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- BIORENEW: White Biotechnology for added value products from renewable plant polymers: Design of tailor-made biocatalysts and new industrial processes. FP6-2004-NI-4-026456-2

- FUNCICEL: Funcionalización de fibras lignocelulósicas mediante sistemas lacasa mediador utilizando compuestos fenólicos de origen natural. MICINN CTQ2009-12904

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Nomenclature

ABTS	2,2’-azinobis-(3-etilbenzotiazolina-6-sulfonat)
AOX	Adsorbable organic halogen
AQ	Anthraquinone
BV	Borohydridre viscosity
c	Consistency
C*	Chroma
CHO	Aldehyde groups
CLD	Coniferyl aldehyde
COD	Chemical oxygen demand
COOH	Carboxyl groups
CS	Cellulose chain scission number
C_n	Numerical average of fibre curl
DP	Degree of polymerization
DRI	Dye removal index
DTI	Dry tensile index
DTPA	Diethylenetiamineoentaacetic acid
ECF	Elementary chlorine free
F_n	Numerical average of fines content
FRC	Ferulic acid
G	Guaiacyl unit
HBT	1-hydroxybenzotriazol

HexA	Hexenuronic acids
HPLC	High performance liquid chromatography
k/s	Kubelka-Munk coefficient
KN	Kappa number
KN_{lig}	Kappa number due to lignin
L_l	Length-weighted average of fibre length
L_n	Numerical average of fibre length
L_w	Weight-weighted average of fibre length
L* a* b*	Chromatic coordinates
LMS	Laccase-mediator system
LT	Laccase-TEMPO
MGCh	Methylglycolchitosan
MS	Methyl syringate
*Mt*L	*Myceliophthora thermophila* laccase
Odp	Oven dried pulp
P	Hydrogen peroxide stage
P_O	Oxygen-reinforced hydrogen peroxide stage
PES-Na	Sodium Polyethensulphonate
Poly-DADMAC	Polydiallyldimethylammonium chloride
PVSK	Potassium polyvinyl sulphate
Py-GC/MS	Pyrolysis coupled with gas chromatography/mass spectrometry
Q	Chelating stage
R_∞	Intrinsic reflectance factor

Rev	Revolutions
Rpm	Revolutions per minute
RT	Pulp refined before an enzyme treatment
°SR	Schopper-Riegler degree
S	Syringyl unit
SLD	Sinapyl aldehyde
SNC	Sinapic acid
TCF	Totally chlorine free
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy free radical
TGA	Thermogravimetry analyses
TMAH	Tetramethylammonium hydroxide
TR	Pulp refined after an enzyme treatment
TU	Toxicity units
*Tv*L	*Trametes villosa* laccase
U	Enzyme activity unit
UV-Vis	Ultraviolet-visible
VA	Violuric acid
W_n	Numerical average of fibre width
Wa	Acidic washing
WRV	Water retention value
WTI	Wet tensile index
WZSTI	Wet zero span tensile index
WZSTS	Wet zero span tensile strength

X	Xylanase stage

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Abstract

Progress in pulp and paper research has led to the increasing adoption of enzyme technology on the grounds of its potential for providing environmentally friendly processes and novel, sustainable products. Among the most widely investigated enzymes in the pulp and paper industry are the oxidoreductases laccases, whose operational flexibility and broad substrate specificity provide a powerful tool for developing cleaner processes and modifying lignocellulose fibres to obtain high-value products. High-priced **non-wood fibres** such as those from **sisal** (*Agave sisalana*), which are used to manufacture specialty papers, are especially amenable to the application of enzymes thanks to the increased profit margins they provide. This research project originated from interest in assessing the potential of enzyme technology (particularly **laccase-based systems**) for the biomodification of sisal specialty fibres by using environmentally friendly processes.

This work was carried out in the CELBIOTECH research group of the Textile and Paper Engineering Department of the Technical University of Catalonia, within the framework of the Spanish MICINN Projects ENZPULP (CTQ2005-08925-C02-01), FUNCICEL (CTQ2009-12904) and BIOFIBERCELL (CTQ2010-20238-CO3-01), and the European Integrated Project BIORENEW (NMP2-CT-2006-026456) (Sixth Framework Programme). The common aim of these projects was to use biotechnology tools to develop new bioprocesses and facilitate the sustainable production of new added-value products by using renewable polymers.

This doctoral work focused on two different research lines, namely: **biobleaching** and **enzymatic functionalization** of sisal pulp fibres. The study was started by assessing the use of natural, potentially cost-effective **phenolic compounds** as substitutes for expensive, potentially toxic laccase mediators. The tendency of natural phenols to either promote delignification or couple onto pulp was examined with a view to assessing their potential for either bleaching or functionalizing sisal fibres. In the **biobleaching** study, **totally chlorine free** (TCF) sequences were implemented in order to compare the efficiency of a selected natural mediator and a well-known synthetic mediator, both in the presence and absence of a **xylanase** pre-treatment. The **effluents** resulting from each stage in the sequence were analysed with a view to assessing the environmental impact of

the laccase treatments —a scarcely explored aspect of biobleaching sequences. The xylanase stage proved highly efficient in reducing the **HexA** content of sisal fibres and in boosting the bleaching effect of the laccase treatments. The proposed TCF sequences provided high-cellulose sisal pulp with brightness above 80% ISO and a reduced HexA content; also, they exhibited improved performance and a reduced impact on effluent properties relative to the use of the synthetic mediator.

Two different approaches to **fibre functionalization** were explored, namely: **lignin modification** (biografting) and **cellulose modification** (laccase–TEMPO oxidation). Biografting of phenolic compounds was for the first time studied in sisal pulp. Covalent binding of the originally assayed phenolic compounds to sisal fibres during the laccase treatment was exposed by a novel analytical approach based on pyrolysis-GC/MS. The phenolic compound showing the highest tendency to couple to fibres was selected to investigate biografting under different reaction conditions and to evaluate the extent of phenol coupling via various pulp properties. Biografting efficiency was enhanced by refining the fibres prior to the enzyme treatment, which provided **improved strength-related properties** in the resulting paper.

The use of the **laccase-TEMPO system** to oxidatively modify cellulose and improve strength-related properties in sisal pulp was for the first time evaluated as an environmentally friendly alternative to existing halide-based systems. The first part of this study revealed that the laccase–TEMPO system considerably **improved wet strength** in sisal pulp by effect of the formation of a substantial amount of aldehyde groups in cellulose chains that facilitated inter-fibre bonding through hemiacetal linkages. The influence of process variables on various properties of the oxidized fibres and resulting paper was assessed by using a **three-variable statistical plan**. The conditions maximizing functionalization and the improvement in paper strength properties were used to design treatments of increased efficiency that exposed the potential of laccase–TEMPO oxidation for **biorefining** pulp fibres.

Analytical methods including pyrolysis-GC/MS, polyelectrolyte titration, conductimetric titration, carbohydrate determination by HPLC, fibre morphology analysis by SEM and thermogravimetry were used to both characterize the raw material and gain a better understanding of the reaction mechanisms behind the different laccase-based treatments. Some of the analyses were performed by collaborating research groups at IRNAS (Seville, Spain) and the Department of Chemical Engineering of the University of

Huelva (Spain). Also, part of this doctoral work was conducted at the Institute of Paper Science and Technology of the Georgia Institute of Technology (Atlanta, USA).

Resumen

Los avances de la investigación en el ámbito de la industria de pasta y papel han llevado a un uso creciente de la tecnología enzimática, atribuible a su potencial para proporcionar procesos más respetuosos con el medio ambiente y productos novedosos y sostenibles. Entre las enzimas más estudiadas están las oxidoreductasas lacasas, que, en virtud de su flexibilidad de uso y amplia especificidad de sustrato, constituyen una herramienta potente para desarrollar procesos más limpios y modificar las fibras lignocelulósicas para obtener nuevos productos de alto valor añadido. Las fibras **no madereras** de alto coste como las de **sisal** (*Agave sisalana*), usadas para la fabricación de papeles especiales, son particularmente adecuadas para la aplicación de enzimas gracias a los mayores márgenes de ganancia que tienen asociados. Así pues, este trabajo de investigación nació del interés en conocer el potencial de la tecnología enzimática, en particular de los **sistemas basados en la lacasa**, para la biomodificación de fibras especiales de sisal a través de procesos amigables con el medio ambiente.

