifica que se han conseguido funcionalizar fibras a través de este sistema enzimático, obteniéndose papeles con propiedades antimicrobianas.

Con la intención de razonar y entender el mecanismo de acción de los compuestos fenólicos oxidados por la lacasa, se emplean diferentes técnicas analíticas, como por ejemplo: pirolisis acoplada a cromatografía de gases/espectrometría de masas, difracción por rayos-X, ATR-FTIR, termogravimetría y fraccionamiento de fibras entre otras. Estas técnicas, además de que corroboran el acoplamiento de los fenoles por acción de la lacasa, permiten una mejor comprensión y conocimiento de los procesos que ocurren durante los tratamientos con lacasa y estos compuestos fenólicos en fibras de lino.

Chapter 1

INTRODUCTION AND OBJECTIVES

SUMMARY

This chapter presents an introduction to the key topics dealt with in this thesis which focuses on the use of flax fibres to produce paper, specifically, the use of biotechnology to improve TCF bleaching sequences, as well as functionalise flax fibres in order to create new bioproducts. The enzyme systems used to this end and their mechanism of action are examined. Finally, it presents the objectives of the thesis and the work plan.

1.1 Raw materials

1.1.1 Non-wood fibres

Wood is currently the biggest raw material input in the global pulp and paper industry. However, significant levels of non-wood fibres are used in a handful of countries (mostly in Asia), and there are also stronger indications of an interest in fibre elsewhere, particularly in North America. At present, the most common non-wood fibre is straw. Other non-wood fibres, such as flax, are also becoming more important in the manufacture of pulp and paper (Ashori 2006; Mabee and Pande 1997).

In Spain, the modern use of non-wood fibres in papermaking has largely been the result of their application to the production of specialty papers, i. e. papers that require properties that could not easily be achieved solely through the use of wood fibre

furnish. For instance, such characteristics as porosity and wet strength, air permeability, specific tare, tensile and fold properties can be attributable to the inherent physical structure of such specific non-wood fibres, thus justifying their preferred furnish. Fibre strength and length, the ratio of fibre length to diameter, as well as the quality and profile of the original raw material are primary factors in the consideration of uses (Martínez et al. 1998; McGovern et al. 1987).

1.1.2 Flax pulp

Flax is a group of the bast annual and perennial plants from the Linaceae family that form the genus *Linum*. Several varieties of the species, *L. usitassimum*, are grown primarily for their fibre, which is used to make Linen Flax, or for their seeds, the source of Linseed oil. Although both types are of the same botanical origin, they perform differently due to their selection for different traits over many decades. Linen flax grows taller (0.9 – 1.2 m with a shive diameter of 0.25 - 0.5 cm), branches closer to the top and has a smaller seed yield. Linseed flax is shorter (0.3 – 0.6 m), but has increased branching and seed yield. Despite these differences, seed produced from linen flax does find its way to the oil crusher and some fibre is extracted from linseed flax shive (García Hortal 2007; Martínez et al. 1998).

Flax is one of the oldest cultivated plants. Today, the major producers of flax fibre are China, France, Russia, Belarus, the United Kingdom, the Czech Republic and Spain (FAO 2005). Flax fibre for papermaking has its origin in the residue from the textile industry and flax straw from the manufacture of seed flax.

Flax has traditionally been used as the primary furnish for cigarette paper (burning tube), where strength, opacity and control of air permeability are required. Banknote paper often incorporates flax as a component of the furnish, largely to enhance fold and general strength characteristics.

The stems of the flax plant have a woody core (known as the “shive”), with a hollow centre. The woody core constitutes approximately 70% of the stem. It has very short fibres and produces a pulp not unlike a rather weak hardwood pulp. The long fibres used for the production of linen and for papermaking are located in the bark or bast of the stems. The bast fibres are long and slender. Fibre length ranges from 10 to 55 mm,

the average being 28-30 mm. Fibre diameters range from 12 to 30 um, the average being 20-22 um (McGovern et al. 1987).

The most frequently cooking process used with flax is that based on sodium hydroxide and anthraquinone (NaOH–AQ). Refining the resulting pulp to obtain paper requires a large amount of energy, which is needed to produce thin paper (García Hortal 2007).

One additional difficulty in using flax fibres is their tendency to stick together or become entangled (Lisson et al. 2001); this hinders their handling in both industrial and laboratory processes.

1.1.3 Major chemical components

The major chemical components of flax pulp are carbohydrates (cellulose and hemicelluloses) and lignin.

Cellulose is a high molecular weight linear polymer composed of D-glucopyranose units linked by β-1,4-glycosidic bonds. The repeating unit of cellulose is cellobiose (Figure 1- 1).

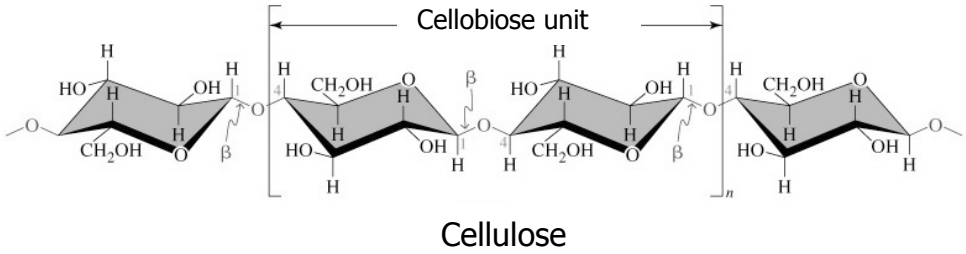

Figure 1- 1. Cellobiose molecule in the cellulosic chain (García 2003).

Hydroxyl groups present in cellulose macromolecules are involved in a number of intra- and intermolecular hydrogen bonds, which result in various ordered crystalline arrangements. Cellulose I is the most abundant form in nature. Several different models of cellulose I have been proposed; however, its structure is still not fully understood because of its complexity. It is known that the crystalline structure of cellulose I is a mixture of two distinct crystalline forms: celluloses I_α (triclinic) and I_β (monoclinic). The relative amounts of cellulose I_α and I_β vary with the source of the cellulose, with

the I_β form being dominant in higher plants. I_β is the most thermodynamically stable form (Park et al. 2010).

Intermolecular hydrogen bonds facilitate the establishment of an ordered fibre network and hence increase the crystallinity of cellulose. Highly crystalline zones are difficult for solvents and reagents to penetrate. By contrast, amorphous zones are relatively more disordered and easier for chemical reagents to access; they also facilitate swelling, elongation and flexibility of fibres (Dence and Reeve 1996; García Hortal 2007).

Cellulose chains aggregate on the cellular wall to form microfibrils, which are the basic elements of cellulosic materials. Dimensions of these microfibrils vary as a function of their origin and position within the cellular wall.

The properties of cellulosic materials are related to the degree of polymerization (DP) of the cellulose molecule. Paper's resistance is due, in part, to the individual resistance of the cellulose chains, and falls when they degrade. Pulp bleaching seeks to remove the colour due to other components (*viz.* lignin), but cellulose can be degraded, and this leads to a decrease in yield and a potential drop in the mechanical properties of the fibres. Cellulose degradation can be hydrolytic, oxidizing, alkaline, thermal, microbiological or mechanical (Dence and Reeve 1996).

Hemicelluloses are chemically heterogeneous polysaccharides, constituted by combinations of monosaccharides of five (xylose and arabinose) or six randomly bonded carbon atoms (glucose, mannose and galactose). Some hemicelluloses are associated to the cellulose moiety while others are associated to lignin. Hemicelluloses constitute the support of cellulose microfibrils in cell walls; they are also more readily accessed, degraded and dissolved than cellulose. In addition, hemicelluloses are amorphous and highly hydrophilic, so they can absorb abundant water during pulp refining and result in extensive fibrillation, thereby improving the mechanical properties that depend on the fibres bonding site.

Lignin structure is very different from that of cellulose and hemicelluloses. It is an aromatic polymer formed by oxidative condensation of phenol precursors. It is very branched, tridimensional and amorphous, and its main role is to act as a cementing material between the middle lamella and the fibre walls. Lignin is highly hydrophobic,

so its presence in pulp inhibits water absorption and fibre swelling, thereby hindering refining. Its content and distribution in cell walls depend on its origin. For instance, conifers present a higher percentage of lignin compared to hardwood. Figure 1-2 illustrates the complexity of lignin structure.

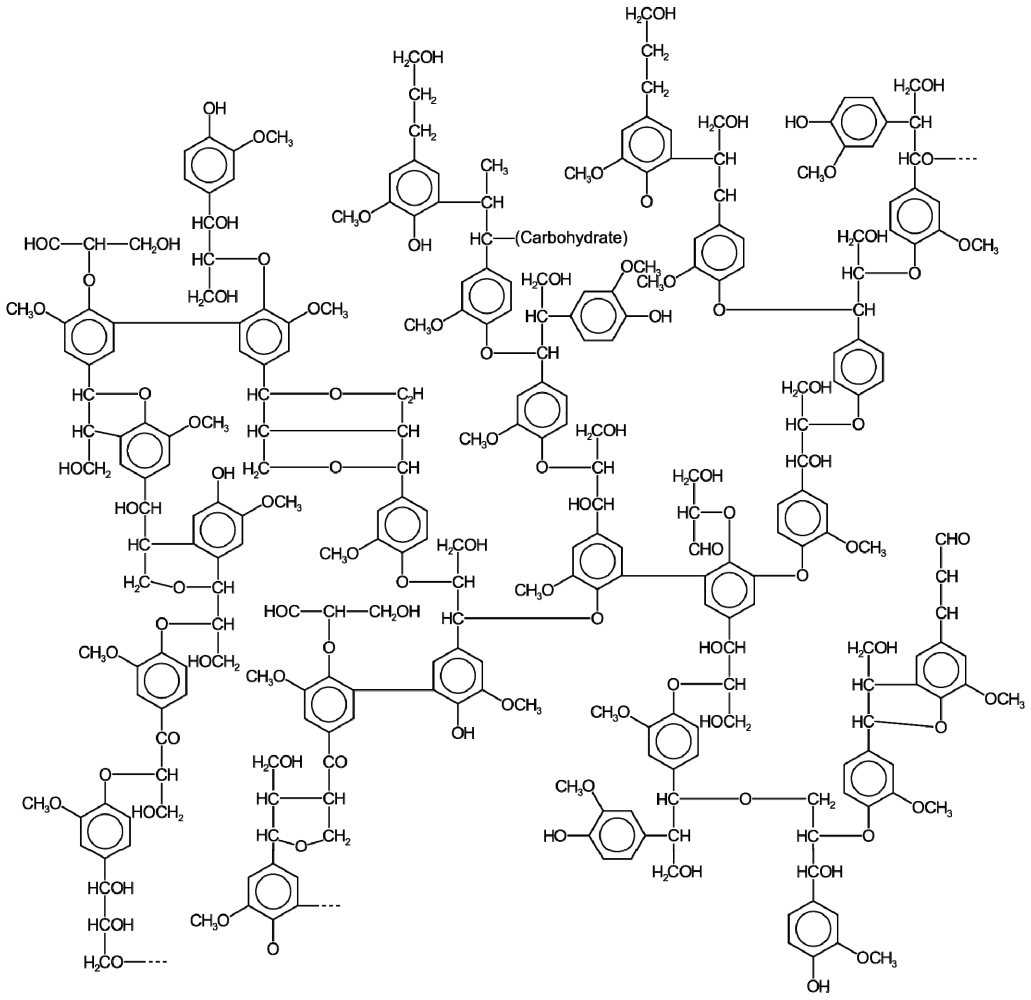

Figure 1- 2. Lignin structure (Glazer 1995).

The basic structure of lignin differs between conifers and hardwood trees. In conifers, lignin basically consists of Guaiacyl (G) units, which possess a single methoxy group on the phenylpropane ring and originate from coniferyl alcohol. On the other hand, lignin in hardwoods is a copolymer or mixture of Guaiacyl and Syringyl (S) units; the latter contain two methoxy groups per phenylpropane ring and originate from sinapyl

alcohol (Parham 1983) (Figure 1-3). S units are known to be more reactive than G units, so lignin containing S units predominantly is easier to remove (del Río et al. 2001; Dence and Reeve 1996). Thus, the lignin S/G ratio directly affects the delignification behaviour. Higher S/G ratios would imply higher delignification rates, less alkali consumption and, therefore, higher pulp yield. Bast fibres from flax have a predominance of G-lignin (S/G ratio of 0.1). The low S/G ratio of the lignin from flax, despite having very low lignin contents, makes it fairly resistant to alkaline delignification (Marqués 2010).

The properties of lignin in non-wood materials vary widely. Thus, lignin in herbaceous plants consists of *p*-hydroxyphenyl units (H) from coumaryl alcohol in addition to S and G units in variable proportions. Lignin in other plants is more similar to softwood or hardwood lignin depending on whether G units or a combination of S and G units prevails.

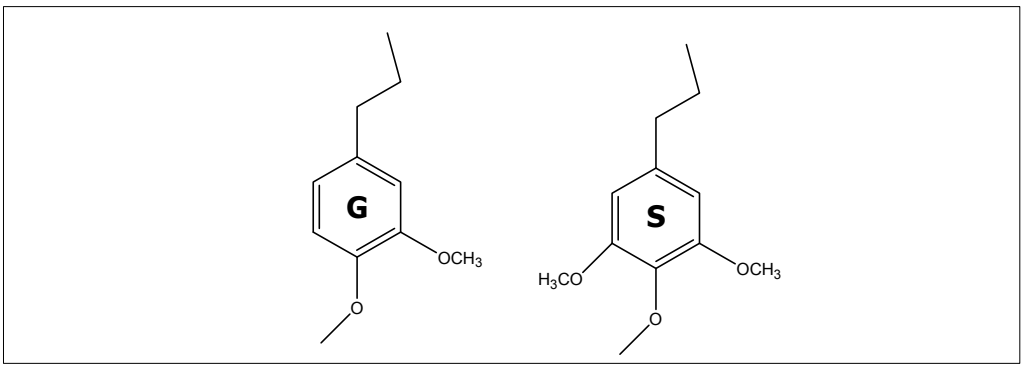

Figure 1- 3. Lignin units: Guaiacyl (G) and Syringyl (S).

Vegetal cells have cellular walls that contain cellulose microfibriles that form the skeleton, which at the same time is surrounded by other substances that act as a matrix (hemicelluloses), and cementing material (lignin). Figure 1-4 shows the general model of the cellular wall. Fibres comprise several layers, namely: the primary wall (P), external secondary wall (S_1), middle secondary wall (S_2) and internal secondary wall (S_3).

The inner part of the ripe cells is empty and is called lumen. The various layers differ in thickness, structure, chemical composition and microfibril orientation with respect to the fibre axis. Lignin in the middle lamella is largely removed during cooking where

fibres are individualised. Residual lignin in the fibres to be bleached concentrates in the middle secondary wall (S_2), since this constitutes the main portion of the cellular wall (Parham 1983).

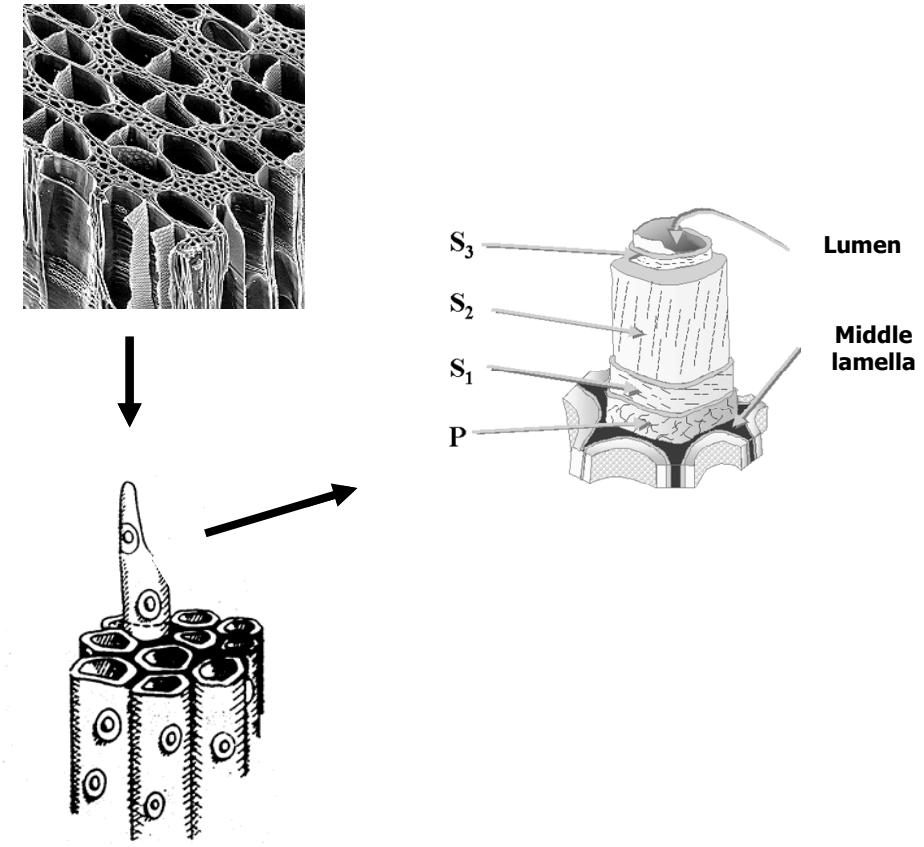

Figure 1- 4. Simplified structure of the cellular wall (Kirk and Cullen 1998).

Flax fibres have the following **chemical composition** (García Barneto et al. 2011), in percentage over dry weight of fibres: 74.4% glucan; 10.2% xylan; 0.5% araban; 0.7% ramnose; 2.1% galacturonic acid; 0.8% glucuronic acid and 7.9% Klason lignin. Figure 1- 5 shows the structure of a xylan molecule with its branches.

Vegetal **extractables** are those compounds that are extracted with organic solvents. They are important as they cause environmental and production problems during the manufacture of pulp and paper. Thus, the known "pitch" deposits originate in the lipophilic components of the raw material (wood) that are not affected during cooking and bleaching processes (Gutiérrez et al. 2007).

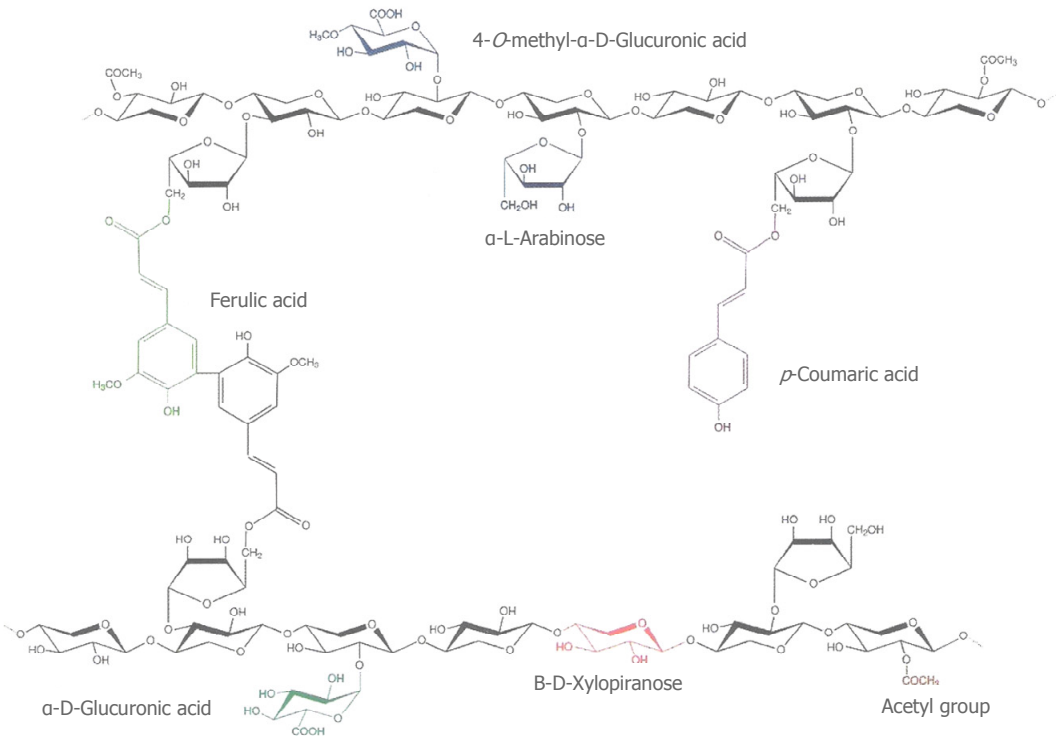

Figure 1- 5. Structure of a xylan molecule with its branches (Gallardo 2007).

Flax pulps also contain **hexenuronic acids**. HexA are formed during kraft cooking, where 4-*O*-methyl glucuronic acid present in xylans is converted to its corresponding unsaturated hexenuronic acid (4-deoxy-β-L-threo-hex-4-enopyranosyluronic acid) by losing methanol, as shown in Figure 1-6 (Costa and Colodette 2007; Danielsson et al. 2006).

The importance of the presence of HexA groups is their influence on the bleaching process and the final properties of the pulp. It is known that HexA have the following effects:

- Contribution to kappa number. HexA interfere in the standard method of determination of kappa number, which is carried out with potassium permanganate in acidic conditions. This chemical agent can react by oxidizing HexAs and giving an incorrect determination of the lignin amount (Costa and Colodette 2007; Li et al. 2002; Roncero 2001).

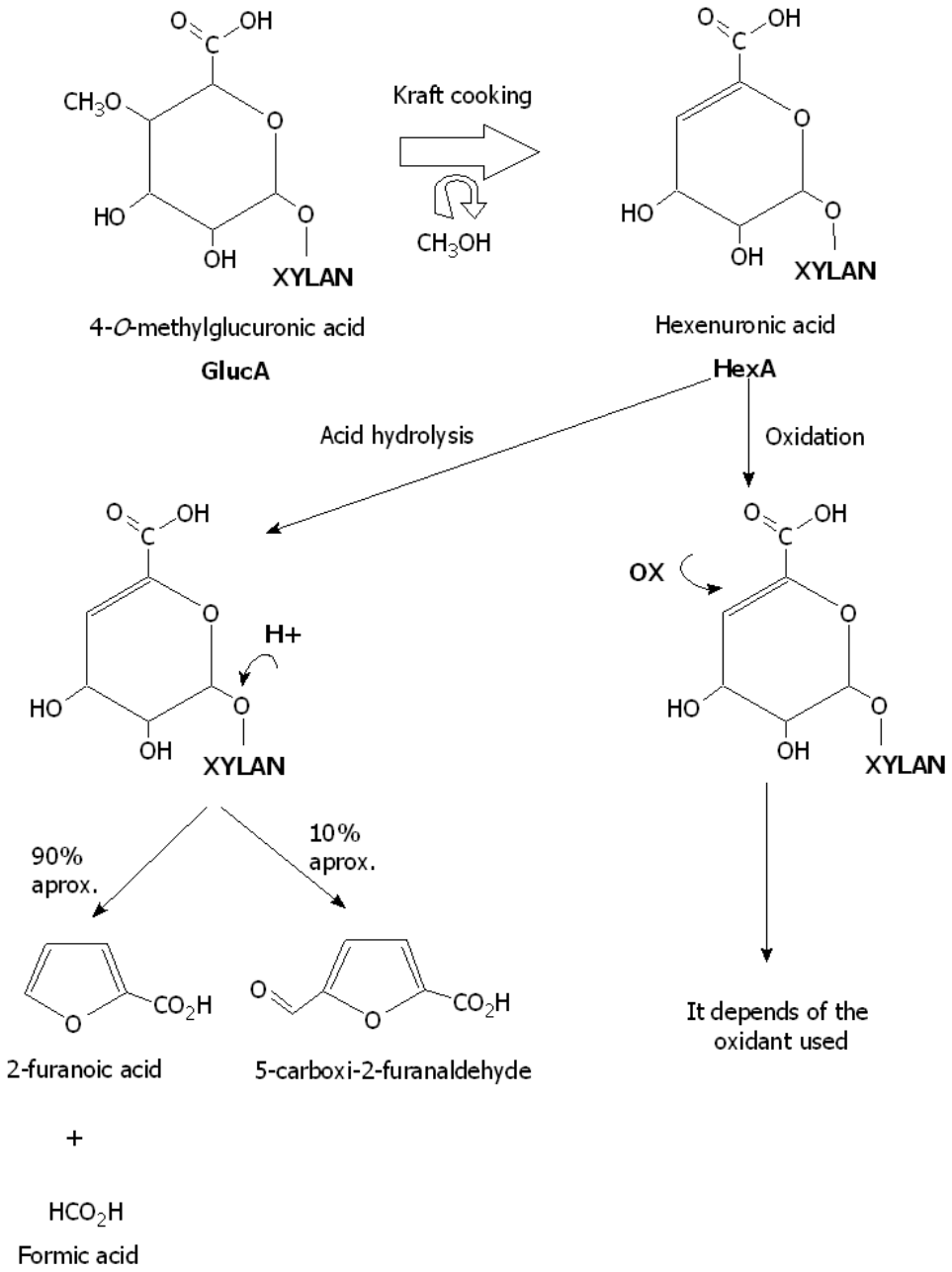

Figure 1- 6. Hexenuronic acid formation (HexA) and its destruction by hydrolysis or oxidation (Roncero 2001).

- Use of bleaching reagents. HexA consume a large amount of chemical agents in the bleaching process (Jiang et al. 2000; Malinen 2006).

- Participation in the retention of metallic ions. Certain metallic ions present in the pulp catalyze the decomposition of some bleaching agents, for example hydrogen peroxide. HexA have a strong chelating effect, and may contribute to the presence of a high content of metallic ions in the pulp (Vuorinen et al. 1999).

- Contributions to brightness reversion. Several works suggest that ageing is related with the HexA amount in bleached pulps (Cadena et al. 2010; Sevastyanova et al. 2006; Vuorinen et al. 1999).

- Contribution to oxalic acid formation. Some authors consider that part of the oxalic acid formation is also due to the HexA acid content (Elsander et al. 2000; Vuorinen et al. 1999).

For this reason, investigations on methods to eliminate these HexA groups have appeared recently (Aracri and Vidal 2011; Valls et al. 2010a). Hexenuronic acids can be eliminated by acid hydrolysis. The bond between HexA acid and the xylan chain is more susceptible to acid hydrolysis than other sugar bonds, due to the specific localization of the double bond. Under acid hydrolysis HexA are converted to 2-furanoic and formic acids, because the presence of the double bond HexA can also be eliminated using electophilic oxidizers such as chlorine, chlorine dioxide, ozone and peracids, giving way to different reaction products that depend on the reagent used.

1.2 Pulp and paper production

The history of paper can be traced back to as early as 4000 B.C. when ancient Egyptians first utilized papyrus, from which the word ‘paper’ was derived, meaning a material on which information is recorded. Pulp and paper manufacturing is now one of the largest industries in the world with enormous annual sales. The manufacture of pulp and paper is generally divided into two main processes: the production of pulp and the production of paper. Pulp produced by chemical digestion contains separated fibres and black liquor. For high-quality papers the elimination of the residual lignin is essential. This removal is achieved in the bleaching process (Stanko and Angus 2006).

1.2.1 Pulp bleaching

The main objective of this process is to increase the brightness degree by removing or modifying the residual lignin and its degradation products (Dence and Reeve 1996; García 1984; Sjostrom 1981).

Brightness is the reflectance of visible light from pulp fibres formed into sheets. Bleaching increases the capacity of paper for accepting printed or written images and so increases its usefulness. It is also a means of purifying pulp, thereby extending its application, increasing its stability, and enhancing some of its properties. In recent years, there has been an extremely rapid evolution of processes for production of bleached pulp. Environmental concern, supported by new laws has put pressure on industries, which are being forced to change, adapt, and/or improve their processes, with the goal of developing processes that are more respectful with the environment, with less environmental impact; that is, sustainable production processes.

Since the pulp bleaching plant is the most contaminating fraction of the paper producing process, the paper industry has made in it the highest number of modifications. Traditional-bleaching sequences for chemical pulps, based on the highly effective and cost-effective but environmentally unsound bleaching with chlorine free (Cl_2), have been replaced by elemental chlorine free (ECF) and totally chlorine free (TCF) sequences. Both processes employ oxygen based-bleaching agents, mainly O_2, alkaline hydrogen peroxide (H_2O_2), and ozone (O_3), while ECF bleaching may also make use of chlorine chemicals other than Cl_2, mainly chlorine dioxide (ClO_2) and hypochlorite (ClO^-) (Widsten and Kandelbauer 2008). The use of TCF sequences involving oxygen or hydrogen peroxide to bleach non-wood fibres is far from new.

The use of biotechnology in pulp bleaching has attracted considerable attention and achieved interesting results in recent years. Enzymes of the hemicellulolytic type, particularly xylan-attacking enzymes, xylanases are now used commercially in the mills for pulp treatment and subsequent incorporation into bleach sequences. The use of oxidative enzymes from white-rot fungi, which can directly attack lignin, is a second-generation approach, which could produce larger chemical savings than xylanase but has not yet been developed to the full scale (Bajpai 2004).

1.2.2 Ligninolytic enzymes: laccases

Laccase is a multicopper oxidase, one of the extracellular glycoprotein enzymes expressed by white-rot fungi and other organisms that play a crucial role in the terrestrial carbon cycle by helping to degrade lignocellulosic material such as wood. In co-operation with other ligninolytic (lignin-degrading) fungal enzymes, such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP), laccases oxidizes lignin. Laccase acts on phenolic substrates by catalyzing the oxidation of their phenolic hydroxyl groups to phenoxy radicals while oxygen (O_2) is reduced to water (Widsten and Kandelbauer 2008).

Laccase is a large molecule which cannot penetrate deep into wood; moreover, due to its rather low-redox potential (~0.5-0.8 V), it is unable to oxidize non-phenolic lignin units, which have a high-redox potential (>1.5 V) (Riva 2006). Because of these limitations, laccase alone can only oxidize phenolic lignin units (<20% of all lignin units in native wood) on the substrate surface. Therefore, laccase is often applied with an oxidant mediator, a small molecule able to extend the effect of laccase to non-phenolic units and to overcome the accessibility problem. In these so-called LMS, the mediator is first oxidized by laccase and then diffuses into the cell wall, oxidizing lignin inaccessible to laccase (Figure 1-7) (Galli and Gentili 2004, Xu et al. 2001).

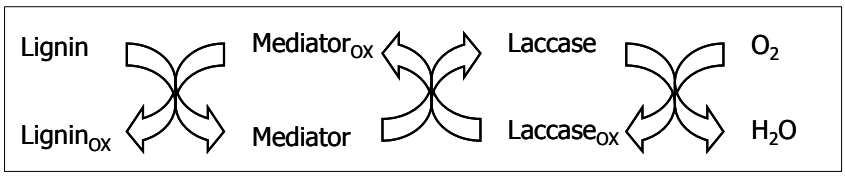

Figure 1- 7. Laccase-mediator system

The first synthetic mediator found to be able to oxidize high-redox potential non-phenolic lignin model compounds is 2,2'-azinobis-(3-ethylbenzenthiazoline-6-sulfonic acid) (ABTS) (Bourbonnais and Paice 1990). The most widely investigated synthetic mediators for application in the forest products industry are N-OH compounds, such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA). However, the cost of synthetic mediators tends to be prohibitive for implementation in biobleaching. This has generated interest in mediators obtainable from plants or as industrial by-products, the so-called natural mediators (Fillat and Roncero 2010; Sigoillot et al. 2005).

In this sense, the term "natural" would not only stand for the natural origin (lignocellulosic material, fruit seeds, etc) of these compounds, but also for their role in nature, most likely being the usual (true) mediators of laccase activities during the biodegradation of lignin polymer carried out by white-rot fungi (Camarero et al. 2005; Cañas et al. 2007). Natural and synthetic laccase mediators used in this thesis are depicted in Figure 1-8.

1.2.3 Hydrolytic enzymes: xylanases

Xylanases are hydrolytic enzymes that catalyze hydrolysis of xylans. Xylans are complex heteropolysaccharides containing β-1,4 bonds of d-xylopyranose units that are highly substituted. Biodegradation of xylan, which is a polysaccharide abundant in nature, is a complex process that requires the coordinated action of several enzymes. Amongst them, β-1,4-endoxylanases play the main role, breaking internal bonds such as that of β-1,4-xylose (Beg et al. 2001).

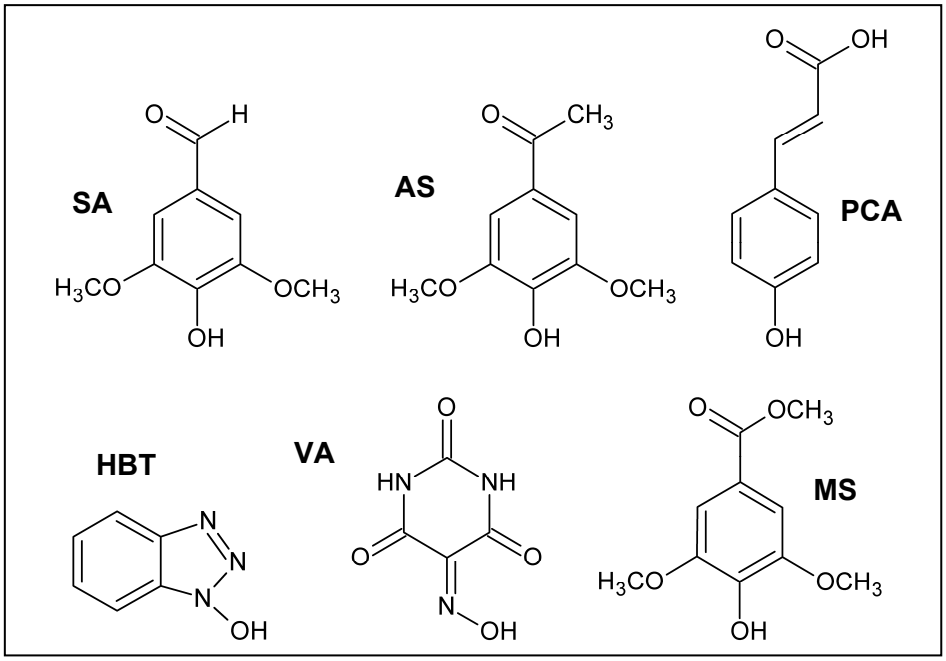

Figure 1- 8. Chemical structure of some mediators. Syringaldehyde (SA), Acetosyringone (AS), p-coumaric acid (PCA), HBT, Violuric acid (VA) and Methyl Syringate (MS).

During alkaline cooking, short chains of xylans precipitate in a crystalline form on the surface of cellulose fibres, causing a reduction in the accessibility to the fibre wall. There are several theories regarding the working mechanism of xylanases on pulp (Pham et al. 1995; Wong et al. 1997). Its positive effect is generally attributed to the elimination of those xylans that are part of the hemicelluloses present on cellulosic fibres, and that are found, in a certain way, in an intermediate position between the structured cellulose chains and the amorphous lignin fraction. When these xylans are eliminated, the existing bond between cellulose and lignin disappears, and facilitates the elimination of lignin, which is now freer, in the subsequent bleaching stages (Pham et al. 1995; Roncero et al. 2000; Valls et al. 2010b).

A number of studies on xylanase and its application in pulp bleaching, have promoted its simple and economically feasible industrial application, since high-density towers can be used to carry out enzymatic pretreatment with xylanase (Fillat 2008; Roncero 2001, Valls 2008). A high number of factories in Europe, North America, South America and Japan currently use technology based on xylanases for continuous pulp bleaching.

1.3 Functionalisation of lignocellulosic materials

The use of different enzymes in fibre modification or “fibre engineering”, rather than as process aids, is an interesting and potential field of application. Through target modification of fibre surface by enzymatic or combined enzymatic and chemical treatments, improved fibre properties or completely new fibre characteristics for various applications can be created. Fibre engineering could be used both in improving the paper and board manufacturing properties of pulp fibres, as well as for modifying of fibres suitable for non-paper applications (Viikari 2002).

In addition, enzymatic treatments offer the potential to selectively modify pulp fibre surfaces to yield new products that cannot be manufactured via chemical and/or mechanical methods. The ability to tailor the surface of pulp fibres will provide pulp manufacturers with new opportunities to develop differentiated, high-value-products for the consumer (Ragauskas 2002).

1.3.1 Application of laccases in functionalisation

While LMS have attracted much attention for biobleaching, where the goal is to obtain high-pulp brightness with minimal losses in paper strength, some research has also been conducted into improving or creating paper properties by laccase or LMS treatment of the pulp. Another approach aimed at improving paper properties is the use of laccase for co-polymerizing low-molecular weight phenols with the pulp (i.e., biografting) before papermaking (Widsten and Kandelbauer 2008).

Laccase-mediated biografting of phenols or certain other types of low-molecular weight compounds provides a method for tailoring the surface of lignocellulosic or isolated lignin under mill conditions and usually without harmful solvents. Depending on the choice of laccase substrate, properties such as increased antimicrobial resistance or hydrophilicity/hydrophobicity can be imparted to lignocellulosic materials, or the grafting could improve bonding in paper and composite board products. The grafted molecules could also act as anchor groups for further fibre modifications (Kudanga et al. 2011; Witayakran and Ragauskas 2009).

This ability could be used in the future to attach chemically versatile compounds to the fibre surfaces, possibly resulting in fibre materials with completely novel properties (Rodríguez Couto and Toca Herrera 2006).

1.4 Objectives of the thesis

This thesis is framed within one of the research lines of the Department of Textile and Paper Engineering of the Universitat Politècnica de Catalunya, addressed at the **Application of Biotechnology for Pulp Fibre Modification**. One of the targets of such a research line is the biobleaching of fibres for pulp and paper production; another important research line is enzymatic functionalisation to provide new properties of interest to lignocellulose fibres.

The ***main objective*** of this study was **to modify flax** (*Linum usitatissimum*) **pulp using new enzymatic processes**, involving the use of laccases and xylanase.

Therefore, the ***first goal*** was **to develop TCF sequences for bleaching flax pulp based on the use of chemical reagents or biotechnological** methods with a view to

obtaining bleached pulp of industrial quality on a par with that provided by conventional bleaching processes, using **less polluting technologies**. The enzymes used in the biotechnological treatments were different laccases and xylanases, and their influence on carbohydrates and hexenuronic acids content, pulp and paper properties, and effluent quality was evaluated. Important targets of this research were saving reagents and/or avoid bleaching steps using enzymatic treatments, as well as applying alternative mediators to HBT, such as VA, and researching new natural laccase mediators.

The ***second purpose*** was **to study the enzyme-assisted grafting of low molecular weight phenols onto flax pulp fibres**, aiming to impart better or novel properties to obtain high-value cellulose products. The capacity of laccase systems to confer antimicrobial properties to paper products was evaluated, trying to elucidate the interactions between pulp fibres and laccase-phenol systems. Moreover, a specific objective was to gain an insight into the mechanism of the laccase-induced coupling of natural phenols onto flax fibres. Some techniques were used for this purpose, such as size classification and Pyrolysis-GC/MS.

The *interest*, *innovation* and *relevance* of the objectives are as follows:

- The use of flax, as non-wood fibres, to obtain bleached pulp of high added value.
- The use of biotechnological techniques to improve bleaching with TCF sequences, by testing new natural mediators, resulting in a more environmentally friendly technology.
- The effect of diverse enzymatic treatments with xylanases and laccases in the elimination of HexA from flax pulps.
- A better understanding of the mechanisms of action of the enzyme systems on the pulp, by classifying enzymatically modified pulps to different size fibres.
- The application of alternative systems to graft phenolic compounds to flax fibres using biotechnology.