Este estudio se llevó a cabo dentro del grupo de investigación CELBIOTECH del Departamento de Ingeniería Textil y Papelera de la Universidad Politécnica de Catalunya, y se presenta enmarcado en los Proyectos nacionales del MICINN ENZPULP (CTQ2005-08925-C02-01), FUNCICEL (CTQ2009-12904) y BIOFIBERCELL (CTQ2010-20238-CO3-01), y en el Proyecto Europeo Integrado BIORENEW (NMP2-CT-2006-026456), del Sexto Programa Marco. El objetivo común de estos proyectos es el uso de herramientas biotecnológicas para el desarrollo de nuevos bioprocesos, y la producción sostenible de nuevos productos de alta calidad a partir de polímeros vegetales renovables.

La presente tesis se desarrolló siguiendo dos líneas de investigación diferentes: el **bioblanqueo** y la **funcionalización enzimática** de las fibras de sisal. El estudio empezó con la evaluación del uso de **compuestos fenólicos** naturales potencialmente económicos como sustitutos de los mediadores costosos y potencialmente tóxicos utilizados en el sistema enzimático lacasa-mediador. Se examinó la tendencia de los fenoles naturales a promover la deslignificación o a acoplarse a las fibras con el fin de evaluar su potencial para el bioblanqueo o la funcionalización de las fibras de sisal. Para el estudio del **bioblanqueo**, se realizaron secuencias **totalmente libres de cloro** (TCF**)** con y sin pre-

tratamiento con **xilanasa,** para comparar la eficacia de uno de los mediadores naturales y un mediador sintético de uso común. Los **efluentes** generados se caracterizaron a lo largo de la secuencia con el fin de evaluar el impacto ambiental de los sistemas basados en la lacasa, un aspecto poco explorado en los estudios de bioblanqueo. La etapa de xilanasa resultó muy eficaz para reducir el contenido de **HexA** (ácidos hexenurónicos) y potenciar el efecto de blanqueo de los tratamientos con sistema lacasa-mediador. Las secuencias estudiadas proporcionaron una pasta de sisal con un alto contenido en celulosa y con valores de blancura superiores a 80% ISO, mostrando mayor eficacia en el proceso y menor impacto en las propiedades de los efluentes en presencia del mediador sintético.

Se estudiaron dos métodos diferentes de **funcionalización de las fibras**, basados en la **modificación de la lignina** (biografting) y en la **modificación de la celulosa** (oxidación con sistema lacasa-TEMPO). El estudio de biografting de compuestos fenólicos en una pasta no maderera de bajo contenido en lignina, como es la de sisal, era la primera vez que se estudiaba. Se demostró el acoplamiento covalente de los compuestos fenólicos a las fibras en el tratamiento con lacasa a través de un nuevo método de análisis basado en el uso de pirolisis acoplada a cromatografía de gases/espectrometría de masas. El compuesto fenólico con mayor tendencia a acoplarse a las fibras fue seleccionado para investigar el biografting bajo diferentes condiciones de reacción y evaluar el grado de acoplamiento a través de varias propiedades de la pasta. La eficacia del biografting se mejoró aplicando la etapa de refino previamente al tratamiento enzimático, obteniéndose papeles de **mayor resistencia mecánica**.

En búsqueda de una alternativa más respetuosa con el medio ambiente a los sistemas convencionales basados en halogenuros, se evaluó, por primera vez en pasta de sisal, el uso del **sistema lacasa-TEMP**O para mejorar las propiedades de resistencia mecánica a través de una modificación oxidativa de la celulosa. Los primeros estudios mostraron una importante **mejora de la resistencia en húmedo** en la pasta tratada por el sistema lacasa-TEMPO atribuido a la formación de una considerable cantidad de grupos aldehídos en las cadenas celulósicas capaces de generar enlaces inter-fibras a través de la formación de hemiacetales. A continuación se estudió la influencia de las variables del proceso en varias propiedades de las fibras oxidadas y de los papeles finales usando un **plan estadístico a tres variables**. Las condiciones que dieron lugar al mayor grado de funcionalización e incremento de propiedades de resistencia mecánica se usaron para

realizar tratamientos de mayor eficacia, lo que sacó a la luz el potencial del sistema lacasa-TEMPO para el **biorefino** de las fibras.

Varias técnicas de análisis –pirolisis acoplada a cromatografía de gases/espectrometría de masas, valoración polielectrolítica, valoración conductimétrica, determinación de hidratos de carbono mediante HPLC, análisis de la morfología de las fibras mediante microscopía electrónica de barrido (SEM), termogravimetría- se usaron tanto para caracterizar la materia prima como para obtener una mejor comprensión de los mecanismos de reacción que tienen lugar en los diferentes tratamientos basados en la lacasa. Estos análisis son en parte el resultado de la colaboración con grupos de investigación del IRNAS (Sevilla, España) y del Departamento de Ingeniería Química de la Universidad de Huelva (Huelva, España). Además, una parte de este trabajo se realizó durante una estancia en el Institute of Paper Science and Technology del Georgia Institute of Technology (Atlanta, EE.UU.).

Riassunto

Il progresso della ricerca nel settore cartario ha condotto a un crescente uso delle tecnologie enzimatiche in virtù della loro capacità di favorire lo sviluppo di processi più rispettosi dell' ambiente e di prodotti innovativi e sostenibili. Tra gli enzimi più studiati nel campo cartario vi sono le ossidoriduttasi laccasi, che, in virtù della loro flessibilità d'uso e ampia specificità di substrato, costituiscono un potente strumento per sviluppare processi più puliti e modificare le fibre lignocellulosiche per ottenere prodotti di alto valore aggiunto. Le **fibre di origine non legnosa**, di alto costo, come quelle di **sisal** (*Agave sisalana*), usate per la fabbricazione di carte speciali, sono particolarmente adatte per l'applicazione di enzimi grazie ai maggiori margini di profitto che offrono. Sulla base di queste premesse, questo progetto di ricerca è nato dall'interesse di studiare l'applicazione della tecnologia enzimatica, in particolare di **sistemi basati sulla laccasi**, per la biomodificazione delle fibre speciali di sisal attraverso processi rispettosi dell'ambiente.

Questo lavoro è stato realizzato nel gruppo di ricerca CELBIOTECH del Dipartimento di Ingegneria Tessile e Cartaria dell'Università Politecnica della Catalogna, ed è inquadrado nei progetti spagnoli del Ministero di Scienza e Innovazione ENZPULP (CTQ2005-08925-C02-01), FUNCICEL (CTQ2009-12904) e BIOFIBERCELL (CTQ2010-20238-CO3-01), e nel progetto Europeo Integrato BIORENEW (NMP2-CT-2006-026456), del Sesto Programma Quadro. L'obiettivo comune dei progetti è l'uso di strumenti biotecnologici per lo sviluppo di nuovi bioprocessi, e la produzione sostenibile di nuovi prodotti di alta qualità a partire da polimeri vegetali rinnovabili.

Il presente lavoro di tesi è stato sviluppato seguendo due linee di ricerca differenti: il **bioimbianchimento** e la **funzionalizzazione enzimatica** delle fibre di sisal. Lo studio è iniziato con la valutazione dell'uso di **composti fenolici** naturali come sostituti di mediatori della laccasi costosi e potenzialmente tossici. Si è esaminata la tendenza dei fenoli naturali a promuovere la delignificazione o a unirsi alle fibre al fine di valutare il loro possibile uso per l'imbianchimento o la funzionalizzazione delle fibre di sisal. Per quanto riguarda lo studio del **bioimbianchimento**, si sono realizzate sequenze **totalmente libere di cloro** (TCF) per confrontare l'efficacia di uno dei mediatori naturali

e un comune mediatore sintetico, sia in assenza che in presenza di un pretrattamento con **xilanasi**. Gli **effluenti** generati si sono analizzati lungo tutta la sequenza al fine di valutare l'impatto ambientale dei trattamenti laccasi-mediatore, un aspetto poco esplorato finora negli studi di bioimbianchimento. Lo stadio di xilanasi è risultato molto efficace per ridurre il contenuto di **HexA** e potenziare l'effetto di imbianchimento dei trattamenti con sistema laccasi-mediatore. Le sequenze studiate hanno permesso di ottenere pasta di sisal d'alto contenuto in cellulosa e con gradi di bianco superiori all'80% ISO, mostrando una maggiore efficacia e un minore impatto sulle proprietà degli effluenti in presenza del mediatore sintetico.

Si sono studiati due approcci differenti di **funzionalizzazione delle fibre**, basati sulla **modificazione della lignina** (biografting) e la **modificazione della cellulosa** (ossidazione con sistema laccasi-TEMPO). Il biografting di composti fenolici è stato studiato per la prima volta in una pasta di tipo non legnosa con un basso contenuto in lignina come quella di sisal. Attraverso un nuovo metodo di analisi basado sull'uso di pirolisi combinata con gas cromatografia/ spettrometria di massa è stata dimostrata l'unione covalente dei composti fenolici alle fibre nel trattamento con la laccasi. Il composto fenolico con la maggiore tendenza a incorporarsi nelle fibre è stato selezionato per investigare il biografting sotto differenti condizioni di reazione e valutare il grado di funzionalizzazione ottenuto attraverso l'analisi di varie proprietà della pasta. L'efficacia del biografting è stata migliorata applicando l'operazione della raffinazione prima del trattamento enzimatico, ottenendo in questo modo fogli di **maggiore resistenza meccanica**.

Alla ricerca di un'alternativa più rispettosa dell'ambiente ai sistemi convenzionali basati su alogenuri, si è valutato, per la prima volta con una pasta di sisal, l'uso del **sistema laccasi-TEMPO** per migliorare le proprietà di resistenza meccanica attraverso una modificazione ossidativa della cellulosa. La prima parte di questo studio ha mostrato un importante **miglioramento della resistenza in umido** nella pasta trattata con sistema laccasi-TEMPO attribuito alla formazione di un considerevole numero di gruppi aldeidici nelle catene cellulosiche capaci di generare legami inter-fibra attraverso la formazione di emiacetali. Successivamente, si è studiata l'influenza delle variabili del processo in varie proprietà delle fibre ossidate e delle carte finali usando un **piano statistico a tre variabili**. Le condizioni che hanno dato luogo al maggior grado di funzionalizzazione e incremento delle proprietà di resistenza meccanica si sono usate per realizzare trattamenti

di maggiore efficacia, i quali hanno messo in luce l'effetto di **bioraffinazione** prodotto dal sistema laccasi-TEMPO sulla pasta fibrosa.

Diverse tecniche analitiche, quali pirolisi combinata con gas cromatografia/spettrometria di massa, titolazione polielettrolitica, titolazione conduttimetrica, determinazione di carboidrati mediante HPLC, analisi della morfologia delle fibre mediante microscopia elettronica di scansione, termogravimetria, si sono usate sia per caratterizzare la materia prima che per ottenere una maggiore comprensione dei meccanismi di reazione che hanno luogo nei differenti trattamenti basati sulla laccasi. Queste analisi sono in parte il frutto della collaborazione con gruppi di ricerca dell'istituto IRNAS (Siviglia, Spagna) e del Dipartimento di Ingegneria Chimica dell'Università di Huelva (Huelva, Spagna). Una parte di questo lavoro si è realizzato nell' Institute of Paper Science and Technology del Georgia Institute of Technology (Atlanta, USA).

Introduction

1.1. Raw materials for papermaking

The primary raw material for manufacturing paper consists of cellulose fibres, which are present in all plants, particularly in wood and annual plants, where they aggregate as elemental cells forming plant tissues. Cellulose fibres are elongated, dead, hollow cells that provide mechanical support and facilitate conduction in plants.

Paper is defined as a thin, flexible web of cellulose fibres randomly deposited on each other from a dilute water suspension and then dried to form inter-fibre hydrogen bonds (García Hortal 2007c). Therefore, manufacturing paper requires reducing plant tissue to a form consisting of individual fibres (pulp).

1.1.1. Structure and chemical composition of fibres

Generally, the wood and plants of interest for papermaking are highly heterogeneous materials whose chemical composition, anatomy and physical properties vary over wide ranges. In fact, the distribution and composition of their chemical constituents vary between and within species, an even across fibre walls in each plant. Fibre walls provide rigidity to cells and mechanical support to the plant body. Rigidity is imparted by a series of coaxial layers of *cellulose* microfibrils (a skeleton) dispersed in an amorphous matrix of *hemicellulose* and *lignin*. Fibres additionally contain minor components including polymeric substances (pectins, starch and proteins) and low-molecular weight compounds (extractives and inorganic salts) (Sjöström 1981b).

Cellulose, the principal component of all plant cells, determines the nature of fibres and their usefulness for papermaking. Cellulose is an unbranched homopolysaccharide consisting of β-D-glucopyranose units in a $^4C_1$ conformation linked by (1→4) glycoside bonds (Marchessault and Sundararajan 1983, Purves 1954). The degree of polymerization (DP) of cellulose varies with the origin of the raw material and the way it is processed. In wood pulp, DP typically ranges from 300 to 1700. By contrast, cotton and other plant fibres have DP values in the 800–10 000 range, depending on the particular treatment applied (Klemm *et al.* 2005). The repeating unit of cellulose is cellobiose (Figure 1-1).

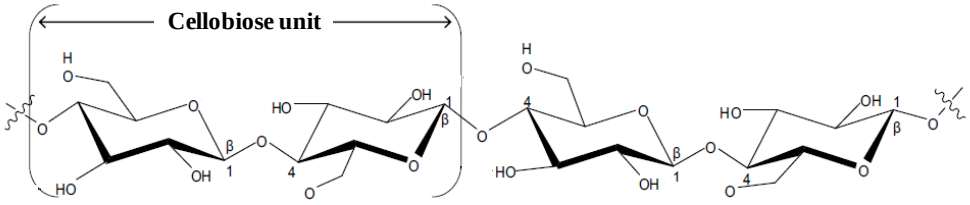


Figure 1-1. Cellobiose molecule in a cellulose chain

Cellulose molecules tend to form intramolecular and intermolecular hydrogen bonds (Figure 1-2), the presence of which influences the morphology, stiffness, orientation, resistance and reactivity of cellulose chains. Intermolecular hydrogen bonds result in the formation of an organized fibrillar structure (microfibrils) that is crystalline. An increased crystallinity usually implies also increased fibre density, hardness, stiffness, rigidity, tensile strength and dimensional stability. Whereas highly crystallized zones are difficult to penetrate by reagents and solvents, relatively less organized (amorphous) zones are more readily accessed and susceptible to all chemical reactions; also, the latter facilitate swelling, stretching and bending of fibres (Annergren 1996, García Hortal 2007b). Many paper properties are affected by these fibre properties.

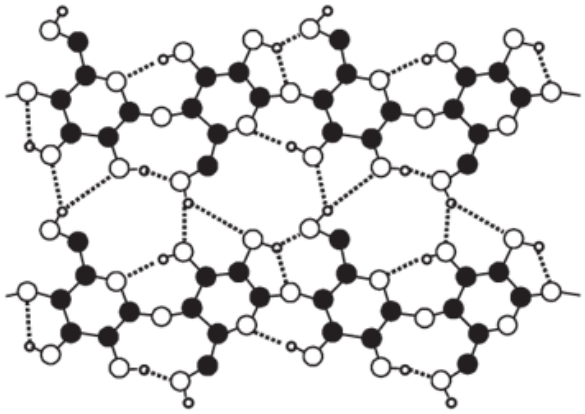


Figure 1-2. Intramolecular and intermolecular hydrogen bonds in cellulose molecules (Sturcova *et al.* 2004).

The intrinsic tensile strength and stretching ability of fibres depend largely on their DP, and paper strength, which is partly related to individual fibre strength, is reduced by

effect of the degradation of cellulose chains (Clark 1978). Cellulose degradation can be hydrolytic, oxidative, alkaline, thermal, microbiological or mechanical (García Hortal 2007b).

Microfibrils in fibre walls are usually present as small bundles called "macrofibrils". These, in turn, can be organized into thin sheets ("lamellae") which give walls a layered architecture.

Hemicelluloses are the second most abundant plant polysaccharides after cellulose. Together with lignin, these carbohydrates form an amorphous matrix in which cellulose microfibrils are embedded; also, they seemingly distribute in a relatively uniform manner in fibre walls. Hemicelluloses differ substantially from cellulose in that they are combinations of different five- or six-carbon monosaccharides (Figure 1-3), possess side groups on the chain molecules, are easy to dissolve and degrade, and amorphous in fibre walls. Also their DP ranges from only 50 to 300 (Sears *et al.* 1978).

In contrast to cellulose, hemicelluloses vary in nature between plant species (García Hortal 2007b). Thus, the composition and structure of hemicelluloses in softwood differ in a characteristic manner from those in hardwood. In fact, softwood contains greater amounts of hexosans (particularly *galactoglucomannans* and *glucomannans*, but also *arabinoglucuronoxylans*). Xylose is more abundant in hardwood, where pentosans such as glucuronoxylans prevail, but glucomannans are also present (Sjöström and Westermark 1999). In non-wood plants, hemicelluloses exhibit a widely variable composition depending on the particular species, with mannose and galactose as the dominant sugars in plants such as flax and hemp, and xylose in others such as kenaf and sisal (Marques *et al.* 2010).

Hemicelluloses are very hydrophilic and play a major role in water absorption by fibres during pulp beating and refining. Consequently, they provide internal lubrication to fibres, thereby increasing flexibility and bonding capacity, ease of mechanical refining, and sheet density (Haun 1970).