- The functionalisation of flax fibres with laccase and natural phenols, obtaining antimicrobial papers.

In order to fulfil these objectives, a ***methodological approach*** was adopted. Thus, preliminary tests were conducted with **natural mediators** in order to compare them with HBT. Once the best natural mediator had been identified, it was applied to a complete **TCF sequence** LQPo, and compared with other potential sequences. Furthermore, the introduction of a **xylanase** pre-treatment was also investigated. Special importance was given to the evolution of pulp properties, effluent load and the evolution of hexenuronic acids throughout the diverse treatments.

In the second part of this study, various enzyme treatments were assessed with a view to selecting the best in terms of **grafting** capacity. Some analytical studies were carried out to elucidate the coupling mechanism. Moreover, **antimicrobial activity** was determined on fibres functionalised with laccase.

The different methods and techniques used in this work facilitated an understanding of the action of the modifying agents and enzyme systems used.

The ***benefits*** derived from the results can have an immediate industrial impact as they are bound to enable the manufacture of special paper of high added value and the bleaching of pulp using less polluting, more environmentally benign processes. The benefits are also sure to have a favourable impact on enzymes and mediator manufacturers and suppliers.

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Chapter 2

MATERIALS AND METHODS

2.1 Raw material

The raw material used in this thesis is flax pulp supplied by the firm CELESA (Tortosa, Spain). The original plant material used to produce the pulp consists of a mixture of bast and core fibres, and is cooked with NaOH-AQ (sodium hydroxide and anthraquinone) by CELESA. The unbleached pulp samples received are washed with H_2SO_4 at pH 4 to remove impurities, reduce the content in metal ions and adjust the pH to the requirements for the enzymatic stage. The pulp is supplied in three different batches, so the initial properties of the samples are different. Its properties are shown in Table 2- 1.

Flax-a is used for the studies of laccase stability in Chapter 3, whereas flax-c is used in Chapter 6 (fibre fractionation). Flax-b is used for the rest of assays.

Table 2- 1. Initial properties of the different pulps used.

Raw material	Kappa number	Brightness (%ISO)	Viscosity (ml/g)
Flax-a	11.0	33.6	970
Flax-b	7.0	38.8	816
Flax-c	10.5	40.1	783

2.2 Enzymes

2.2.1 Laccases

Two different laccases are used in this thesis. In most of the experiments, the laccase from *Pycnoporus cinnabarinus* (PcL) is applied. PcL is produced by INRA (Marseille) from the monokaryotic hyperproducing strain ss3 (Herpoël et al. 2000). In Chapter 4 a laccase from *Myceliophthora thermophila* (MtL) is also applied; MtL is a commercial laccase produced by Novozymes® in Denmark. Laccase activity is followed by measuring the ABTS oxidation in 0.1 M sodium acetate buffer (pH 5) at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}). One activity unit is defined as the amount of laccase that transforms 1 μmol/min of ABTS at 25ºC.

2.2.1.1 Mediators and phenolic compounds

The synthetic mediators used are: 1-hydroxybenzotriazol or HBT and violuric acid or VA, whereas the natural mediators used are: syringaldehyde or SA, acetosyringone or AS, p-coumaric acid or PCA and methyl syringate or MS. All these phenolic compounds have been supplied by Sigma-Aldrich except MS that has been supplied by Novozymes®.

2.2.2 Xylanase

A commercial xylanase is used (Pulpzyme® HC). It is supplied by Novozymes®. One unit of enzymatic activity is defined as the amount of enzyme that transforms 1 μmol of xylane reducing sugar (measured as xylose equivalents) per minute, at pH 5 and 50ºC.

2.3 Biobleaching assays

2.3.1 Laccase stability assays

In Chapter 3, *P. cinnabarinus* laccase (200 mU/mL initial activity) is incubated in 50 mM sodium tartrate buffer during 5 h at pH 4 containing a 1.65 mM concentration of mediator at 30, 40, 50 and 60ºC to determine its stability at those temperatures. Laccase activity is also followed during 5 h in the presence of 0.83 mM mediator at

50ºC. Control tests are conducted by using laccase under the same conditions in the absence of mediator. The effect of the pulp is evaluated by monitoring laccase activity (initial value = 20 U/g pulp) for 5 h in the presence of the mediator at a rate of 3% w/w (equivalent to 1.65 mM) and its absence, using 1 g (dry weight) of pulp at 1% odp consistency in sodium tartrate buffer (pH 4) at 30, 40, 50 or 60ºC. Laccase activity is also examined in the presence of a low mediator concentration (1.5% w/w) at 50ºC. Tween 80 from Sigma is used at 0.05% w/v as surfactant in all tests. Residual activity values are expressed as percentages of initial activity, which is measured at the outset (time 0) of the incubation period. All treatments are carried out in 500 mL flasks under atmospheric O_2 in a thermostatic shaker.

2.3.2 Laccase treatments (L stage)

Biobleaching assays are carried out in Chapter 3 and Chapter 4. In the first Chapter, PcL is used in combination with the natural mediators SA, AS, PCA and the synthetic one HBT; two different rates mediator dosages are used: 1.5 a 3% w/w. In Chapter 4, PcL is applied with SA and VA at 1.5% w/w, whereas MtL is applied with MS at 1.5% w/w. Treatments L are performed at 3% consistency and pH 4 with a 50 mM sodium tartrate buffered solution (for PcL treatments) or pH 7 with 50 mM sodium phosphate buffered solution (for MtL treatments).

Figure 2- 1. Pressure reactor.

The assays are carried out in the pressure reactor (Figure 2- 1) at 0.6 MPa, at 50ºC and 60 rpm stirring; 0.05% w/v Tween 80 is added as a surfactant. After stage L, the liquors are recovered and the pulp is rinsed twice with decalcified water and, finally, once with distilled water. In Chapter 7, flax pulp is treated with PcL and VA at the same conditions that the adopted in Chapter 4. Control treatments are performed in the absence of mediator for comparison.

2.3.3 Xylanase treatment (X stage)

The X treatment is performed in polyethylene bags in a water thermostatic bath, using 3 U/g odp of Pulpzyme® HC in Tris-HCl buffer (pH 7), at 5% consistency, at 50ºC for 2 hours. The pulp is manually stirred every 20 minutes. When the reaction time is completed, the pulp is filtered collecting the remaining liquors. Next, the pulp is washed three times with decalcified water and, finally, with distilled water. The pulp is chopped and its properties determined.

2.4 Bleaching sequence

In Chapter 3, the bleaching sequence consists in two stages: LP. In Chapter 4, the L stage is followed by a Q stage and a Po stage using pressure (LQPo sequences); furthermore, an X pretreatment is inserted in one of the sequences (XLQPo sequence).

2.4.1 Chelating stage (Q stage)

A Q stage is carried out in Chapter 4. This step involves the use of a chelating agent to reduce the content in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) liable to degrade the bleaching agents and cellulose during the peroxide bleaching treatment. Application conditions are shown in Table 2- 2.

This treatment is carried out in a thermostatic bath in polyethylene bags at pH 5-6. The pulp is manually stirred every 20 minutes. When the reaction time is completed, the pulp is filtered collecting the remaining liquors. Next, the pulp is washed three times with decalcified water and, finally, with distilled water. The pulp is chopped and its properties determined.

Table 2- 2. Conditions of application of various stages of bleaching.

	Q	P	Po
Consistency (%)	5	5	5
Temperature (ºC)	85	90	90
Time (hours)	1	2	4
H_2O_2 (% odp)	-	3	1+1+2
NaOH (% odp)	-	1.5	1.5
DTPA (% odp)	1	1	0.3
$MgSO_4$ (% odp)	-	0.2	0.2

2.4.2 Hydrogen peroxide treatment (P or Po stage)

A hydrogen peroxide stage is carried out in Chapter 3 and Chapter 4. The conditions of both stages are very similar. In Chapter 3, a P stage is conducted with small amounts of pulp in the absence of pressure; these treatments are performed in the individual oscillating reactor Easydye AHIBA of Datacolor (Figure 2- 2).

Figure 2- 2. Reactor Easydye AHIBA of Datacolor.

This equipment allows carrying out several treatments at a time. Pulp and the necessary reagents are added at the pre-determined conditions. Once the reaction time is elapsed,

reactors are cooled with cold water. Chapter 4: Using pressure (stage P_0) called for increased amounts of pulp (25 g dry mass or more). Tests are conducted in the pressurized reactor (0.6 MPa) under stirring. Application conditions for both stages are shown in Table 2- 2. Po is performed in 3 steps (t_1 = 1h, t_2 = 1h, t_3 = 2 h), each including the addition of 1% odp H_2O_2. Finally, the pulp is filtered, liquors are collected and the pulp is washed three times with decalcified water and finally, with distilled water.

2.5 Biografting assays

Different enzyme treatments are carried out in order to graft phenolic compounds onto the fibres.

2.5.1 Enzyme treatments

First signals of grafting are observed after laccase-natural mediators treatments in Chapter 3. Further studies are done to explore the coupling mechanism. Application conditions of the biografting assays are reproduced in Table 2- 3. Treatment conditions have changed during the thesis in order to increase the coupling of the phenolic compounds onto the fibres or to adapt to the amount of sample needed. Laccase-grafting treatments are carried out in the individual oscillating reactor Easydye AHIBA of Datacolor (assays performed in absence of pressure) or in a pressurised reactor (0.6 MPa). In all cases, PcL is used as laccase. Tween 80 (0.05% w/v) is added as a surfactant.

Table 2- 3. Conditions of application of the biografting assays.

	Chapter 5	Chapter 6
Pulp amount (g odp)	10	100
Consistency (%)	5	4
Temperature (ºC)	50	50
Time (hours)	4	4
Mediator dose (% odp)	3,5	3,5
Laccase (U/g opd)	40	40
Pressure (MPa)	-	0,6

2.5.2 Soxhlet extraction

Pulp extractives are eliminated with acetone in a Soxhlet extractor apparatus (Figure 2-3). 2 g odp of pulp are added on a cellulose extraction thimble and placed in the extractor. 50 ml of acetone are added in a flask and assembled to the extractor. Acetone is allowed to boil (140ºC) during 135 min. Finally, acetone is recovered and the solvent in the pulps is evaporated.

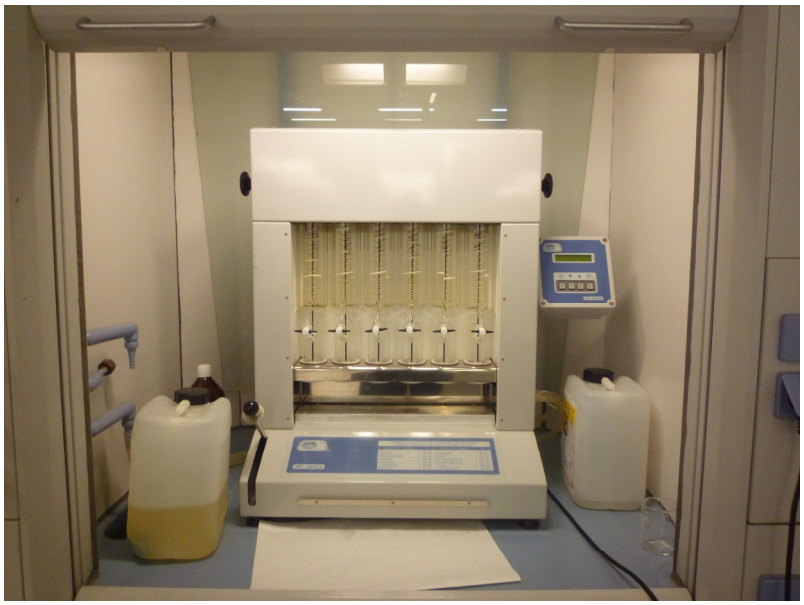

Figure 2- 3. Soxhlet extraction apparatus.

2.6 Pulp characterisation

2.6.1 Kappa number

The pulp is characterised in terms of kappa number following ISO 302:2004. Some modifications are made due to the special morphology of flax pulp: the amount of pulp sample used is 0.8 g odp, moreover, 600 ml of de-ionized water are added to sample vessel before starting the titration with sodium thiosulphate.

2.6.2 Brightness and other optical properties

Brightness is measured following ISO 3688:1999. A Technydyne PC Color Touch (Figure 2-4) is used. Some modifications are executed due to the special morphology of the flax fibres: the amount of sample to make the handsheets is 0.8 g odp and the pulp suspension is firstly filtered at atmospheric pressure with slight stirring to assure a proper formation. Finally, the sample is vacuum-filtered, pressed and dried in the handsheet former at 90ºC during 5 minutes.

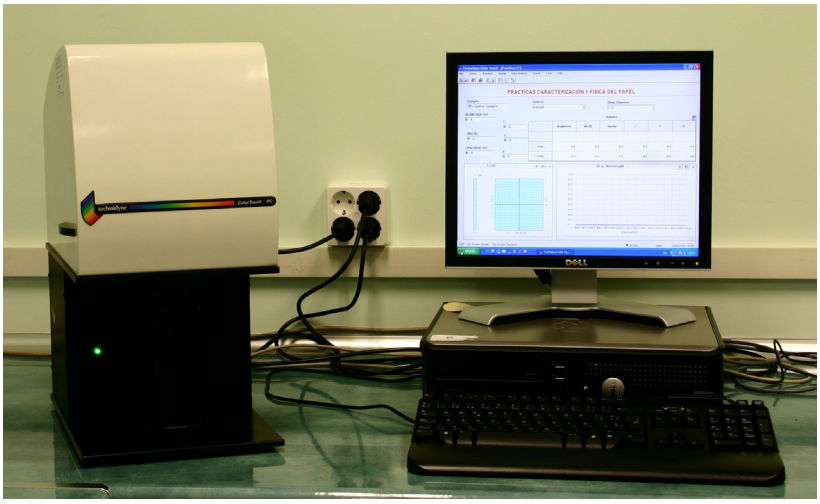

Figure 2- 4. Technydyne PC Color Touch.

The optical properties of pulp are analyzed using the same apparatus at standard illuminant D65 (LAV/Spec.Excl., d/8, D_{65}/10º). The color of the samples is described according to the CIEL*a*b* color system, where L*, and a* and b* are the coordinates of the color in the cylindrical colour space (Figure 2-5), based on the theory that colour is perceived as L* (Lightness, which varies from 100 for a perfect white to 0 for absolute black), a* (which varies from greenness to redness), and b* (which varies from blueness to yellowness, from negative to positive values) (Hunt 1998). The reflectance spectra of handsheets are obtained from scattering (s) and absorption (k) coefficients using the Kulbelka-Munk theory (Dence and Reeve 1996).

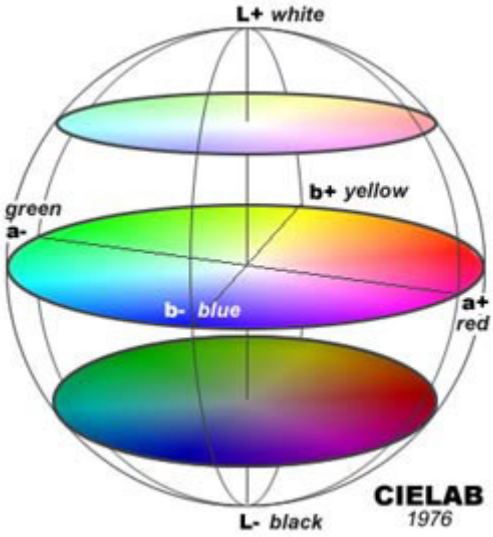

Figure 2- 5. CIEL*a*b* colour space.

Other optical parameters used are:

- Chroma (C*):

Perpendicular distance from lightness axis (Eq. 2-1), measure of color saturation,

$$C^* = (a^{*2} + b^{*2})^{1/2} \qquad \text{Eq. 2- 1}$$

- Dye Removal Index (DRI) (Fluet and Shepperd 1997):

The percentage of original color removed by the treatment (Eq. 2-2/4),

$$DRI = -100[\Delta R^2/R_1^2] \qquad \text{Eq. 2- 2}$$

where,

$$R^2 = a^2 + b^2 + (100 - L)^2 \qquad \text{Eq. 2- 3}$$

Is the geometric distance from the pulp CIEL*a*b* location to the ideal bleach point where a* = b* = 0, and L*= 100,

$$\Delta R^2 = R_2^2 - R_1^2 = R^2 \text{ (for treated pulps)} - R^2 \text{ (for reference pulp)} \qquad \text{Eq. 2- 4}$$

- Bleaching Index (BI):

Considers the volume of the cone formed by the height ($100 - L_1$) and the radius of the base equal to the square root of ($a_1^2 + b_1^2$), and compares it to the corresponding volume formed after bleaching (Eq. 2-5/7).

$$BI = 100 (V_1 - V_2) / V_1 \quad \text{Eq. 2- 5}$$

where,

$$V_1 = (100 - L_1) (a_1^2 + b_1^2), \text{ and} \quad \text{Eq. 2- 6}$$

$$V_2 = (100 - L_2) (a_2^2 + b_2^2) \quad \text{Eq. 2- 7}$$

Pulps treated with laccase alone (in the absence of phenolic compounds) are used as reference, therefore positive values represent colour removal and negative ones represent colouration.

2.6.3 Viscosity

ISO 5351:2010 is followed to determine viscosity. Due to the flax morphology some modifications are introduced: reaction time between pulp and cupriethylenediamine is at least 20 hours and shaking time of the sample before viscosity measurement is 4 hours.

2.6.4 Hexenuronic acids (HexA)

The method used for the quantitative determination of hexenuronic acids (HexA) in pulp is based on the procedure suggested by Li (Chai et al. 2001; Gellerstedt and Li 1996; Valls 2008) in which the analysis is carried out by UV spectroscopy after a HexA hydrolysis by sodium acetate and mercury acetate.

2.6.5 Carbohydrate analysis by HPCL

The sugar composition of the pulp samples is determined by high performance liquid chromatography (HPLC) following Soxhlet extraction with acetone to remove extractives. The samples are ground to a particle size < 0.5 mm and two aliquots from each hydrolysed with sulphuric acid in two steps, namely: (*a*) *pre-hydrolysis with*

concentrated sulphuric acid, using an amount of *ca.* 50 mg of sample that is impregnated with 5 ml of 72% sulphuric acid in a test tube which is placed in a thermostatic shaker at 30ºC for 1 h; and (b) *final hydrolysis with dilute sulphuric acid*, using distilled water to wash the test tubes and dilute the samples to a final sulphuric acid concentration of 4%. The collecting flasks are then placed in an autoclave at 121ºC for 60 min. Before HPLC analysis, the solid residue from the post-hydrolysis process is recovered by filtration and taken to represent Klason lignin (*viz.* lignin insoluble in sulphuric acid). The high performance liquid chromatograph used is fitted with a refractive index detector. The chromatographic determination is performed with an Agilent 1100 HPLC instrument furnished with a column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 0.006 mol/L sulphuric acid; flow rate, 0.6 mL/min; column temperature, 60ºC. Measurements are interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from Sigma-Aldrich). Because the column fails to resolve xylose, manose and galactose, their combined content is expressed as xylose (Garrote et al. 2001).

2.6.6 ATR-FTIR spectroscopy

FTIR spectra for handsheets made from cellulose fibre samples in accordance with ISO 5269 are recorded on a Nicolet Model 6700 FTIR spectrometer equipped with a Smart Orbit ATR (attenuated total reflectance) accessory fitted with a diamond crystal. Spectra are recorded with a resolution of 4 cm^{-1} over the wavenumber range 4000–400 cm^{-1}, using 64 scans per sample. The relative cellulose crystallinity is assessed through the intensity peak ratios $A_{1375}/_{2902}$ (Nelson and O'Connor 1964), whereas for relative order the ratio is $A_{1427}/_{898}$ (Focher et al. 2001).

2.6.7 Antimicrobial activity

These analyses have been performed at the Department of Microbiology of the University of Barcelona in Barcelona, Spain. They have been carried out to determine the antimicrobial activity of the grafted pulps and the results are shown in Chapter 5.

2.6.7.1 Antimicrobial properties of the natural phenols

The antimicrobial properties of some natural phenols studied are tested on three microorganisms: *Staphylococcus aureus* (Gram+), *Pseudomonas aeruginosa* (Gram-) and *Klebsiella pneumoniae* (Gram-). The bacterial strains are inoculated in 5 ml of LB medium supplemented with increased concentrations, 0 to 25 mM, of the natural phenols and the cultures are incubated overnight at 37ºC (Figure 2-6). After overnight incubation, OD_{600nm} is measured as an estimation of the growth of the bacterial strain. The percentage of growth inhibition is calculated using the following formula (Eq. 2-8):

$$\text{Growth inhibition } \% = 100 - \left(\frac{I}{B} \times 100 \right) \qquad \text{Eq. 2- 8}$$

where: $I = OD_{600nm}$ of the culture of a bacterial strain with a natural phenol at a given concentration; $B = OD_{600nm}$ of the culture of the same strain without added phenol.

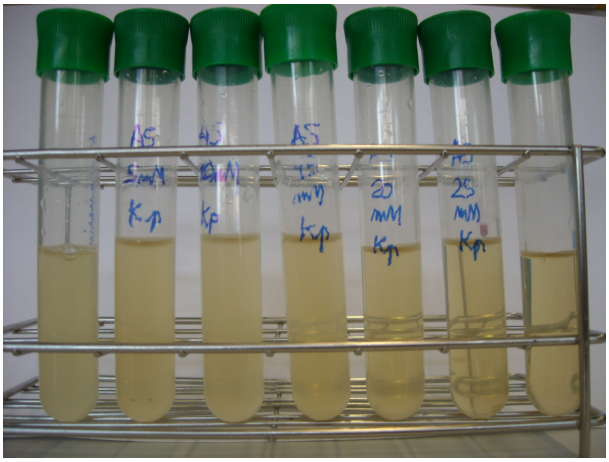

Figure 2- 6. Study of antimicrobial properties of natural phenols.

2.6.7.2 Antimicrobial properties of laccase-phenols treated paper

Antimicrobial activity test of the papers treated with laccase and phenol is based on the ASTM Standard Test Method E 2149-10. Overnight shaken cultures of *Staphylococcus aureus* (Gram+), *Pseudomonas aeruginosa* (Gram-) or *Klebsiella pneumoniae* (Gram-)

are diluted in KH_2PO_4 buffer (working bacteria dilution). 1 g odp of the treated paper handsheets or control papers cut into small portions are added to flasks containing 50 ml of working bacterial solution and shaken 200 rpm for 1 h at 37ºC. Samples are taken before or after 1h incubation with papers, and bacterial concentration as colony forming units (CFU/ml) is determined by standard viable plate count in TGE Agar (from Scharlau). The percentage of reduction in CFU is calculated using the following equation (Eq. 2-9):

$$\text{CFU/ml Reduction (\%)} = \frac{B - A}{B} \times 100 \qquad \text{Eq. 2- 9}$$

where: A = CFU/ml after 1 h contact time; B = CFU/ml before the contact.

The presence of antimicrobial leaching from treated pulps is determined by evaluating the production of growth inhibition haloes on agar plates inoculated with the microorganisms studied. Treated paper samples (1 g odp) are added to 50 ml of sterile buffer solution and shaken in flasks at 200 rpm for 1 hour at 37ºC. 100 µl of these samples are poured into 8 mm diameter holes made in the centre of TGE agar plates previously inoculated with a confluent lawn of the bacteria studied ($1x10^5$ CFU/ml) and incubated at 37°C overnight. Finally, the presence of a zone of inhibition surrounding the agar holes is recorded.

2.6.8 Pyrolysis analytical conditions

Py–GC/MS analyses are performed by the IRNAS (Institute of Natural Resources and Agrobiology) of the CSIC in Seville, Spain.

Pyrolysis of pulps (approximately 1 mg) is performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a DB-5MS (Agilent J&W) fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 µm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis is performed at 500ºC. The oven temperature is programmed from 40ºC (1 min) to 300ºC at 6ºC min^{-1} (10 min) and the carrier gas (He) is set at 1 ml·min^{-1}. In addition, pulp samples are analysed by pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), as a base and methylating reagent. For the Py/TMAH, 1 mg of pulp sample are mixed with approximately 0.5 µL TMAH (25%,

w/w, aqueous solution) and the pyrolysis is carried out as described above. The compounds are identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature (Faix et al. 1990; Ralph and Hatfield 1991).

2.6.9 Pulp fibre fractionation

A laboratory Bauer-MacNett classifier (Figure 2-7) with screen sizes of 30 and 200 mesh is used to separate long and short fibres of flax pulp according to TAPPI T233-cm-06. For fibre separation, 10 g of pulp is disintegrated in 3,000 mL of water and poured in the first chamber. A continuous flow of water passes through this chamber. The short fraction passed this first chamber is collected in the screen of 200 mesh. The fraction passing through the screen with the 200 mesh sieve is considered as fines. After 20 min of operation the flow of water is stopped and the two fractions are recovered and the content of each fraction is determined.

Figure 2- 7. Bauer-MacNett classifier.

2.6.10 Morphological properties of fibres

2.6.10.1 Fibre length analysis

Morphological properties of fibres such as fibre length, fibre width, and content in fines are determined with a Kajaani FS300 fibre analyser (Figure 2-8). TAPPI T271-om-07 method is followed to measure these properties. Measurements are based on the ability of the fibres to change the direction of polarized light. The FS300 is used extensively in the paper and pulp industry because it is fast and simple to use.

20 mg of pre-soaked sample are added to 1 L of de-ionized water and put in an automatic disintegrator (30,000 rev.). After making sure that fibres are properly separated, and the no fibre bundles or fibre-to-fibre bonds remains in the test specimen, samples are diluted by using de-ionized water until obtain a final volume of 4 L. 50 mL of the dilution are taken using a pipette with a tip opening of al least 2 mm in diameter. When the sample is taken, the dilution must be agitated continuously (by moving the pipette both horizontally and vertically during sampling).

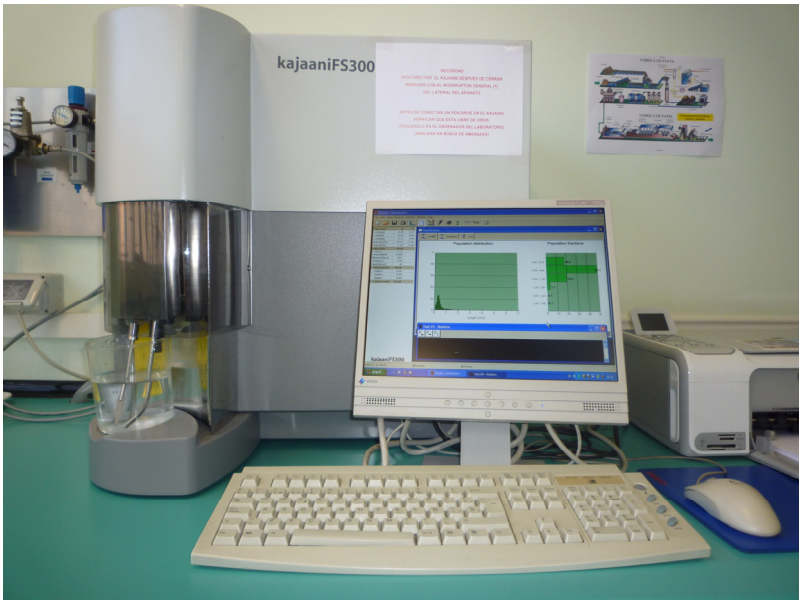

Figure 2- 8. Kajaani FS300 fibre analyser.

Two different kinds of average are calculated; L(n) or numerical average length (Eq. 2-10) and L(l) or length-weighted average length (Eq. 2-11), defined as:

$$L(n) = \frac{\sum_{i=1}^{N} n_i \, l_i}{\sum_{i=1}^{N} n_i} \qquad \text{Eq. 2- 10}$$

$$L(l) = \frac{\sum_{i=1}^{N} n_i l_i^2}{\sum_{i=1}^{N} n_i l_i} \qquad \text{Eq. 2- 11}$$

where,

n_i, is the number of fibres measured in different length fraction,

l_i, is the average length of the fraction,

i, is the total number of fractions,

N, is the maximum number of fractions greater or equal to 144.

Fibre width is calculated following the same equations (Eq. 2-10 and Eq. 2-11) but changing length for width.

2.6.10.2 Optical microscopy analysis of fibres

Individual fibres are analysed by optical microscopy, according to ISO 9184-3:1990. Images are captured with a DeltaPix (Infinity X) digital camera integrated on a microscope. DeltaPix Viewer Software is used to digitalize the pictures taken. The procedure used is: individual fibres in a small amount are stained with 2 to 3 drops of Herzberg stain (a solution of zinc chloro-iodide); the stained fibres are placed under a microscope and are examined using a magnification of x40 and x100.

2.6.11 Thermogravimetric analysis

This analysis has been performed at the Department of Chemical Engineering of the University of Huelva (UHU) in Huelva, Spain. Termogravimetric runs are carried out with a Mettler Toledo model TGA/SDTA85e/LF1600 on samples of around 5 mg

under synthetic air (N2:O2 4:1) environment using three heating rates (5, 10, and 20ºC/min) from 25 to 900ºC. The pyrolysis runs are carried out under nitrogen atmosphere (García Barneto et al. 2011).

2.6.12 X-ray diffraction

This analysis has been performed at the Department of Chemical Engineering of the University of Huelva (UHU) in Huelva, Spain. X-ray diffraction patterns for dry pulp samples pressed into tablets are obtained on a Bruker D8 Advance diffractometer using Ni-filtered Cu K_α radiation (λ = 0.1542 nm) generated at 40 kV and 20 mA. The scanned range is from 2θ = 5º to 50º. Experimental XRD signals are fitted to Gaussian peaks, which include an amorphous background. Pulp crystallinity is determined as the ratio of the surface under the crystalline cellulose peaks to the total surface, which includes the amorphous background contribution. The equatorial dimension of crystallites is determined from the (2,0,0) reflection. The full width at half maximum (FWHM) of the diffraction peaks is used to determine crystallite width, B_{hkl}, using the Scherrer equation (Eq. 2-12):

$$B_{hkl} = \frac{0.94\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta_0)^2}\cos\theta} \qquad \text{Eq. 2- 12}$$

where λ is the X-ray wavelength, $\Delta 2\theta$ the FWHM of each peak, $\Delta 2\theta_0$ the apparatus broadening parameter and θ the Bragg angle.

The crystallinity index (CI) is obtained by using the method of Segal et al. (1959) (Eq. 2-13):

$$CI = (1 - I_{am}/I_{002})100 \qquad \text{Eq. 2- 13}$$

where, I_{am} is the intensity of the minimum between the 002 and 101 peaks (approximately at 2θ = 19º), and I_{002} that of the maximum for the 002 peak (approximately at 2θ = 23º).

2.6.13 ^{1}H NMR analysis of models experiments

This analysis has been performed at the Wood Chemistry and Pulp Technology Department of the Royal Institute of Technology (KTH) in Stockholm, Sweden. In

model studies, pulp xylan isolated from the TCF-bleached eucalypt pulp by extraction with DMSO is used for laccase treatments. Xylan is treated with 40 U/g of laccase together with 4.0% of PCA in 50 mM tartrate buffer at pH 4. After 4 h of treatment at 50ºC the sample is filtered and washed throroughly before drying under vacuum at room temperature. ^{1}H NMR spectra of the isolated xylan before and after laccase-PCA treatment are recorded after dissolution in 10% NaOD in D_2O on a Bruker Avance 400 MHz instrument using the standard Bruker pulse program at room temperature.

2.7 Effluent characterization

Effluents are characterized in terms of chemical oxygen demand (COD) and colour, by following ASTM D1252-06 and ASTM D1209-05(2011) methods, respectively. Toxicity and residual laccase activity are also determined. Moreover, thin layer chromatography tests are carried out after some enzymatic treatments in Chapter 4.

When necessary, parameters are determined by using a Shimadzu UV-1603 or a Thermo Scientific Evolution 600 spectrophotometer. Results are corrected with the dilution factor of each sample. In each stage, the final pH of effluents is also measured.

2.7.1 Thin layer chromatography

For thin layer chromatography tests, 200 µl dropwise of each effluent is applied, using a 10 µL pipette, on a silica gel plate which constitutes the solid phase. Next, 10 µL of the Xyloses standard mixture and 5 µL of the Glucoses standard mixture are applied on the same plate. The Xyloses standard mixture is formed by: X1 (xylose); X2 (xylobiose); X3 (xylotriose); and X4 (xylotetraose). The Glucoses standard mixture is formed by: G1 (glucose) and G2 (cellobiose). After that, the plate is introduced in a glass cuvette that contains the mobile phase, constituted by chloroform, glacial acetic acid and H_2O in a 6:7:1 ratio, respectively. The plate is eluted until a height of 5 cm below the upper edge. Next, the plate is removed and let air-dry in the hood until all eluent is evaporated; repeat the elution twice. Afterwards, the silica gel plate is sprayed with the developing solution (spray Fungilab), which is a 5% solution of H_2SO_4 in ethanol. Heat in the oven at 100ºC for a minimum of 5 minutes that can be extended for up to 1 hour. The various compounds dissolved in the effluent can be identified by comparison with the standards, after development of the plate.

2.7.2 Toxicity

Effluent toxicity is determined with the Microtox method, using the marine luminescent bacterium *Vibrio fischeri* in a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental) in accordance with the UNE-EN ISO 11348-3:2009 standard. This test is based on the difference before and after contact with a toxic substance. In order to prevent pH effects, each sample is adjusted to pH 6-8 with an NaOH solution. Ecotoxicity is 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) or equitox/m^3. The reference toxicant $ZnSO_4 \cdot 7H_2O$ is used to control *V. fischeri* batch quality in accordance with the Basic Test Procedure. Toxicity measurements are colour-corrected as per the recommendations of the equipment manufacturer.

2.7.3 UV-visible absorbance spectrum

The effluents from the enzymatic treatments (Chapter 5) are diluted to 1:20 and their absorbance measured between 200 and 400 nm.

The test consists in obtaining the UV-visible absorbance spectrum of the effluents between 190 nm and 900 nm wavelengths, in an UV spectrophotometer (Shimadzu model UV-1603).

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Chapter 3

A NEW APPROACH TO THE BIOBLEACHING OF FLAX PULP WITH LACCASE USING NATURAL MEDIATORS

SUMMARY

The phenols syringaldehyde (SA), acetosyringone (AS) and *p*-coumaric acid (PCA) were used as natural laccase mediators in combination with a laccase from *Pycnoporus cinnabarinus* to bleach flax fibres. Their performance was compared with 1-hydroxybenzotriazole (HBT) in terms of enzyme stability, and pulp and effluent properties. The potential inactivation of laccase in the absence of pulp by HBT and the natural mediators was examined, and HBT and PCA found to inactivate the enzyme. In the presence of unbleached flax pulp, however, the enzyme exhibited substantially increased stability; thus, with PCA and unbleached pulp, laccase retained 77% of its initial activity, in contrast with the complete inactivation in the absence of pulp. This suggests a protective effect of the pulp against denaturalization of the enzyme. All natural mediators resulted in a reduced kappa number after the subsequent alkaline treatment with hydrogen peroxide; the reduction being especially marked with SA (about 2 units- with respect to the control sample) and comparable to that obtained by HBT. Brightness was significantly increased by all natural mediators, but especially by AS and SA (23% with both), which performed very similarly to HBT in this respect. Natural mediators therefore might constitute an effective alternative to synthetic mediators for flax pulp biobleaching. This study demonstrates for the first time the use of natural mediators in the laccase-assisted delignification of flax pulp and their effect on the properties of the resulting effluents.

3.1 Introduction

The paper industry has undergone marked changes over the past decades, particularly as regards the pulp bleaching section. Environmental pressure has fostered the reduction or total avoidance of chlorine and chlorine-containing by-products in bleaching processes. Obtaining TCF (Totally Chlorine Free) pulps entails using chemical reagents such oxygen, hydrogen peroxide or ozone; however, these combinations are less efficient than chlorination. The use of enzyme systems in TCF bleaching sequences appears to be a good alternative here (Kenealy and Jeffries 2003; Roncero 2001). In fact, enzymes have high potential for improving traditional pulp manufacturing processes by virtue of their high specificity and environmental friendliness. Several studies have shown the use of xylanases in various bleaching sequences to reduce consumption of chemicals and contamination (Bajpai 2004; Roncero et al. 2003; Roncero et al. 2005; Valls and Roncero 2009). Lignin-oxidizing enzymes (*viz.* lignin peroxidases, manganese peroxidases, versatile peroxidases and laccases) directly attack the lignin structure of pulp; as a result, these oxidative enzymes improve the efficiency of kraft pulp bleaching (Bajpai 2004; Call and Mücke 1997; Martínez et al. 2005). The combination of a fungal laccase and a redox mediator, which constitutes a "laccase-mediator system", was first reported 20 years ago (Bourbonnais and Paice 1990), and affords the bleaching of various types of pulp thanks to its ability to oxidize phenolic and non-phenolic lignin (Li et al. 1998; Sigoillot et al. 2004). An ideal redox mediator must be inexpensive and a good laccase substrate, its oxidized and reduced intermediates forms must be stable but must not inhibit the enzymatic reaction. In addition, its redox conversation must be cyclic and without side reactions. However, the mechanism of these processes is insufficiently understood in many cases (d'Acunzo and Galli 2003; Morozova et al. 2007).

Sigoillot et al. (2005) compared various fungal enzymes in the bleaching of high-quality paper pulps and found the laccase-mediator system to possess the highest pulp delignification potential. Also, the joint use of laccase from *Pycnoporus cinnabarinus* and the mediator HBT has proved a highly efficient choice for delignifying flax pulp (Camarero et al. 2004; García et al. 2003; Fillat and Roncero 2009a).

Since natural mediators are phenols which can be readily obtained from pulping liquors (Gutiérrez et al. 2007), effluent streams (Ismail et al. 2005) and plant materials, their

use can provide major environmental and economic advantages. Thus, lignin-derived phenols used as laccase mediators have been found to perform similarly to or even better than the best synthetic mediators, with increased activity and more modest useful rates (Johannes and Majcherczyk 2000). These natural mediators take part in the natural degradation of lignin by white-rot fungi and may be derived from oxidized lignin units or, directly, from fungal metabolism (Eggert et al. 1996; Johannes and Majcherczyk 2000). Some lignin-derived phenols mediate laccase decolourization of dyes (Camarero et al. 2005), oxidation of polycyclic aromatic hydrocarbons (Johannes and Majcherczyk 2000), removal of lipophilic extractives (Gutiérrez et al. 2007) or delignification of paper pulp from wood (Camarero et al. 2007; Moldes et al. 2008) and non-wood fibres (Aracri et al. 2009).

Wood is by far the main raw material for paper pulp in developed countries. However, non-wood plants are being widely used for this purpose in Asia and some countries such as Spain in order to exploit agricultural crops surpluses and meet their increasing demand for specially paper (Ashori 2006; Saijonkari-Pahkala 2001; Sigoillot et al. 2005). Oxidative enzymes have scarcely been used to bleach non-wood pulp; also, most studies in this area have focused on synthetic laccase mediators (Camarero et al. 2002; Fillat and Roncero 2009a; Fillat and Roncero 2009b; García et al. 2003).

In this work, we assessed for the first time the potential of various lignin-derived phenolic compounds (syringaldehyde, acetosyringone and *p*-coumaric acid) for delignifying flax fibres in order to identify the most efficient and ecofriendly choice among them. In addition, the lack of studies on the properties of effluents from the treatment of non-wood pulp with laccase and these natural mediators led us to examine pulp and effluent properties upon treatment with various laccase–natural mediator systems where the enzyme was obtained from *P. cinnabarinus*. The results thus obtained were compared with those provided by the synthetic mediator 1-hydroxybenzotriazole (HBT).

3.2 Materials and methods

3.2.1 Raw material

The raw material consisted of two samples of flax pulp with different delignification degree, supplied by CELESA mill (Tortosa, Spain) and obtained by soda-anthraquinone cooking. The unbleached pulp samples received were washed with H_2SO_4 at pH 4 to remove impurities, reduce the content in metal ions and adjust the pH to the requirements for the enzymatic stage. Following acid washing, pulp of kappa number 11, 33.6% ISO brightness and 970 mL/g viscosity was obtained that was used to study laccase stability. The pulp samples used in the bleaching treatments had a kappa number of 7, 38.8% ISO brightness and 816 mL/g viscosity.

3.2.2 Enzyme and laccase mediators

Pycnoporus cinnabarinus laccase (PcL) was produced by INRA (France) from the monokaryotic hyperproducing strain ss3 (Herpoël et al. 2000). Activity was followed by measuring ABTS oxidation in 0.1 M sodium acetate buffer (pH 5) at 436 nm (ε_{436} = 29300 M^{-1} cm^{-1}). One activity unit was defined as the amount of laccase capable of converting 1 μmol/min of ABTS at 25ºC. All measurements were made on a Shimadzu UV-vis 1603 spectrophotometer. The natural mediators used were acetosyringone (AS), syringaldehyde (SA) and *p*-coumaric acid (PCA), which were compared in performance with 1-hydroxybenzotriazole (HBT). All mediators were purchased from Sigma-Aldrich.

3.2.3 Enzyme stability assays

P.cinnabarinus laccase (200 mU/mL initial activity) was incubated in 50 mM sodium tartrate buffer at pH 4 containing a 1.65 mM concentration of mediator at variables temperatures (30, 40, 50 and 60ºC) for 5 h. The enzyme was also incubated in the presence of 0.83 mM mediator at 50ºC for 5 h. Control tests were conducted by using laccase under the same conditions in the absence of mediator. The effect of the pulp was evaluated by monitoring laccase activity (initial value = 20 U/g pulp) for 5 h in the presence of the mediator at a rate of 3% w/w (equivalent to 1.65 mM) and its absence, using 1 g odp of pulp at 1% consistency in sodium tartrate buffer (pH 4) at 30, 40, 50 or 60ºC. Laccase activity was also examined in the presence of a low mediator

concentration (1.5% w/w) at 50ºC. Tween 80 from Sigma was used at 0.05% w/v as surfactant in all tests. All treatments were performed in duplicate and carried out in 500 mL flasks under atmospheric O_2 in a thermostatic shaker.

3.2.4 Enzymatic bleaching of flax pulp

Delignification tests with the laccase-mediator system were carried out with 25 g (dry weight) of brown pulp at 3% odp consistency in an oxygen pressurized (0.6 MPa) reactor containing 50 mM sodium tartrate buffer (pH 4), 20 U/g of *P. cinnabarinus* laccase, the mediator at either of two different rates dosages (1.5 or 3% w/w, both relative to pulp dry weight) and Tween 80 as surfactant at 50ºC. The mixture was shaken at 60 rpm for 5 h. Pulp treated under identical conditions in the absence of either the mediator or both the enzyme and mediator was used for control purposes. Once treated, the pulp samples were filtered and residual liquor collected for subsequent analysis.

3.2.5 Hydrogen Peroxide stage

In this bleaching step, the pulp samples, at 5% odp consistency, were treated with 3% H_2O_2, 1.5% NaOH, 1% DTPA (diethylenetriaminepentaacetic acid) and 0.2% $MgSO_4$ (all relative to pulp dry weight) in a Datacolor Easydye AHIBA oscillating individual reactor at 90ºC for 2 hours. Once treated, the samples were filtered and residual liquor collected for subsequent analysis (Aracri et al. 2009; Camarero et al. 2004; Fillat and Roncero 2009b).

3.2.6 Evaluation of pulp and effluents properties

Pulps samples from laccase-mediator treatments (L) and pulp samples after laccase-mediator treatments followed by a hydrogen peroxide stage (LP) were characterized in terms of brightness, kappa number (an estimation of lignin content) and viscosity in accordance with the following standards: ISO 3688:1999, ISO 302:2004 and ISO 5351:2010, respectively. Chemical Oxygen Demand (COD) and Colour were determined in duplicate following ASTM D1252-06 and ASTM D1209-05(2011), respectively. Toxicity was measured in a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental), following UNE-EN ISO 11348-3:2009. This test is based on the difference between the amount of light emitted by *Vibrio fischeri*

bacteria before and after contact with a toxic substance. One equitox/m^3 is defined as the reciprocal of wastewater dilution that reduces light emission by 50% after 15 min of contact. Toxicity tests were conducted in duplicate.

3.3 Results and discussion

3.3.1 Influence of temperature on enzyme stability

Enzyme stability is important for most biotechnological applications. The stability of laccase could obviously have a substantial effect on the oxidation rate so it should be considered. The temperature stability of laccase from *Pycnoporus cinnabarinus* (PcL) was assessed by measuring its activity at 30, 40, 50 and 60ºC at hourly intervals for 5 h. As can be seen in Figure 3-1, the enzyme was stable at 30 and 40ºC, both in the presence of pulp and in its absence. Laccase activity increased during the first hour of incubation and then levelled off to 106% the initial value at 30ºC and 91% at 40ºC.

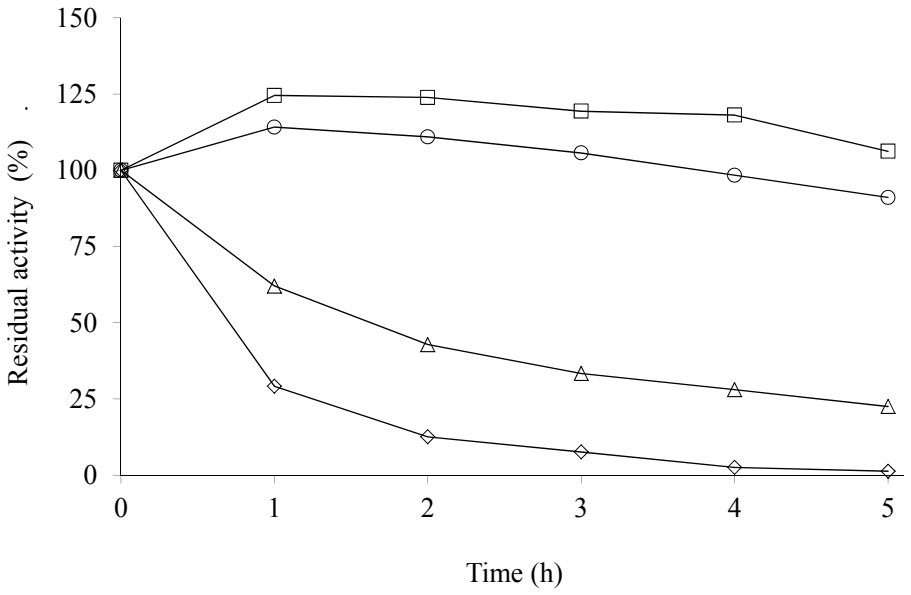

Figure 3- 1. Variation of PcL residual activity at 30ºC (□), 40ºC (○), 50ºC (△) and 60ºC (◇) in the absence of pulp over a 5 h treatment.

On the other hand, the tests conducted at 30ºC in the presence of pulp (Figure 3-2) revealed a gradual rise in activity during the first 4 h of treatment (the enzyme

exhibited 150% residual activity after that time). At 50 and 60ºC, enzyme activity decreased with time, especially in the absence of pulp —so much so that no residual activity was observed after 5 h at 60ºC. Based on the foregoing, the presence of pulp has a stabilizing effect on PcL activity.

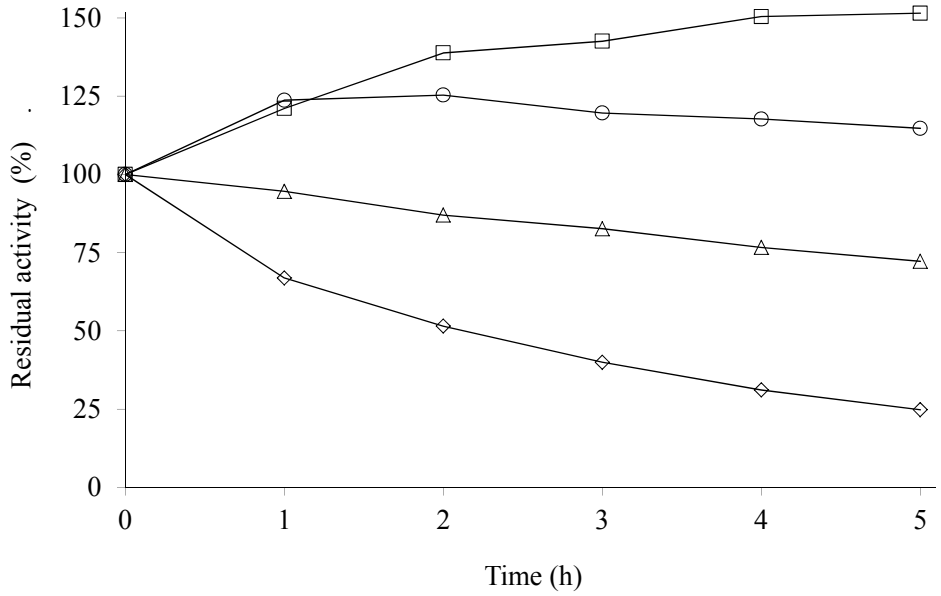

Figure 3- 2. Variation of PcL residual activity at 30ºC (□), 40ºC (○), 50ºC (△) and 60ºC (◇) in the presence of pulp over a 5 h treatment.

3.3.2 Laccase inactivation by the mediators

Laccase activity was monitored at variable temperatures (30, 40, 50 and 60ºC) in the presence of a mediator and both the presence and absence of pulp. Figure 3-3 shows the variation of the enzyme residual activity during a 5 h treatment at 50ºC (temperature of the pulp delignification assays) with a 1.65mM concentration (3% odp) of mediator. In the absence of pulp, the mediators SA and AS increased the enzyme stability and led to a higher residual activity than in the absence of mediator; the increase in activity with respect to the control sample amounted to 19% with SA and 12% with AS. On the other hand, laccase was strongly inactivated by PCA and also, to a lesser extent, by HBT. In fact, both mediators caused virtually complete inactivation of PcL; thus, HBT reduced its residual activity to 3% after 5 h and PCA to 3% after 2

h.

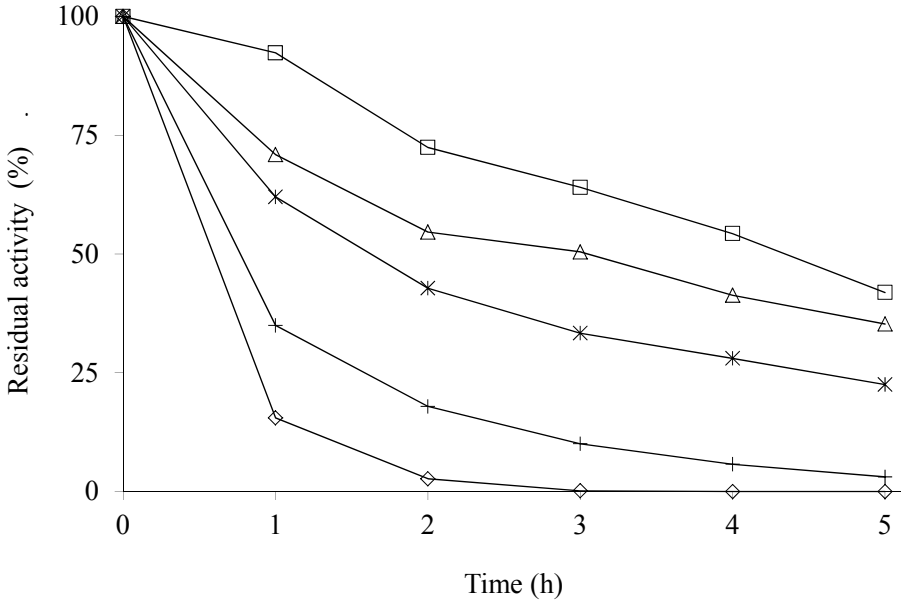

Figure 3- 3. Variation of residual activity in *P. cinnabarinus* laccase incubated at 50ºC in the absence of pulp for 5 h, and effect of the addition of various mediators: HBT (+), SA (□), AS (△) and PCA (◇) at a 1.65 mM or 3% odp concentration. Laccase and pulp-laccase (✳) are incubated in the absence of mediator.

In the presence of pulp (Figure 3-4), HBT and PCA led to residual activity levels similar to those of the control test, and AS to 10% lower values. The reduction in laccase activity was maximal with SA (the enzyme retained only 47% of its initial activity after 5 h). In any case, residual activity was higher in the presence of pulp than in its absence. Laccase activity at 30, 40 and 60°C (data not shown) in the presence of the mediators followed the same behaviour than at 50°C, although the inactivation/stabilisation rate varied depending on the temperature: at lower temperatures inactivation was slower and stabilisation was faster.

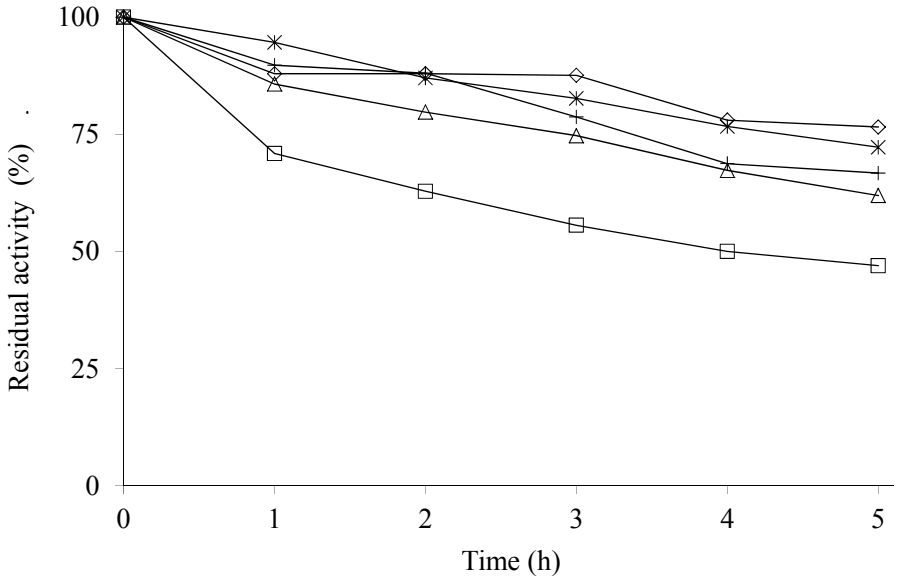

Figure 3- 4. Variation of residual activity in *P. cinnabarinus* laccase incubated at 50ºC in the presence of pulp for 5 h, and effect of the addition of various mediators: HBT (+), SA (□), AS (△) and PCA (◇) at a 1.65 mM or 3% odp concentration. Laccase and pulp-laccase (✳) are incubated in the absence of mediator.

3.3.3 Influence of the mediator rate on laccase activity

The influence of the mediators on laccase activity was examined at two different rates (1.65 mM and 0.83 mM), using an incubation temperature of 50ºC. In the absence of pulp, HBT (Figure 3-5) exhibited disparate effects at the two studied rates; thus, the lower rate stabilized the enzyme —its residual activity was 15% higher than that for the control sample—, whereas the higher rate resulted in substantial inactivation (20% relative to the control test). In the presence of pulp, however, the mediator rate had no effect on residual activity.

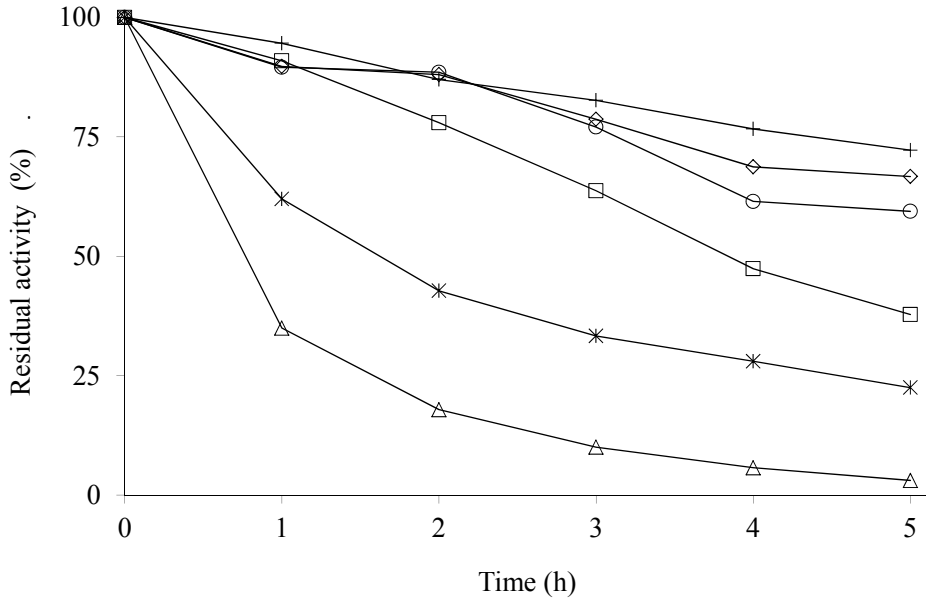

Figure 3- 5. Variation of the activity of *P. cinnabarinus* laccase during incubation in the presence of the mediator HBT. Residual activity in the absence of pulp: L-HBT at a mediator rate of 1.5% (□) or 3% (△) and laccase control without mediator (✳); and effect of the addition of pulp: Pulp+L-HBT at a mediator rate of 1.5% (○) or 3% (◇) and pulp-laccase control without HBT (+).

With 0.83 mM SA (Figure 3-6), laccase activity in the absence of pulp was similar to that for the control sample; a higher SA concentration, however, had a stabilizing effect quite similar to that observed in the presence of pulp and the enzyme residual activity amounted to 19% that in the control test. No differences in this respect between SA rates were observed in the presence of pulp, however.

The mediator AS (Figure 3-7) stabilized PcL in the absence of pulp, to a greater extent (11%) at 0.83 mM than at the higher rate. In the presence of pulp, AS led to a lower residual activity than in the control test, with no differences between mediator rates. Finally, the PCA rate (Figure 3-8) had no effect on laccase activity. Laccase is inactivated by effect of the attack of a mediator radical on susceptible amino acids at the enzyme surface (Amann 1997; Li et al. 1999).

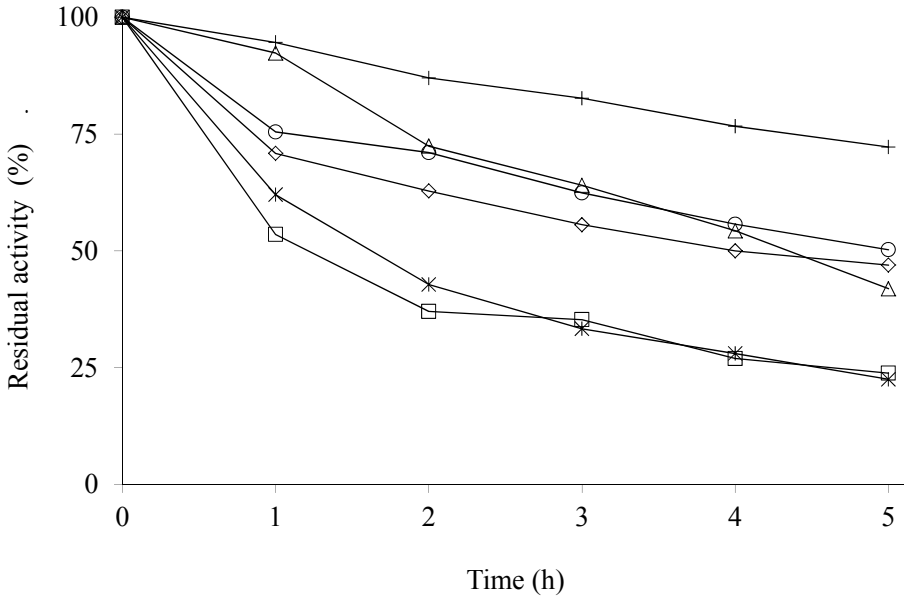

Figure 3- 6. Variation of the activity of *P. cinnabarinus* laccase during incubation in the presence of the mediator SA. Residual activity in the absence of pulp: L-SA at a mediator rate of 1.5% (□) or 3% (△) and laccase control without mediator (✳); and effect of the addition of pulp: Pulp+L-SA at a mediator rate of 1,5% (○) or 3% (◇) and pulp-laccase control without SA (+).

In this work, inactivation was only observed with HBT and PCA; in fact, SA and AS had the opposite effect (stabilizing the enzyme) in the absence of pulp, probably as a result of a lower reactivity in their radicals —both mediators possess a similar structure. A similar stabilizing effect was previously observed by Aracri et al. (2009), who found the natural mediators sinapic acid and sinapyl aldehyde to prevent denaturation of *Trametes villosa* laccase. Also, Mai et al. (2003) found some phenolic compounds acting as enzyme substrates to stabilize laccase by binding to the active sites or suitable points in the protein chain of the enzyme. Our two stabilizing mediators possess a higher affinity constant for *P. cinnabarinus* laccase than do our two inactivating mediators (Camarero et al. 2005; Li et al. 1999).

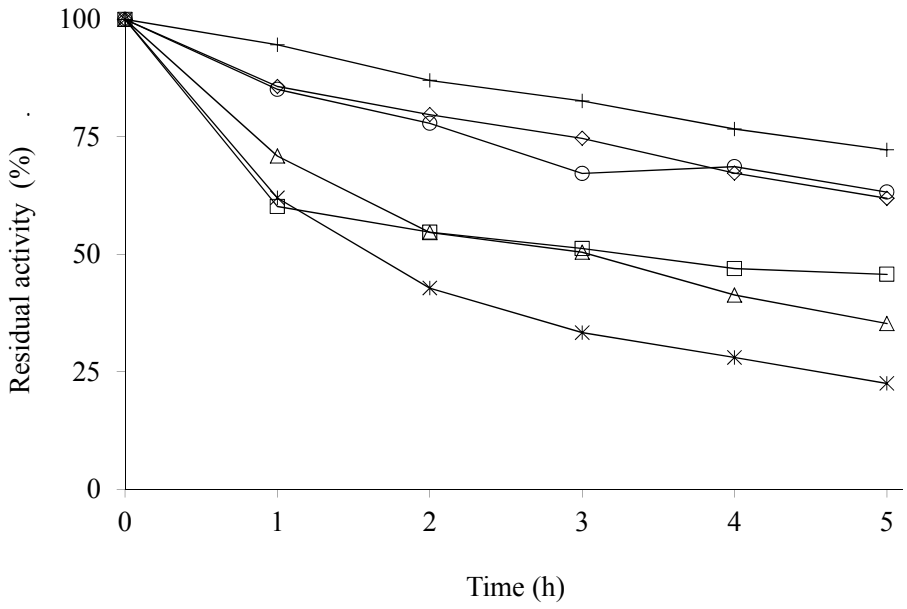

Figure 3- 7. Variation of the activity of *P. cinnabarinus* laccase during incubation in the presence of the mediator AS. Residual activity in the absence of pulp: L-AS at a mediator rate of 1.5% (□) or 3% (△) and laccase control without mediator (✳); and effect of the addition of pulp: Pulp+L-AS at a mediator rate of 1,5% (○) or 3% (◇) and pulp-laccase control without AS (+).

A protective effect of pulp on enzyme activity was observed in several studies (Ibarra et al. 2006; Sigoillot et al. 2005), that could have resulted from the pulp acting as a reductive substrate for free mediator radicals and preventing laccase inactivation as a consequence.

In this work, PCA was the only mediator not inactivating laccase in the presence of pulp. As shown in discussing pulp properties below, PCA treated pulp samples exhibited an increased kappa number suggestive of mediator condensation on the pulp. PCA condensation may have prevented the mediator free radicals from inactivating the enzyme. We observed no laccase adsorption on the pulp here, however.

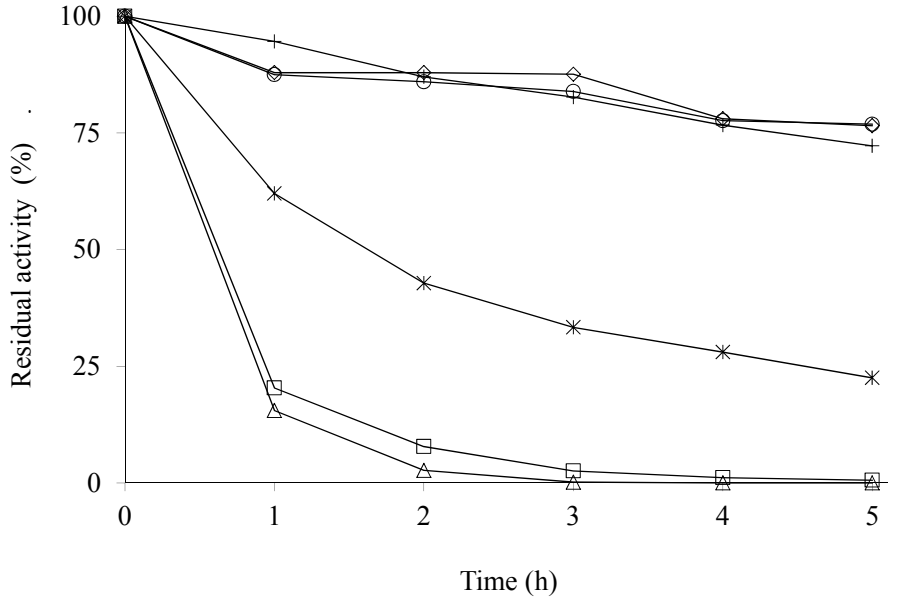

Figure 3- 8. Variation of the activity of *P. cinnabarinus* laccase during incubation in the presence of the mediator PCA. Residual activity in the absence of pulp: L-PCA at a mediator rate of 1.5% (□) or 3% (△) and laccase control without mediator (✳); and effect of the addition of pulp: Pulp+L-PCA at a mediator rate of 1,5% (○) or 3% (◇) and pulp-laccase control without PCA (+).

3.3.4 Enzymatic bleaching of flax pulp

Flax pulp was submitted to a laccase-mediator treatment (L) and a subsequent hydrogen peroxide stage (LP). Both delignification stages were followed by measurement of kappa number and brightness. Figure 3-9 illustrates the delignifying effect of the different enzyme treatments at two different rates of HBT and the natural mediators, as well as the contribution of each bleaching step to reduce the kappa number. The use of HBT and AS in the L stage reduced the kappa number by almost 2 and 1 units, respectively, with respect to the control sample; with SA, the kappa number was quite similar to the control values —treatment L-SA1.5 even led to a 0.6 units greater value. On the other hand, PCA increased the kappa number by more than 1 unit at low rates and more than 10 at high rates. This was probably a result of partial condensation of the phenoxy radical of *p*-coumaric acid on fibres (Camarero et al. 2007). The tendency of the natural mediators to couple to fibres was previously observed in sisal, the fibre surfaces of which were assumed to covalently bond the mediators (Aracri et al. 2009). These results clearly expose the need to insert a

hydrogen peroxide stage after the enzyme treatment (Fillat and Roncero 2010). In fact, all LMS treatments resulted in substantial delignification after the P stage; this was especially so with HBT, which reduced the kappa number by 0.6–1.6 units. The best results among the natural mediators were those for SA, which reduced the kappa number by 57% (approximately 2 units) with respect to the control sample. In both cases, the higher was the mediator rate, the smaller was the kappa number, the difference amounting to 0.5–1 units. AS and PCA led to a similar reduction in kappa number and exhibited less marked differences between rates.

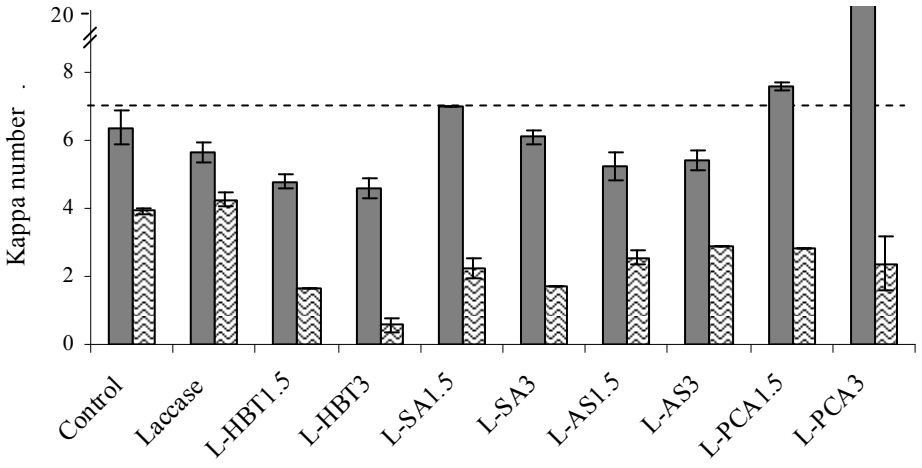

Figure 3- 9. Effect of natural mediators and HBT on kappa number after a laccase-mediator treatment (L, grey bars) and a subsequent peroxide treatment (LP, dashed bars). Laccase and control pulps samples were treated in the absence of either the mediator or both the mediator and enzyme, respectively. The dashed line corresponds to the initial pulp. Kappa number values correspond to two measurements in different samples of a single experiment.

Figure 3-10 shows the pulp brightness level obtained after each bleaching stage. All treatments except that with SA at a 3% odp rate increased brightness relative to the control after the L step. The best results were provided by the synthetic mediator, HBT, at a 3% odp rate (6 percent points higher than in the control test). AS and PCA exhibited no substantial differences between rates. On the other hand, HBT resulted in higher brightness at its higher rate; by contrast, SA led to higher brightness at its lower rate. All treatments resulted in markedly increased brightness after the P stage. The highest brightness level was again obtained with 3% odp HBT (76% ISO, which was nearly 20 percent points higher than in the control test). HBT, AS and SA, all at a 1.5%

odp rate, provided good brightness values as well: 71–72% ISO, which is approximately 15 percent points higher than for the control sample. Based on these results, brightness after the L stage depends on the particular type of mediator used and its rate. The treatment may cause the formation of chromophores in the pulp and/or, based on the kappa number results, cross-linking or cross-coupling reactions in the lignin structure of pulp may also take place.

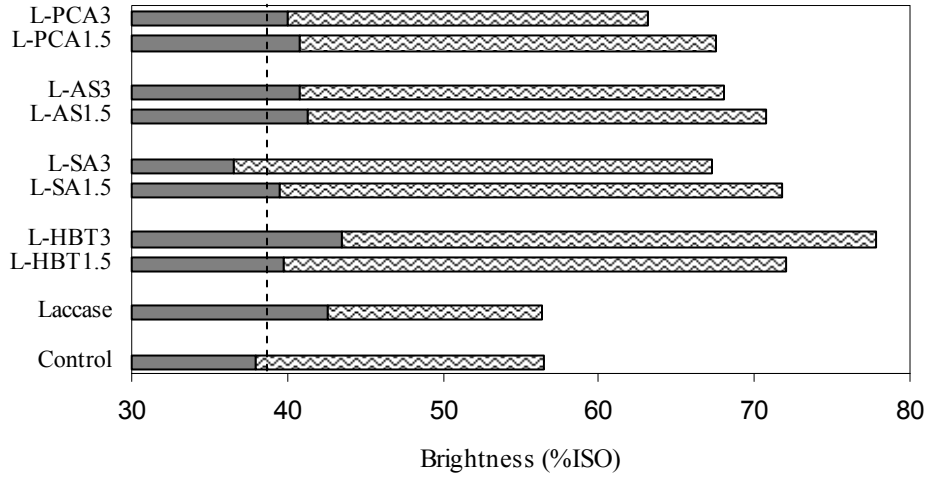

Figure 3- 10. Effect of natural mediators and HBT on brightness after a laccase-mediator treatment (L, grey bars) and a subsequent peroxide treatment (LP, dashed bars). Laccase and control pulps samples were treated in the absence of either the mediator or both the mediator and enzyme, respectively. The dashed line corresponds to the initial pulp.

The final viscosity values obtained after the LMS treatments (L stage) were slightly lower than those for the control samples (Figure 3-11). Except in the control test in the absence of enzyme and mediator, the viscosity was lower after the LP sequence than it was after the L stage. The decreased viscosity obtained in the presence of laccase may have resulted from the copper it contains being adsorbed onto pulp fibres and causing hydrogen peroxide to decompose during the P stage. The hydroxyl radicals thus formed may cause cellulose depolymerization; in the presence of transition metals in the cellulose matrix, such radicals may be generated close enough to cellulose chains to react with them (Lachenal 1996). The samples treated with HBT and the natural mediators exhibited no substantial differences from the control sample treated with laccase; however, HBT3% and SA1.5% treated pulp exhibited better viscosity retention (~100 mg/L over the laccase control sample). The L stage may have two

different effects on the pulp, namely: direct degradation of cellulose and alteration of functional groups in it leading to easier degradation in a subsequent alkaline stage (P stage). Because viscosity measurements are made in an alkaline medium, the viscosity after L was a measure of both degrading effects. The P stage was performed in a hot, strongly alkaline medium, which may have resulted in modified cellulose being more markedly degraded than it was during the viscosity measurements. As a result, the viscosity differences between L and LP may have resulted from degradation of the cellulose modified by L (Fillat and Roncero 2009a).

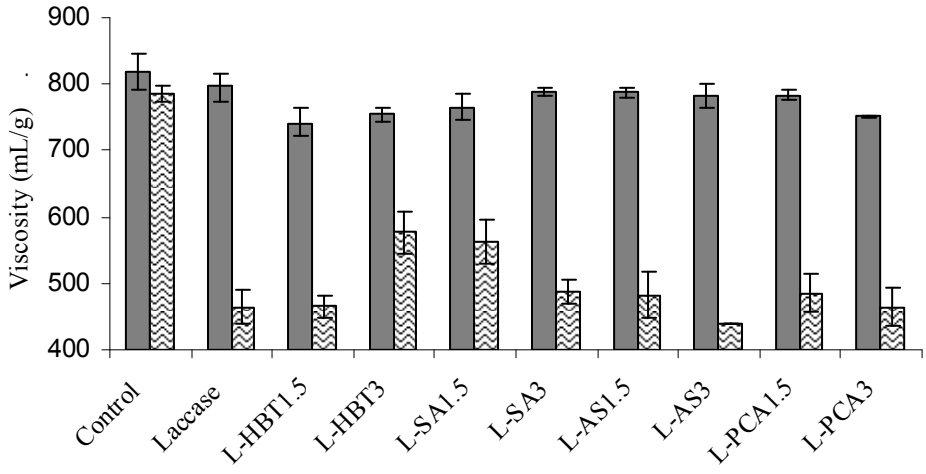

Figure 3- 11. Comparison of natural mediators and HBT in terms of the viscosity of flax pulp after an enzymatic treatment (L, grey bars) and a subsequent peroxidase stage (LP, dashed bars). The control sample was pulp treated with neither laccase nor a mediator. Laccase sample was treated with the enzyme alone. Viscosity values correspond to two measurements in different samples of a single experiment.

3.3.5 Characterisation of bleaching effluents

Although the effluents from TCF bleaching sequences contain no organochlorine pollutants, they should be monitored for at least COD, colour and toxicity in order to facilitate total circuit closure. Table 3-1 shows the COD and colour values of the effluents from the L and P stages. As can be seen, COD after L was markedly higher than it was after P. The control treatment (no enzyme and no mediator) exhibited an increased COD after L, probably as a result of the presence of sodium tartrate buffer in

the effluent –COD measurements of this solution at the treatment concentration provided a high value close to that for the control test. The highest COD value was ~300 kg O_2/dpt —twice as high the control value— and provided by the AS3% treatment. Chemical oxygen demand increased in the sequence HBT < PCA < SA ≈ AS, which is consistent with COD measurements of the mediators at the treatment concentrations in solution (results not shown). The effluents recovered after the peroxide stage exhibited very low COD values (19–35 kg O_2/dtp) that were higher at the higher mediator rates. COD was essentially due to the presence of sodium tartrate buffer and the natural mediators. The laccase–mediator system was found to contribute to effluent colour after the L stage. With PCA, the effluents exhibited no colour and the pulp no delignification —no reduction in kappa number— after the L stage. The highest colour value was that for HBT, followed by those for AS and SA, the higher mediator rate having a stronger effect than the lower one in all cases. The increase in colour may have been caused by the formation of coloured oxidation products of the mediator and also by the presence of an increased amount of degraded lignin in the effluents.

Table 3- 1. COD and colour of effluents after L and LP sequence. Variation coefficient values were below 7%.

	L stage effluent		LP sequence effluent	
	COD (kg O_2/t_{pulp})	Colour (kg Pt/t_{pulp})	COD (kg O_2/t_{pulp})	Colour (kg Pt/t_{pulp})
Control	134	0	19	12
Laccase	149	0	20	20
L -HBT1.5	193	128	25	9
L-HBT3	141	219	32	11
L -SA1.5	242	63	19	11
L -SA3	231	75	25	17
L-AS1.5	199	93	20	13
L-AS3	302	130	35	20
L-PCA1.5	206	0	20	13
L-PCA3	234	0	34	23

Figure 3-12 illustrates the inversely proportional relationship between kappa number and colour; therefore, kappa number correlates with effluent colour and colour must result from the presence of an increased amount of chromophoric groups formed by

oxidation and/or degradation of lignin and the mediators (Fillat and Roncero 2009b). Both the control tests and all treatments provided coloured effluents after the LP sequence. Colour was approximately 10 times lower than after the L stage and higher at the higher mediator rate.

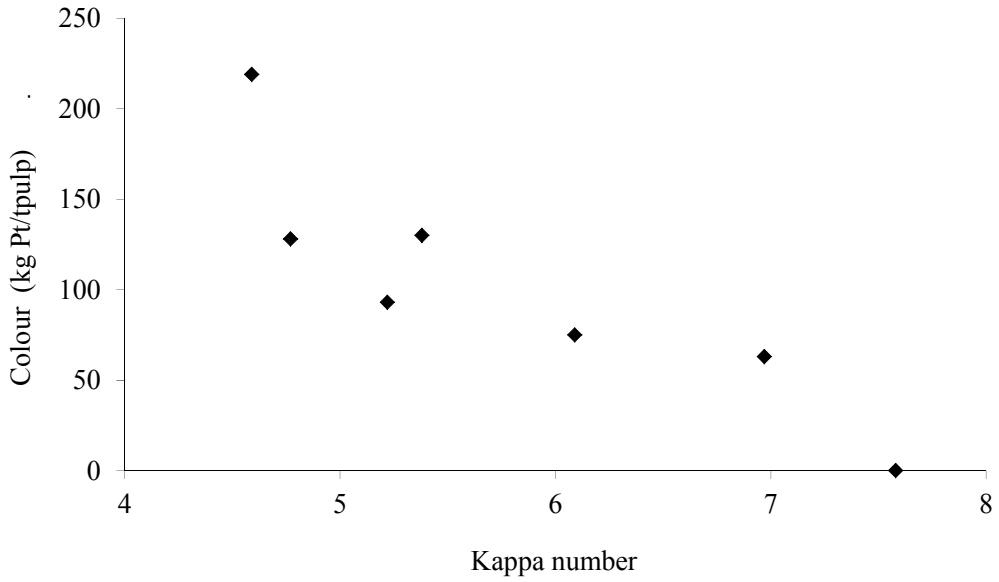

Figure 3- 12. Relationship between effluent colour and kappa number after the L stage.