Lignin is produced by maturing cells and permeates fibre walls and intercellular regions (middle lamellae), rendering plant tissue rigid and cohesive as a result. Also, it plays a role in the natural defence mechanisms of plants against degradation by microbial enzymes (Fengel and Wegener 1989a).

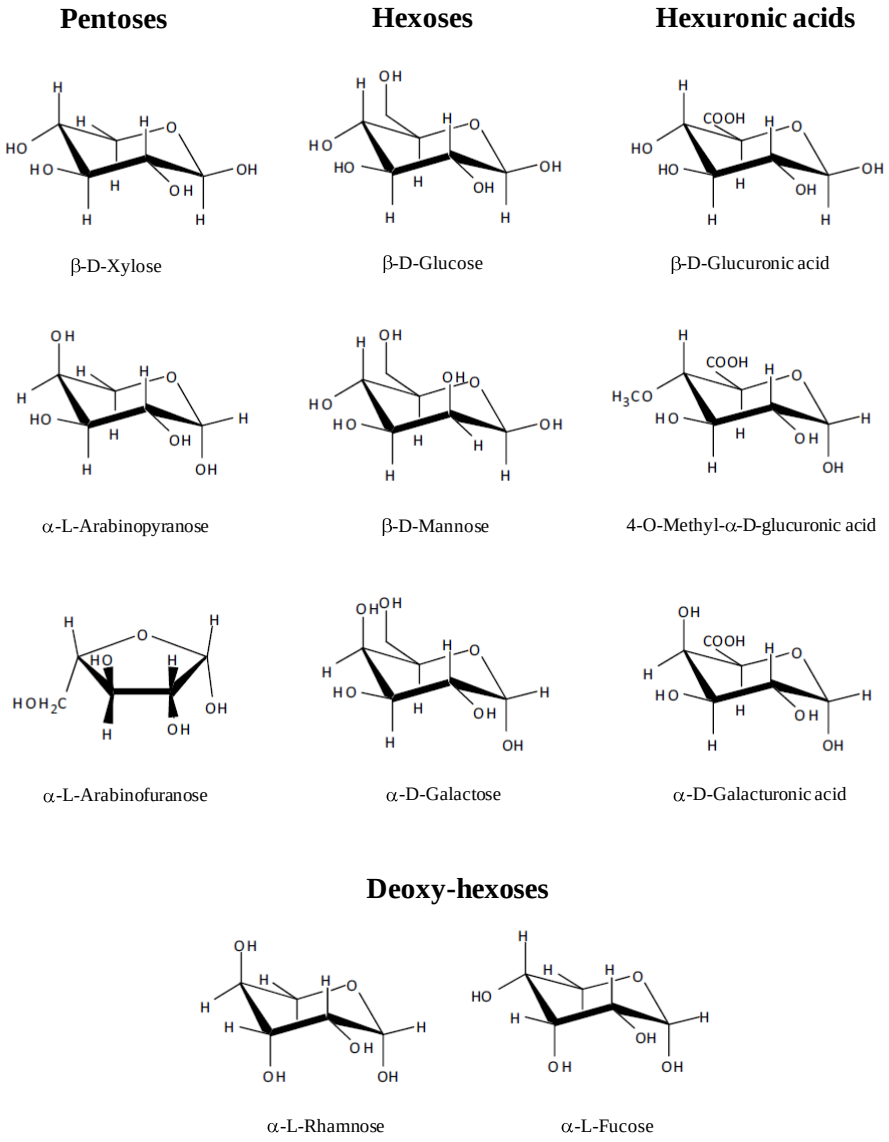


Figure 1-3. Monosaccharide constituents of hemicelluloses (adapted from Fengel and Wegener 1989b).

Chemically, lignin is an aromatic heteropolymer with an irregular three-dimensional structure resulting from enzymatic dehydrogenative polymerization of three phenylpropane units, namely: coniferyl, sinapyl and *p*-coumaryl alcohols (Sjöström 1981a) (Figure 1-4). By way of example, Figure 1-5 shows a phenoxy radical formed from coniferyl alcohol and its resonance forms.

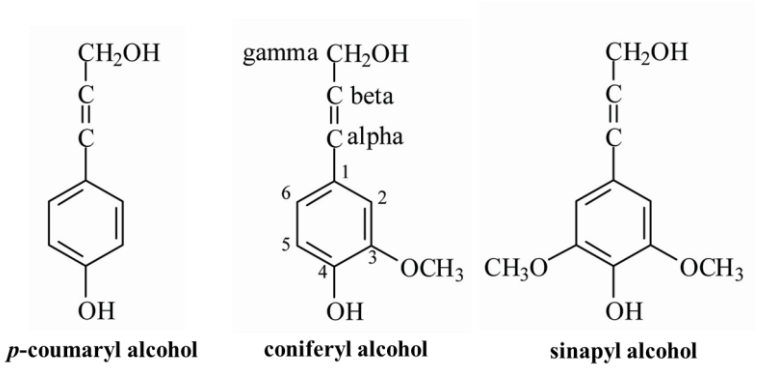


Figure 1-4. Phenylpropane precursors of lignin (Sjöström 1981a).

Phenylpropane units exhibit variable substitution patterns and a wide variety of linkages; however, interunit linkages, which impart lignin a high resistance to degradation (Hammel 1997), are generally of the ether bond or carbon–carbon bond type. Figure 1-6 shows the main types of lignin bonds and Figure 1-7 the overall lignin structure originally proposed by Adler (1977) and later modified by Karhunen *et al.* (1995).

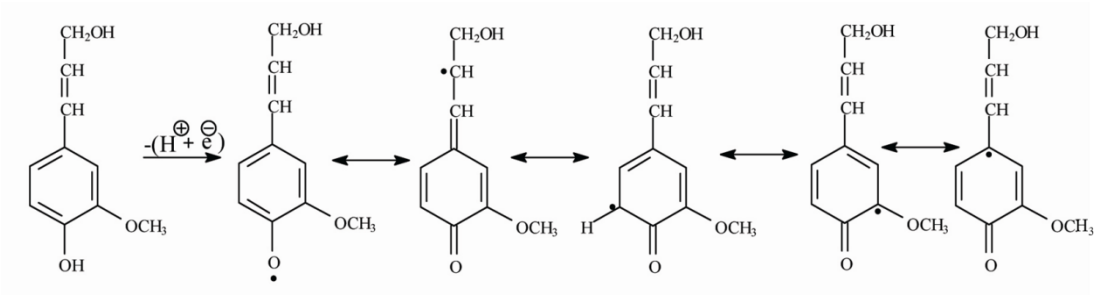


Figure 1-5. Phenoxy radicals formed by dehydrogenation of coniferyl.

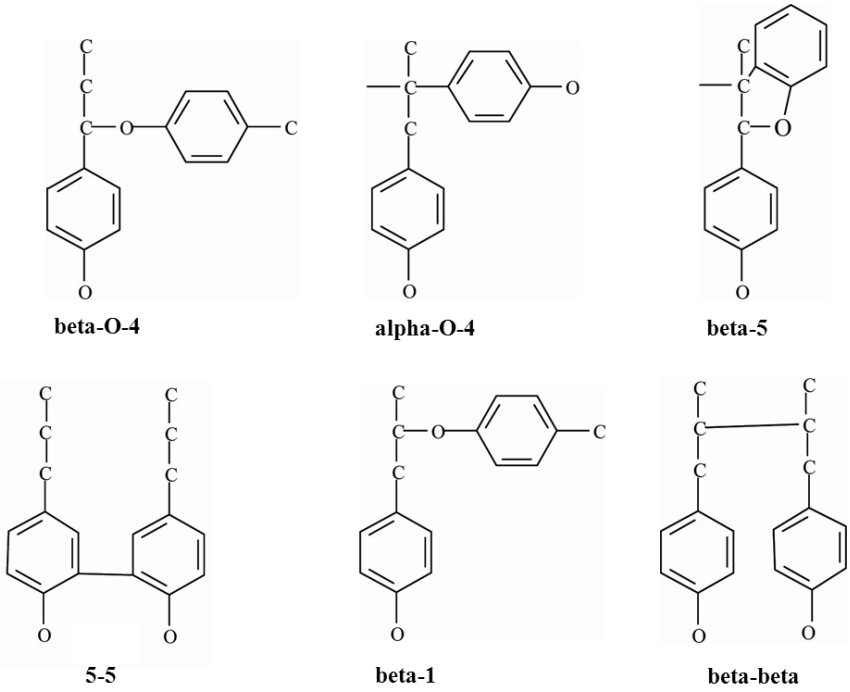


Figure 1-6. Major linkages in lignin (Sjöström 1981a).