The bleaching stage is the source of most of the environmentally harmful organic contaminants a paper manufacturing process can produce (Stanko and Angus 2006). Toxicity tests provide a valuable tool for assessing the potential deleterious effects of wastewater on aquatic organisms. Spanish legislation has imposed a limit on the composition of dumped inhibitory matter (IM); thus, Decree 130/2003 of the Catalonian regional government has set an emission limit for public wastewater of 25 equitox/m^3. Table 3-2 only shows toxicity values for the effluents from the L stage since those from the LP sequence exhibited very low levels. Control (with laccase and without mediator) showed the lowest value. The mediators that obtained a toxicity value under the emission limit were HBT, AS and PCA (at 1.5% odp rate). The natural mediators SA in LMS caused a high toxicity in the treatment effluents; SA treatments stood up from the rest (~10-fold more toxicity than legal limit). Some extra controls were carried out, thus flax pulp was treated in the same conditions that the delignification assays but without laccase (mediators were not oxidized); this test was

performed in order to evaluate the contribution of the non-oxidized mediators on ecotoxicity: these controls clearly showed a lower toxicity than the obtained in the LMS treatments and lower than the Catalonian emission limit. Therefore, the toxicity enhance in some laccase-mediator assays effluents could be caused by the generation of intermediate species (oxidized or radicals) and degradation products from the mediators.

Table 3- 2. Ecotoxicity values. Variation coefficient values were between 4 and 10%.

	Ecotoxicity (T.U.)	
	Mediator solution	L stage effluent
Laccase	-	1
L -HBT1.5	2	9
L-HBT3	-	23
L-SA1.5	-	176
L-SA3	13	239
L-AS1.5	-	22
L-AS3	5	17
L-PCA1.5	-	20
L-PCA3	7	122

3.4 Conclusions

In this work, unbleached flax pulp was treated with laccase and natural mediators in order to compare their bleaching potential with the synthetic mediator HBT. Bleaching efficiency was assessed in terms of pulp and effluent properties. Based on the results, the natural mediators are promising alternatives as delignifying agents to HBT (particularly syringaldehyde). Enzyme stability is also important for most biotechnological applications. Although some mediators inactivated laccase, the presence of the pulp was found to have a protective effect on the enzyme activity. In conclusion, the combination of PcL and a natural mediator affords efficient biobleaching of flax pulp in a TCF sequence including a subsequent peroxide stage.

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Chapter 4

ASSESSING THE USE OF XYLANASE AND LACCASES IN BIOBLEACHING STAGES OF A TCF SEQUENCE FOR FLAX PULP

SUMMARY

The use of enzymes appears as a promising approach for clean pulp bleaching process. In this study, two different laccases were used in combination with various mediators as biobleaching agents in a TCF sequence for flax fibre and pulp. Thus, non-commercial laccase from *Pycnoporus cinnabarinus* (PcL) was used with the synthetic mediator violuric acid (VA) or the natural mediator syringaldehyde (SA), and commercial laccase from *Myceliophthora thermophila* (MtL) was used in combination with the natural mediator methyl syringate (MS). Each laccase–mediator treatment was used as an L stage in the following TCF bleaching sequence: LQPo (Q denoting a chelating stage and Po a pressurized alkaline peroxide multi-step treatment). Also, a xylanase pretreatment was carried out for first time here to assess its effect on flax fibre. Pulp (kappa number, viscosity and brightness) and effluent properties (COD, colour, residual activity and toxicity) were determined after each bleaching stage. The hexenuronic acid (HexA) content of the pulp was also determined to assess the effect of different enzyme systems on its integrity. The best L stage towards enhancing pulp properties was PcL+VA, which provided a final brightness and kappa number of 81% ISO and 1.3, respectively. Using a xylanase pretreatment was found to efficiently remove HexA and enhance delignification by laccase (final kappa number, 0.7). These results warrant upscaling any of these biobleaching sequences for flax pulp as they

provide environmentally sustainable flax fibre with a high cellulose content and brightness above 80% ISO (see Annex I).

4.1 Introduction

Market demands and the environmental problems raised by the extensive use of wood have elicited technological efforts including the development of faster growing species as sources of high-quality fibre and more environmentally friendly bleaching processes (González-García et al. 2010; Skals et al. 2008). Flax fibre provides heavyweight, strong, permanent paper highly suitable for making tough, fine products such as tea bags, smoking paper and bank notes, as well as high-quality ledger paper. However, its chemical and morphological properties make flax pulp usually more difficult to delignify than wood pulp (García Hortal 2007).

Although pulp cooking removes most lignin, a residual amount invariably remains in pulp fibres that must be eliminated by oxidative bleaching. The pulp bleaching section is the most polluting part of the paper manufacturing process. However, the use of enzymes holds promise for clean bleaching by virtue of their high specificity and environmental friendliness (Bajpai 2004; Call and Mücke 1997). Xylanases are gaining importance as alternatives to toxic chlorine-containing chemicals; these enzymes can induce morphological changes in cellulose fibres by hydrolysing xylans on fibre surfaces and making fibres more readily accessible to the reagents as a result (Roncero et al. 2000; Viikari et al. 1994). Fungal laccases (*p*-diphenol oxygen oxidoreductase, EC 1.10.3.2) in combination with mediators are especially useful for bleaching pulp on account of their ability to act directly on lignin. Thus, laccase–mediator systems (LMS) have proved efficient in the bleaching of wood and non-wood pulp (Aracri et al. 2009; Fillat and Roncero 2010; Moldes and Vidal 2008; Valls et al. 2010b). Various laccases and synthetic mediators have been tested in depth and found to be highly effective towards enhancing pulp delignification; however, the enormous potential of LMS has promoted a search for new, cost-effective, natural mediators readily obtained from plants and spent pulping liquors or directly from fungal metabolism, as well as for alternative, more effective synthetic mediators (Astolfi et al. 2005; Camarero et al. 2007; Oudia et al. 2008; Sigoillot et al. 2005). Several studies have shown the potential of laccase–mediator stages in environmentally friendly whole bleaching sequences (Camarero et al. 2002; Eugenio et al. 2010; Fillat et al. 2010; Ibarra et al. 2006).

One highly innovative use of xylanases and laccases is for reducing the hexenuronic acid (HexA) content of pulp, whether by releasing xylans (xylanases), direct oxidation (laccases) or via an as yet unknown mechanism. Hexenuronic acids, which form during alkaline cooking of pulp, have adverse effects on pulp bleaching; thus, they contribute to kappa number, cause brightness reversion and oxalic acid formation, consume bleaching agents and retain metal ions by chelation (Cadena et al. 2010; Valls and Roncero 2009).

In this work, we assessed the use of various enzyme delignification stages in an industrial bleaching sequence. The ensuing totally chlorine free (TCF) sequence comprised various LMS treatments (L stage) followed by a chelating treatment (Q stage) and a subsequent bleaching step with hydrogen peroxide (Po stage). A xylanase pretreatment was additionally carried out in one of the sequences. Laccase from *Pycnoporus cinnabarinus* (PcL) was used in combination with the natural mediator syringaldehyde (SA) or the synthetic mediator violuric acid (VA), whereas laccase from *Myceliophthora thermophila* (MtL) was used with the natural mediator methyl syringate (MS). The primary purposes of this work were to compare the effects of various whole LMS-aided biobleaching sequences on flax pulp, and to explore, for the first time, the introduction of a xylanase pretreatment in order to facilitate the delignification of flax. Changes in lignin and HexA contents during the process were examined, and effluents characterised, in order to identify the most efficient and eco-friendly choice among the enzyme systems.

4.2 Materials and methods

4.2.1 Raw material

Linum usitatissimum unbleached pulp was produced by soda-anthraquinone cooking at the CELESA mill in Tortosa (Spain). Following acid washing with H_2SO_4 at pH 4 to remove impurities, reduce the content in metal ions and adjust the pH to the level required for the enzyme treatments, the pulp had a kappa number of 6.1, 41% ISO brightness and 816 mL/g viscosity, in addition to a hexenuronic acid (HexA) content of 10.7 μmol/g oven-dried pulp (odp).

4.2.2 Laccase–mediator treatment

Two fungal laccases, one from *P. cinnabarinus* (PcL, produced by INRA in Marseille, France) and the other from *M. thermophila* (MtL, produced by Novozymes® in Denmark), were used in combination with various mediators. Syringaldehyde (SA) and violuric acid (VA), purchased from Sigma-Aldrich, were used with PcL, and methyl syringate (MS), supplied by Novozymes®, was used with MtL. Delignification tests with the LMS (L stage) were carried out with unbleached pulp at 3% odp consistency in an oxygen pressurised (0.6 MPa) reactor containing 50 mM sodium tartrate buffer at pH 4 for PcL treatments (50 mM sodium phosphate buffer pH 7 for MtL treatments), 20 U/g laccase and 1.5% (w/w) mediator. Tween 80 was added as surfactant and the mixtures shaken at 60 rpm for 5 h. Control pulp samples were processed in the absence of mediator. Laccase activity was monitored by measuring ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) oxidation at 436 nm (ε_{436} = 29 300 M^{-1} cm^{-1}). The chemicals used in this study were purchased from Sigma-Aldrich.

4.2.3 Bleaching sequences

The TCF bleaching sequences studied were performed with and without a xylanase pre-treatment (X stage) and included an LMS treatment. The L stage was followed by a chelating step (Q stage) and a subsequent hydrogen peroxide stage (Po). The biobleaching sequences studied were thus LQPo and XLQPo. An X stage was inserted before L with PcL and VA in order to assess the ability of xylanase to improve delignification of this non-wood pulp. The enzyme used in the X stage was a commercial crude xylanase preparation (Pulpzyme® HC, from Novozymes®) intended to reduce chemical consumption during the bleaching sequence by effect of it boosting effect (Aracri and Vidal 2011; Valls et al. 2010a). This treatment was performed in polyethylene bags, using 3 U/g odp xylanase in 50 mM Tris-HCl buffer (pH 7), at 5% consistency at 50ºC for 2 h. The Q stage was conducted in the presence of 1% DTPA (diethylenetriaminepentaacetic acid) at 5% consistency, pH 5–6 and 85°C for 1 h. A subsequent bleaching treatment with hydrogen peroxide (Po stage) was conducted by using 3% H_2O_2 in 1.5% NaOH, 0.3% DTPA and 0.2% $MgSO_4$ at 5% consistency in an oxygen pressurised (0.6 MPa) reactor with stirring at 30 rpm at 90°C for 4 h. This stage was performed in 3 steps (t_1 = 1 h, t_2 = 1 h, t_3 = 2 h), each including the addition of 1% odp H_2O_2. Control sequences were performed in the absence of mediator in the L stage for comparison. After each bleaching stage, the pulp samples were filtered to

collect residual liquors for subsequent analysis and thoroughly washed with de-ionized water.

4.2.4 Carbohydrate analysis by HPLC

The sugar composition of the pulp samples after the X, L and Po stages in the different sequences was determined by high performance liquid chromatography (HPLC) following Soxhlet extraction with acetone to remove extractives. The samples were ground to a particle size < 0.5 mm and two aliquots from each hydrolysed with sulphuric acid in two steps, namely: (*a*) *pre-hydrolysis with concentrated sulphuric acid*, using an amount of *ca.* 50 mg of sample that was impregnated with 5 ml of 72% sulphuric acid in a test tube which was placed in a thermostatic shaker at 30º C for 1 h; and (b) *final hydrolysis with dilute sulphuric acid*, using distilled water to wash the test tubes and dilute the samples to a final sulphuric acid concentration of 4%. The collecting flasks were then placed in an autoclave at 121ºC for 60 min. Before HPLC analysis, the solid residue from the post-hydrolysis process was recovered by filtration and taken to represent Klason lignin (*viz.* lignin insoluble in sulphuric acid). The high performance liquid chromatograph used was fitted with a refractive index detector. The chromatographic determination was performed with an Agilent 1100 HPLC instrument furnished with a column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 0.006 mol/L sulphuric acid; flow rate, 0.6 mL/min; column temperature, 60ºC. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from Sigma-Aldrich). Because the column failed to resolve xylose, manose and galactose, their combined content was expressed as xylose (Garrote et al. 2001).

4.2.5 Pulp properties

The treated pulps samples were characterized in terms of kappa number, brightness and viscosity according to ISO 302:2004, 3688:1999 and 5351:2010, respectively. Their hexenuronic acid (HexA) content was determined by UV detection according to Chai et al. (2001). An estimate of the actual lignin content of each sample was obtained by determining the kappa number due to lignin (KN_{lig}) (Li et al. 2001). KN_{lig} was measured by acid hydrolysis with mercury acetate and efficient washing with de-ionized water following removal of HexA. The results were used to estimate the actual

degree of delignification. Kappa number, viscosity and HexA content were analysed in duplicate, whereas brightness was measured in quadruplicate.

4.2.6 Effluent properties

Chemical oxygen demand (COD) and colour were determined in duplicate after each bleaching stage, following ASTM D1252-06 and ASTM D1209-05(2011), respectively. All spectrophotometric assays were carried out in a Thermo Scientific Evolution 600 spectrophotometer. Toxicity was measured in a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental), following UNE-EN ISO 11348-3:2009. This test is based on the difference between the amount of light emitted by *Vibrio fischeri* bacteria before and after contact with a toxic substance. One equitox/m^3 or toxicity unit (TU) is defined as the reciprocal of wastewater dilution that reduces light emission by 50% after 15 min of contact. Toxicity tests were conducted in duplicate.

4.3 Results and discussion

Flax pulp was subjected to a chlorine-free bleaching sequence based on the use of fungal laccases and redox mediators followed by a chelating stage and an alkaline H_2O_2 treatment (LQPo sequence). The industrial and economic feasibility of this sequence depends on a number of factors, namely: (*a*) an efficiency above 80% ISO is difficult to obtain with chemical sequences owing to the high recalcitrance of flax pulp to high brightness bleaching; (*b*) the substantial amounts of enzyme and mediators used for enzymatic bleaching required that they should be economically competitive and readily available. However, the high cost of the enzyme can be assumed in this process since flax pulp is used to make specialty paper and is thus expensive. Previous studies showed the possibility of bleaching flax pulp in a TCF sequence including an enzymatic stage involving the joint use of a laccase and a redox mediator. In this work, an enzyme-based bleaching stage was included in a whole bleaching sequence for non-wood pulp. Two different types of laccase were used, namely: non-commercial laccase from *P. cinnabarinus* or PcL and commercial laccase from *M. thermophila* or MtL. PcL, which features a high redox potential and thermal stability, and was previously tested for bleaching different types of pulp was used in combination with syringaldehyde (SA), a natural mediator with the potential for delignifying flax pulp,

and violuric acid (VA), which is one of the most efficient synthetic mediators but had never previously been applied to flax pulp (Chapter 3; Oudia et al. 2008). MtL (Novozym® 51003) is a robust, thermostable commercial laccase that acts efficiently in the presence of the natural mediator methyl syringate. Six different bleaching sequences were investigated, namely Control PcL and Control MtL, two control sequences where the L stage was carried out with laccase from *P. cinnabarinus* and *M. thermophila*, respectively, in the absence of mediator; PcL+SA and PcL+VA, where L was performed with the combination of PcL and the mediator SA and VA, respectively; Xyl+(PcL+VA), which included a xylanase pretreatment and an L stage with PcL and VA; and MtL+MS, which included an L stage with MtL in the presence of MS as mediator. The chelating stage included in the bleaching sequence was intended to facilitate delignification of the pulp by decomposing H_2O_2 while avoiding too high a decomposition rate in order to ensure efficient peroxide bleaching. Some transition metals present in pulp can accelerate H_2O_2 decomposition, so a strong chelating agent such as DTPA can be used to displace them and facilitate their dissolution and removal by subsequent washing (Lapierre et al. 1995). The hydrogen peroxide stage was performed in three phases (P1, P2 and P3) in order to assess changes in pulp properties with time and chemical addition. All delignification stages were followed by measurements of kappa number, brightness and viscosity.

4.3.1 Analysis of pulp properties

4.3.1.1 Kappa number

Figure 4-1 illustrates the delignifying effect of the different enzyme treatments, as well as the contribution of each stage of the bleaching sequence to reducing kappa number. PcL (Control PcL) caused slight delignification by itself, but the presence of a mediator clearly increased the effect. The greatest reduction in lignin content was obtained with the mediator VA, which decreased kappa number by 40% (after L), even though the combination PcL+SA reduced it 0.7 units more than the L stage in the Control PcL sequence. Kappa number was only slightly reduced by the chelating treatment (0.3–0.5 units with respect to the previous stage in all PcL sequences). With H_2O_2 in the bleaching sequence, the greatest kappa number reduction was again obtained with PcL+VA: 57% with respect the previous enzyme stage in the Po1 step and to only 1.6 at the end of the LQPo sequence. The PcL+SA sequence also improved

the results of the Control PcL sequence (a final kappa number of 2.2 was obtained). MtL was able to delignify brown flax (Control MtL reduced kappa number by 1.3 units with respect to the initial pulp); in fact, the kappa number at the end of the bleaching sequence was very similar to that obtained with Control PcL. Moreover, the MtL+MS combination proved effective in decreasing the lignin content and reduced the kappa number to 2.2 —which is higher than the value obtained with PcL+VA. The Po stage was not so effective in diminishing kappa number in the pulp samples treated with MtL as it was with PcL. The efficiency of a specific laccase–mediator system in degrading lignin depends on the properties of the laccase, the mediator and their combination. Probably, PcL was more effective in oxidizing lignin than was MtL; this oxidized lignin might be water-soluble and hence easily removed during washing. In addition, the mediators were previously found to exhibit electrochemical differences in the presence of flax lignin (Díaz González et al. 2011), thus, an increase in the oxidation current for VA, and a decrease in those for SA and MS, were measured in the presence of lignin.

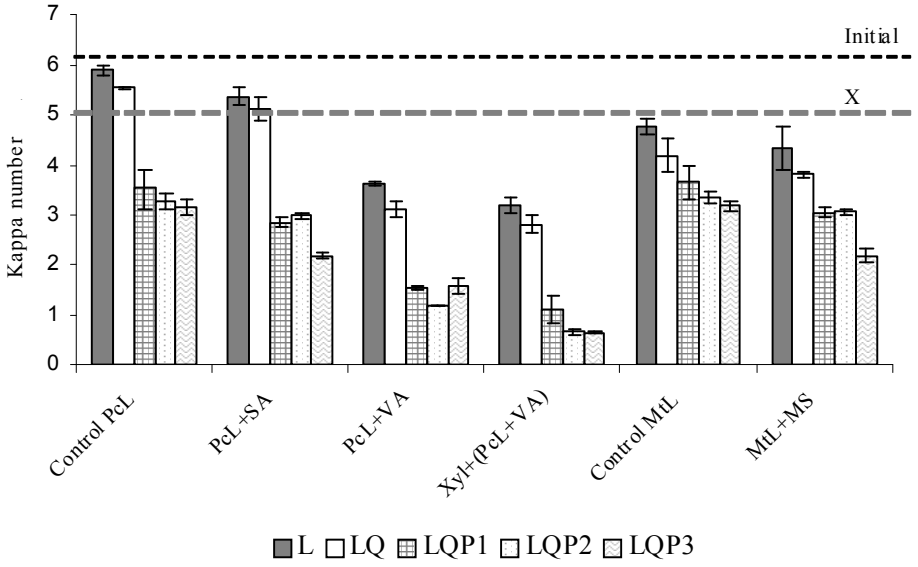

Figure 4- 1. Comparison of kappa number during the different enzyme-based bleaching sequences for flax pulp. Both properties were estimated after each stage of the LQPo sequence (the three steps of H_2O_2 addition in the Po stage included). The following L stages were applied: PcL+SA and PcL+VA were the samples treated with laccase from *P. cinnabarinus* and the mediators SA and VA, respectively; and MtL+MS was the pulp treated with laccase from *M. thermophila* and MS as mediator. The Control PcL and Control MtL sequences included an L stage without mediator. The dashed lines represent the values for the initial pulp and the xylanase-treated pulp (X).

The xylanase pretreatment was applied before the bleaching sequence including an L stage with PcL+VA since this sequence exhibited the best delignification results. The X stage afforded a kappa number reduction of 1 unit with respect to the initial pulp; the reduction can be ascribed to a delignifying effect resulting from the removal of lignin trapped between xylan chains (Roncero et al. 2003), as well as to removal of HexA bonded as side groups to xylans (Aracri and Vidal 2011). Also, the LMS resulted in the highest delignification in the XLQPo sequence: a 47% reduction with respect to the initial pulp. This suggests that the xylanase pretreatment facilitates access of the LMS into the fibre network, thereby increasing the removal of lignin and HexA —HexA content changes are discussed in greater detail below. In fact, residual lignin and HexA are known to be the main substrates contributing to kappa number in pulp fibres. Accordingly, the final kappa number obtained at the end of the sequence including the Xyl+(PcL+VA) treatment was the smallest of all (0.67 units, which is very difficult to obtain in a TCF sequence for flax pulp).

4.3.1.2 Brightness

Figure 4-2 shows the pulp brightness level obtained after each bleaching stage. All enzyme systems caused slight pulp darkening (i.e. a small decrease in brightness relative to the initial pulp) immediately after the LMS treatment. On the other hand, the chelating stage allowed brightness to be slightly increased. The hydrogen peroxide stage was more efficient in the bleaching sequences including an enzyme treatment with laccase from *P. cinnabarinus*, especially during the first hour of treatment; thus, pulp previously treated with PcL+VA exhibited a brightness increase of 38% before Po1 relative to the L stage. The LMS sequences involving the use of PcL exhibited a very high bleaching efficiency; for example, a final brightness of 81% ISO was obtained with the L(PcL+VA)QPo sequence. The commercial enzyme MtL provided worse results; thus, brightness values after L were similar to those obtained with PcL, but the Po stage resulted in no specially high brightness. The sequence including the Control MtL treatment provided a very low final brightness (56% ISO) and a combination of MtL with the mediator MS failed to improve it —rather, it diminished it to a level below that for the Control PcL sequence. The MtL treatment may have caused the formation of chromophores more difficult to remove from the pulp in subsequent stages. The xylanase pretreatment failed to improve brightness immediately, the increase amounting to only 1 percent point with respect to the initial

pulp; however, a subsequent L stage with PcL+VA resulted in the best improvement in brightness (3.5 percent points over the initial pulp). The hydrogen peroxide treatment used in this sequence provided a high brightness gain (40% ISO more than in the initial pulp). As with kappa number, the best bleaching results were obtained with the XLQPo sequence including an LMS stage consisting of a PcL+VA treatment. Using the LMS afforded a reduction in hydrogen peroxide dose. Using SA and VA in combination with PcL resulted in the same brightness as the Control PcL sequence but reduced H_2O_2 consumption by 0.75 and 1%, respectively. The xylanase pretreatment afforded a saving of 2% in H_2O_2. Also, the kappa number was always smaller than with the Control sequence.

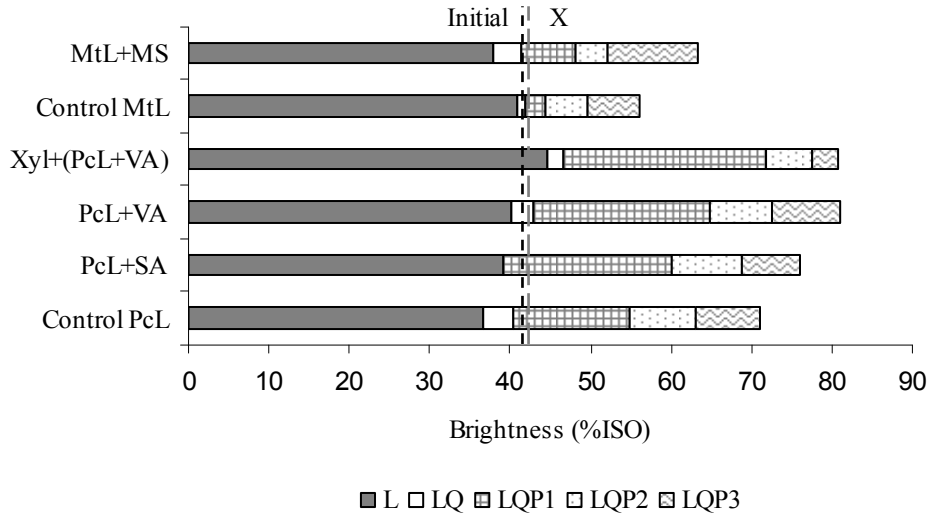

Figure 4- 2. Comparison of and brightness during the different enzyme-based bleaching sequences for flax pulp. Both properties were estimated after each stage of the LQPo sequence (the three steps of H_2O_2 addition in the Po stage included). The following L stages were applied: PcL+SA and PcL+VA were the samples treated with laccase from *P. cinnabarinus* and the mediators SA and VA, respectively; and MtL+MS was the pulp treated with laccase from *M. thermophila* and MS as mediator. The Control PcL and Control MtL sequences included an L stage without mediator. The dashed lines represent the values for the initial pulp and the xylanase-treated pulp (X).

4.3.2 Viscosity

The effect of each bleaching stage on cellulose integrity was assessed via viscosity measurements (Figure 4-3). PcL treatments resulted in a gradual decrease in viscosity during the bleaching sequence. The decrease was greatest in the presence of VA, where

the viscosity after the L was 17% lower than in the initial pulp. No appreciable differences between the L and Q stages were obtained in this respect. On the other hand, a substantial loss of viscosity was observed after the Po stage —the presence of a mediator reduced the loss relative to Control PcL, however, more markedly with PcL+VA than with PcL+SA. No differences between stages were observed in the MtL treatments. Thus, the Control MtL sequence resulted in a viscosity loss of about 50 units, and the MtL+MS in one of ca. 90 units, with respect to the initial pulp. The viscosity decrease was more marked in the pulp samples treated with laccase from *P. cinnabarinus*. The loss in cellulose integrity caused by the LMS treatment was not increased by the xylanase pretreatment. The final viscosity provided by the XLQP sequence was 60 units lower with a xylanase pretreatment (PcL+VA) than without it, but very similar to the value obtained at the end of the Control PcL sequence. PcL and the mediators SA and VA may have had two different effects, namely: (*a*) direct degradation of cellulose and (*b*) oxidization of carbohydrate chains in it to carbonyl groups, which would have made the pulp vulnerable to degradation by the strong alkaline medium used in the bleaching stage (Po).

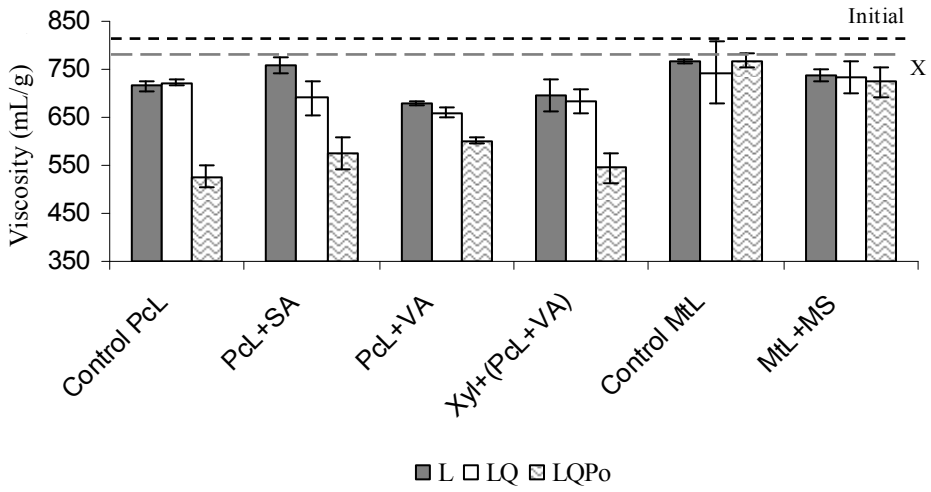

Figure 4- 3. Effect on viscosity of different enzymatic treatments during a bleaching sequence. This property was measured after each stage of the LQPo sequence. See Fig. 1 for further details.

Because viscosity measurements were made in an alkaline medium, the viscosity after L was a measure of both degrading effects. The Po stage was performed in a hot, strongly alkaline medium, which may have resulted in modified cellulose being more

markedly degraded than during the viscosity measurements (Fillat and Roncero 2009). Therefore, the viscosity differences between L and Po may have resulted from degradation of cellulose modified by the LMS. On the other hand, cellulose was probably not oxidized to carbonyl groups by the MtL.

4.3.2.1 HexA content

The initial HexA content of the pulp was quite low (10.7 μmol/odp) compared with unbleached pulp from other non-wood materials such as sisal (41.4 μmol/odp; kappa number 7.9) or unbleached eucalyptus pulp (38 μmol/odp; kappa number 8.0). As can be seen from Figure 4-4, the LMS treatment decreased the HexA content. Both laccases were in fact effective in reducing the amount of HexA; thus, after L, the Control MtL sequence reduced the HexA content by 26% with respect to the initial pulp. This must have resulted from direct oxidative elimination of readily accessed HexA molecules on the outer parts of fibres. However, the mediators altered the HexA removal efficiency: whereas SA had no effect on the HexA reduction caused by laccase from *P. cinnabarinus*, VA enhanced the effect of PcL —probably because the oxidized form of the mediator was able to penetrate deeper into fibre walls and attack HexA molecules there.

On the other hand, the natural mediator MS failed to reduce the HexA content; in fact, hexenuronic acids were not affected by this LMS treatment (MtL+MS). Probably, this was a result of competitive reactions of the mediators and HexA with the enzymes arising from the disparate affinity of laccase for these structures. The greatest reductions in HexA content among the LQPo sequences were those provided by PcL+VA, Control MtL and Control PcL, which decreased it to similarly low levels (below 7 μmol/odp). The hydrogen peroxide stage decreased the HexA content in all sequence probably due to the extraction of those HexA bound to xylan-lignin complexes under the alkaline conditions used in this stage. According to Li et al. (2002), HexA groups can be more or less efficiently removed from pulp depending on the particular bleaching agent. Hexenuronic acids are unsaturated compounds and hence amenable to oxidation by strongly electrophilic bleaching agents (particularly acid reagents such as chlorine dioxide, ozone and peracids) (Valls and Roncero 2009). The enzyme pretreatment with xylanase was very efficient in removing a substantial fraction of HexA by releasing it from fibres; in fact, as expected, it was more efficient

than the LMS —the X stage afforded a reduction of 50% with respect to the initial pulp. No synergistic effect with the two subsequent bleaching stages (L and Po) was observed in the XLQPo sequence. Finally, the pulp samples from the sequence including an X stage exhibited the lowest final HexA contents. The chemical analysis of the initial pulp after acidification revealed a high glucan content (94.2 g/100g odp), a low xylan content (4.4 g/100g odp) and a rather low Klason lignin content (1.3 g/100g odp), all expressed as percentages of dry matter.

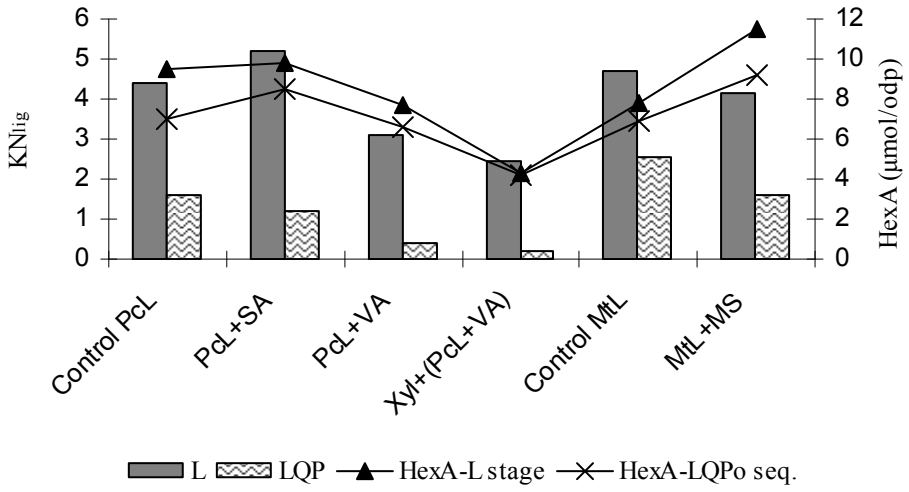

Figure 4- 4. Kappa number due to lignin (KN_{lig}, measured following removal of hexenuronic acids) after a laccase–mediator treatment (L, grey bars) and subsequent chelating and peroxide treatments (LQPo, dashed bars). The control pulp samples were treated in the absence of mediator. The HexA content was also determined after the L stage (▲) and LQPo sequence (X). The kappa number after the xylanase pretreatment was 5.0. The initial HexA content (10.7 µmol/g odp) and KN_{lig} (5.3) were reduced to 5.5 (µmol/g odp) and 5.0, respectively, by the xylanase pretreatment.

Thin layer chromatography confirmed that xylanase had some effect on xylans in the pulp. By contrast, HPLC (results not shown) revealed no appreciable differences between treatments along the bleaching sequences, probably because of the high glucan content of the pulp. Klason lignin (CLK) is a measure of the insoluble fraction collected upon reaction with concentrated sulphuric acid. As can be seen from Table 4-1, the xylanase pretreatment failed to diminish it, whereas all LMS treatments did. The hydrogen peroxide stage reduced Klason lignin, especially in the sequences including an LMS treatment with PcL.

Table 4- 1. Klason lignin (CLK) in the initial and xylanase-treated pulp samples, and after the L and Po stages, all expressed as percentages of dry matter.

	CLK (g/100 g odp)
Initial	1.3 ± 0.0
X	1.4 ± 0.0

	CLK (g/100 g odp)	
	L	Po
Control PcL	1.5 ± 0.1	0.5 ± 0.1
PcL+SA	1.4 ± 0.1	0.3 ± 0.0
PcL+VA	0.9 ± 0.1	0.1 ± 0.0
Xyl+(PcL+VA)	0.9 ± 0.2	0.5 ± 0.1
Control MtL	1.1 ± 0.1	0.8 ± 0.1
MtL+MS	1.3 ± 0.0	0.5 ± 0.1

4.3.2.2 "Actual" kappa number (KNlig)

Residual lignin and HexA proved the main substances contributing to kappa number; however, other minor, oxidizable species known as "non-lignin structures" may have influenced kappa number measurements. Like lignin, false lignin consumes bleaching chemicals and will presumably add to the environmental load of the bleaching effluent. In addition, false lignin may influence some pulp properties (Li et al. 2002). The "actual" kappa number (KN_{lig}) was determined after removing HexA from the pulp. KN_{lig} for the initial pulp was 5.3, which suggests that HexA contributed to KN measurements. Figure 4-4 shows the KN_{lig} values measured after the L and Po stages in the different bleaching sequences. As can be seen, KN_{lig} was smaller than KN in all tests; therefore, false lignin contributed to KN measurements. Also, these results further confirm that PcL was more efficient than MtL in delignifying flax pulp, and also that the synthetic mediator VA, used in combination with PcL, provides a highly effective biobleaching system. The sequence including a xylanase pretreatment exhibited the smallest KN_{lig} value at the end; therefore, delignification by the LMS was enhanced after X stage.

4.3.3 Effluents characterisation

The bleaching stage is the source of most organic environmental pollutants produced in the paper manufacturing process. This requires assessing the environmental feasibility of a new industrial bleaching sequence by monitoring appropriate parameters such as the chemical oxygen demand (COD), colour and toxicity of bleaching effluents.

Table 4-2 shows the COD and colour values for the effluents from the X, L, Q and Po stages, as well as the toxicity values for the effluents from the X and L stages. Using a xylanase pretreatment provided the highest COD among the different stages (190 kg O_2/t_{pulp}), probably as a result of efficient removal of HexA-xylooligosaccharides by the enzyme. As can be seen, COD was much greater after L than it was after Q and Po. After the L stage, the effluents from the PcL-containing treatments exhibited a high COD owing to the presence of sodium tartrate buffer and the formation of certain products during the reaction (Chapter 3). The PcL-mediator systems slightly increased COD with respect to the Control PcL treatment by effect of the presence of the mediator and of degradation products of the mediator and lignin; owing to the use of sodium phosphate as buffer, Control MtL resulted in a low COD value, which, however, was increased by the presence of MS. The effluents from the Q stage exhibited COD values from 8–14 kg O_2/t_{pulp}, whereas those measured after the Po stage amounted to 11–15 kg O_2/t_{pulp} in the PcL sequences and were slightly lower in the MtL sequences (7–9 kg O_2/t_{pulp}). The laccase–mediator system was found to contribute to effluent colour after the L stage; thus, all treated pulp samples had higher colour values than the laccase controls (especially those treated with natural mediator SA). Colour values after Q and Po were similar to those obtained after the enzyme stage; however, colour after these two stages was higher with the MtL treatments that with the PcL treatments. Effluents colour must have resulted from the presence of an increased amount of chromophoric groups formed by oxidation and/or degradation of lignin and the mediators. Table 4-2 only shows the toxicity values for the effluents from the L stage since those from the Q and Po stages exhibited very low levels. The laccase control samples had the lowest values (1–3 TU), followed by those subjected to a PcL+VA treatment; both values comply with the Catalonian urban emission limit, which has been set at 25 TU. On the other hand, the PcL+SA treatment introduced a high toxicity in the effluents. The MtL+MS treatment also raised the toxicity level above the Catalonian emission limit; however, effluent toxicity was still much lower

than with the other natural mediator (SA). Based on these results, using a laccase in combination with a natural mediator increases effluent toxicity. The toxicity of the xylanase pretreatment had a low toxicity level complying with the Catalonian emission limit value.

Table 4- 2. . COD and colour of the effluents from the xylanase treatment, and the L, Q and Po stages in each bleaching sequence. Coefficients of variation were all less than 4% and 7% for COD and colour, respectively. Ecotoxicity values for the effluents from the X and L stages. The coefficients of variation ranged from 4% to 10%.

	COD (kg O_2/t_{pulp})			Colour (kg Pt/t_{pulp})			Toxicity (T.U.)
X	190			5			4
	COD (kg O_2/t_{pulp})			Colour (kg Pt/t_{pulp})			Toxicity (T.U.)
	L	Q	Po	L	Q	Po	L
Control PcL	122	9	14	2	0	9	3
PcL+SA	135	10	15	50	2	9	216
PcL+VA	137	12	11	10	4	7	5
Xyl+(PcL+VA)	146	8	15	14	5	11	7
Control MtL	10	11	7	1	4	35	1
MtL+MS	61	14	9	11	9	14	31

4.4 Conclusions

Two fungal laccases and various mediators were used in combination to compare their delignification potential. Bleaching efficiency was assessed in terms of pulp and effluent properties. Based on the results, the laccase–mediator system was especially effective for bleaching high-quality pulp from flax. Integrating it in a TCF sequence can be expected to provide high pulp brightness and a very small kappa number. The potential of a pretreatment with xylanase for improving the bleaching ability of the LMS was assessed and the enzyme found to have a boosting effect by reducing the HexA content of the pulp and facilitating the removal of lignin. Also, the LMS afforded a substantial saving in hydrogen peroxide. The sequence including PcL+VA and an X pretreatment exhibited the best bleaching performance and acceptable effluent characteristics except for COD, which should be further reduced in order to allow circuit closure in the bleaching section of the pulp mill if required. This system provides a useful method for obtaining high-cellulose flax fibre by use of a TCF sequence.

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Chapter 5

ENZYMATIC GRAFTING OF NATURAL PHENOLS TO FLAX FIBRES: DEVELOPMENT OF ANTIMICROBIAL PROPERTIES

SUMMARY

Unbleached flax fibres for paper production were treated with laccase from *Pycnoporus cinnabarinus* and low molecular weight phenols (syringaldehyde –SA, acetosyringone –AS- and *p*-coumaric acid –PCA-) to evaluate the potential of this treatment to biomodify high cellulose content fibres. After the enzymatic treatment with the phenols, an increase in kappa number was found, probably due to a covalent binding of the phenoxy radicals on fibres. Grafting was more evident in pulps treated with PCA (an increase of 4 kappa number points respect to the laccase control was achieved). Paper handsheets from treated pulps showed antimicrobial activity against the bacteria tested: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. An important reduction on microbial count was obtained after incubation of liquid cultures of the bacteria with grafted handsheets. AS and PCA grafted fibres showed a high antibacterial activity on *K. pneumoniae*, getting a nearly total growth inhibition. AS fibres also caused a high reduction in bacterial population of *P. aeruginosa* (97% reduction). Optical properties of handsheets from treated pulps were also determined, showing a brightness decrease and increase in colouration, evaluated by CIE *L*a*b** system, caused by the laccase induced grafting of the phenols. The results suggest that these low molecular weight phenols, covalently bound to the flax

fibres by the laccase treatment, can act as antimicrobial agents and produce handsheets with antimicrobial activity.

5.1 Introduction

Laccases (oxidoreductases) are one of the most important enzymes in terms of application versatility in the forest products industry. Laccases are multicopper oxidases secreted by white-rot fungi and other organisms that play a crucial role in the terrestrial carbon cycle by helping the synthesis and the degradation of lignocellulosic materials. In co-operation with other ligninolytic (lignin-degrading) fungal enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Bajpai 2004; Call and Mücke 1997; Martínez et al. 2005), laccase oxidizes lignin, an aromatic polymer that together with the polysaccharides cellulose and hemicelluloses are the principal components of wood.

As a result, laccase biotechnology is applicable in those sectors of the forest products industry where the goal is to either remove, e.g., in pulp bleaching (Aracri and Vidal 2011; Bourbonnais et al. 1997; Fillat and Roncero 2010; Sigoillot et al. 2004; Valls et al. 2010) or co-polymerize lignin, e.g., in grafting (Chandra and Ragauskas 2002; Widsten and Kandelbauer 2008).

Recently, laccase research has shifted towards fibre modification, a new research field of increasing interest (Aracri et al. 2009; Liu et al. 2009). In the last years several authors have shown that laccase treatments can improve physical and chemical properties of different fibres for a better performance, or to create new value-added products. Laccase-catalyzed biografting is a versatile method of functionalisation due to the enzyme's nonspecific substrate requirements, which allow bonding a wide range of phenolic compounds to fibres (Chandra et al. 2004; Kenealy and Jeffries 2003). Wood surfaces have been enzymatically modified either for aesthetic purposes or for preservation (Kudanga et al. 2008). Lignocellulosic fibres have been grafted with laccases and phenolic compounds (Aracri et al. 2009; Chandra and Ragauskas 2002; Gronqvist at al. 2006) in order to confer them hydrophobicity, antimicrobial properties or to improve mechanical properties as wet tensile strength (Chandra et al. 2004; Elegir et al. 2008; Garcia-Ubasart at al. 2011; Lund and Felby 2001; Schroeder et al. 2007).

In recent years, interest has grown in the preparation of materials with antibacterial properties, for use in a wide range of fields such as food packaging, sanitary materials, and household, medical, and military items (Hou et al. 2009). The best way to obtain antimicrobial surfaces is by incorporating antimicrobial agents through covalent bonding (Roy et al. 2008). Fibre modification by an eco-friendly approach, such as the enzymatic grafting of natural antimicrobial organic molecules to lignocellulosic fibres, can represent a valid solution to meet the growing consumers' expectation of higher hygiene standards and safer products together with environment protection concerns (Elegir et al. 2008).

Natural phenols are potential antibacterial agents of low molecular weight. In a previous study (Chapter 3), treatment of flax pulp with laccase and natural single phenols such as syringaldehyde (SA), acetosyringone (AS) and *p*-coumaric acid (PCA) as mediators, improved pulp properties (decrease in kappa number and rise in brightness) after a bleaching sequence. However, the cross-linking of some of these natural compounds to fibres will evidence after an enzymatic stage with *Pycnoporus cinnabarinus* laccase (Chapter 7).

In this study, we have evaluated the capacity of laccases to graft natural phenols (syringaldehyde, acetosyringone and *p*-coumaric acid) on unbleached flax fibres and analysed the antimicrobial properties conferred to the paper products obtained.

5.2 Materials and methods

5.2.1 Pulp sample, enzyme and natural phenols

Unbleached soda-anthraquinone pulp from *Linum usitatissimum* was provided by CELESA mill in Tortosa (Spain). Pulp was washed with sulphuric acid at pH 4 for 30 min to remove impurities, reduce the content in metal ions and adjust the pH to the requirements for the enzymatic stage. Initial high cellulose content pulp had kappa number of 7.00 and 38.8% ISO brightness.

Laccase from *Pycnoporus cinnabarinus* (PcL) was produced by the INRA (Marseille, France) from the monokaryotic hyperproducing strain ss3 (Herpoël et al. 2000). Activity was monitored by measuring the ABTS oxidation at 436 nm (ε_{436} = 29,300 M^{-}

^{1} cm^{-1}). One laccase activity unit was defined as the amount of enzyme that transforms 1 μmol/min of ABTS at 25ºC. All measurements were carried out using a Thermo Scientific Evolution 600 spectrophotometer. The natural phenols acetosyringone (AS), syringaldehyde (SA) and *p*-coumaric acid (PCA) were purchased from Sigma-Aldrich.

5.2.2 Laccase assisted grafting treatments

Washed flax pulp was firstly extracted with acetone in a Soxhlet extractor for 2 h and 15 min. Grafting treatments with laccase and simple phenols were carried out with an Ahiba Spectradye apparatus (Datacolor) in closed vessels containing 10 g (dry weight) of pulp at 5% consistency in 50 mM sodium tartrate buffer (pH 4), 40 U/g of *P. cinnabarinus* laccase and 3.5% (w/w) natural phenols (relative to dry pulp weight). Tween 80 from Sigma (0.05% w/v) was added as surfactant. Samples were incubated with shaking (30 rpm) at 50ºC for 4 h. Control pulps were treated under identical conditions in the absence of either the phenolic compound (lacasse control) or both the phenols and laccase (initial control). Once treated, pulps were extensively washed with water and filtered, and the residual liquor was collected for subsequent analysis.

5.2.3 Kappa number and optical properties

Kappa number and brightness were determined following ISO 302:2004 and ISO 3688:1999, respectively. Other optical properties were analysed by a paper reflectance measuring Technidyne Colour Touch apparatus at standard illuminant D65 (LAV/Spec. Excl., d/8, D_{65}/10º). The reflectance spectra of paper sheets were obtained from scattering (*s*) and absorption (*k*) coefficients using the Kulbelka-Munk theory (Dence and Reeve 1996). The colour of the samples was described according to the CIE *L*a*b** colour system, where *L**, *a** and *b** are the coordinates of the colour in the cylindrical colour space, based on the theory that colour is perceived by black-white (*L**=lightness), red-green (*a**) and yellow-blue (*b**) sensations (Hunt 1998). Other optical parameter used was the Chroma (C^*) = $(a^{*2} + b^{*2})^{1/2}$, that represents the perpendicular distance from lightness axis.

5.2.4 Antimicrobial properties of the natural phenols

The antimicrobial properties of the natural phenols studied were tested on three microorganisms: *Staphylococcus aureus* (Gram+), *Pseudomonas aeruginosa* (Gram-)

and *Klebsiella pneumoniae* (Gram-). The bacterial strains were inoculated in 5 ml of LB medium supplemented with increased concentrations, 0 to 25 mM, of the natural phenols and the cultures were incubated overnight at 37ºC. After overnight incubation, OD_{600nm} was measured as an estimation of the growth of the bacterial strain. The percentage of growth inhibition was calculated using the following formula (Eq. 5-1):

$$\text{Growth inhibition \%} = 100 - \left(\frac{I}{B} \times 100 \right) \qquad \text{Eq. 5- 1}$$

where: I=OD_{600nm} of the culture of a bacterial strain with a natural phenol at a given concentration; B=OD_{600nm} of the culture of the same strain without added phenol.

5.2.5 Antimicrobial properties of laccase-phenols treated paper

Antimicrobial activity test of the papers treated with laccase and phenol was based on the ASTM Standard Test Method E2149-10. Overnight shaken cultures of *Staphylococcus aureus* (Gram+), *Pseudomonas aeruginosa* (Gram-) or *Klebsiella pneumoniae* (Gram-) were diluted in KH_2PO_4 buffer (working bacteria dilution). 1 g (dry weight) of the treated paper handsheets or control papers cut into small portions were added to flasks containing 50 ml of working bacterial solution and shaken 200 rpm for 1 h at 37ºC. Samples were taken before or after 1h incubation with papers, and bacterial concentration as colony forming units (CFU/ml) was determined by standard viable plate count in TGE Agar (Scharlau). The percentage of reduction in CFU was calculated using the following equation (Eq. 5-2):

$$\text{CFU/ml Reduction (\%)} = \frac{B - A}{B} \times 100 \qquad \text{Eq. 5- 2}$$

where: A = CFU/ml after 1 h contact time; B = CFU/ml before the contact.

The presence of antimicrobial leaching from treated pulps was determined by evaluating the production of growth inhibition haloes on agar plates inoculated with the microorganisms studied. Treated paper samples (1 g odp) were added to 50 ml of sterile buffer solution and shaken in flasks at 200 rpm for 1 hour at 37ºC. 100 µl of these samples were poured into 8 mm diameter holes made in the centre of TGE agar plates previously inoculated with a confluent lawn of the bacteria studied ($1x10^5$

CFU/ml) and incubated at 37°C overnight. Finally, the presence of a zone of inhibition surrounding the agar holes was recorded.

5.2.6 Effluent properties: spectrophotometric curves and residual activity

The effluents from enzymatic treatments were diluted to 1:20 and their absorbance measured between 200 and 400 nm in a UV–vis Thermo Scientific Evolution 600 spectrophotometer. The residual activity of the laccase on the effluents was also measured.

5.3 Results and discussion

In preliminary assays, some intrinsic pulp interference that made complicated the measurement of the antimicrobial properties of enzyme treated fibres was observed. We assumed that pulp extractives could cause unreal outcomes. Extractives are relatively small molecules that can be removed using solvents. These molecules are non-cell wall components and some of them protect the plant against bacterial or fungal attack. Gutierrez and del Río (2003) identified the main extractives present on bast fibres of flax (long fibres from the stem used for manufacturing specialty papers). Their results revealed that waxes, series of long chain n-fatty alcohols, n-aldehydes, n-fatty acids, and n-alkanes were present in the pulps. To eliminate extractives and avoiding the interference of these substances in the determination of antimicrobial properties of grafted papers, unbleached pulps used in the studies were washed with acetone in a Soxhlet extractor before the lacasse – phenol grafting stage.

5.3.1 Kappa number

Treatment conditions were previously optimized in order to get the maximum possible amount of phenolic substrates coupled onto the fibres. So, some variables were modified respect to the conditions used in the laccase mediator bleaching treatments reported before. In this way, the pulp consistency, phenolic compounds dose and laccase units were increased (laccase units were doubled respect to the bleaching assays), while the reaction time was reduced by 1 h. Chandra and Ragauskas (2002) observed that oxidative polymerisation and grafting copolymerisation of phenoxy radicals in lignin caused an increase of kappa number. For this reason, kappa number

and optical properties were measured after the enzymatic treatment in order to assess the tendency of the natural phenols to couple to fibres.

Treatments with laccase and simple phenols produced an increased kappa number of pulps when compared to control samples (Figure 5-1). These results suggest that laccase leads to the crosslinking or grafting of these phenolic compounds on fibres. The highest degree of grafting was obtained with PCA (4 points respect to the laccase control), while treatments with SA and AS also produced a higher kappa number than that of the laccase control (1.5 and 1.3 points, respectively). These results match up with previous studies where biobleaching capacity of this enzymatic system was evaluated (Chapter 3). Laccase-induced grafting will be also reported for *P. cinnabarinus* and these phenols in Chapter 7 where are evidenced the incorporation of these compounds onto the fibres by pyrolysis analysis coupled to gas chromatography/mass spectrometry in the absence and/or in the presence of TMAH as methylating agent. Therefore, natural phenols may be involved simultaneously in oxidative degradation and grafting reactions.

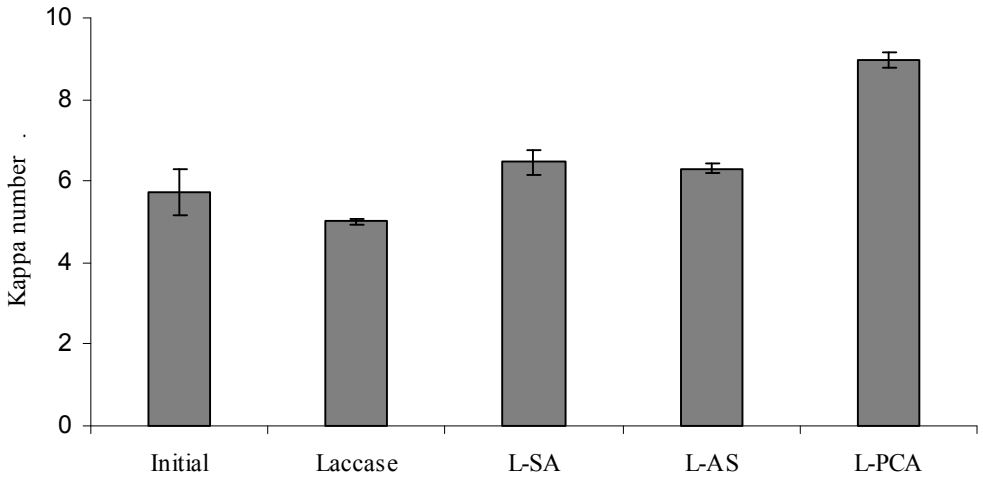

Figure 5- 1. Kappa number of flax pulp after laccase-assisted grafting of phenols. Initial sample corresponds to flax pulp after the acetone extraction; laccase control sample corresponds to flax pulp treated with laccase in the absence of phenols.

The higher grafting degree caused by PCA is probably due to both the higher *pKa* of its phenolate group and the lower stability of its phenolic radical, that facilities condensation reactions at the C_3 and C_5 atoms with residual lignin (Camarero et al. 2007). Furthermore, PCA phenoxyl radicals could form several kinds of dymers and olygomers with a lower phenolic content (Camarero et al. 2008). On the other side SA and AS have similar structure: two methoxy radicals with steric hindrance that protect the phenolic groups (Chandra and Ragauskas 2002). These phenols present lower *pKa* values and form more stable radicals. Their substituents increase the lifetime of their phenoxy free radicals by preventing the coupling reactions with lignin, resulting in less grafting (Astolfi et al. 2005) but higher bleaching efficiency (Chapter 3).

5.3.2 Brightness and optical properties

After the enzymatic treatment with phenols, brightness decreased when compared to the laccase control pulp (7-10% decrease) suggesting again a possible grafting of the phenolic compounds (Figure 5-2). However, pulps treated with laccase alone resulted in an increase of brightness and a decrease of kappa number respect to the initial pulp, showing the potential of the laccase used in flax pulp delignification. So, it could be assumed the coexistence of two simultaneous reactions during the treatments: lignin oxidation and phenol grafting.

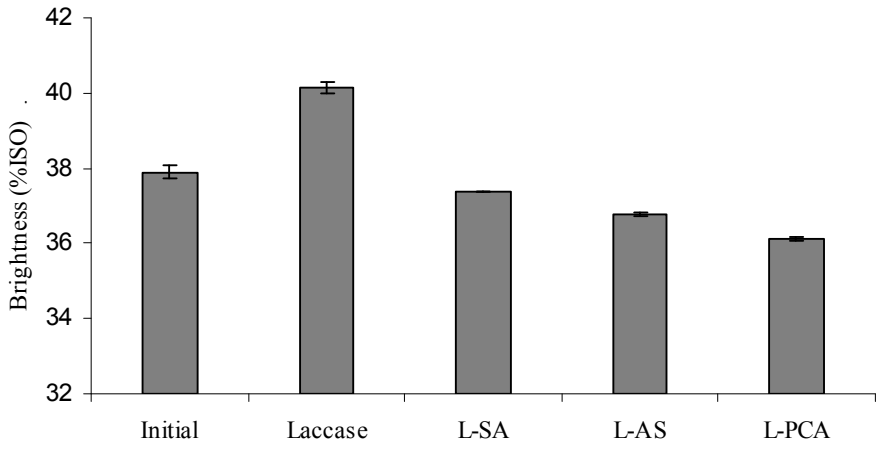

Figure 5- 2. Brightness of flax pulp after laccase-assisted grafting of phenols. Initial sample corresponds to flax pulp after the acetone extraction; laccase control sample corresponds to flax pulp treated with laccase in the absence of phenols

In the previous chapters, it was demonstrated the capacity of *P. cinnabarinus* laccase and these natural phenols in flax delignification by getting higher final delignification rates, after a chemical bleaching stage, than the laccase alone. Because of this ability, the differences of the laccase-phenol treated pulps respect to the laccase control pulps (without phenol) do not reflect the total grafting of phenols on pulps.

The optical properties of treated pulps were further analysed. The CIE L*a*b* is defined as a three-dimensional space based on opposite colours. The L* coordinate (Lightness) indicates the amount of light present in a given colour, in our case; whether the pulp was lighter or darker. A positive a* coordinate is indicative of red colour and a negative one of green colour; a positive b* coordinate is indicative of yellow colour and a negative one of blue colour. The Chroma (C*) is a parameter based on the CIE L*a*b* system and it is indicative of strong or weak colour (intensity). Treated pulps with phenols were darker than laccase control pulps, L* coordinate was smaller in all the pulps grafted with simple phenols (Figure 5-3). Simultaneously, these pulps presented a higher colour saturation (C*) than the laccase control.

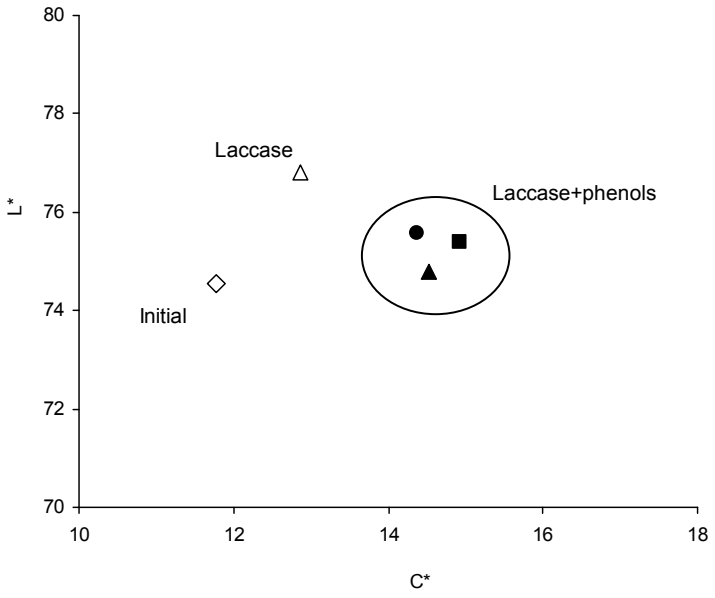

Figure 5- 3. CIE L*a*b* coordinates. Variation of the chromatic values L* and C* of the flax papers. L-SA paper (●), L-AS paper (■) and L-PCA paper (▲).

In all the treatments, the chromatic coordinates *a** and *b** were positive (Figure 5-4). Furthermore, pulps treated with laccase and phenols showed an increase of *a** and *b** coordinates respect to laccase control (except in the case of PCA treated pulp, where only a *b** coordinate increase was observed), which supposed an increase in red and yellow colour, respectively.

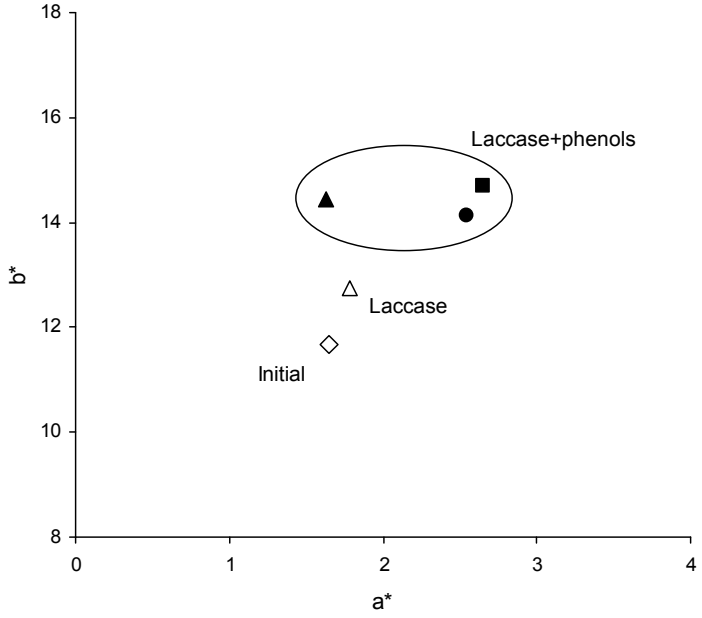

Figure 5- 4. CIE *L*a*b coordinates. Variation of coordinates *a** and *b** of the flax papers. L-SA paper (●), L-AS paper (■) and L-PCA paper (▲).**

The *k/s* index allows evaluating the amount of chromophoric groups presents in pulp. Papers obtained from pulps treated with laccase and natural phenols presented higher *k/s* values than initial and laccase control papers (Figure 5-5), mainly at 400 nm, confirming the formation or addition of chromophores in these pulps. Oppositely, laccase control papers resulted in a *k/s* values decreased respect to the initial control papers, showing a loss of chromophoric groups (lignin removal) caused by the action of the laccase.

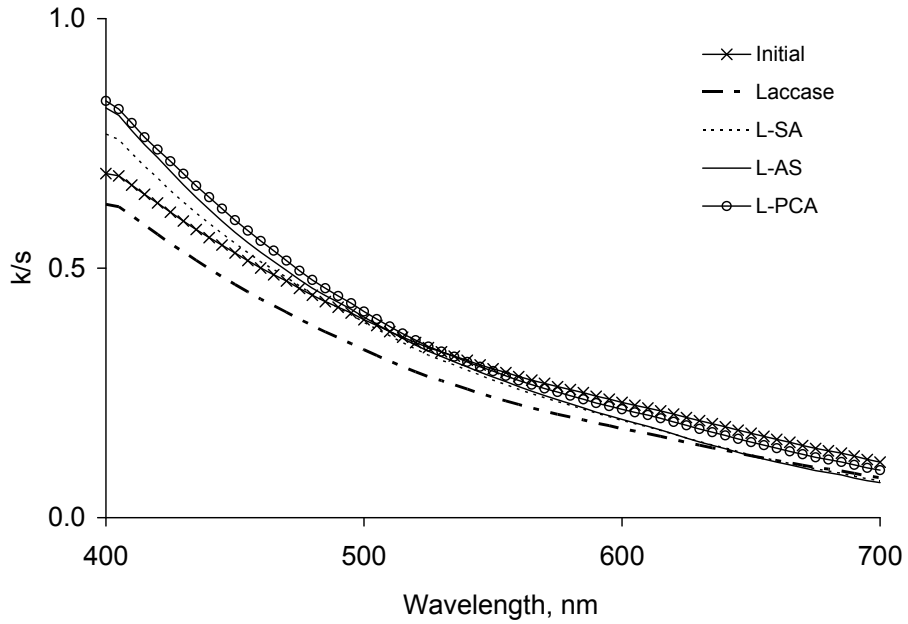

Figure 5- 5. *k/s* curves of paper obtained from: initial pulp, laccase control pulp and laccase-phenol treated pulps.

5.3.3 Antimicrobial properties of the grafted fibres

The antimicrobial properties of the grafted flax fibres against three bacteria: *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were tested.

Phenolic compounds are known to exhibit antimicrobial activity against a variety of microorganism. They have been reported some studies about antifungal and antibacterial activity of SA (de Souza et al. 2005; Zaldivar et al. 1999) and PCA (Salomão et al. 2008; Wen et al. 2003).

Some of the requirements for an "ideal" antimicrobial agent would include effectiveness against a wide range of microorganism, low cost, easiness to apply and resistance to leaching from the material. According to these needs, natural phenols are potentially good antimicrobial substrates to test in grafting.

The method used for evaluate the antimicrobial activity effectiveness of an antimicrobial fibre was based on the ASTM E2149 Standard Test Method. The type of test challenge applied in this method is extreme, and very effective to test antimicrobials that are covalently bonded to the fibres. Furthermore, this method ensures good contact of inoculums to treated fibres by constant agitation during the test period.

The results showed that incubation of the tested microorganisms with the enzyme-phenol treated fibres caused a decrease in the microbial viable count, indicating an antibacterial activity of the grafted papers. The Gram negative bacteria tested, *K. pneumoniae* and *P. aeruginosa* showed an important growth inhibition by the phenol grafted papers, as a notable reduction in the number of bacterial cells (colony forming units, CFU) was caused by the contact with these fibres. AS and PCA grafted fibres showed an important antibacterial activity on *K. pneumoniae*, producing a nearly total growth inhibition, while SA fibres caused a less pronounced effect. AS fibres also caused a high reduction in bacterial population of *P. aeruginosa* (97% reduction), while SA and PCA coupled fibres reduced the bacterial population around 70%. Contact with the phenol grafted fibres produced a lower reduction in the bacterial cell count of the Gram positive bacterium tested, *S. aureus* (Table 5-1). The major reduction was caused by the PCA grafted fibres (73% reduction), while AS and SA fibres caused a reduction between 40 and 55% in the microbial population.

Table 5- 1. Antibacterial activity against different bacteria of flax fibres grafted with natural phenols.

Bacterial population reduction (%)			
	S. aureus	*K. pneumoniae*	*P. aeruginosa*
Control	17	5	0
Laccase-Syringaldehyde (L-SA)	55	61	71
Laccase-Acetosyringone (L-AS)	40	99	97
Laccase-ρ-coumaric acid (L-PCA)	73	97	70

Immobilized antimicrobial agents, as surface bonded materials, are not free to diffuse or be released into the environment under normal conditions of use. The method used to determine the antimicrobial activity of these materials ensures good contact between the microorganisms and the treated sample. To verify the suitability of this

methodology for the specimens tested, the presence of antimicrobial leaching was determined evaluating the effect on bacterial growth of the supernatants obtained from incubation of the grafted fibres in sterile buffer, as detailed under Material and Methods. The absence of inhibition in every of the bacterial lawns cultured on agar plates indicated the absence of leaching, i.e. all the phenolic compounds tested remained bound to the fibres during the test.

Previous results reported by Elegir et al. (2008) showed that laccase mediated grafting of softwood kraft pulps can give antimicrobial properties to fibres. The phenols used in this report were different to those tested in our work. We have shown the antibacterial activity of pulp flax fibres grafted with acetosyringone, syringaldehide or *p*-coumaric acid. To our knowledge this is the first report that shows the antimicrobial activity of grafted pulp flax fibres, and also the first reported study on the antimicrobial effect of these natural phenols when grafted to lignocellulosic materials. The results obtained show the potential of functionalised fibres to produce safer paper products for sanitary and food uses, even with high quality fibres, as those from flax, of important alimentary use.

5.3.4 Antimicrobial properties of the natural phenols

To analyse the correlation between the antimicrobial properties of the grafted flax fibres and the antimicrobial properties of the corresponding natural phenols used, the antibacterial effect of these compounds against *K. pneumoniae*, *P. aeruginosa* and *S. aureus* was tested.

PCA was the most effective growth inhibitor, leading to inhibitions greater than 90% at 10 mM concentration for *K. pneumoniae* and *S. aureus*, and at 15 mM for *P. aeruginosa* (Figure 5-6).

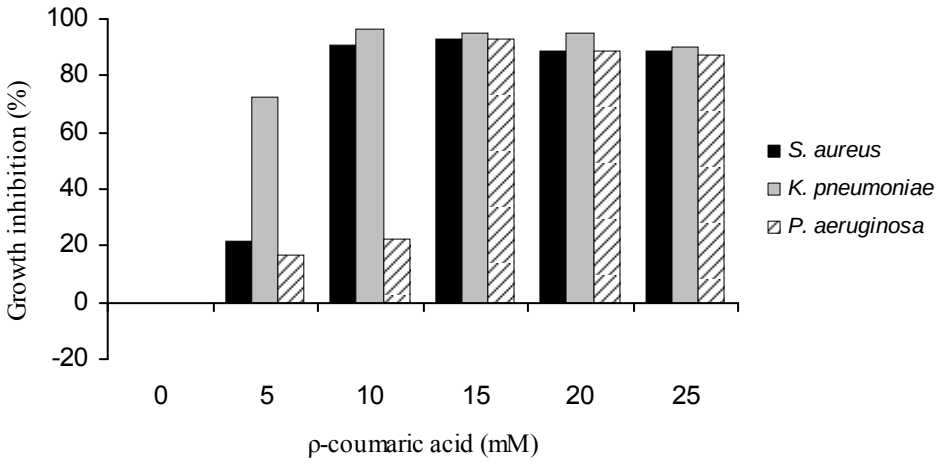

Figure 5- 6. Antimicrobial properties of the natural phenol PCA against *S. aureus* (black bars), *K. pneumoniae* (grey bars) and *P. aeruginosa* (striped bars).

SA caused more than 50% growth inhibition on *K. pneumoniae* at 10 mM, and on all the tested strains at 25 mM, the highest concentration assayed (Figure 5-7).

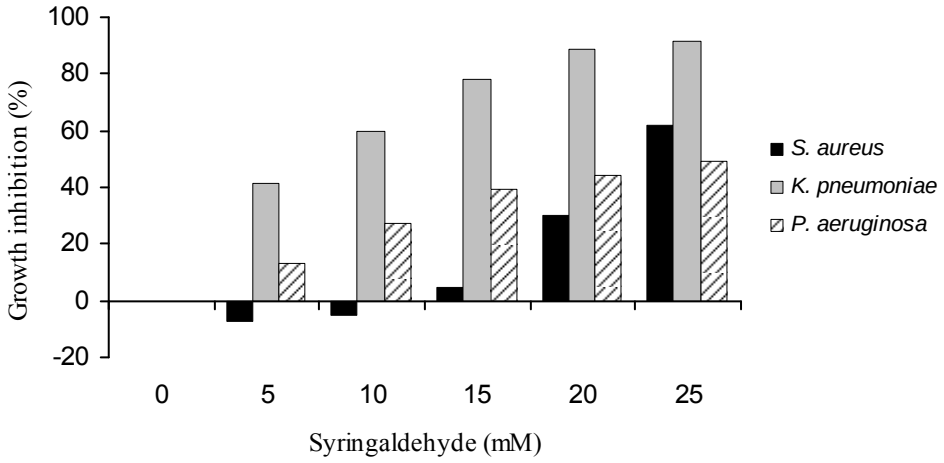

Figure 5- 7. Antimicrobial properties of the natural phenol SA against *S. aureus* (black bars), *K. pneumoniae* (grey bars) and *P. aeruginosa* (striped bars).

On the other hand, AS had a lower inhibition effect, only showing high antimicrobial effect on *K. pneumoniae* (more than 50% inhibition above 15 mM). At 25 mM concentration AS reduced the growth of *S. aureus* around 3%, and that of *P. aeruginosa* by 35% (Figure 5-8).

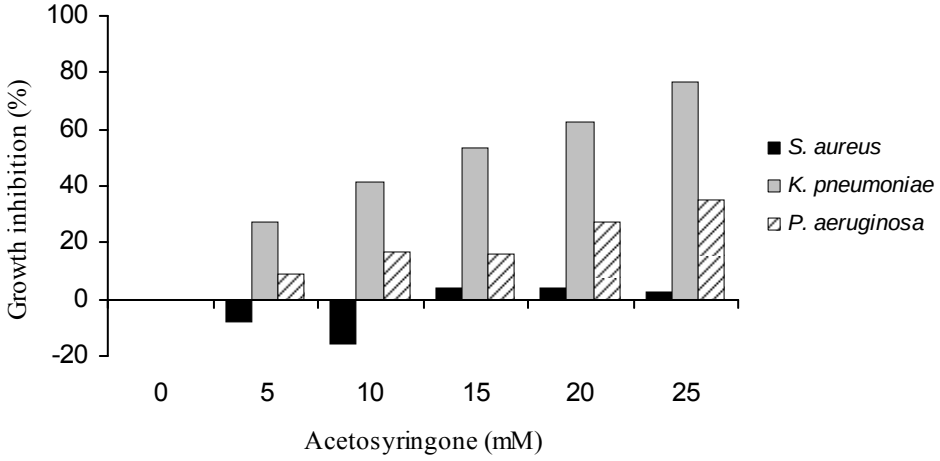

Figure 5- 8. Antimicrobial properties of the natural phenol AS against *S. aureus* (black bars), *K. pneumoniae* (grey bars) and *P. aeruginosa* (striped bars).

It is interesting to indicate that the lower concentration tested with the free phenols (5 mM) is higher than that used in the grafting experiments (3.5 mM). Growth inhibition values caused by 5 mM free phenols, with the exception of PCA and SA on *K. pneumoniae,* are below 30%, while inhibition caused by grafted fibres was always above 40%. The differences observed in the antibacterial behaviour between the free natural phenols, and those coupled onto laccase treated flax fibres, may be explained due to the modifications produced in these compounds by coupling to fibres by the laccase treatments. At this regard while PCA was the most effective antimicrobial agent both free or grafted onto fibres, AS showed a pronounced effect in grafted pulps, while it showed the lower antimicrobial activity when tested as a free compound.

5.3.5 UV spectra and residual activity in effluents from grafting

It was not possible to estimate the amount of simple phenols not grafted onto the fibres or the quantity of dissolved lignin on the effluents, as information given by spectra is only qualitative, and the signals that appeared overlapped and corresponded to different

products. Figure 5-9 shows the effluents spectra from the enzymatic stage. In the graph for each simple phenol three spectra are shown: laccase control, phenol control (effluents from pulp incubated with phenol but without laccase) and laccase assisted grafting of phenols. As an additional measurement laccase was incubated with the phenols studied without pulp to monitor their oxidation (data not showed). The reduced state of the phenols produced a spectrum with the same peaks that those exhibited by the effluents of the phenol controls. Laccase control spectrum (pulp treated with laccase, 40 U/g, but without phenol) showed a very low signal; though a signal increase was observed under 250 nm (caused by the buffer used on the treatments). SA control exhibited a peak at 308 nm corresponding to SA solution spectra (Figure 5-9a). This signal diminished strongly in the laccase SA treatments, though a lower peak was detected at 287 nm. This peak could be due to a SA not oxidized that remained in the effluents or/and SA degradation and/or oxidation products as well as the presence of dissolved lignin. On its side, AS control showed a peak at 300 nm also detected in AS solution (Figure 5-9b). Furthermore, AS oxidation produced an increasing peak at 362 nm which was not detected in Laccase-AS samples, indicating AS grafting onto the pulp or AS degradation, although a signal below 330 nm was detected. Figure 5-9c shows the PCA control spectrum exhibiting a high peak at 286 nm that corresponds to PCA reduced form. Laccase-PCA treatments exhibited a very low signal below 330 nm suggesting high grafting to pulps. The signal changes between the laccase-phenols treatments effluents and the control-phenols would indicate a phenol grafting onto the pulp, as well as degradation and/or oxidation products, although an increased concentration of dissolved lignin can not be excluded.

The inactivation of the laccase after the treatments was also investigated (Table 5-2). The control experiment without phenol resulted in 50% remaining activity. Treatments with AS and SA caused a laccase inactivation of 10% and 32%, respectively. In contrast, only 22% of the initial laccase activity was found after PCA treatments. This high laccase inactivation rate matched up with a high grafting rate caused by PCA. The loss of laccase activity caused by SA and AS would allow longer treatment time and maybe higher amount of grafted phenols onto the pulps, as well as the reutilization of the laccase.

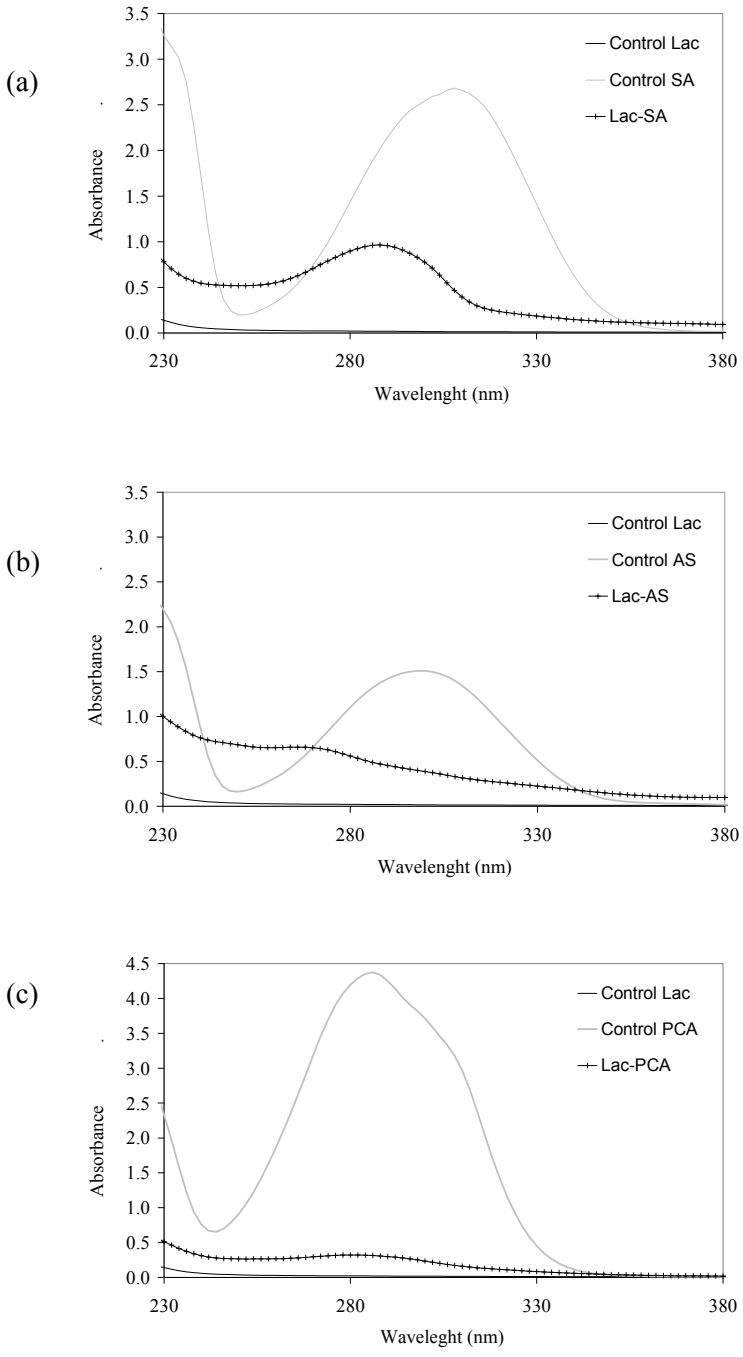

Figure 5- 9. Spectra of effluents from enzymatic treatments with SA (a), AS (b) or PCA (c). Control SA, AS or PCA: effluents from control phenols. Control Lac: control laccase. All spectra were carried out with 1:20 dilution of the effluents.