The amount of lignin present, its distribution across fibre walls and its chemical structure vary considerably between species. For instance, softwood contains more lignin than does hardwood; also, the basic structure of lignin differs considerably between the two. Thus, the dominant structure in softwood lignin is the guaiacyl unit (G), which contains a single methoxy group on the phenylpropane ring and derives from coniferyl alcohol. On the other hand, hardwood lignin consists mainly of guaiacyl and syringyl (S) units, the latter containing two methoxy groups per phenylpropane ring and deriving from sinapyl alcohol (Parham 1983). Lignin in non-wood plants varies markedly in structure depending on the particular species. Thus, lignin in herbaceous plants consists of *p*-hydroxyphenyl units (H) from coumaryl alcohol in addition to S and G units in variable proportions. S units are known to be more reactive than G units, hence lignin rich in S units is easier to remove by pulp delignification (del Río *et al.* 2001, Dence 1996). The S/G ratio has a direct effect on delignification efficiency: the higher S/G is, the higher is the delignification rate, the less alkali is needed and the higher is the resulting pulp yield.

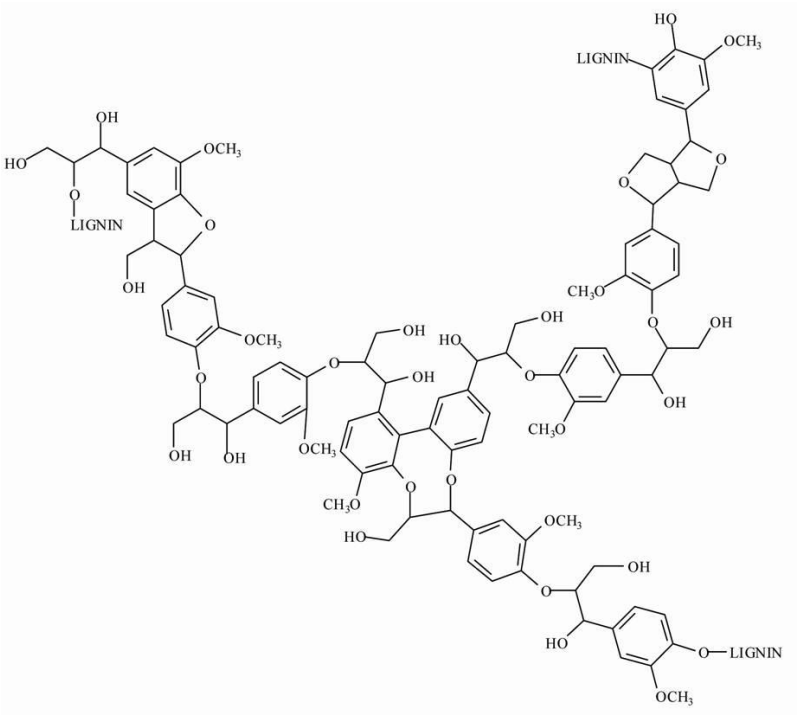


Figure 1-7. Fragment of softwood lignin (Adler 1977, Karhunen *et al.* 1995).

Because of its hydrophobicity, lignin inhibits water absorption and fibre swelling, which can make fibres less responsive to mechanical refining. However, because it is thermoplastic, lignin possesses a feature that can be used to advantage in mechanical pulping to soften it at high temperatures (Parham 1983).

Fibre walls possess a layered structure comprising various regions, namely: the primary wall (P), external secondary wall (S1), middle secondary wall (S2) and internal secondary wall (S3) (Figure 1-8). The inner part of mature cells, which is empty, is called the "lumen". In the thin primary wall, microfibrils form a more or less irregular, interwoven pattern that is embedded in a matrix of pectic materials, other hemicelluloses and lignin. In the secondary wall, cellulose microfibrils exhibit an ordered arrangement and each region is distinguished by a preferred orientation with respect to the fibre axis of its corporate lamellae. Lignin in the middle lamella is largely removed during pulp cooking, whereby fibres are individualized. Residual lignin in fibres to be bleached concentrates in the middle secondary wall (S2), which constitutes the main portion of the cell wall (Parham 1983).

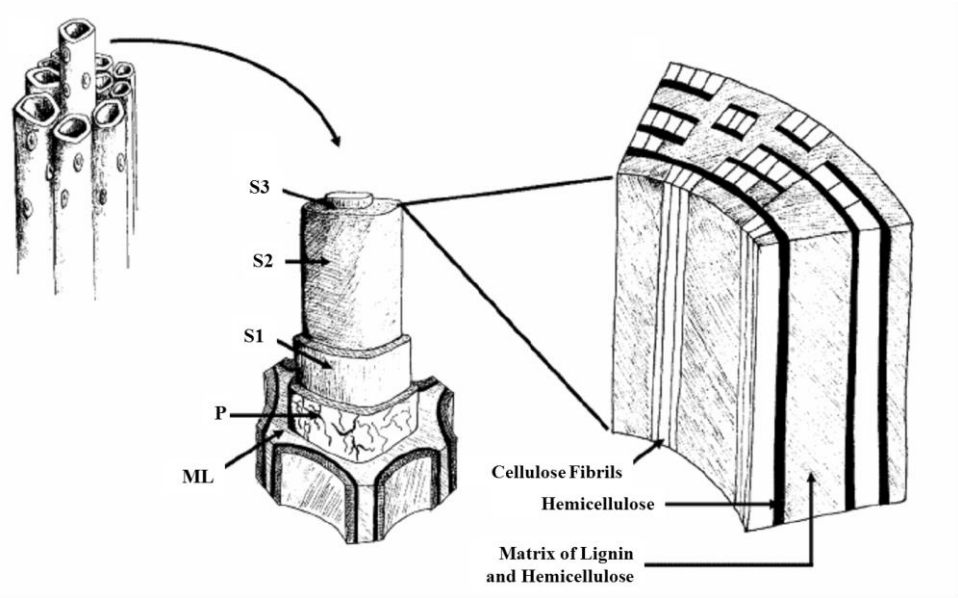


Figure 1-8. Simplified structure of the cell wall (Kirk and Kullen 1998).

Extractives are minor components of low molecular weight which have no influence on the morphological structure of plant cells but do play an important role during the processing of lignocellulosic materials. These substances, which are largely organic in nature, can be extracted from plant tissue or fibre walls with either water or various organic solvents, the choice varying with the nature of the particular extractive. Lipophilic extractives (alkanes, fatty alcohols, fatty acids, free and conjugated sterols, terpenoids, triglycerides and waxes) have and adverse impact on pulp manufacturing and the paper end-product by effect of their forming insoluble deposits known as "pitch" (Gutiérrez *et al.* 2007). On the other hand, polar extractives (phenolic compounds, tannins and flavonoids) can increase reagent consumption during pulping and hinder penetration and diffusion of pulping liquors into the raw material (Parham 1983).

1.1.2. Non-wood fibres

Wood is by far the most widely used raw material for pulp and paper making at present; in fact, non-wood plants account for only 9% of the total amount of fibre used for papermaking worldwide (Table 1-1) (Leponiemi 2008). However, some countries abound with non-wood plants, which are the primary source of papermaking fibre in

those with inadequate forest supplies (particularly China and India) (López *et al.* 2004). In addition, environmental pressure, restrictions on forest use, significant increases in wood and recycled fibre cost, and the increasing world demand for paper are forcing manufacturers in the traditionally forest-rich countries to take a renewed look at non-woods (Kissinger *et al.* 2007).

Table 1-1. Non-wood pulp and wood pulp production in the year 2004 (Leponiemi 2008).

	Non-wood pulp production (million tons)	Wood pulp production (million tons)	Non-wood pulp production of total pulp production (%)
EUROPE	0.6	50.0	1.3
NORTH AMERICA	0.3	80.9	0.4
SOUTH AMERICA	0.4	14.1	2.7
ASIA	15.5	24.7	38.6
- China	12.2	4.1	74.9
- India	1.9	1.8	50.4
AFRICA	0.4	2.7	11.5
OCEANIA	0.0	2.7	0.0
WORLD	17.4	175.4	9.0

The term “non-wood fibre” encompasses a range of plants with widely differing characteristics. Non-wood fibres currently in use and others with the potential for future use by the papermaking industry include agricultural residues such as sugar cane bagasse and straw; natural growing plants such as bamboo, reeds and grasses; and fibres grown specifically for their high fibre content such as kenaf, true hemp, jute, sisal, abaca, cotton linters and flax (Atchinson 1998). Most non-wood plants are annual plants that develop full fibre potential in one growing season. Table 1-2 compares the physical and chemical properties of selected non-wood and wood species.