Table 5- 2. Laccase residual activity after the enzymatic treaments.

	Laccase	L-SA	L-AS	L-PCA
Residual activity (%)	50	68	90	22

5.4 Conclusions

In this study, a laccase was used to initiate the grafting of three different antimicrobial phenol structures onto unbleached flax fibres with high cellulose content. Pulp and paper properties of the treated samples were analysed to assess the tendency of the natural phenols to couple to fibres, suggesting that laccase lead to the crosslinking of these compounds. Furthermore, grafted pulps presented high antimicrobial activity against three bacteria analysed and non-leaching of these phenolic compounds to the media; demonstrating the immobilization of the antimicrobial agents. This system can be seen as a valid method to create covalently bound bio-active papers with a new high added value: antimicrobial activity.

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Chapter 6

ELUCIDATING THE EFFECTS OF LACCASE-PHENOL TREATMENTS ON BAST AND CORE FIBRES FROM FLAX PULP

SUMMARY

Unbleached flax pulp is being studied as a raw material target for biotechnology innovation. Laccases have been tested in presence of several phenolic compounds in order to obtain high-value environmentally friendly paper products. In order to better understand the enzymatic effects of violuric acid (VA) and *p*-coumaric acid (PCA) on flax, changes in the chemical composition of the two main types of fibre that compose this pulp were assessed. After classification of initial pulp, two fractions according to fibre size were obtained (bast or long fibres and core or short fibres). There was a significant effect of the fibre dimension on the pulp properties and on the response to the application of different laccase-phenol treatments. PCA and laccase treatments resulted in a high increase in kappa number and darkening in both fibre fractions, indicating a grafting of this compound onto the fibres. Whereas, VA treatments produced long fibres with low lignin content (kappa number of 1.3) and high brightness (5 units higher than the control fraction), proving its bleaching capacity. Both biotreatments resulted in long fibres with high cellulose crystallinity and in a HexA removing in global and short fibres. Furthermore, no morphological changes were observed after laccase treatments, thus preserving the integrity of the fibres. In this study, we demonstrate that laccase acts as a polymerization agent for PCA and as a

delignification agent for VA and that the action of each enzyme system differs between bast and core fibres.

6.1 Introduction

Non-wood fibres have a long history as papermaking raw materials. Although wood fibres continue to be much more widely used, some developing countries employ annual plants as their major source of fibres for this purpose. Moreover, in developed countries, non-wood fibres are currently being used to manufacture high-quality pulp for speciality papers (Moore 1996; Sigoillot et al. 2005). Flax (*Linum usitatissimum*) is an herbaceous annual plant that is highly suitable for the production of thin, strong sheets such as those used in cigarette, bible and light-weight bond paper. The specific properties of the fibres used mean that they are likely to always remain part of the furnish required to manufacture these products (Focher et al. 2001).

Flax pulp usually consists of two different types of plant material, namely: so-called "bast fibres", which come from the bark and are long and strong, and "core fibres", which come from the log or xylem. The reason for using both types of plant material is purely economic (bast fibres are up to four times more expensive than core fibres). The higher the core fibre content in a pulp, the lower the tear index and tensile strength, the lower the porosity and the smaller the amount of energy required to refine the resulting paper (Martínez et al. 1998). Bast fibres are typically 10–55 mm long and 12–30 μm thick. On the other hand, core fibres are more similar to leafy fibres as they range from 0.05 to 0.5 mm in length and from 10 to 30 μm in thickness (García Hortal 2007; McGovern et al. 1987). Core fibres contain much more lignin than bast fibres, so they are much more difficult to bleach (deJong et al. 1999). Current interest in the use of biotechnology in pulp and paper production processes has been aroused both by the high potential of biological treatments and by increasing environmental restrictions. The use of enzymes has emerged as a very promising choice not only for implementing clean bleaching processes, but also for developing novel, high-added value products (Bajpai 2004; Kenealy and Jeffries 2003). Laccases are multicopper oxidases secreted by white-rot fungi and other organisms that play a crucial role in nature by participating in the lignin metabolism. Laccase technology is applicable to those sectors of the pulp and paper industry where the aim is to either remove (bleaching) or

(co-)polymerize lignin (grafting) (Garcia-Ubasart et al. 2011; Widsten and Kandelbauer 2008).

Fungal laccases have been extensively studied in the presence of redox mediators in order to assess their ability to degrade lignin; this makes them useful for environmentally friendly pulp bleaching. These redox mediators are phenolic compounds that are easily oxidized by the enzyme and in turn oxidize other substrates with higher than laccase redox potential or inappropriate size to fit the active centre of the enzyme. Some synthetic mediators such as HBT or violuric acid showed high potential for non-wood pulp delignification (Chapter 3 and Chapter 5; Aracri and Vidal 2011); in any case, the search for natural compounds as new and environmentally safe mediators has gained much interest in recent years (Andreu and Vidal 2011; Cañas and Camarero 2010). The advantage of using natural compounds resides in their low cost and lack of toxicity since they are obtained from natural, renewable sources. When natural phenols are applied as laccase mediators to perform pulp bleaching, the delignification effect can be hindered by adverse reactions involving the phenoxy radicals generated upon the mediator enzymatic oxidation, such as depleting reactions (i.e. homopolymerization and cross-coupling reactions in the lignin structure) or fragmentations (d'Acunzo and Galli 2003; Moldes and Vidal 2008). In consequence, the treatment of lignocellulosic fibres and phenolic compounds with laccases are likely to result in a variety of oxidation and coupling products which are difficult to predict due to the complexity of the lignocellulosic matrix and the nature of free radical reactions (Kenealy and Jeffries 2003). In previous works, we successfully applied the synthetic mediator violuric acid (VA) in combination with a laccase from the white-rot fungus *Pycnoporus cinnabarinus* (PcL) for biobleaching of flax pulp achieving a high delignification rate (Chapter 4); furthermore, a natural phenolic compound, *p*-coumaric acid (PCA), was tested with PcL on unbleached flax fibres obtaining the coupling of the PCA onto the fibres by covalent bonding and getting a new-value product (paper with antimicrobial activity) (Chapter 5 and 7). Our current knowledge of oxidized phenolic compounds activity on the different types of fibre that form unbleached flax pulp has many shortcomings, and these need to be resolved through both fundamental and applied research. The pulp used in this study consisted of a mixture of the two types of fibre, which differ in chemical composition (García Barneto et al. 2011; Sain and Fortier 2002).

For this purpose, in order to better understand the enzymatic effects on fibres, unbleached flax pulp was submitted to different biotreatments that involved the use of a laboratory laccase (PcL) in conjunction with two phenolic compounds with different modifying behaviours (VA and PCA). Initial and biotreated flax pulp were classified for the first time in two-size fractions consisting of bast and core fibres and characterized in depth in order to elucidate the different performance of these active compounds on each fraction of fibres.

6.2 Materials and methods

6.2.1 Raw material

The raw material consisted of unbleached pulp from flax (*Linum usitatissimum*) produced at the CELESA mill in Tortosa (Spain) by soda-anthraquinone cooking. This pulp was formed by a mixture of two types of fibres (bast and core fibres). Before classification and enzyme treatments, the pulp was washed with H_2SO_4 at pH 4 to remove impurities and reduce the metal ion content. The unbleached pulp had 40.1% ISO brightness, a kappa number of 10.5 and viscosity of 783 mL g^{-1}.

6.2.2 Pulp fibre fractionation

A laboratory Bauer-MacNett classifier with screen sizes of 30 and 200 mesh was used to separate long and short fibres of flax pulp according to TAPPI T233-cm-06. For fibre separation, 10 g of pulp was disintegrated in 3,000 mL of water and poured into the first chamber. A continuous flow of water passes through this chamber. The short fraction passed through this first chamber was collected in the screen with the 200 mesh. The fraction passing through the screen with the 200 mesh sieve was considered to be fines. After 20 min of operation the flow of water was stopped and the two fractions were recovered and the content of each fraction was determined. The initial unbleached flax pulp (W_a) was fractionated –W_a long size or bast fibre (W_aL) and W_a short size or core fibre (W_aS)- and the three samples were characterized.

6.2.3 Laccase treatments

The initial non-classified pulp was submitted to two different enzyme treatments. The enzyme used was a laccase from the white-rot fungus *Pycnoporus cinnabarinus* (PcL)

produced by the INRA (Marseille, France) and the phenolic compounds applied were the natural lignin-derived *p*-coumaric acid (PCA) and the synthetic phenol violuric acid (VA). Both laccase treatments were carried out in an oxygen pressurized (0.6 MPa) reactor in 50 mM sodium tartrate buffer (pH 4) at 50ºC. Tween 80 (0.05% w/v) was added as surfactant. The specific conditions for each pulp treatment are as follows: 80 g of pulp were treated at 4% consistency for 4 h at 30 rpm shaking with a PCA dose of 3.5% (w/w) and 40 U/g odp of laccase; 80 g of pulp were submitted to the same treatment although the consistency, shaking, time, VA dose and laccase dose were 3%, 60 rpm, 5 h, 1.5% (w/w) and 20 U/g odp. One activity unit of laccase was defined as the amount of enzyme needed to convert 1 µmol of the substrate ABTS per minute. Oxidation of ABTS was monitored via the absorbance increase at 436 nm (ε_{436} = 29,300 M^{-1} cm^{-1}) in a Shimadzu 1603 UV-vis spectrophotometer. The reaction mixture contained 5 mM ABTS and 100 mM sodium acetate buffer at pH 5 and 24ºC. After both enzyme treatments, pulps were filtered and extensively washed with de-ionized water. Afterwards, the treated pulps were fractionated in the Bauer-MacNett classifier and global pulp and classified fibres were characterized. Both globally treated pulps were so-called L_{PCA} (pulp treated with PcL in presence of PCA) and L_{VA} (pulp treated with VA in presence of PcL), whereas, long size classified fibres were designed as $L_{PCA}L$ and $L_{VA}L$ and short size classified fibres were identified as $L_{PCA}S$ and $L_{VA}S$.

6.2.4 Analysis of pulps

The initial unbleached global pulp and its two fractions as well as laccase treated pulps and their corresponding long and short fibre fractions were characterized in terms of pulp properties; carbohydrate content was analysed by HPLC; Klason lignin was determined by gravimetry of the solid residue and FTIR spectra of handsheets were recorded to analyse cellulose. Furthermore, morphological properties of fibres were determined.

6.2.4.1 Morphological properties of fibres

Morphological properties of fibres such as fibre length, fibre width, and fines content (TAPPI T271-om-07) were determined with a Kajaani FS300 fibre analyser. Individual fibres were also analysed by optical microscopy, according to ISO 9184-3:1990.

Images were captured with a DeltaPix (Infinity X) digital camera integrated on a microscope. DeltaPix Viewer Software was used to digitise the pictures taken.

6.2.4.2 ATR-FTIR spectroscopy

ATR-FTIR spectra of paper handsheets were recorded on a NICOLET 6700, using an ATR Smart Orbit adapter (attenuated total reflectance). The spectra wavenumber range was 4000–400 and the number of scans was 64, with a wavelength resolution of 4 cm^{-1}. The relative cellulose crystallinity was assessed through the intensity peak ratios $A_{1375/2902}$ (Nelson and O'Connor 1964), whereas for relative order the ratio was $A_{1427/895}$ (Focher et al. 2001).

6.2.4.3 Carbohydrate analysis by HPLC

Carbohydrates were characterized by quantitative acid hydrolysis and the resulting hydrolysates were analysed for glucan and xylan content (HPLC determination of the glucose and xylose, respectively). Chromatographic determination was performed using an Agilent 1100 HPLC equipped with an ion-exchange resin Aminex HPX-87H column under the following conditions: mobile phase, 0.006 mol/L of sulphuric acid; flow rate, 0.6 ml/min; and column temperature, 60ºC. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all purchased from Sigma-Aldrich). Because the column failed to resolve xylose, manose and galactose, their combined content was expressed as xylose (Garrote et al. 2001). Before HPLC analysis, the solid residue from the post-hydrolysis process was recovered by filtration and considered Klason lignin (CLK, insoluble in sulphuric acid lignin).

6.2.4.4 Pulp characterization

In this way, kappa number, brightness and viscosity were determined according to ISO 302:2004, 3688:1999 and 5351-1:2010, respectively. The hexenuronic acid content (HexA) was analysed by UV-detection (Chai et al. 2001). Additionally, kappa number was measured again in Hex-A free pulps (Hex-A removed by acid hydrolysis with mercury acetate) to estimate the actual lignin content (KN_{lig}). The optical properties of pulp were analysed using a reflectance measuring Technidyne Colour Touch apparatus. The colour of the samples was described according to the CIE L*a*b* colour system.

Other optical parameters used were Chroma (C*), Dye Removal Index (DRI) and Bleaching Index (BI). The Lightness (L*) varies from 100 for perfect white to 0 for absolute black, a* varies from greenness to redness, and b* from blueness to yellowness; C* is a measure of colour saturation. The DRI and BI measure the efficiency of colour removal; both use the CIE L*a*b* measurements, but employ different equations to obtain a single, easier-to-use value of colour removal (Fluet and Shepperd 1997).

6.3 Results and discussion

Usually the raw material for industrial flax pulp production is a blend that contains flax fibres (bast fibres) and a small proportion of shives (core fibres). Fibre and shive compositions are very different; fibre is richer in cellulose and shives are richer in hemicellulose and lignin (García Barneto et al. 2011). The flax fibres used to produce our flax pulp contained approximately 15% wt of shives.

Consequently, industrial flax pulp contains fibres of different size and properties. By using two consecutive filtration steps, 77% wt of the initial pulp was retained by a 30 mesh screen (considered long fibres) and 23% wt by a 200 mesh screen (short fibres). Fines, which pass through the 200 mesh screen, were discarded. In order to better understand the mechanism of two different phenols used in combination with a laccase, both biotreated fibres were also classified and then analysed in terms of pulp properties and composition. Flax pulp treated with *Pycnoporus cinnabarinus* laccase (PcL) and *p*-coumaric acid (PCA) resulted in a 75% wt of long fibres ($L_{PCA}L$) and 25% wt of short fibres ($L_{PCA}S$), whereas pulp submitted to a treatment with PcL and violuric acid (VA) presented 78% wt of long fibres ($L_{VA}L$) and 22% wt of short fibres ($L_{VA}S$).

6.3.1 Morphological properties of fibres

The FS300 unit (Kajaani Electronics Ltd.) is specifically designed to evaluate fibre-length distributions of cellulosic fibres. Measurements are based on the ability of these fibres to change the direction of polarized light and are computed-controlled. The FS300 is used extensively in the pulp and paper industry because it is fast and simple to use.

Although this part of the study was mainly dedicated to measuring of fibre length, fibre width and fines content were also evaluated. The average fibre lengths L(n), L(l) are represented in Table 6-1 for the global pulps and for the various fractions. The arithmetic average fibre length L(n) is not always the most commonly used indicator of the fibre length because the effect of short fibres is emphasized. The commonly used expression is the length weighted average fibre length L(l). As expected, smaller screen meshes (short fractions) produced lower average fibre length, confirming that the classifiers divide the pulp into fractions that vary in length. It can be seen that larger deviations are found between the weighted and numerical averages as fibre size increases (highly evident in global pulp samples and also in long fibres); the result of the large number of fines. Relative fines content –Fines(n)- varies enormously between global pulps and classified pulps; global pulps obviously presented the higher proportion of fines: pulps were not fractionated and consequently all the fines are still in these samples. As can be seen, some fines were retained during fractionation and recovered with both long and short fibre fractions, due to the tendency of flax fibres to stick together or become entangled; this hinders their handling in both industrial and laboratory processes. In any case, length weighted average fines content values –Fines(l)- showed that in samples that have been fractionated this retention was not very significant.

Table 6- 1. Fibre length, content of fines and fibre width for non-classified pulps and classified fractions measured by a Kajaani FS300 fibre analyser. Variation coefficient values were below 5%.

	Global pulp			Long fibres			Short Fibres		
	W_a	L_{PCA}	L_{VA}	W_aL	$L_{PCA}L$	$L_{VA}L$	W_aS	$L_{PCA}S$	$L_{VA}S$
L(n) mm	0.40	0.44	0.44	1.04	1.24	1.17	0.39	0.41	0.32
L(l) mm	1.43	1.49	1.58	2.53	2.79	2.46	0.62	0.65	0.46
Fines (n) %	46	40	44	27	24	20	20	19	24
Fines (l) %	14	11	12	3	2	2	7	6	10
W(n) µm	17	17	17	23	22	21	16	16	16
W(l) µm	20	19	20	27	25	23	17	17	16

Concerning fibre widths (W), global pulp samples showed similar values to those obtained for the short sized fibre fractions, whereas higher values were observed for long fibre fractions.

Morphological fibre analyses using a Kajaani FS300 revealed clear evidence of the differences between global and size-classified samples. However, no important differences were found between biotreatments, showing that laccase-phenolic compound treatments did not affect the morphology of the fibres. It is important to emphasize that treatments with cellulases (hydrolase enzymes that act on the surface and inner layers of cellulose fibres) also failed to directly alter the morphology of the fibres although they were able to facilitate mechanical refining of pulp (Cadena et al. 2010; Garcia-Ubasart et al. 2010).

The Kajaani FS300 can be considered an optical method, although it is based on an indirect technique. For this reason, further conventional morphology analyses of fibres were carried out using optical microscopy. This microscopic study revealed the presence of the two types of fibre occurring in the plant. Upon staining with Herzberg dye, bast fibres (long) acquired a reddish brown colour, whereas core fibres (short) took on lighter, bluish colours. Core fibres are shorter and smaller than bast fibres. Figure 6-1 shows a photograph of a flax fibre sample as seen at x100 magnification under the light microscope.

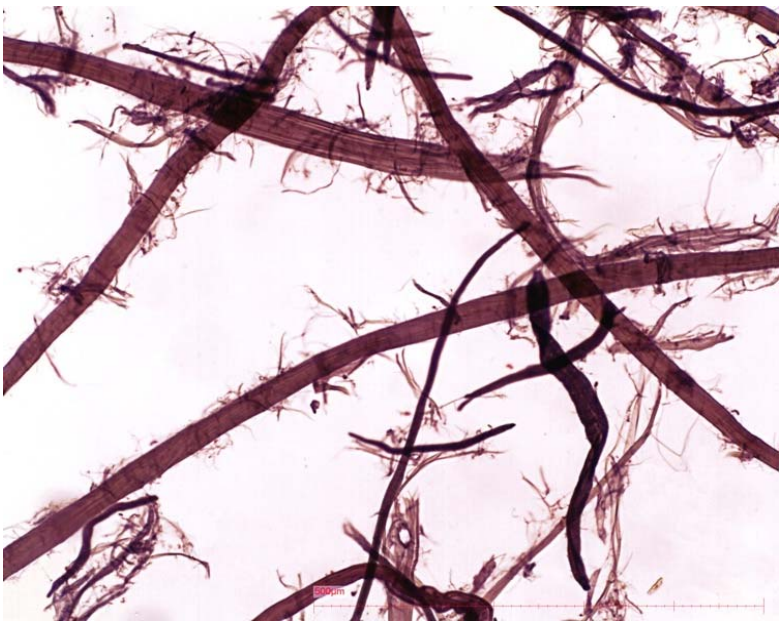

Figure 6- 1. Picture of flax fibres obtained by optical microscopy. Initial global sample x100.

As can be seen in the image of global pulp (Wa), two different types of fibre were observed that differ clearly in size. Photographs from long and short fractions were also taken (Figure 6-2). Both type of fibre showed high degree of fibrillation. No differences were found between biotreatments and untreated samples.

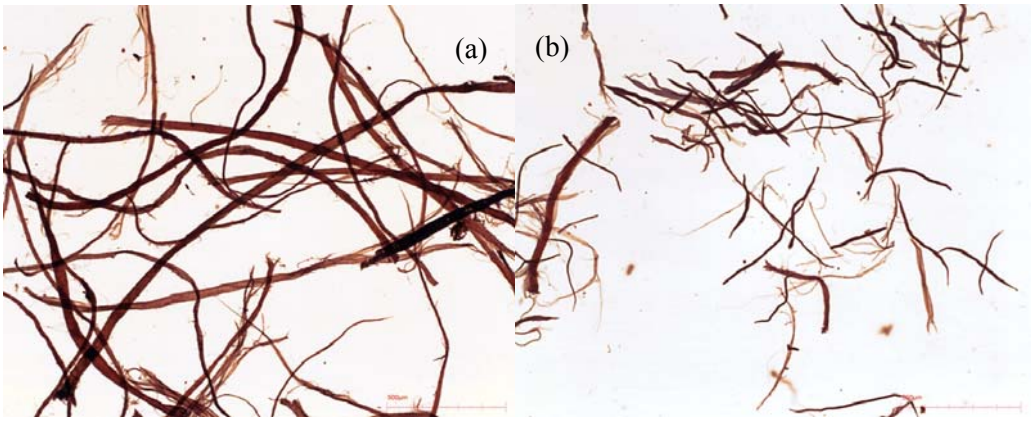

Figure 6- 2. Pictures of flax fibres obtained by optical microscopy. Long fibre fraction, x40 (a) and short fibre fraction, x40 (b).

6.3.2 FTIR spectroscopy

FTIR spectroscopy is a powerful tool for studying the structure of constituents and chemical changes in lignocellulosic materials. The relative absorbance of different bands was determined via the baseline correction method for making a comparative study of the spectra. It can be assumed the existence of general difficulties in pulp analyses principally arises from the numerous components with different chemical characteristics. The FTIR spectra of several flax pulp samples are shown in Figure 6-3; initial unbleached flax pulp (W_a), and both fibre fractions of the biotreated pulps are represented in the graph. All the FTIR spectra showed similar pattern, although some differences in the band intensities of the samples were observed. Two information-rich regions of the FTIR spectra, namely 3600-2800 cm^{-1} (Figure 6-3a) and 1600-600 cm^{-1} (Figure 6-3b) are represented. The most representative bands in the first region are 3333, 3290 and 3270 cm^{-1}, which are those assigned to –OH vibrations as well as 2896 cm^{-1} attributed to asymmetric methoxyl C-H stretching; these peaks are associated to cellulose. The region 1600-600 cm^{-1} (Figure 6-3b) is complicated and complex. Bands assigned to different lignin groups (1594, 1510, 1263, and 1130 cm-1) were not found. This is because the lignin content is very low. Bands common to lignin and cellulose

such as: 1427, 1370, 1334, 1160, 1108 and 1030 cm^{-1}, as well as bands assigned to cellulose such as: 1314, 1280, 1053 and 898 cm^{-1} were evident in the spectra.

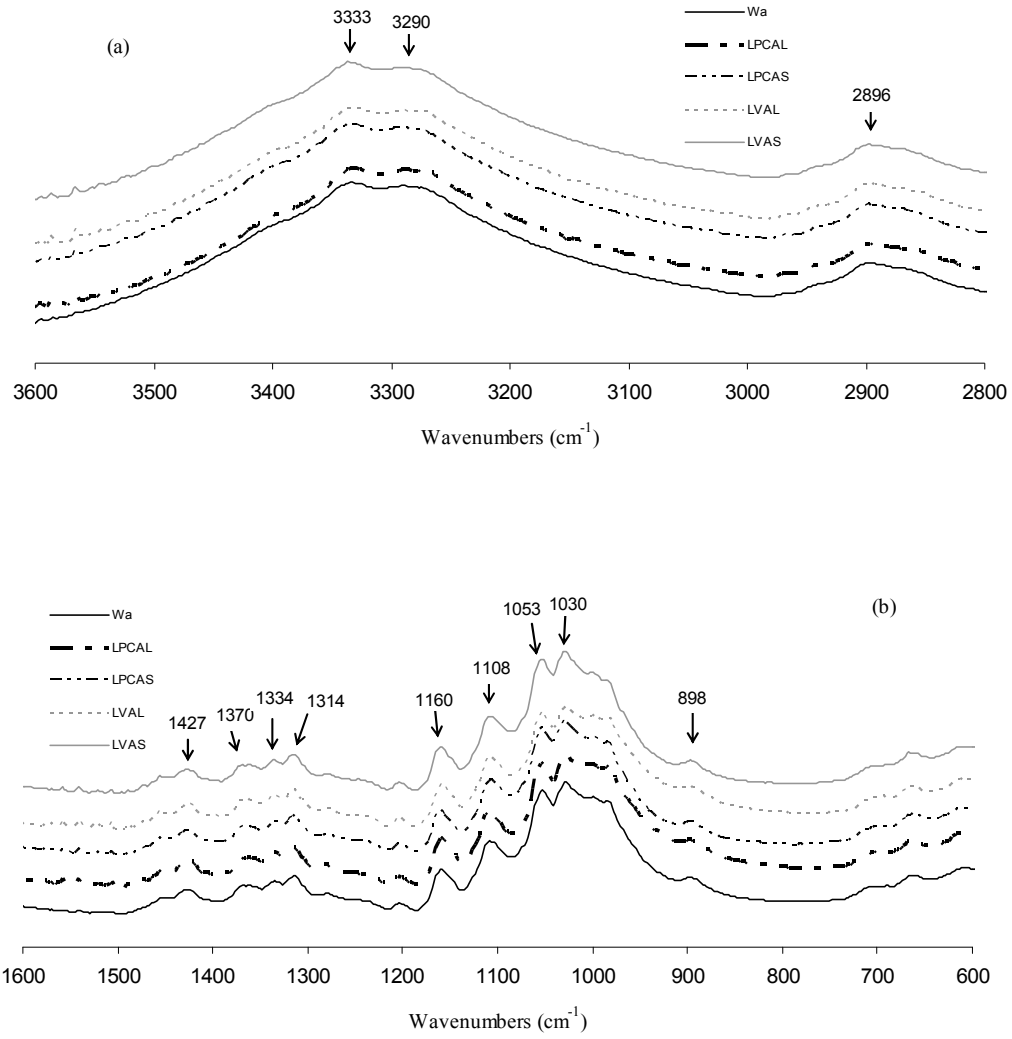

Figure 6- 3. ATR-FTIR spectra of the 3600-2800 cm^{-1} (a) and 1600-600 cm^{-1} regions of handsheets from some pulp samples: Initial global pulp (W_a) and long ($L_{PCA}L$ and $L_{VA}L$) and short fibres ($L_{PCA}S$ and $L_{VA}S$) of laccase-treated pulps.

The main bands are assigned to C-H asymmetric deformation in $–OCH_3$, H-O-C in plane bending of alcohol groups in cellulose (1426 cm^{-1}); C-H bending from $–OCH_3$, O-H and C-O of phenol and tertiary alcohol and C-H bending in cellulose (1370 cm^{-1}); C-O aromatic ring (1334 cm^{-1}); $C-H_2$ wagging (1314 cm^{-1}); C-H bending (1280 cm^{-1}); C-O-C asymmetric stretching (1160 cm^{-1}); ring asymmetric stretching (1108 cm^{-1}); C-O stretching (1053 cm^{-1}); C-O stretching (1030 cm^{-1}) and C-O-C stretching at β-glycosidic linkages between glucose (898 cm^{-1}) (Carrillo et al. 2004).

The ordered region of native cellulose is a mixture of two crystalline modifications (cel I_α and cel I_β) that vary in proportion, depending on the source of cellulose; the monoclinic I_β allomorph is the most thermodynamically stable form. Due to the metastability of the triclinic I_α form, the allomorph composition may change during pulping. Characteristic IR absorption bands for the two allomorphs of native cellulose were identified earlier. The bands characteristic of cellulose I_α and cellulose I_β in the -OH region are reported to be found at 3240 and 3270 cm^{-1}, respectively (Åkerholm et al. 2004); a peak was marked at 3269 cm^{-1}, peculiar to the I_β form. A peak characteristic for cellulose I_α at 750 cm^{-1} was not found, whereas a peak at 710 cm^{-1} characteristic of cellulose I_β was present in all the samples (Sugiyama et al. 1991). Table 6-2 shows the values of the total crystallinity index (TCI) and lateral order index (LOI) of all the samples. In the unbleached flax samples, the highest TCI value was calculated for long fibres (W_aL). This result agrees with a previous fibre-fractionation study (García Barneto et al. 2011). After enzymatic treatments, TCI of long fibre fractions increased in both biotreatments. The total crystallinity index is proportional to the crystallinity degree of the cellulose (Carrillo et al. 2004). An increase in the TCI was expected after biotreatment; probably due to the removal of substances adhered to cellulose (such as lignin and hemicellulose) and giving a cleaner, more ordered microfibril surface as a result (Chapter 7).

Concerning LOI, slight differences were observed between samples; it has been used to reflect the cellulose I fraction (crystalline versus amorphous cellulose). No differences in cellulose I fraction in the cellulose structure were found. The determination of CI using FTIR is the simplest method, but gives only relative values, because the spectrum always contains contributions from both crystalline and amorphous regions (Park et al. 2010).

Table 6- 2. Relative intensities of infrared bands (ratios): total crystallinity index (TCI, 1375/2902 cm^{-1}) and lateral order index (LOI, 1427/898 cm^{-1}).

	TCI ($A_{1375/2902}$)	LOI ($A_{1427/898}$)
W_a	0.44	0.69
W_aL	0.46	0.72
W_aS	0.39	0.69
L_{PCA}	0.35	0.68
$L_{PCA}L$	0.54	0.67
$L_{PCA}S$	0.39	0.74
L_{VA}	0.40	0.70
$L_{VA}L$	0.51	0.69
$L_{VA}S$	0.41	0.75

6.3.3 Carbohydrate content

As expected, long and short fibres showed different carbohydrate content (see Table 6-3). Initial global pulp (W_a) presented a composition of: glucan 90.1 g/100 g, xylan 5.5 g/100 g and Klason lignin 4.4 g/100g. After fractionation, it could be seen that long fibres (W_aL) were richer in glucan (cellulose) and short fibres (W_aS) were richer in xylan. Both biotreatments make it possible to decrease the Klason lignin content, except $L_{PCA}S$ fibres and especially fibres treated with laccase and VA (L_{VA}). When VA was used in combination with PcL, global pulp showed a decrease in xylan content; furthermore, the relative glucan content increase observed could be caused by the drop in the other fibre components. Concerning PCA treatments, a lower decrease in xylan content was observed; moreover glucan content was very similar to that obtained for the initial samples. The xylan content reduction occurred simultaneously with a decrease in HexA content, especiallly in short fractions of both biotreatments. It is important to remark that Klason lignin did not represent total lignin and xylan is not the sole component of the hemicellulose fraction.

Table 6- 3. Glucan, xylan and Klason lignin (CLK) content of pulps expressed as a percentage of dry matter, as well as viscosity measurements. HPLC variation coefficient values were below 3%; viscosity measurements had a variation coefficient between 1 and 9%.

	Glucan (g/100g)	Xylan (g/100g)	CLK (g/100g)	Viscosity (mL/g)
W_a	90.1	5.5	4.4	783
W_aL	95.0	4.1	0.9	821
W_aS	88.5	7.9	3.7	711
L_{PCA}	91.4	5.6	3.0	792
$L_{PCA}L$	95.6	3.8	0.6	842
$L_{PCA}S$	89.1	7.1	3.8	727
L_{VA}	95.4	3.7	0.9	788
$L_{VA}L$	94.8	4.7	0.4	831
$L_{VA}S$	91.3	7.2	1.5	712

Intrinsic viscosity values are a tool for assessing the polymerization degree of cellulose chains in fibres, in other words, the extent of cellulose degradation. As expected, long fibres presented high viscosity values, whereas short fraction fibres showed more than 100 viscosity units less than the long ones. Laccase-assisted biotreatments did not affect the cellulose integrity of any of the samples.

6.3.4 Pulp properties

As mentioned above, the pulp properties of fibre fractions of different size were studied. Figure 6-4 compares the kappa number of the different pulps analysed. As expected, long and short fibres of initial flax pulp presented evident differences in kappa number. Our initial pulp had a kappa number of 10.5, whereas long fibres (W_aL) showed a low kappa number (only 2.4) and the short (W_aS) ones a kappa number of 8.7. The difference between global pulp and both fractions may be caused by the loss of fines during fractionation and washing of the samples. Furthermore, unbleached flax pulp presents some impurities that can pass through the screen with the 200 mesh. Enormous differences in lignin content between fibres were clearly visible. Regarding biotreated pulps, differences between the phenolic compounds used were evident. In

the case of pulps treated with PCA, global pulp reflected the partial condensation of the phenoxy radicals of *p*-coumaric acid on fibres (increase of 4.5 points in kappa number with respect to W_a). Similarly, classified fibres showed an increase in kappa number with respect to the unbleached classified pulp; both fractions resulted in an increase of around 2 points, although the augment was more marked in the case of long fibres ($L_{PCA}L$) where the kappa number rose in 67% respect to W_aL.

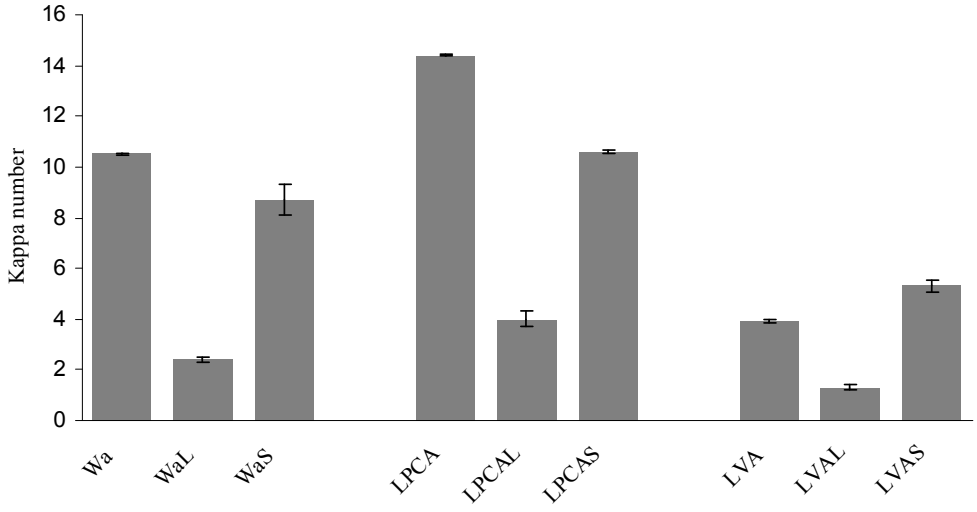

Figure 6- 4. Kappa number of global and classified pulp samples.

The tendency of some natural phenols oxidized by laccases to couple to fibres is studied depth and in different ways. In the next Chapter it is demonstrated by Py-GC/MS that PCA is covalently bound to the pulp fibres; furthermore, in other study it is verified that the PCA bounded to flax fibres by a laccase can act as an antimicrobial agent and produce handsheets with antimicrobial activity (Chapter 5).

The most efficient laccase mediators for lignin degradation are >N-OH mediators, such as violuric acid (VA). The oxidation of this type of mediator by laccase generates a highly reactive nitroxyl radical, due to the enzymatic removal of an electron followed by release of a proton. Nitroxyl radicals oxidize the target substrate by hydrogen atom transfer (Bourbonnais et al. 1997). The laccase-VA system resulted particularly effective in woody and non-woody pulp bleaching and delignification (Chapter 4; Aracri and Vidal 2011; Moldes et al. 2008). Non-classified VA treated pulp showed a

reduction in kappa number value of 6.6 points with respect to the initial global pulp; between the two fibre sizes, the highest net kappa number reduction was observed in short fibres ($L_{VA}S$), whose kappa number dropped 3.4 units. Long fibres ($L_{VA}L$) decreased by almost 50% with respect to W_aL. Consequently, the synthetic mediator VA used in combination with PcL caused pulp delignification in both fibres.

Residual lignin and hexenuronic acids (HexA) are known to be the main substances contributing to kappa number in pulp fibres (Chai et al. 2001). HexA form during alkaline cooking of plants by elimination of methanol from the 4-O-methylglucuronic acid group bonded as a side group to xylans; the significance of HexA relies on their adverse effects on pulp bleaching. It has been found that some laccases and laccase-mediator systems can reduce the HexA content of pulp (Valls et al. 2010). HexA content and contribution to kappa number were measured in all the samples, in order to evaluate the real delignifying effect of the different biotreatments in each fibre fraction (Figure 6-5). Initial unbleached pulp had an HexA content of 12.5 μmol/odp, whereas long (W_aL) and short (W_aS) fibre fractions presented 4 and 9.1 μmol/odp, respectively. The difference between global and classified fibres may be caused by the loss of fines and other impurities during classification and washing. Enzyme treatments were able to reduce the HexA content; as can be seen in global pulp measurements, pulps treated with the laccase-VA system exhibited a reduction of 4 units; the decrease in L_{PCA} was less important. The results suggest that these laccase-phenol systems can destroy HexA by oxidizing their double bonds in similar fashion to electrophilic bleaching agents (Ventorim et al. 2008). Comparing treated fibre fractions, both laccase-phenols treatments assayed failed to reduce the HexA content of long fraction fibres (probably due to the initially low HexA content of this type of fibres or to difficult accessibility that does not make any action of the enzyme or the oxidized phenol possible); on the other hand, short fibres showed a ~20% diminution with respect to the initial short fraction (W_aS).

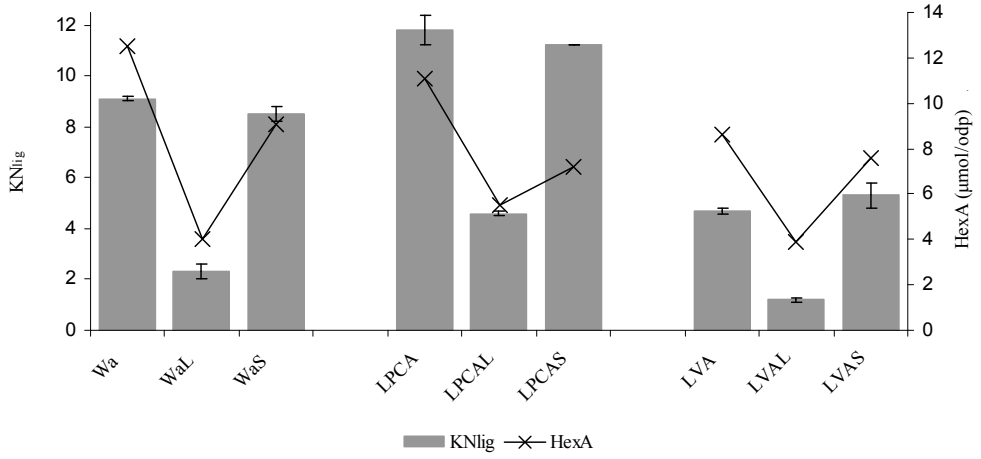

Figure 6- 5. Kappa number due to lignin KN_{lig} (measured after the removal of hexenuronic acid) and HexA content of the global and classified samples. Variation coefficient values of HexA were below 7%.

The possible contribution of HexA to kappa number was also estimated (Figure 6-5). The KN_{lig} of L_{PCA} presented the main decrease with respect to the initial KN measurement (~3 units); likewise this sample presented the tallest KN_{lig} and a high HexA content: these observations were probably related to the coupling of PCA to fibres. The other global pulps (W_a and L_{VA}) also had lower KN in HexA-free pulps with respect to initial measurements. Interestingly, no changes in KN_{lig} were observed in any of the classified samples.