Table 1-2. Chemical and morphological characteristics of selected non-wood fibres as compared to hardwood and softwood fibres (García Hortal 2007a, Page 1989).

	Chemical composition (% of total)				Morphological characteristics		
	Cellulose	Lignin	Pentosan	Ash	Fiber length (mm)	Fiber width (μm)	Average L/w ratio
Flax (bast)	70-80	1-6	2-6	1-2	9-70	5-38	1837
Hemp	57-77	9-13	14-17	<1	5-55	10-51	983
Kenaf	44-57	15-19	22-23	2-5	2-6	14-33	170
Sisal	47-62	7-9	21-24	0.6-1	1-8	8-14	409
Hardwoods	38-49	23-30	19-26	<1	0.85-1.8	15-55	38
Softwoods	40-45	26-34	7-14	<1	2.9-6.3	22-58	115

The renewed interest in non-wood fibre sources is unsurprising as they provide several advantages to the pulp and paper industry. One is their ability to grow in annual cycles (*i.e.* their renewability) rather that the typically long cycles of wood plants. Also, the comparatively small amounts of lignin present in non-woods can be removed by using more environmentally benign chemical treatments for pulping than with wood sources (Madakadze *et al.* 2010, Madakadze *et al.* 1999, Paavilainen 1998). Moreover, non-food applications can provide farmers with additional income from food crops or cattle raising (Kissinger *et al.* 2007, Rousu *et al.* 2002). In addition, non-wood fibres can be used to make all grades of paper and board, fibreboard (Hurter 1998, Ververis *et al.* 2004) and composite materials (Sain and Panthapulakkal 2006). This has resulted in a substantial increase in the use of non-wood raw materials, from 12 000 tons in 2003 to 850 000 tons in 2006 (FAO 2009, López *et al.* 2010). Unlike wood sources, non-wood fibres are subject to seasonal (rather than year-round) availability and cumbersome to handle owing to their high volume and low density; also, they require the removal of large amounts of silica during their processing (Pande 1998).

In developed countries, non-wood fibres for papermaking are largely used to manufacture specialty paper (*i.e.* paper with special properties which cannot be easily obtained from wood fibre furnish). For instance, some characteristics of paper such as porosity, wet strength, air permeability, tear and tensile strength, and foldability are a result of the intrinsic physical structure of some non-wood fibres. The specific uses of

fibres are primarily dictated by their strength, length, and length-to-diameter ratio (Martínez 1998).

1.1.2.1. Sisal fibres

Sisal fibres are hard fibrous material isolated from the leaves of sisal (*Agave sisalana*), a monocotyledonous plant of the family *Agavaceae*. Though endemic to Mexico, sisal has successfully thrived to tropical regions of Africa, South America and the West Indies, Brazil and Tanzania being the two main producing countries (Chand *et al.* 1988). Sisal is one of the most widely used natural fibres by virtue of its ease of cultivation and short renewal times (Mukherjee and Satyanarayana 1984, Samal *et al.* 1994). It has traditionally been used to manufacture natural ropes, twines, sacking and carpet backing. In recent years, stiff competition from synthetic materials and the lack of technological development has eroded the traditional sisal markets, however (Hurter 1997).

The commodity development strategy adopted by the FAO Intergovernmental Group on Hard Fibres in 1996 stressed the importance of expanding the demand for sisal, particularly for manufacturing non-traditional products such as paper (Moir 2001). At present, sisal pulp is being increasingly used by the specialty paper sector. Sisal pulp has certain characteristics such as high tear resistance, high alpha cellulose content, high bulk, absorbency and folding endurance, which make it an excellent raw material for manufacturing dielectric, plug wrap, laminating substrate, vacuum bag, tea bag and filtration paper. The physical properties of sisal pulp are superior to those of softwood kraft pulp. This provides many opportunities for expanding its use to the production of commodity paper grades. For example, sisal pulp may be used as a reinforcing fibre in high recycle content paper, or to obtain basis weight reductions while maintaining product quality. Also, meeting the ever increasing worldwide production of paper is bound to require using greater amounts of all types of fibres, but particularly fibre from recovered paper (Hurter 2001). This phenomenon can be viewed as an opportunity for sisal pulp to be established as a reinforcing additive in paper with a high recovered fibre content (Hurter 2001).

A sisal plant typically produces 200–250 leaves, and each leaf contains 1000–1200 fibre bundles 60–150 cm long and 100–400 μm wide which are composed of 4% fibre,

0.75% cuticle, 8% dry matter and 87.25% water (Mukherjee and Satyanarayana 1984). A single sisal bundle (Figure 1-9) is formed by a large number of elongated fusiform cells known as "ultimate fibres" (single cells), which are 1.5–4 mm long and 20–30 μm thick (Li *et al.* 2000). Sisal fibres contain 85% holocellulose (70% cellulose and 15% hemicellulose), 6–9% lignin, 1% ash and 0.5% lipophilic extractives (Marques *et al.* 2010). In a recent study, sisal lignin was reported to have a high S/G ratio (3.4). This is an advantage for delignification since S lignin is relatively unbranched and has a lower condensation degree than G lignin, which makes it more susceptible to depolymerization reactions (del Río *et al.* 2007).

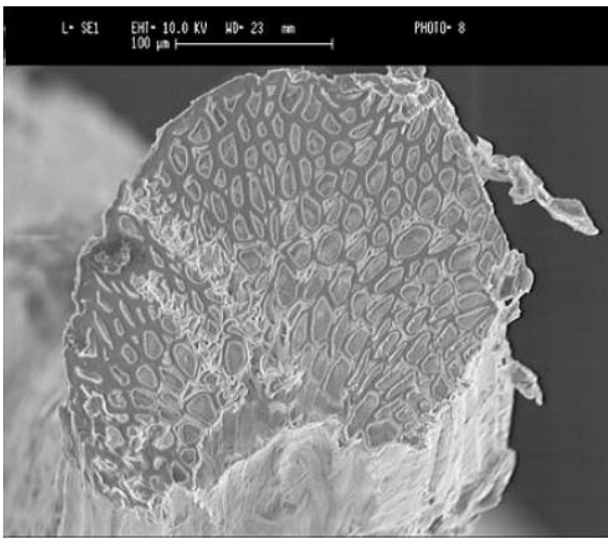


Figure 1-9. Typical cross-section of a sisal bundle at ×300 magnification (Carr *et al.* 2006).

1.2. Pulp and paper production

Paper has been a key factor in the progress of civilization, especially during the past 100 years. Today, paper is an indispensable element of everyday life. Beyond its use as the basic material for written and printed communication, paper in its various forms has a dazzling array of applications that are under continuous development to meet society's ever-changing needs.

Paper has a long history stretching back to ancient Egypt in the third millennium BC. The word "paper" is derived from the name of the reedy plant *Cyperus papyrus*, once abundant along the Nile delta and whose stem was used to produce papyrus; this is a textured material which was to be the chief writing material in the ancient world until the III century, when it was gradually replaced with the less expensive *vellum* (parchment) (Curley 2009).

The earliest production of paper, as we know today, is credited to T. S'ai Lun in 105 AD in China. This first paper was apparently made from textile wastes, old rags and used fishnets, which consisted of the fibres of true hemp and China grass (ramie) (Atchinson and McGovern 1993). The papermaking technique was learnt by the Arabs in the VIII century and paper mills proliferated from the Middle East to Europe under Arab domination. Linen and cotton rags were the chief source of paper fibres until the introduction of papermaking machinery in the early XIX century, when it became possible to obtain papermaking fibers from wood. Since then, the papermaking technique has been increasingly refined, streamlined and polished to the extent of becoming a highly automated and sophisticated process. Today, the pulp and paper manufacturing industry is among the largest in the world and commands vast annual sales.

1.2.1. Pulping and bleaching

The paper manufacturing process begins with pulping, which is intended to break down the bulk structure of the fibre source into its constituent fibres. Pulp can be manufactured by using mechanical, semi-chemical or fully chemical methods. The type of pulping process applied has an important effect on the characteristics of the resulting paper (García Hortal 2007c).

Mechanical pulping produces a high yield of fibres (around 95%) dependent on the original weight of the processed raw material. The process uses very little or no chemicals, but is extremely energy-intensive. Mechanical pulp is high-lignin, low-strength, stiff pulp used in products such as newsprint and tissue paper.

Semi-chemical pulping includes a mild chemical pretreatment intended to aid the mechanical separation of fibres with a reduced loss of strength in the subsequent mechanical process (refiner plates). Many different processes are possible that lead to a

variety of pulp qualities, the most common being neutral sulphite semi-chemical pulp (NSSP), whose stiffness and low water absorbency make it suitable for manufacturing corrugated boards.

Chemical pulping, the most commonly used pulping process, achieves fibre separation by dissolving the non-cellulosic components in the fibrous raw material (mainly lignin binding fibres together). This process removes upwards of 80% lignin and some concomitant carbohydrate (mainly hemicellulose and some cellulose in soluble form); as a result, the overall pulp yield (45–60%) is much lower than with mechanical processes. On the other hand, chemical pulp typically possesses a high mechanical strength.

Sulphite pulping and kraft (sulphate) pulping are two major chemical processes. Sulphite pulping, which can be carried out at a variable pH and with various bases, provides pulp that is easy to bleach and refine to obtain paper. Kraft pulping is an alkaline process that uses sodium hydroxide and sodium sulphide as cooking chemicals. Kraft pulp possesses higher mechanical strength and a darker colour than other types of pulp; also, it is obtained in lower yields than sulphite pulp.

The most commonly used pulping process for non-wood fibres is the soda process, where the addition of anthraquinone has become commonplace in the last few decades by virtue of the ability of this catalyst to increase delignification selectivity, carbohydrate protection and pulp yield (Blain 1993). The soda–AQ pulping process is relatively simple and requires little capital investment.

Following the pulping process, pulp stock is put through the **bleaching process**, a multi-stage process that increases the cleanliness and brightness of the raw pulp. The objective is to dissolve (chemical pulp) or modify (mechanical pulp) the brown-coloured lignin that was not removed during pulping while preserving the integrity of the pulp (Dence 1996, García Hortal *et al.* 1984). Brightness is a measure of the light reflectance of pulp in the blue region of the visible light spectrum, which is the most sensitive to the effects of bleaching. Bleaching is a mandatory treatment for a number of paper grades (particularly writing and printing paper).

Mechanical pulp has a high lignin content and is bleached in such a way as to selectively alter the chromophoric groups responsible for its brown colour without

significantly removing lignin in order to save chemical reagents and avoid substantial yield losses. Bleaching treatments for mechanical pulp are often referred to as "lignin-preserving treatments" (Biermann 1996). The bleaching agents used for this purpose are reducing agents such as dithionite or oxidants such as hydrogen peroxide, often used in a one-stage process.

Bleaching of chemical pulp is achieved by removing lignin and requires several consecutive stages. The early stages can be considered a continuation of the delignification process started in cooking, and the last have the effect of eliminating residual colour (chromophoric groups). The bleaching agents most commonly applied to pulp are oxidants producing acid groups in residual lignin (chlorine, chlorine dioxide, ozone); these are usually employed in the first stages and followed by an alkaline treatment with oxygen, hydrogen peroxide or sodium hypochlorite to remove acid degradation products of lignin. Removing lignin from chemical pulp increases the flexibility and strength of paper fibres. However, a chemical treatment can result in the loss of hemicelluloses, which aid fibre swelling and interfibre bonding.

In the last two decades, environmental concern in the pulp and paper industry, and pressure by new, more stringent legislation, have promoted strong efforts at changing, adapting and/or improving technology to develop more environmentally friendly production processes (Bajpai 2005). The pulp bleaching plant, which is the most contaminating section of the pulp and paper industry, has obviously undergone the greatest changes towards accomplishment of this goal. Traditional bleaching sequences for chemical pulp based on highly effective and economical, but environmentally unsound bleaching with elemental chlorine have been gradually replaced with elemental chlorine free (ECF) and totally chlorine free (TCF) sequences in order to minimize the discharge of organochlorine compounds, measured as adsorbable organic halogen (AOX). Both ECF and TFC processes use oxygen-based bleaching agents (particularly oxygen, alkaline hydrogen peroxide or and ozone), and some ECF sequences also use chlorine chemicals other than elemental chlorine (chlorine dioxide mainly).

1.2.1.1. Hexenuronic acids

Bleaching efficiency and the final pulp properties can be adversely affected by the presence of hexenuronic acids (HexA) in pulp. HexA are formed by β-elimination of

methylglucuronic acid, which is randomly distributed on the sides of xylan chains, during alkaline pulping (Figure 1-10) (Teleman *et al.* 1995). The reaction takes place rapidly during the heating phase at the early stages of pulping, at temperatures of 110–150 ºC and pH 12–13 (Törngren and Gellerstedt 1997).

HexA are known to have the following effects:

- They contribute to kappa number. In fact, HexA interfere with the standard method for determining kappa number; this is based on estimation of the lignin content with potassium permanganate in an acid medium. This chemical oxidizes HexA and gives rise to spurious results in the determination of lignin (Costa and Colodette 2007, Li *et al.* 2002).

- They consume large amounts of bleaching reagents (Jiang *et al.* 2000).

- They retain metal ions. Thus, some metal ions present in pulp catalyse the decomposition of some bleaching agents (*e.g.* hydrogen peroxide) and HexA can have a strong chelating effect, thereby contributing to the presence of large amounts of metal ions in pulp (Vuorinen *et al.* 1999).

- They increase brightness reversion. Several studies have suggested that paper ageing is related to the presence of HexA in bleached pulp (Cadena *et al.* 2010, Sevastyanova *et al.* 2006, Vuorinen *et al.* 1999).

- They facilitate the formation of oxalic acid. Some authors believe that oxalic acid formed in pulp is partly due to the presence of HexA (Elsander *et al.* 2000, Vuorinen *et al.* 1999).

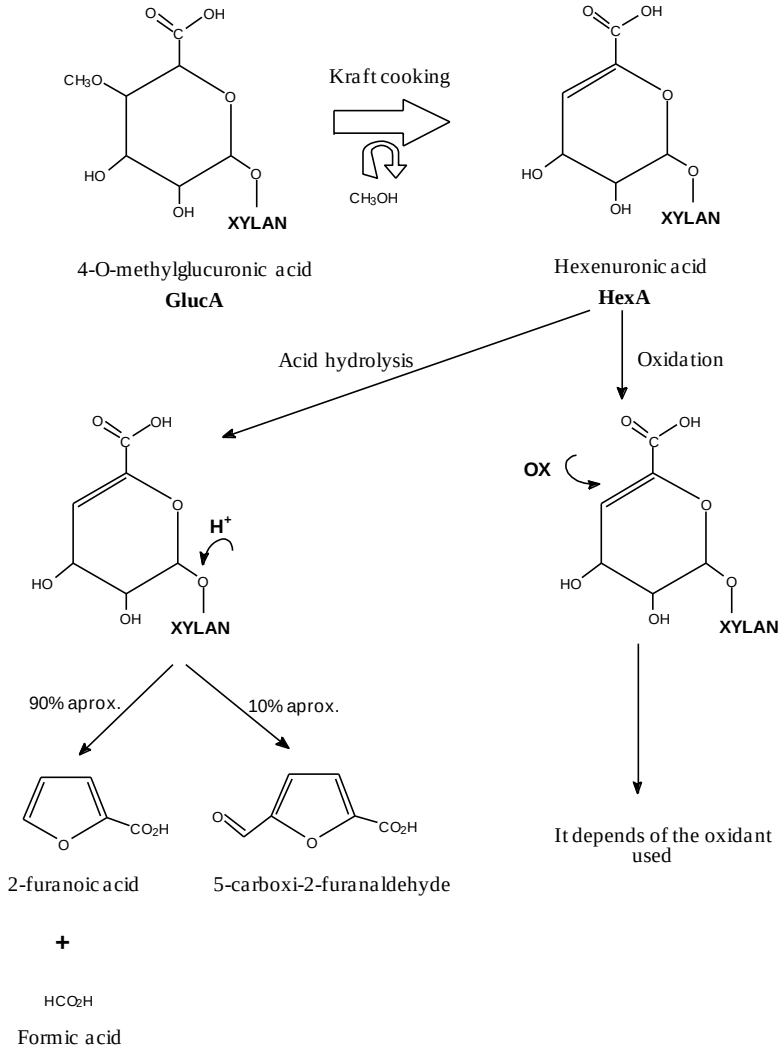


Figure 1-10. Hexenuronic acid (HexA) formation and destruction by hydrolysis or oxidation (Roncero 2001).

According to Jiang (2000), HexA protects xylans from terminal depolymerization; therefore, early removal of HexA during pulping could lead to a significantly reduced yield. In any case, HexA can be removed by using an appropriate agent prior to bleaching. HexA contain enol–ether and unsaturated carboxyl functional groups, so they exhibit both nucleophilic and electrophilic reactivity (Jiang *et al.* 2000). These groups are relatively stable under mild acid conditions; however, HexA are hydrolysed to furan derivatives in the presence of strong acids (Allison *et al.* 1999). Hydrolysis with sulphuric

acid can remove substantial amounts of HexA; unfortunately, this results in considerable degradation of cellulose owing to the poor selectivity of the acid (Henricson 1997). By virtue of their containing double bonds, HexA can also be eliminated by using electrophilic oxidizers such as chlorine, chlorine dioxide or ozone, which, however, produce undesirable organic by-products (Vuorinen *et al.* 1999).