So, in the light of these results, it is evident that each enzymatic system caused different effects on pulp properties. PCA used in combination with PcL mainly induce the grafting of the same on the fibres; differences in KN response between fibre fractions may indicate that the higher grafting degree was carried out in the fines fraction. We can assume that fines presented a high lignin and xylan content and consequently a high HexA content. So, as it has been observed (Chapter 4), phenolic compounds aided by laccases attack HexA; nevertheless, some interaction with other fibre compounds could be supposed. HexA were probably modified by the action of the laccase and PCA. PCA seems to couple to the xylan-HexA double bond, giving rise to an adduct (that will be discussed on Chapter 7). So, HexA coupled to PCA were not detected. On the other hand, an evidence of certain delignification action was also

found (CLK diminished in PCA treatments) indicating that PCA was involved simultaneously in oxidative degradation and grafting reactions. Concerning VA behaviour, a clear delignification activity was found. In all treated fractions, KN diminished, and so did HexA content (except in long fraction). Furthermore, no influence of HexA on KN measurements was found.

Regarding brightness (Figure 6-6), unbleached global flax pulp presented a value of 40.1% ISO, very similar to the obtained in short-sized fibres (W_aS); on the contrary, initial long fibres (W_aL) gave an elevated brightness: 7 points higher than W_a pulp. In the case of pulp treated with PCA+PcL, all samples -global and classified fibres- showed a darkening. The decrease in brightness was more marked in the post-classification fibres; both types of fibre lost brightness (4-6 points with respect to the initial pulp), although long fibres ($L_{PCA}L$) showed the higher decrease in this optical property (13% less than initial long fibres –W_aL-). On the other hand, the laccase-VA system promoted an increase in brightness; in spite of the fact that L_{VA} pulp presented the same value as the control pulp, long fibres ($L_{VA}L$) rose to higher levels (10% higher than W_aL) and the short-sized ($L_{VA}S$) increased in brightness by 3 points. Usually, the laccase-mediator system may cause the formation of chromophores in pulp due to the oxidative action of the enzyme treatment and, based on the kappa number results, cross-linking or cross-coupling reactions in the matrix structure of pulp may also take place.

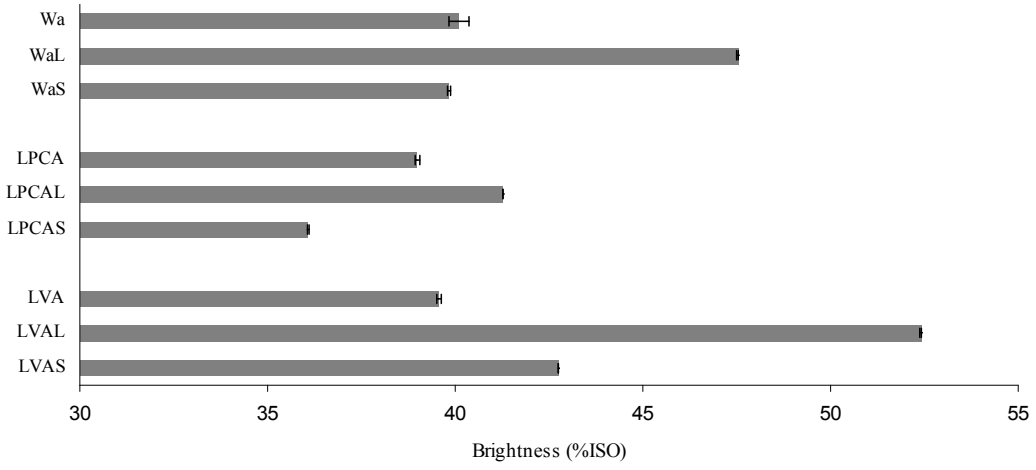

Figure 6- 6. Brightness of global and classified pulp samples.

Complementary optical properties were assessed, Chroma (*C**, gives information about colour saturation) and Lightness (*L**, represents the light amount in a colour) are shown in Figure 6-7. Some of the samples did not present important changes either in *C** or in *L** with respect to the initial global pulp (W_a); on the contrary, $L_{PCA}S$ fibres clearly increased their *C** and decreased in *L**, while, W_aL and especially $L_{VA}L$ experienced an important gain in L* and a fall in colour saturation (*C**).

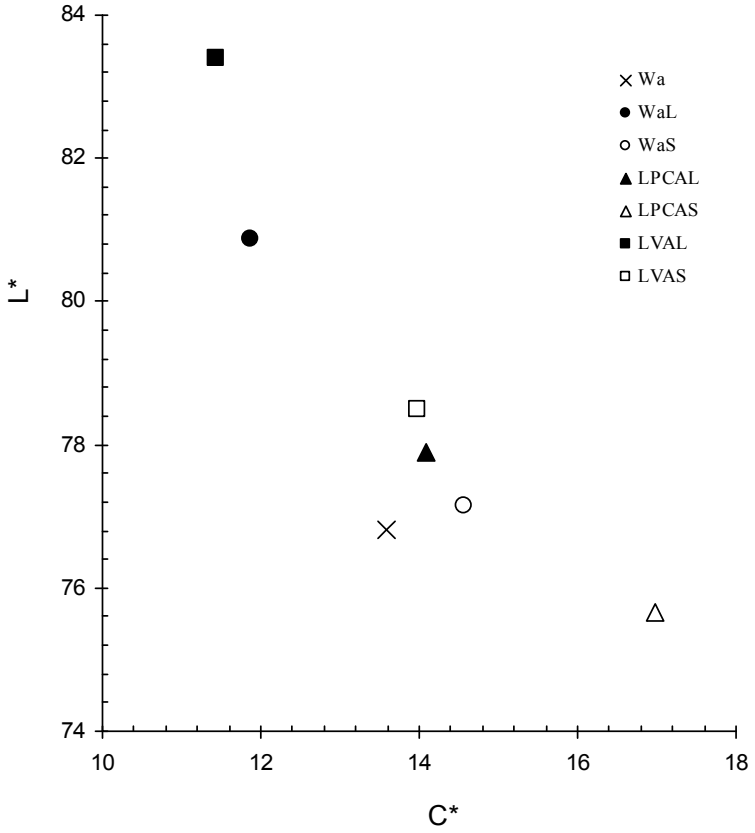

Figure 6- 7. Chromatic coordinates *L vs. *C** of global initial (W_a) and classified (W_aL and W_aS) pulp and laccase-treated-classified pulp.**

Regarding *a** and *b** colour coordinates (Figure 6-8), a similar effect can be observed; $L_{PCA}S$ suffered an important yellowness (increase in *b** coordinate) and a slight displacement to red. Once more, W_aL and $L_{VA}L$ showed the best values in terms of colour coordinates; the treated sample –$L_{VA}L$- was able to improve *a** and *b** values by diminishing them. The remaining trials did not show important differences with respect to the initial pulp (W_a), although all were darker than the control.

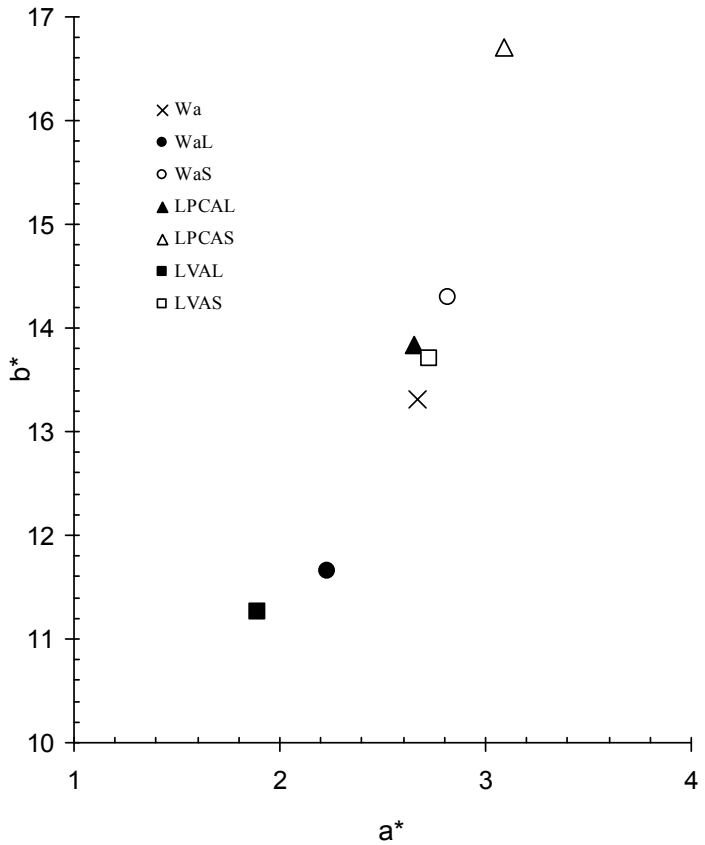

Figure 6- 8. Chromatic coordinates *a vs. *b** of global initial (W_a) and classified (W_aL and W_aS) pulp and laccase-treated-classified pulp.**

These results match with brightness measurements, where the best improvements were obtained with $L_{VA}L$ fibres. Optical properties revealed clear differences between long and short fractions, as well as evident differences between biotreaments: long fibres present better optical properties than global and short fibres; moreover, PcL+PCA caused an evident darkening and colouration of the fibres, whereas PcL+VA act as a good bleaching system (mainly in the long fraction).

In an attempt to measure the efficiency of colour removal, two commonly used colour indices were evaluated: Dye Removal Index (DRI) and Bleaching Index (BI). Both indices use the CIEL**a*b** measurements, but employ different equations to obtain a single, easier-to-use value of colour removal. The results obtained were shown in Table 6-4. Both indices used the initial global pulp (W_a) as a reference (DRI, BI = 0%). Positive values indicate colour removal, and negative ones indicate colour gain or darkening. As expected, the starting classification showed different sign, long fibres

(W_aL) were positive, whereas short fibres (W_aS) were negative. Concerning pulps treated with laccase and phenolic compounds, both treated global pulps did not show any colour removal; when classified fibres were considered, PCA biotreatments gave negative values, especially in short fibres ($L_{PCA}S$), again indicating colour gain. VA-classified fibres values showed positive values, rather high in the case of long fibres ($L_{VA}L$), these observations again confirm the ability of VA-laccase treatments for flax biobleaching. These results confirm the disparate behaviour of the enzymatically oxidized phenols towards fibres.

Table 6- 4. Dye Removal Index (DRI) and Bleaching Index (BI) of initial classified flax pulp and laccase-phenol global and classified pulp.

	DRI (%)	BI (%)
W_aL	29.89	37.05
W_aS	-1.76	-13.49
L_{PCA}	-6.31	-26.49
$L_{PCA}L$	4.90	-2.54
$L_{PCA}S$	-21.99	-64.36
L_{VA}	-4.70	-27.68
$L_{VA}L$	43.79	49.33
$L_{VA}S$	8.93	1.82

Initial Global Pulp (W_a) used as reference (DRI, BI = 0%).

6.4 Conclusions

Flax pulp from industrial alkaline cooking is a mixture of mainly two sizes of fibre. Long fibres (or bast fibres) are richer in glucans, whereas short fibres (or core fibres) are richer in hemicellulose and lignin. Consequently, long fibres presented a higher viscosity and lower kappa number and HexA content, as well as better optical properties (higher brightness). Two different enzymatic systems were applied to unbleached flax fibres; afterwards these fibres were classified and their properties evaluated. The characterization of the fractionated fibres revealed that the laccase acts as a polymerization agent for the natural phenol PCA and as a delignifying agent for

the mediator VA. The highest grafting degree of PCA was produced on the short size fibres and presumably on the fines. On the contrary, the long fibres fraction showed better bleaching performance when VA was used as an enhancing compound in the presence of laccase. The fact that the response to the biotreatments changes between both different types of flax fibres demonstrates that an initial characterisation of the fibres in terms of size could be considered to achieve different goals using laccases and phenolic compounds.

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Chapter 7

AN EXPANDED INVESTIGATION INTO THE BLEACHING AND GRAFTING EFFECT ON FLAX FIBRES OF LACCASE-PHENOL TREATMENTS

SUMMARY

This thesis is focused on the application of different enzymatic systems to flax pulp in order to modify flax fibres and obtain novel environmentally friendly high-value paper products. These enzyme systems are based on the use of laccases and natural or synthetic phenolic compounds. In order to better understand the enzymatic effects of several phenols on flax, further characterisation was assessed in collaboration with different research centres. The present study shows the use of several analytical techniques as an effective approach to studying bleaching and fibre functionalisation and using laccase-phenols treatments on flax pulp.

7.1 Introduction

Among the most investigated enzymes in the field of pulp and paper are laccases (EC 1.10.3.1), multi-copper oxidases, produced by microorganisms and plants, which participate in nature in both the biosynthesis and degradation of lignin (ten Have and Teunissen 2001). Laccases catalyse the oxidation of various substrates, including phenols, diphenols, aminophenols, polyphenols, and polyamines, with concomitant reduction of oxygen to water (Yaropolov et al. 1994). Most of the earlier research focused on the potential of laccase for the biobleaching of pulp (Bourbonnais and Paice

1990; Camarero et al. 2004; Fillat and Roncero 2010; Valls and Roncero 2009). In Chapter 3, several lignin-derived phenols were assayed as laccase mediators for aiding pulp delignification in a bleaching sequence. Although no immediate improvement or even a slight loss of pulp properties, in terms of kappa number and brightness, was observed immediately after the enzymatic treatment, the delignification effect was observable at the end of the bleaching sequence.

When natural phenols are applied as laccase mediators to perform pulp bleaching, the delignification effect can be hindered by adverse reactions involving the phenoxy radicals generated upon the mediator enzymatic oxidation, such as depleting reactions (*i.e.* homopolymerization and cross-coupling reactions in the lignin structure) or fragmentations (d'Acunzo and Galli 2003; Moldes et al. 2008). As a consequence, the treatment of lignocellulosic fibres and phenolic compounds with laccases are likely to result in a variety of oxidation and coupling products which are difficult to predict due to the complexity of the lignocellulosic matrix and the nature of free radical reactions (Kenealy and Jeffries 2003).

On the one hand, radical coupling reactions competing with delignification represent an adverse and undesirable phenomenon in biobleaching processes (Camarero et al. 2007). On the other hand, they have been drawing increasing attention due to being the key mechanism behind the laccase-assisted grafting of low-molecular weight phenols onto pulp fibres. This is a new approach to the use of these compounds, aimed at imparting better or novel properties to pulps and papers (Chandra and Ragauskas 2002; Liu et al. 2009). Fibre modification, especially with the assistance of enzymes, is a rapidly growing field of research and interest. Laccase-catalyzed bio-grafting is a versatile functionalisation method due to the enzyme's nonspecific substrate requirements, which enabled the bonding of a wide range of phenolic compounds and thus the incorporation of several desired properties in the fibre matrix (Chandra et al. 2004; Elegir et al. 2008; Gronqvist et al. 2006). The feasibility of this approach has been demonstrated in numerous studies; however, the interest has focused mainly on wood materials and lignin-rich fibres.

In this study, a novel approach of analysis was adopted to gain an insight into the mechanism of the laccase-phenol system (including natural and synthetic mediators) on flax pulps. In particular, the treated pulps were analysed by thermogravimetry, X-ray diffraction, analytical pyrolysis and ^{1}H NMR.

The chapter is divided into two parts:

- In the first part, changes in cellulose during enzymatic bleaching were analysed at the Department of Chemical Engineering of the UHU (University of Huelva, Spain). Initial flax pulp and ezymatically treated flax pulp (laccase+HBT) were compared in terms of X-ray diffraction spectroscopy and thermogravimetry.

- The second part, related to the grafting effect, was carried out in collaboration with IRNAS-CSIC (Instituto de Recursos Naturales y Agrobiología in Seville, Spain). The objective of this cooperation was to demonstrate that some natural phenols were covalently incorporated in the flax fibres by enzymatic treatment with laccase. In order to prove this assertion, in addition to pulp properties studies, Py-GC/MS and Py/TMAH analyses were carried out. Moreover, some studies were developed at the KTH (Royal Institute of Technology) facilities in Stockholm, Sweden. This collaborative work was carried out in order to further elucidate the interactions between flax pulp fibres and laccase-*p*-coumaric acid (PCA) treatments in terms of PCA coupling onto pulp fibres.

7.2 Materials and methods

7.2.1 Raw material

In all cases, flax pulp had a kappa number of 7.00, a viscosity of 816 mL/g, and an ISO brightness of 38.8%. Prior to the enzymatic treatments, the pulp was washed with acidified water (pH 4) for 30 min, at 2% pulp consistency, followed by filtration and extensive washing with de-ionized water.

7.2.2 Laccase-treatments

In the first part, thermogravimetric analyses (UHU studies) were developed in flax pulps submitted to the following conditions: 50 g of pulp at 5% consistency were treated with 20 U laccase/g odp, 1.5% odp of 1-hydroxybenzotriazole (HBT) and 0.05% Tween20 in 50 mM tartrate buffer at pH 4 at 50ºC with stirring at 50 rpm at an oxygen pressure of 0.6 MPa in a reactor for 4 h. A control test was carried out under

the same conditions but excluding the mediator. After the enzymatic treatment, pulps were filtered and extensively washed with de-ionized water.

In the second part, (IRNAS studies), flax pulp was treated under the same conditions at those adopted in Chapter 3. Syringaldehyde (SA), acetosyringone (AS) and *p*-coumaric acid (PCA) were applied as phenolic compounds at doses of 3% (w/w odp). Pulp samples treated under identical conditions, but in the absence of the phenolic compound, were used as controls. After the enzymatic treatment, pulps were filtered and extensively washed with de-ionized water. Thereafter, they were extracted with acetone in a Soxhlet apparatus for 2 h and 15 min in order to eliminate the phenolic compounds adsorbed on the pulp. In KTH studies, isolated xylan was treated with laccase and *p*-coumaric (PCA) acid.

7.2.3 Monitoring the effects on cellulose of laccase-phenol treatments used for bleaching of flax pulp (UHU collaboration)

7.2.3.1 Thermogravimetric analysis

Thermogravimetric runs were carried out with a Mettler Toledo model TGA/SDTA851e/LF1600, using ca. 5 mg of sample in each. Pyrolysis and combustion runs were carried out in nitrogen and synthetic air (N_2:O_2 4:1), respectively. The temperature was raised from 25 to 900ºC at three different heating rates (5, 10 and 20ºC/min). Experimental data were fitted by following a number of reported procedures that are described in García-Barneto et al. (2011).

7.2.3.2 X-ray diffraction

X-ray diffraction patterns for dry pulp samples pressed into tablets were obtained on a Bruker D8 Advance diffractometer using Ni-filtered Cu K_α radiation (λ = 0.1542 nm) generated at 40 kV and 20 mA. The scanned range was from 2θ = 5º to 50º. Experimental X-ray diffraction (XRD) signals were fitted to Gaussian peaks, which include an amorphous background. Pulp crystallinity was determined as at the ratio of the surface under the crystallinity cellulose peaks to the total surface, which includes the amorphous background contribution (Andersson et al. 2003). The equatorial dimension of crystallites was determined from the (2 0 0) reflection. The full width at

half maximum (FWHM) of the diffraction peaks was used to determine crystallite width, B_{hkl}, using the Scherrer equation (Eq.7-1):

$$B_{hkl} = \frac{0.94\lambda}{\sqrt{(\Delta 2\theta)^2 - (\Delta 2\theta)^2}\cos\theta} \quad \text{Eq. 7- 1}$$

where λ is the X-ray wavelength, Δ2θ the FWHM of each peak, Δ2θ the apparatus broadening parameter and θ the Bragg angle. X-ray diffraction analyses were performed twice.

The crystallinity index (CI) was obtained using the method by Segal et al. (1959) (Eq. 7-2):

$$CI = (1 - \frac{I_{am}}{I_{002}}) * 100 \quad \text{Eq. 7- 2}$$

where I_{am} is the intensity of the minimum between the 002 and 101 peaks (approximately at 2θ = 19º), and I_{002} that of the maximum for the 002 peak (approximately at 2θ = 23º).

7.2.4 Further elucidation of the interactions between flax pulp fibres and the laccase-phenol grafting treatments (IRNAS - KTH collaboration)

7.2.4.1 Evaluation of pulp properties

Brightness and kappa number of pulps before and after acetone extraction were assessed according to ISO 3688:1999 and ISO 302:2004, respectively. A straightforward method was developed to obtain an estimation of the amount of grafted phenol: kappa numbers of phenol solutions, where the presence of 1 g of totally bleached pulp was supposed, were measured for different phenol concentrations. Thus, a calibration line (Eq. 7-3/5) was originated, providing the amount of grafted phenol in correspondence to the increase in kappa number produced by this compound with respect to laccase control (samples coming from the acetone extraction). Calibration lines were:

$$y = 166.55x + 0.6554 \text{ (PCA)}, \quad \text{Eq. 7- 3}$$

$$y = 141.77x + 0.2969 \quad (SA), \qquad \text{Eq. 7- 4}$$

$$y = 130.65x + 0.2876 \quad (AS), \qquad \text{Eq. 7- 5}$$

If the coexistence of grafting and delignification reactions is supposed, this method will provide the *minimum* amount of phenol onto fibres.

7.2.4.2 Pyrolysis-Gas Chromatography/Mass Spectrometry (Py-GC/MS)

Pyrolysis of pulps (approximately 1 mg) was performed with a 2020 micro-furnace pyrolyzer (Frontier Laboratories Ltd.) connected to an Agilent 6890 GC/MS system equipped with a DB-5MS (Agilent J&W) fused-silica capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis was performed at 500ºC. The oven temperature was programmed from 40ºC (1 min) to 300ºC at 6ºC min^{-1} (10 min) and the carrier gas (He) was set at 1 $ml \cdot min^{-1}$. In addition, pulp samples were analysed by pyrolysis in the presence of tetramethylammonium hydroxide (TMAH), as a base and methylating reagent. For the Py/TMAH, 1 mg of pulp sample was mixed with approximately 0.5 μL TMAH (25%, w/w, aqueous solution) and the pyrolysis was carried out as described above. The compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries and reported in the literature (Faix et al. 1990; Ralph and Hatfield 1991).

7.2.4.3 Model experiments with isolated xylan

In model experiments, xylan was treated with 40 U/g of laccase together with 4% of PCA in 50 mM tartrate buffer at pH4. After 4 h of treatment at 50ºC the sample was filtered and thoroughly washed before drying under vacuum at room temperature.

^{1}H NMR spectra of the isolated xylan before and after laccase-PCA treatment were recorded after dissolution in 10% NaOD in D_2O on a Bruker Avance 400 MHz instrument using the standard Bruker pulse program at room temperature.

7.3 Results and discussion

7.3.1 Monitoring the effects on cellulose of laccase-phenol treatments used for bleaching of flax pulp (UHU collaboration)

Enzymatic effects on pulp are usually studied via changes in chemical composition. However, this approach excludes potential structural changes in microfibril surfaces when slight composition changes may in fact result in substantial changes in cellulose surface chains. For this reason it fibre changes were examined using thermal degradation to fully expose surface-based processes resulting from application of the enzyme treatment.

7.3.1.1 Effects of the enzyme treatment on thermal degradation of pulp

Figure 7-1 compares the thermal degradation profile of the Initial and HBT pulp samples as obtained in an inert and oxidative environment, respectively. As can be seen, the thermogravimetric profile was sensitive to the changes caused by the enzyme treatment. Under pyrolitic conditions (i.e. in the absence of oxygen), lignocellulosic materials such as pulp are known to exhibit a single mass loss step due to the volatilization of major components (hemicellulose, cellulose and lignin). The process leaves a carbonaceous residue (char) that remains on the thermobalance. As can be seen in Figure 7-1a, the pulp treated with HBT volatized to a greater extent than the untreated pulp (88.7% wt versus 82.3% wt) and exhibited a higher mass loss rate (17.2 min^{-1} at 370ºC versus 14.3 min^{-1} at 358ºC).

These results are consistent with the laccase-mediator treatment removing substances adhered to cellulose and leaving a cleaner, more ordered microfibril surface as a result. Since hemicellulose, lignin and extractives produce comparatively more char than cellulose; their removal must reduce char production and increase volatile release by pulp. Also, cleaner, more ordered (crystalline) cellulose is bound to decompose over a narrower temperature range and exhibit an increased mass loss rate as a result.

The thermal degradation profile for lignocellulosic materials under oxidative conditions exhibited two mass loss steps. The first was a volatilization step similar to that observed under an inert atmosphere except that the DTG curve (the mass loss profile: mass loss rate versus temperature) was shifted to lower temperatures and

reached higher mass loss rates. The second step was due to the oxidation of previously formed char. The resulting broad peak changed to a sharp, narrow peak when the experimental conditions (mainly a high heating rate) caused char to ignite (Figure 7-1b).

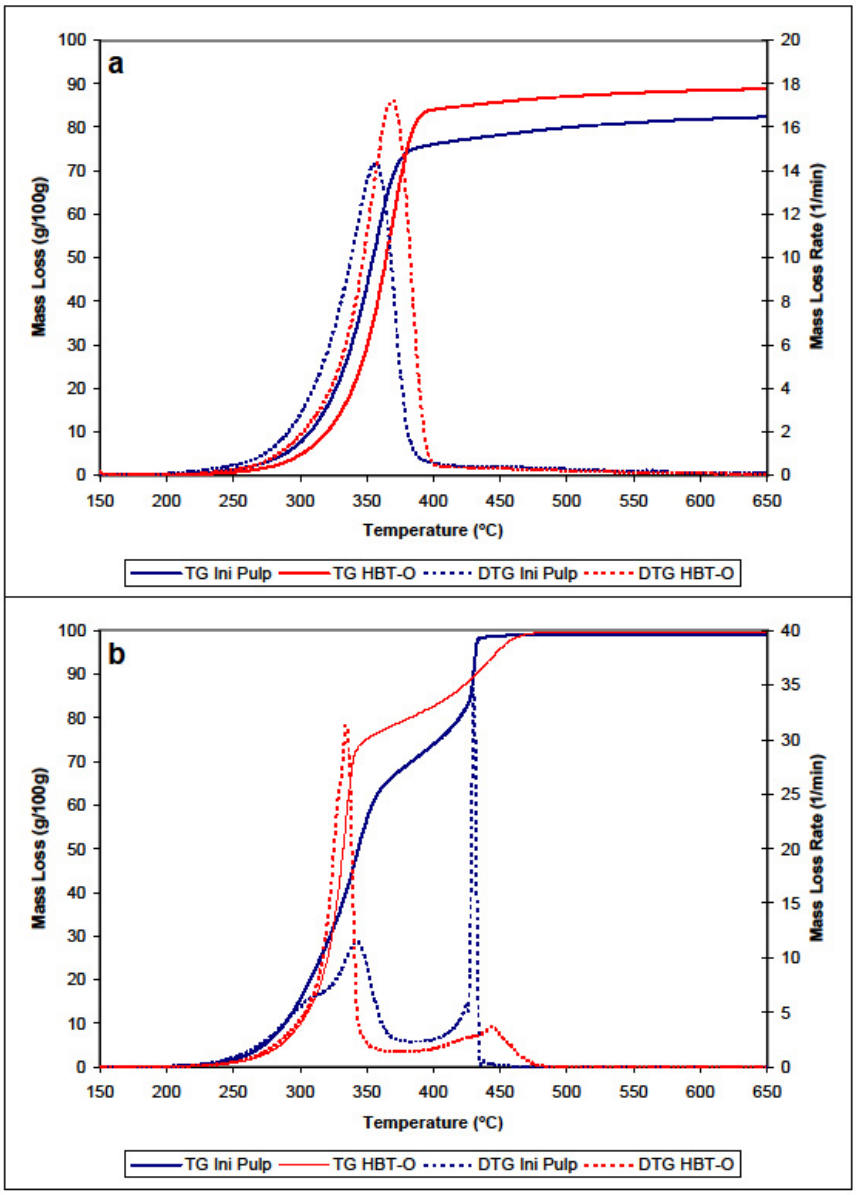

Figure 7- 1. Thermal degradation profiles of Initial and HBT treated pulp (referred as HBT-O): (a) in nitrogen atmosphere, (b) in air atmosphere.

A test conducted under atmospheric air provided additional information about amorphous and crystalline cellulose. As shown in Figure 7-1b, the differences between the Initial and HBT pulp samples were greater in atmospheric air than under a nitrogen atmosphere. Under oxidative conditions, differences in thermal degradation for amorphous and crystalline cellulose caused the volatilization step for Initial to exhibit two sub-steps at temperatures close to 300 and 350ºC which were not observed under pyrolytic conditions. Since disordered cellulose is more accessible than crystalline cellulose, the presence of oxygen must have mainly affected the volatilization of amorphous cellulose; as a result, its peak was shifted to a lower temperature and separation between the respective mass losses (separate peaks) increased. No peak for amorphous cellulose was observed in the profile for HBT because the proportion of amorphous cellulose remaining after the enzyme treatment was significantly lower than in Initial.

Based on the above, the laccase-HBT system removes substances adhered to cellulose, alters microfibril surfaces and increases the proportion of crystalline cellulose as a result. These changes affect the thermal degradation of pulp by increasing the production of volatiles, reducing that of char and raising the maximum mass loss rate during volatilization.

7.3.1.2 Pulp crystallinity

Enzyme treatment with HBT increased the crystallinity index (CI = 88.7%) with respect to its initial value (CI = 84.3%) and the control pulp. The CI as determined with the Segal method is useful for comparing relative differences between samples; however, it underestimates amorphous cellulose and overestimates cellulose crystallinity as a result (Park et al. 2010).

Table 7-1 shows pulp crystallinity (i.e. the proportion of crystalline cellulose in the pulp) and microfibril equatorial size as obtained by deconvoluting the XRD patterns (data not shown). Overall, the enzyme treatment increased pulp crystallinity and reduced microfibril equatorial size without altering the interlayer spacing [0.39 nm in the (2,0,0) plane]. These effects were strengthened by the presence of the mediator HBT.

Pulp crystallinity (Table 7-1) differed in terms of the analytical method used to determine it (XRD or TGA) by effect of their disparate response to changes in microfibril surfaces. Thermogravimetric analysis is sensitive to the thermal behaviour of sample components; thus, if cellulose surface fibres contain adhered substances, they end up in a disordered state and are thermally degraded as amorphous cellulose. Based on XRD patterns, however, the position of nodes in the cellulose lattice does not change; as a result, XRD measurements of pulp crystallinity invariably exceed TGA measurements of this property.

Table 7- 1. Pulp properties obtained from TGA or XRD measurements. TGA derived values are obtained from kinetic parameters (data not shown).

	Initial	Control	HBT
Pulp crystallinity (%) TGA[a]	-	55.1	69.2
Pulp crystallinity (%) XRD	66.9	68.2	69.4
Equatorial size microfibril B (2 0 0) (nm)[b]	6.7	6.2	5.7

[a] Pulp crystallinity, obtained from volatilization and charing of *Cellulose1* pseudo-component

[b] Measured by deconvoluting the XRD patterns

7.3.2 Further elucidation of the interactions between flax pulp fibres and the laccase-phenols grafting treatments

The flax pulp was treated with the laccase from *P. cinnabarinus* (PcL) in the presence of syringaldehyde (SA), acetosyringone (AS) and *p*-coumaric acid (PCA). After the laccase treatments, flax pulps showed an increase in kappa number (Figure 7-2) when treated with SA, and especially when treated with PCA. In the case of laccase treatment with AS, the kappa number obtained was very similar to the control pulp. In order to eliminate the contribution of low molecular-mass phenols to kappa number, a Soxhlet extraction with acetone was carried out to remove these compounds adsorbed on the pulp. After acetone washing, the kappa number decreased in all cases, although it was still higher than the control pulp when using SA and PCA. These results suggest the occurrence of crosslinking or cross-coupling reactions of these phenolic compounds into the fibres. The highest increase in kappa number was observed with PCA (*ca.* 3 points higher than the control pulp), indicating a higher degree of PCA incorporation into the flax fibres.

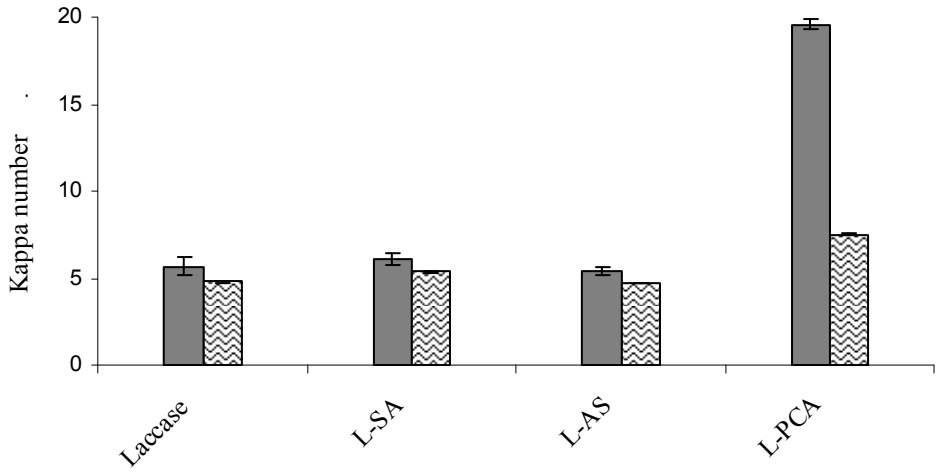

Figure 7- 2. Kappa number of flax pulps after the enzymatic treatment (grey bars) and the subsequent Soxhlet extraction with acetone (dashed bars). Laccase control sample was treated in the absence of phenolic compounds.

7.3.2.1 Study of enzymatic grafting of simple phenols on flax using analytical pyrolysis (IRNAS collaboration)

The minimum amount of grafted compound was estimated from the increase in kappa number with respect to the control pulp in the laccase treated pulps after acetone extraction.

The minimum amount, determined by the calibration line was estimated to be 12.3 μmols/g of pulp for PCA treatments (Figure 7-3) and 2.1 μmols/g of pulp for SA treatments. Interestingly, in a previous study (Chapter 3), we demonstrated the capacity of some of these phenolic compounds (AS and SA) to delignify flax pulp, when a subsequent hydrogen peroxide stage was applied.

Besides the increase in kappa number, the pulp treatment also showed a decrease of pulp brightness (Figure 7-4), which suggests the formation of chromophore groups due to the oxidative action of the enzyme treatment and/or the grafting of the phenolic compounds onto the pulp.

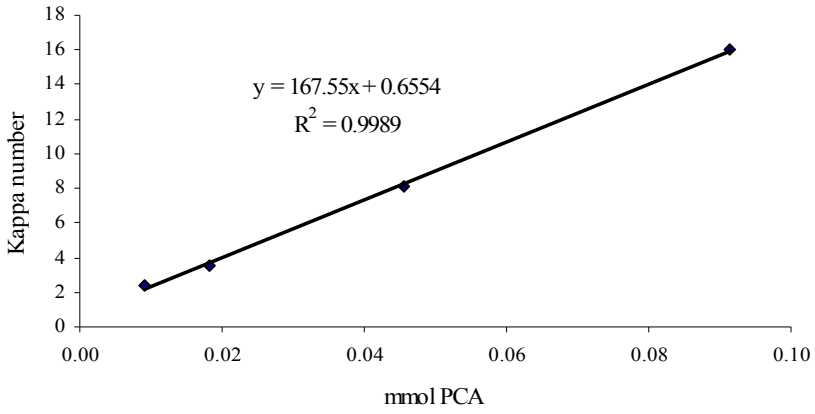

Figure 7-3. Kappa number of different concentrations of *p*-coumaric acid solution (from 1.5 mg to 15 mg). The presence of 1 g of totally bleached pulp was supposed. Inset shows the calibration line of PCA .

After acetone extraction, the brightness remained lower than the control pulp, similar to the kappa number results, except in the case of the SA treated pulp, in which the brightness increased by just 6% with respect to the non-extracted sample. This may be explained on the basis of the radical coupling reactions between the natural phenols and the fibres.

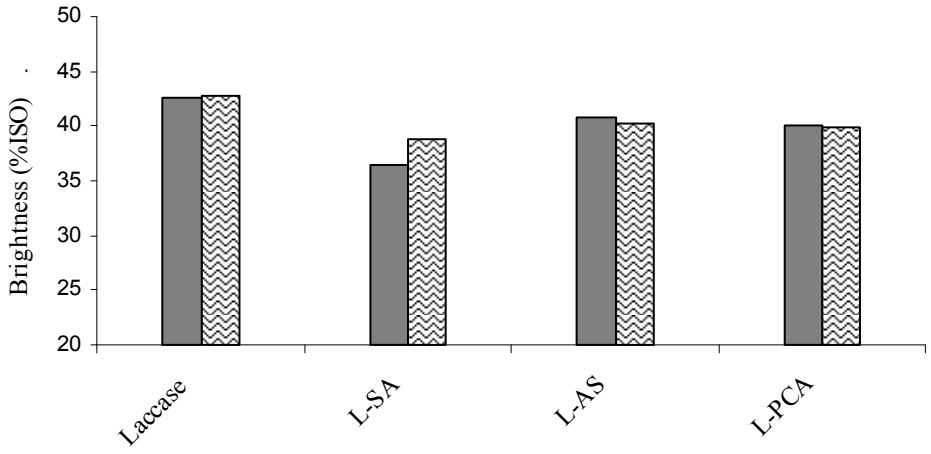

Figure 7- 4. Brightness of flax pulps after the enzymatic treatment (grey bars) and the subsequent Soxhlet extraction with acetone (dashed bars). Laccase control sample was treated in the absence of phenolic compounds.

In order to confirm the incorporation of the different simple phenols assayed here into the flax pulp fibres, the acetone extracted laccase treated pulps were analysed by Py-GC/MS, both in the absence and presence of tetramethylammonium hydroxide (TMAH).

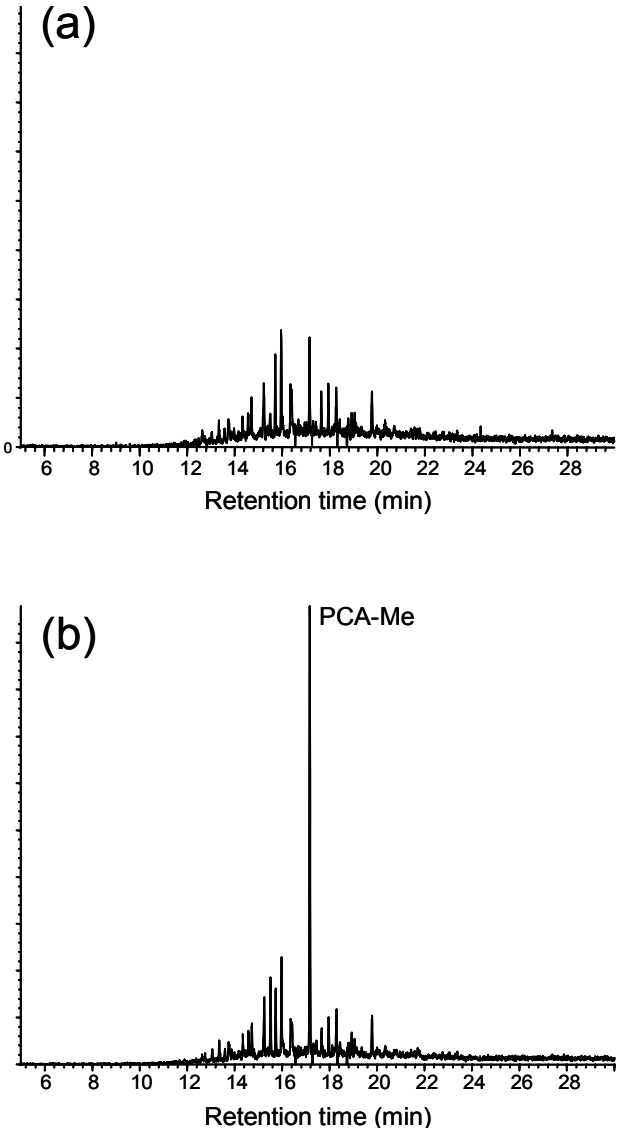

Figure 7- 5. Analysis by Py-GC/MS in the presence of TMAH (Py/TMAH) of (a) flax pulp control (treated with laccase alone), after acetone extraction, and (b) flax pulp treated with laccase and PCA, after acetone extraction. Single ion chromatograms of the fragment at *m/z* 192, characteristic of *p*-coumaric acid methyl derivative (PCA-Me) are shown.

Py-GC/MS is a powerful and sensitive tool for analysing residual lignin in pulp without the need of previous isolation (del Río et al, 2001). Among the different laccase treated flax pulps, the Py-GC/MS of the pulp treated with PCA released high amounts of 4-vinylphenol (Figure 7-5), a compound arising from the decarboxylation of PCA during pyrolysis (del Rio et al. 1996), and which was absent in the control pulps. In addition, pyrolysis in the presence of TMAH (Py/TMAH) of this pulp released high amounts of intact PCA as its methyl derivative (i.e. the methyl ester of 4-methoxycinnamic acid), and which is absent in the control pulp (treated with laccase alone) (Tabla 7-2).

These data clearly demonstrate that PCA is covalently bound to the pulp fibres, and are in agreement with the high increase observed in the kappa number of this fibre. In the case of pulps treated with SA and AS, the analysis by Py-GC/MS and Py/TMAH also demonstrated the grafting of a part of these phenolic compounds onto the pulp fibres (Table 7-2). However, the lower kappa number of the pulps treated with PcL and AS or SA indicates a lower degree of grafting in comparison with that treated with PCA.

Table 7- 2. Products released upon Py-GC/MS and Py/TMAH of the flax pulps treated with laccases and different phenolic compounds.

Flax pulp		
Phenolic compound	Py-GC/MS products	Py/TMAH products
Acetosyringone (AS)	Acetosyringone	Acetosyringone (methyl derivate)
Syringaldehyde (SA)	Syringaldehyde	Syringaldehyde (methyl derivate)
p-coumaric acid (PCA)	4-vinylphenol	p-coumaric acid (methyl derivate)

7.3.2.2 Further elucidation of the interactions between flax pulp fibres and the laccase-*p*-coumaric acid system (KTH collaboration)

A coupling reaction between the phenolic compound and pulp should involve at least one of the components cellulose, hemicellulose or lignin.

Treatment of the xylan with laccase+PCA resulted in no or very little elimination of HexA, while a substantial amount of polymeric aromatic structures could be observed giving rise to ^{1}H NMR signals in the region 6-8 ppm (Figure 7-6). These signals (which were absent in the ^{1}H NMR of the xylan) were assigned to polymerization

products of the phenol itself caused by the laccase, thus showing that PCA has a higher reactivity to laccase than HexA.

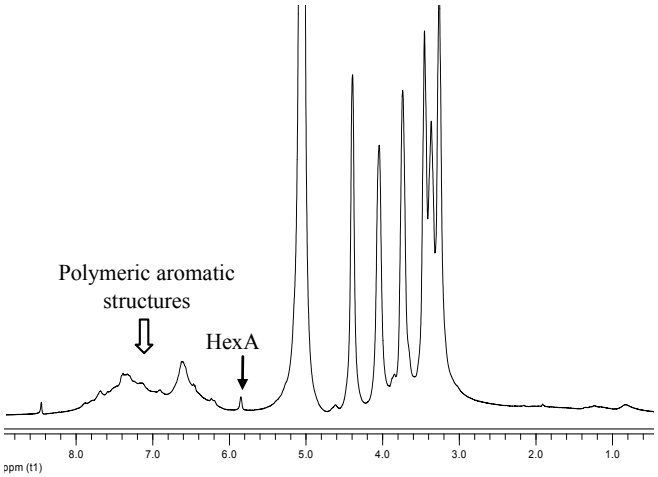

Figure 7- 6. ^{1}H NMR of laccase PCA treated isolated xylan from Eucalyptus kraft black liquor.

7.4 Conclusions

Laccase-HBT treatment for bleaching flax pulp caused a slight reduction in the microfibril equatorial size and increase in pulp crystallinity. The laccase treatment essentially removes substances adhered to cellulose and helps recover crystallinity in microfibril surfaces as a result. The changes involved affect the thermal degradation of pulp and can be monitored by TGA.

The enzymatic treatment for grafting flax pulp with laccase in the presence of the simple phenols syringaldehyde, acetosyringone and *p*-coumaric acid resulted in their covalent incorporation into the pulp. This assertion is based on the pulp properties obtained, and on the Py-GC/MS and Py/TMAH results. The laccase-PCA treatment of isolated xylan did not present much HexA removal, and upon oxidation by laccase, polymerization of PCA was the preferred reaction.

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Chapter 8

GENERAL SUMMARY AND MAIN CONCLUSIONS

In this section a general summary of the results obtained in this thesis is presented. A screening for selecting alternative natural mediators for the LMS system is studied; furthermore, some natural mediators are included in a complete TCF sequence and compared with a synthetic mediator that has been used with flax pulp for the first time. Pulp properties, as well as effluent properties are monitored along these sequences. The use of laccases as grafting agents is also studied, the grafting degree is examined by different techniques; furthermore, this capacity is used to produce papers with antimicrobial properties. Finally, an in-depth study on the mechanism of action of the enzyme systems on pulp is carried out.

- **Screening of natural mediators for flax pulp biobleaching**

The natural mediators evaluated are AS, SA and PCA; their performance is compared with the synthetic HBT. Some mediators (HBT and PCA) inactivate the laccase of *Pycnoporus cinnabarinus*, whereas SA and AS have a stabilizing effect. In any case, the presence of pulp is found to have a protective effect on enzyme stability. Laccase is inactivated by effect of the attack of a radical mediator on susceptible amino acids on the enzyme surface; on the contrary, the stabilization of the laccase may be the effect of a mediator binding to the active site or suitable point in the protein chain of the enzyme. The pulp can act as a reductive substrate for free radicals mediator, preventing laccase inactivation as a consequence. PCA was the only mediator that did not

inactivate laccase in the presence of pulp, this phenolic compound is condensed on the pulp; this condensation may prevent the free radicals mediators from inactivating PcL.

Natural mediators represent a promising alternative as delignifying agents, although after the enzyme stage only HBT and AS are able to reduce the kappa number with respect to the laccase control. So, it is necessary to introduce a hydrogen peroxide stage after the enzymatic treatment, in order to assess the real bleaching potential of the different laccase-mediator systems. Therefore, all LMS treatments result in substantial delignification after the P stage, this is especially so with HBT. The best results among the natural mediators in terms of KN are for SA, and in terms of final brightness are for SA and AS. It is interesting to remark that immediately after the L stage, SA and above all PCA increase the KN over the laccase control, indicating a condensation of these phenols onto the pulp. Viscosities after the L stage do not show major differences between enzyme treatments and control tests; however, the P stage causes an important loss of viscosity. The decreased viscosity obtained in the presence of laccase may have resulted from the accessible copper it contains being adsorbed onto the pulp fibres and causing hydrogen peroxide to decompose during the P stage. The hydroxyl radicals thus may cause cellulose depolymerisation; in the presence of transition metals in the cellulose matrix, such radicals may be generated close enough to cellulose chains to react with them. Furthermore, the L stage may have two different effects on the pulp, namely: direct degradation of cellulose and alteration of functional groups in the same that lead to easier degradation in a subsequent alkaline stage (P stage). Because viscosity measurements are made in an alkaline medium, the viscosity after L was a measure of both degrading effects. The P stage was performed in a hot, strongly alkaline medium, which may have resulted in modified cellulose being more markedly degraded than it was during viscosity measurements. As a result, the viscosity differences between L and LP may have resulted from degradation of the cellulose modified by L.

Concerning the effluent properties, COD values are markedly higher after L than they are after the P stage. These high values are the result of the presence of sodium tartrate buffer in the effluent. In whatever case, natural mediators present a higher COD than HBT. The effluents recovered after the P stage exhibit very low COD values. Therefore, the demand for chemical oxygen is essentially due to the presence of sodium tartrate buffer and the natural mediators. The LMS contribute to effluent colour

after the enzymatic stage (except when PCA is used as a mediator). HBT cause the higher colour value. This colour may have been caused by the formation of coloured mediator oxidation products and also by the presence of an increased amount of chromophoric groups formed by oxidation and/or degradation of lignin and the mediators. After the P stage colour is approximately 10 times lower than after the L stage and higher at the higher mediator rate.

SA exhibits the higher toxicity load. PCA also shows elevated toxicity. HBT presents the lowest toxicity after the L stage. The non oxidized mediators show a lower toxicity than that obtained in the LMS treatments. Therefore, the toxicity enhancement in some laccase-mediator assays of effluents could be caused by the generation of intermediate species (oxidized or radicals) and degradation products from the mediators.

The combination of PcL and a natural mediator affords efficient biobleaching of flax pulp in a TCF sequence including a subsequent peroxide stage.

- **Effects of LMS on flax pulp by thermogravimetry**

The thermal degradation profile is sensitive to the changes caused by the enzyme treatment. Under pyrolytic conditions, the pulp treated with HBT volatilizes to a greater extent than the untreated pulp and exhibits a higher mass loss rate. The results are consistent with the LMS removing substances adhered to cellulose and leaving a cleaner, more ordered microfibril surface as a result. Since hemicellulose, lignin and extractives produce comparatively more char than cellulose; their removal must reduce char production and increase volatile release by pulp. Also, a cleaner, more ordered (crystalline) cellulose is bound to decompose over a narrower temperature range and exhibits an increased mass loss rate as a result. Tests conducted under atmospheric air provide additional information about amorphous and crystalline cellulose. Since disordered cellulose is more accessible than crystalline cellulose, the presence of oxygen must have mainly affected the volatilization of amorphous cellulose. No peak for amorphous cellulose is observed in the profile for HBT because the proportion of amorphous cellulose remaining after the enzyme treatment is significantly lower than in the initial pulp.

So, laccase-HBT treatment removes substances adhered to cellulose, alters microfibril surfaces and increases the proportion of crystalline cellulose as a result. These changes affect the thermal degradation of flax pulp by increasing the production of volatiles, reducing that of char and raising the maximum mass loss rate during volatilization.

- **Comparing TCF sequences**

The laccase from *P. cinnabarinus* is used with VA and SA, and the laccase from *M. thermophila* is used in combination with MS. After the L stage, VA in the presence of PcL causes the greatest reduction in lignin, even though the combination PcL+SA also gives a decrease in kappa number. MtL is able to delignify flax pulp; moreover, MtL+MS prove effective in decreasing the kappa number over a larger extension than that caused by VA. The Po stage is not as effective in diminishing kappa number in the samples treated with MtL as it is with PcL. The efficiency of a specific LMS in degrading lignin depends on the properties of the laccase, the mediator and their combination. PcL is probably more effective in oxidizing lignin than MtL; this oxidized lignin might be water-soluble and hence easily removed during washing. In addition, these mediators exhibit electrochemical differences in the presence of flax lignin, so there is an increase in the oxidation current for VA, and a decrease in those for SA and MS, as measured in the presence of flax lignin. The LMS results in the highest delignification stage in the bleaching sequence. Xylanase treatment is also applied together with the LMS system. The pretreatment with xylanase provides the smallest kappa number at the end of the sequence. Moreover, the X stage can reduce the kappa number; this reduction can be ascribed to a delignifying effect resulting from the removal of lignin trapped between xylan chains, as well as the removal of HexA bonded as side groups to xylans.

It is important to remark that all enzyme systems cause slight pulp darkening immediately after LMS treatment. The Po stage is more efficient in bleaching sequences including the use of PcL, especially with VA. MtL provides the worst results, maybe due to the formation of chromophores more difficult to remove from the pulp in subsequent stages. The X pretreatment fails to improve brightness immediately, although the best bleaching results are obtained with the XLQPo sequence including a

PcL+VA treatment. Furthermore, using the LMS affords a reduction in H_2O_2 dose; xylanase pretreatment afford a saving of 2% in hydrogen peroxide.

PcL treatments result in a gradual decrease in viscosity. No differences are observed in the MtL treatments. The loss in cellulose integrity caused by the LMS is not increased by the xylanase pretreatment. Cellulose is probably not oxidized in carbonyl groups by the MtL.

The HexA content of flax pulp is fairly low compared with unbleached pulp from other non-wood materials or unbleached eucalyptus pulp. The LMS decreases the HexA content. The several mediators used reduce HexA to different extents, probably as a result of the competitive reactions of these mediators and HexA with the enzymes arising from the disparate affinity of laccase for these structures. The pulp samples from the sequence including an X stage exhibit the lowest final HexA content. The enzyme pretreatment with xylanase is highly efficient at removing substantial fraction of HexA by releasing it from fibres; in fact, it is more efficient than the LMS.

On the other hand, after KN_{lig} measurements it can be seen that false lignin contributes to KN measurements: KN_{lig} is lower in all cases than KN. Again, the sequence including a xylanse pretreatment exhibits the smallest KN_{lig} at the end.

Effluent characterisation shows that the highest COD is provided by using a xylanase pretreatment, probably as a result of efficient removal of HexA by the enzyme. COD is much greater after L than it is after Q and Po. After the L stage, effluents from PcL treatments exhibit a high COD due to the presence of sodium tartrate buffer and the formation of certain products during the reaction. LMS contributes to effluent colour; after the L stage, all treated pulp samples have higher colour than the laccase controls. Concerning toxicity, PcL+SA induces very high toxicity in the effluents; MtL+MS also gives a high toxicity level. Therefore, using a laccase in combination with a natural mediator increases effluent toxicity. Toxicity of the X stage has a low value.

- **Laccase-catalyzed biografting of natural phenols**

Enzyme treatments with natural phenols produce an increase in kappa number compared to control samples. These results suggest that laccase leads to a crosslinking

or grafting of these phenolic compounds on fibres. In order to eliminate the contribution of low molecular-mass phenols to kappa number, a Soxhlet extraction with acetone is carried out to remove these compounds adsorbed on the pulp. After acetone washing, the KN decreases in all cases, although they are still higher than the control pulp when using SA and PCA. The minimum amount of grafted compound, determined by a calibration line is estimated to be 12.3 μmol/g of pulp for PCA treatments and 2.1 μmol/g of pulp for SA. Interestingly, the capacity of these phenols to delignify flax pulp has also been demonstrated.

A decrease in brightness is also observed, which suggests the formation of chromophore groups due to the oxidative action of the enzyme treatment and/or the grafting of phenolic compounds onto the pulp. After acetone extraction, the brightness remains lower than it is for the control pulp. Additional studies of optical properties also suggest coupling reactions and/or the possible generation of quinones.

Py-GC/MS, both in the absence and presence of TMAH, is used as a tool to analyse residual lignin in pulp without the need of previous isolation. PCA treated pulp reveals high amounts of 4-vinylphenol, a compound arising from the decarboxylation of PCA during pyrolysis, and which is absent in the control pulps. In addition, pyrolysis in the presence of TMAH of this pulp releases high amounts of intact PCA as its methyl derivate, and which is absent in the control pulp. These data clearly demonstrate that PCA is covalently bound to the pulp fibres. In the case of pulps treated with SA and AS, the analysis also demonstrated the grafting of a part of these phenolic compounds onto the pulp fibres. However, the lower KN of these treated pulps indicates a lower degree of grafting in comparison with that treatment with PCA.

Treatment of isolated xylan with laccase+PCA reveals a substantial amount of polymeric aromatic structures that could be observed by H NMR. These structures are assigned to polymerization products of PCA.

- **Development of antimicrobial properties**

Some intrinsic pulp interference complicated the measurement of the antimicrobial properties of enzyme treated fibres. It is assumed that pulp extractives could cause

unreal outcomes. To eliminate extractives and avoid interference, unbleached pulps are washed with acetone in a Soxhlet extractor before the laccase-phenol grafting stage.

The results for antimicrobial activity reveal that enzyme-phenol treated fibres cause a decrease in the microbial count, indicating the antibacterial activity of all grafted papers against the three bacteria tested. The Gram– bacteria tested *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* show an important growth inhibition by the phenol grafted papers, as a notable reduction in the number of bacterial cells is caused by the contact with these fibres. The major reduction is caused by the PCA grafted fibres. The non-diffusion to the media of the phenol compounds is verified by the presence of antimicrobial leaching. The absence of inhibition in each of the bacterial lawns cultured on agar plates indicates that all the tested phenolic compounds remain bound to the fibres during the test.

The antimicrobial properties of these phenolic compounds show that PCA is the most effective growth inhibitor, whereas AS has the lowest inhibition effect. The differences observed in the antibacterial behaviour between the free natural phenols and those coupled onto laccase treated flax fibres may be explained by the modifications produced in these compounds by coupling to fibres in the laccase treatments.

- **Effects of laccase-phenol treatments on bast and core flax fibres**

Industrial flax contains fibres of different size and properties, namely bast (77% wt) and core fibres (23% wt). Morphological fibre analysis using a Kajaani FS300 reveals clear evidence of the differences in length and width between global and size-classified samples. However, no important differences are found between biotreatments, showing that laccase-phenolic compound treatments do not affect the morphology of the fibres. Neither does conventional morphology analysis using optical microscopy reveal differences between biotreatments. FTIR analysis exposes no differences between fibres: peaks characteristics of cellulose and cellulose I_β are the main signals. Long fibres present a higher crystallinity index than short fibres; furthermore TCI of long fibre fractions increased in both biotreatments, indicating a removal of substances adhered to cellulose (such as lignin and hemicellulose) and giving a cleaner, more ordered microfilm surface as a result.

Long fibres are richer in glucan (cellulose) and have high viscosity values, whereas short fibres are richer in xylan and have less viscosity than the long ones. In PcL+VA treatments, xylan content decreases with respect to initial pulp. When PCA is used as a phenolic compound a lower decrease in xylan content is observed. The xylan content reduction occurs simultaneously with a decrease in HexA content.

Long and short fibres of initial flax pulp present evident differences in KN. Long fibres have a smaller KN. There are some differences between global and classified fibre results, which may be caused by the loss of fines during fractionation and washing of the samples. Furthermore, unbleached flax pulp presents some impurities that can pass through the screen with the 200 mesh. Differences between biotreatments are evident. In the case of pulps treated with PCA, all fibre fractions reflected the partial condensation of the phenoxy radicals of *p*-coumaric acid on fibres. The laccase-VA system resulted in a KN reduction in all the fractions analysed.

Concerning HexA content, short fibres have a higher content than long fibres. Enzyme treatments are able to reduce HexA content as can be seen in both global biotreated fractions; this decrease is more important in laccase-VA samples. These results suggest that these laccase-phenol systems can destroy HexA by oxidizing their double bond in similar fashion to electrophilic bleaching agents. Both laccase-phenol treatments assayed fail to reduce the HexA content of long fraction fibres, probably due to the initially low HexA content of this type of fibre or to the difficult accessibility that does not make any action of the enzyme or the oxidized phenol possible.

Regarding brightness, again there are differences between fibre fractions. Initial long fibres present a higher brightness than initial short and global fibres. In pulps treated with PcL+PCA, all fractions reveal a darkening of the fibres, mainly in the long fibres fraction. The laccase-VA system promotes an increase in brightness in both classified fractions. Usually, the LMS may cause the formation of chromophores in pulp due to the oxidative action of the enzyme treatment and cross-linking or cross-coupling reactions in the matrix structure of pulp may also take place. Complementary optical properties studies agree with the differences observed in brightness.

In the light of the results, it is evident that each enzymatic system causes different effects on pulp properties. PCA used in combination with PcL mainly induces the grafting of the same on the fibres; differences in KN response between fibre fractions

may indicate that the higher grafting degree is carried out in the fines fraction. It can be assumed that fines present a high lignin and xylan content and consequently a high HexA content. Phenolic compounds aided by laccases can attack HexA; nevertheless, some interactions with other fibre compounds could be supposed, HexA are probably modified by the action of the laccase and PCA. PCA seems to couple to the xylan-HexA double bond, giving rise an adduct. So, HexA coupled to PCA are not detected. On the other hand, an evidence of certain delignification action is also found indicating that PCA is involved simultaneously in oxidative degradation and grafting reactions. Concerning VA behaviour, a clear delignifying activity is found. In all treated fractions, KN diminishes, as does HexA content. Furthermore, no influence of HexA on KN measurements is found.

Concluding remarks

This doctoral thesis aimed to modify flax fibres using laccases and phenolic compounds in order to obtain high-value products in environmentally friendly processes. New biobleaching treatments have been studied with the aim of improving pulp properties, as well as checking the potential of natural lignin-derived phenolic compounds. Novel TCF sequences based on the LMS have been developed. Several sequences are found to provide pulp with properties on a par with those obtained using an industrial ECF sequence. The proposed sequence involves only three steps and provides flax pulp of very high brightness.

Furthermore, laccases are used as tools for fibre modification in an eco-friendly approach. Laccase-catalyzed biografting of phenolic compounds onto flax fibres has been studied. This system enables the covalent binding of several natural phenols and functionalising flax fibres by conferring antimicrobial properties upon them.

The analysis techniques used –determination of carbohydrates by HPLC, different techniques for determination of crystallinity, determination of hexenuronic acids, pyrolysis-GC/MS for analysing residual lignin, fibre fractionation, FTIR studies and thermal degradation profiles –have provided a greater understanding of the reaction mechanisms occurring in the enzyme treatments examined.

The interest and benefits of this doctoral work can be summarized as follows:

- Flax fibres enabled speciality paper to be produced and bleached using more environmentally benign sequences.

- Flax pulp is fractionated to better understand the enzymatic effects on bast and core flax fibres.

- The use of a laccase-mediator system as a bleaching agent provides flax pulp with good properties and enables an upscaling (see Annex I).

- Some mediators inactivate laccase, but the presence of pulp is found to have a protective effect on enzyme activity.

- The proposed TCF sequences enable efficient bleaching of flax pulp and chemical saving, as well as removal of lipophilic extractives (see Annex II).

- The sequence that includes the use of VA as a mediator resulted in higher brightness than 80% ISO. This value is difficult to reach with chemical sequences in flax pulp due to the high recalcitrance of this pulp towards high brightness bleaching. VA is assessed as a flax bleaching mediator for the first time.

- The L treatment not only reacts with the lignin, but also effectively reduces the HexA content of the raw material.

- HexA content measurement is carried out for first time on flax pulp.

- Natural mediators (SA and AS) combined with PcL afford efficient biobleaching of flax pulp in a TCF sequence. These natural mediators are used for the first time as flax bleaching agents.

- Xylanase pretreatment is found to have a boosting effect on the LMS by reducing the HexA content and facilitating the removal of lignin on flax pulp. Flax pulp is submitted for the first time to a xylanase stage.

- The effluents produced by the laccase–mediator system have been characterised and the mediators (HBT and VA) alone have been found to contribute no toxicity.

- TGA can be used to monitor the thermal degradation that is affected by changes caused by enzyme treatments.

- Laccase treatment with HBT removes substances adhered to cellulose and helps recover crystallinity in microfibril surfaces as a result.

- The enzyme system consisting in laccase-phenol treatment could be regarded as a method for the grafting of these compounds onto the fibres to give them improved or novel properties.

- The treatment of flax pulp with laccase and some natural phenols results in their covalent incorporation into the fibres demonstrated by Pyrolysis-GC/MS.

- Bioactive papers have been created: grafted fibres present high antimicrobial activity against the three bacteria analysed.

- The antimicrobial agents grafted are immobilized in the flax fibres.

- The role of laccase–phenols as a modifying agent has been clarified.

ANNEX I

UPSCALING OF FLAX PULP TCF BIOBLEACHING (CTP)

SUMMARY

Biorenew is the short name for the research project "White Biotechnology for added value products from renewable plant polymers: Design of tailor-made biocatalysts and new industrial bioprocesses", an Integrated Project (IP) funded by the EC involving the participation of 3 Research Institutes, 11 Universities and 12 Companies from 13 European countries. One of the goals of the project is to analyse the industrial feasibility of new bleaching sequences based on the use of biotechnology, specifically by using the laccase-mediator system.

In order to validate the results obtained by the UPC research group, the CTP (Centre Tecnique de Papier in Grenoble, France) was involved in some pilot trials to explore the actual potential of a laccase-mediator system to remove lignin-derived products responsible for colour from a high-quality flax pulp.

A.1 Materials and methods

The raw material consisted of unbleached flax pulp, supplied by CELESA (Tortosa, Spain); this pulp was cooked by NaOH-AQ.

The pulp was bleached with the CTP bleaching pilot plant (Figure An-1), which was able to treat up to 100 kg of pulp (odp) in any conditions and which is totally reliable

and transferable to industrial units (consistency: from 3 to 25%; temperature: up to 120ºC, pressure: up to 6 MPa).

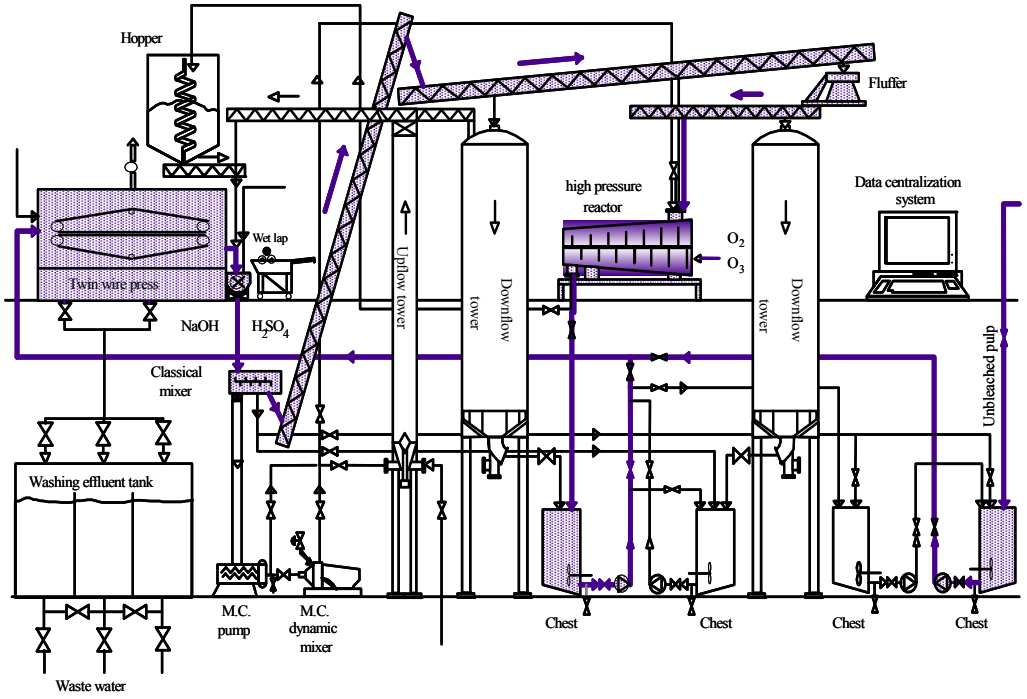

Figure An- 1. The CTP bleaching pilot plant used for bio-bleaching the flax pulp (BIORENEW Project Brochure).

The bleaching sequences applied consisted of: $K_{PcL}QPo$, $L_{HBT}QPo$ and KQPo. Firstly, pulp was submitted to acidic washing with H_2SO_4 at pH4 for 30 min at room temperature (18ºC) to remove impurities, reduce the metal ion content and adjust the pH to the requirements for the following enzymatic stage. In the L (or laccase) stage a laccase from *Trametes villosa* (NS51002) supplied by Novozymes® was used combined with HBT as a mediator. The conditions for the treatment were: 15 U/ g odp of laccase, 1.5% (w/w) of mediator, Neodol 25-3 was used as a surfactant (0.1 g/L), at 4% consistency for 4 h at 50ºC at a pressure of 5.2 MPa of oxygen. The L stage was followed by a chelating step (Q stage) and a subsequent hydrogen peroxide stage (Po). Control pulps were processed in the absence of mediator (K_{TvL}) or in the absence of both mediator and laccase (K). The application conditions for the biobleaching sequence are shown in Table An- 1.

Table An- 1. Application conditions of the different bleaching stages used to upscale the biobleaching of flax pulp (UPC conditions).

	Laccase stage			Q	Po
	K	K_{TvL}	L_{HBT}		
Consistency (%)	4	4	4	5	5
Temperature (ºC)	50	50	50	85	90
Time (hours)	4	4	4	1	2
Laccase (U/ g odp)	-	15	15	-	-
HBT (% odp)	-	-	1.5	-	-
H_2O_2 (% odp)	-	-	-	-	3
NaOH (% odp)	-	-	-	-	1.5
DTPA (% odp)	-	-	-	1	0.3
$MgSO_4$ (% odp)	-	-	-	-	0.2
Pressure (Mpa)	5.2	5.2	5.2	-	5.2

All bleaching stages were followed by measurements of kappa number, pulp brightness, polymerization degree of the cellulose and chemical consumption.

A.2 Results and discussion

The pulp brightness mainly increased during the peroxide stage Po, whereas the kappa number decreased regularly throughout the bleaching sequence. The acidic stage enabled a slight decrease in kappa number. The laccase stage in the presence of HBT drastically decreased the kappa number with no significant impact on the brightness. An alkaline stage was needed to see the impact of the enzymatic stage on the pulp brightness. The L_{HBT}QPo sequence made it possible to reach a higher final brightness than the KQPo one at an equivalent peroxide consumption. Brightness higher than 80% ISO was reached and the objective requested by CELESA was achieved. The comparison between the L_{HBT} and K results revealed the significant impact of the laccase system on the delignification of flax pulp.

The introduction of the L_{HBT} stage to the bleaching sequence had no significant effect on the degree of polymerisation of the cellulose:

A.3 Conclusions

The design of the laccase-mediator stage for the bleaching of a nonwoody pulp has been validated at pilot scale. According to the results obtained, it is possible to implement a laccase mediator system (*Trametes villosa* laccase + HBT) stage in the bleaching of flax pulp to enable improvement of final brightness and pulp quality. This sequence made it possible to reach brightness higher than 80% ISO with a similar peroxide consumption. The final brightness was higher than that of the control sequence KQPo. No significant effect on pulp quality was observed after biobleaching.

Concerning the industrial and economical feasibility of developing this TCF sequence, the following aspects have to be taken into account:

- Higher values than 80% ISO of brightness are difficult to reach with chemical sequences in flax pulp due to the high recalcitrance of this pulp towards high brightness bleaching.

- The important amounts of enzyme and mediators used in the enzymatic bleaching force these compounds to be economically competitive (HBT) and commercially available (*Trametes villosa* laccase). In any case, a higher enzyme cost could be assumed in this process due to the higher price of flax pulp (used for specialty papers).

- Low consistency and high reaction times in the enzymatic stage have been used. Nevertheless, these operational conditions could be adjusted to fit industrial requirements without significant variations in the final properties.

- Analysis of the effluent properties showed that very low toxicity values are obtained with the HBT synthetic mediator, making its industrial application feasible.

A.4 References

BIORENEW Project Brochure. Available in: www.biorenew.org

ANNEX II

EFFECT OF TCF BIOBLEACHING SEQUENCES ON LIPOPHILIC EXTRACTIVES

SUMMARY

The main lipophilic extractives present in unbleached flax pulp include different classes of compound, such as alkanes, fatty acids, fatty alcohols and free and conjugated esterols (such as esters and glycosides) (Marques et al. 2010). These lipophilic compounds, even present in low amounts in the raw material, may play an important role in industrial wood processing since they are the origin of "pitch" deposits. These deposits are formed during the pulp and paper manufacturing processes and contribute to the drastic decrease of the in the quality of the final product and negatively affect the work of the paper machine and are responsible for some interruptions to industrial operations, resulting in economic losses in this industrial sector. The aim of this work was to study behaviour during the TCF bleaching sequences studied in the present thesis (Chapter 4) that include enzymatic stages.

A.1 Materials and methods

It has been demonstrated that treatment with a laccase-mediator system is capable of releasing sterols from eucalyptus pulp, not only using mediator HBT (Gutiérrez et al. 2006), but also using natural mediators (Babot el al. 2011). For this reason, the content of flax pulp lipophilic extractives after different bleaching stages has been monitored.

The pulp samples studied in Chapter 4 (TCF biobleaching sequence tests) were analysed by GC/MS. The lipophilic extractives were determined after the X, L and Po stages of the different sequences. Control pulps (K) were processed in the absence of mediator.

Flax pulps were submitted to several bleaching sequences. These sequences included treatments with laccases from *Pycnoporus cinnabarinus* (PcL) and *Myceliophthora thermophila* (MtL) in the presence of several redox mediators (SA, VA and MS), as well as a pretreatment with a commercial xylanase.

These analyses were performed at the IRNAS in Seville, Spain. The GC and GC/MS analyses were performed following the methodology previously developed by Gutiérrez et al. (1998). The GC analyses were carried out in an Agilent 6890N Network GC system using a short fused silica capillary column (DB-5HT; 5 m x 0.25 mm I.D., 0.1 μm film thickness) from J&W Scientific. The temperature program was started at 100ºC with a 1 min hold, and then raised to the final temperature of 350ºC at 15ºC/min, and held for 3 min. The injector and flame-ionization detector (FID) temperatures were set at 300ºC and 350ºC, respectively. The carrier gas was helium at a rate of 5 mL/min, and the injection was performed in spotless mode. Peaks were quantified by area in the GC chromatograms.

The GC/MS analyses were performed with a Varian 3800 chromatograph equipped with an ion trap-detector (Varian 4000) using a medium-length (12 m) capillary column of the same characteristics described above. The oven was heated from 120ºC (1 min) to 380ºC at 10ºC/min and held for 5 min. The transfer line was kept at 300ºC. The injector was temperature programmed from 120ºC (0.1 min) to 380ºC at a rate of 200ºC/min and held until the end of the analysis. Helium was used as carrier gas at a rate of 2 mL/min. Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries, by mass fragmentography, and compared with standards.

A.2 Results and discussion

The main compounds identified by GC/MS in unbleached flax pulp are shown in Table An-2.

Table An-2. List of the main lipophilic extractive compounds present in unbleached flax pulp.

Lipophilic extractives	Main compound	mg/100 g odp
Alkanes	*n*-nonacosane	72.61
Fatty acids	*n*-octadecanoic acid	96.27
Fatty alcohols	*n*-octacosanol	24.3
Free sterols	sitosterol	8.36
Conjugated sterols		
sterols esters	sitosterol ester	1.23
sterol glycosides	sitosterol 3β-D-glucopyranoside	2.95

Concerning the sequences that include an enzymatic stage using the laccase of *P. cinnabarinus*, L_{PcL+VA} treatment showed higher effectiveness in the removal of the lipophilic extractives than L_{PcL+SA}; the content of alkans, fatty alcohols, fatty acids, free sterols, sterol esters and sterol glycosides decreased as shown in Table An-3. When the enzymatic treatments were followed by a hydrogen peroxide stage (LQPo) a different behaviour between both mediators used and lipophilic compound changes was observed. L_{PcL+SA}QPo showed the best performance for diminishing alkans, fatty acids, fatty alcohols and sterols esters; whereas the L_{PcL+VA}QPo sequence was more effective in the removal of free sterols and sterol glycosides. On the contrary, when a xylanase pretreatment was added to the better sequence (in terms of pulp properties), an enhancement on the elimination of most of the lipophilic compounds after the L and Po stages was achieved.

Table An-3. Removal (percentage reduction) of the main lipophilic extractives from flax pulp after treatment with *P. cinnabarinus* laccase (PcL) in the absence (control) and presence of syringaldehyde (SA) or violuric acid (VA) after a laccase stage (L stage) or after a hydrogen peroxide stage (Po stage), as well as after a xylanase pretreatment and a subsequent L and Po stage.

	Control PcL		PcL+SA		PcL+VA		X+(PcL+VA)		
	L	Po	L	Po	L	Po	X	L	Po
Alkanes	30	50	21	66	50	40	39	81	92
Fatty acids	21	8	25	23	39	3	100	86	83
Fatty alcohols	5	48	0	59	18	52	79	50	81
Free sterols	0	21	5	44	38	64	0	72	8
Conjugated sterols									
sterols esters	0	48	54	81	46	45	100	41	70
sterol glycosides	0	57	0	55	43	87	0	79	5

Treatments with the laccase of *M. thermophila* are shown in Table An-4. L_{MtL+MS} gave efficient elimination of alkanes, free and conjugated sterols and fatty acids. After a subsequent Po stage, the removal of lipophilic extractives was not as important.

Table An-4. Removal (percentage reduction) of the main lipophilic extractives from flax pulp after treatment with *M. thermophila* laccase (MtL) in the absence (control) and presence of methyl syringate (MS) after a laccase stage (L stage) or after a hydrogen peroxide stage (Po stage).

	Control MtL		MtL+MS	
	L	Po	L	Po
Alkanes	39	60	51	64
Fatty acids	2	11	26	0
Fatty alcohols	38	68	19	72
Free sterols	16	42	42	55
Conjugated sterols				
sterols esters	17	38	31	43
sterol glycosides	4	58	21	63

A.3 Conclusions

The laccase mediator system makes it possible to alter the lipophilic extractive content of flax pulp. The response depends on the laccase applied, as well as on the mediator used in combination with. The removal of the lipophilic extractives is always higher when a laccase is applied in the presence of a mediator. PcL leads to better performance than MtL. The mediator VA gives high reduction, especially when this mediator is applied after a xylanase pretreatment. Furthermore, xylanase also enables a high reduction (total removal of fatty acids and sterol esters). A lipophilic extractive reduction is also observed at the end of all the sequences, this removal varies between the different enzyme treatments applied. It is important to remark that natural mediators are also able to remove lipophilic extractives in flax pulp treatments.

The combination of substantial bleaching benefits and effective control of pulp extractives may provide important economic advantages for the enzymatic process as a potential alternative to current industrial processes.

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STANDARD METHODS

ASTM D1209 - 05(2011)	Standard Test Method for Color of Clear Liquids (Platinum-Cobalt Scale)
ASTM D1252 - 06	Standard Test Methods for Chemical Oxygen Demand (Dichromate Oxygen Demand) of Water
ASTM E2149 - 10	Standard Test Method for Determining the Antimicrobial Activity of Immobilized Antimicrobial Agents under Dynamic Contact Conditions
ISO 302:2004	Pulps - Determination of Kappa Number
ISO 3688:1999	Pulps - Preparation of Laboratory Sheets for the Measurement of Diffuse Blue Reflectance Factor (ISO Brightness)
ISO 5351:2010	Pulps - Determination of Limiting Viscosity Number in Cupri-ethylenediamine (CED) Solution
ISO 9184-3:1990	Paper, Board and Pulps - Fibre Furnish Analysis - Part 3: Herzberg Staining Test
TAPPI T233-cm-06	Fiber Length of Pulp by Classification
TAPPI T271	Fiber Length of Pulp and Paper by Automated Optical Analyzer using Polarized Light
UNE-EN ISO 11348-3:2009	Calidad del Agua. Determinación del Efecto Inhibidor de Muestras de Agua sobre la Luminiscencia de *Vibrio fischeri* (Ensayo de Bacterias Luminiscentes). Parte 3: Método utilizando Bacterias Liofilizadas. (ISO 11348-3:2007).