1.2.2. Papermaking

Prior to sheet-formation, pulp is subjected to **refining**, which is an essential step towards developing the potential of pulp fibres to obtain specific products of the required quality in terms of strength and surface. Pulp refining is a mechanical treatment which alters the cell wall structure to improve interfibre bonding capacity in order to improve sheet formation and the mechanical properties of the resulting paper.

In the refining process, chemical pulp enters the refiner suspended in water at a consistency of 2%–6%. The pulp is treated between conical or flat rotor and stator plates equipped with metal bars to inflict mechanical energy on fibres (Figure 1-11). Most of the fibres in the refining gap are not in contact with the refiner bars, but with one another, which results in frictions between fibres that create shear forces.

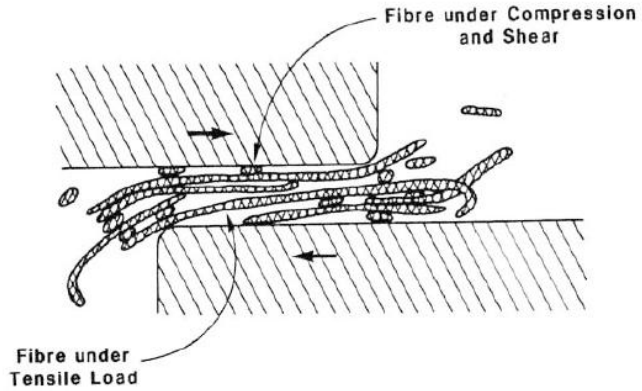


Figure 1-11. Impression of fibres trapped between the passing stator and rotor bars (Page 1989).

Figure 1-12 shows the effects of refining at different intensities on sisal pulp fibres. Refined fibres exhibit increased swelling, flexibility, conformability and surface area, all of which promote interfibre bonding (Kerekes 2005, Lumiainen 2000). Increased bonding in sheets leads to increased density and tensile, burst and fold strengths. On the other hand, tear strength is adversely affected by bonding, and so is opacity through a decreased ability of the paper to scatter light (Kline 1982).

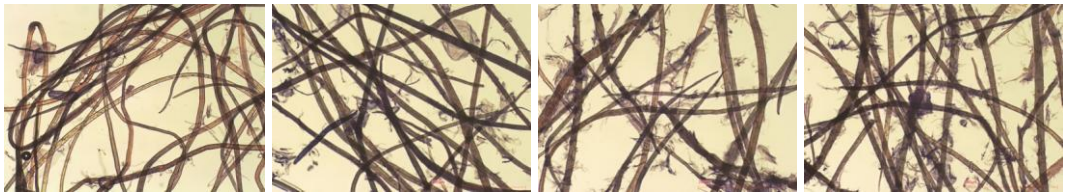


Figure 1-12. Light microscopy images (100× magnification) of unrefined sisal pulp, and after refining for 1500, 3000 and 4500 revolutions in a PFI refiner.

Refining is only a part of the preparation of stock for use on the paper machine: various chemicals and mineral additives may be added to achieve the desired final product. Papermaking operations are performed in two sections, namely: the "wet end" and the "dry end". The wet end comprises all equipment and operations between the machine chest, where the stock is stored, and the press section. The dry end begins after the press section and includes the drying section and the finishing operations. Figure 1-13 depicts a typical papermaking process.

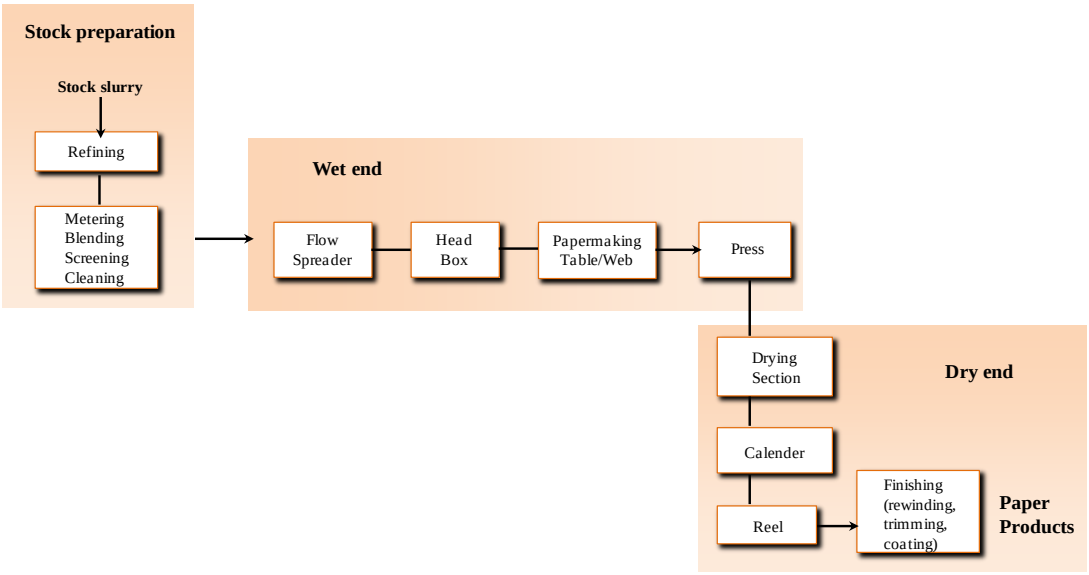


Figure 1-13. Flow chart of a typical papermaking process.

1.2.2.1. Paper strength

The strength of paper is a crucial property dictating its grade. The strength-related properties of paper are dependent on the chemical and physical nature of the pulp fibres forming sheets. The physical properties include fibre length, strength, curl and surface area, and the chemical properties the degree of cellulose polymerization, lignin content, and amount of hemicelluloses and their distribution.

The two main factors governing the strength of the fibre network that makes up a sheet of paper are the bonds between fibres and the intrinsic strength of the individual fibres. Hydroxyl groups in cellulose are widely regarded as the main source of hydrogen bonds in paper (Robinson 1980). Hemicelluloses are also capable of forming hydrogen bonds and pulp with an increased hemicellulose content usually provides paper with good strength-related properties (Li *et al.* 2011, Schönberg *et al.* 2001). On the other hand, lignin lacks the advantageous hydrogen bonding properties of the carbohydrate fraction in fibres. Pulp with a high lignin content is difficult to refine and exhibits poor interfibre bonding, possibly as a result of its containing very few hydrophilic groups.

Paper sheets can be imparted acceptable strength-related properties by adding strength additives to the stock prior to web formation in order to facilitate gluing or

bonding of fibres. The most common additives for enhancing interfibre hydrogen bonding are starches (whether natural or modified), plant and animal gums, and synthetic polymers (polyacrylamide, polyamide and latex), which are generally referred to as "dry-strength" additives.

Wet strength

Hydrogen bonding is very sensitive to water: as water wets paper, it breaks bonds in it and reduces its dry strength to 3–10% of the original value. Wet-strength additives, which are generally chemically reactive, water-soluble polymers, can be used to facilitate retention of some strength in paper subjected to a high humidity or soaking in water. Wet strength development in paper can be accomplished in basically two different ways, namely: (*a*) by strengthening existing bonds or creating new ones; and (*b*) by protecting existing bonds. Thus, the polymers can react with cellulose or hemicellulose (co-cross-linking) via a "reinforcement" mechanism, or with one another (homo-cross-linking) via a "preservation" mechanism (Espy 1995). Examples of wet-strength additives include urea–formaldehyde resins, melamine–formaldehyde resins, epoxidized polyamide resins and glyoxalated polyacrylamide resins. The paper industry generally classifies wet-strength resins into two groups: temporary and permanent. Chemically, permanent resins can generally form covalent bonds that are not readily hydrolysed in water, whereas temporary resins are usually based on reactive aldehydes that form acetal or hemiacetal linkages with paper (Chen *et al.* 2002). It is generally accepted that paper with a wet-tensile strength exceeding 15% of its dry tensile strength should be considered wet-strength paper (Dunlop-Jones 1991).

In the last few decades, environmental concerns have restricted the use of some classes of wet-strength additives on the grounds that they release formaldehyde during paper manufacturing and from the end product, and imposed the alteration of others contributing to AOX emissions from paper mills (Stange 1994). Environmental pressure continues to influence research into new chemicals or methods for developing wet strength (Lonsky and Negri 2002, Lund and Felby 2001, Saito and Isogai 2005) and a need exists for new products that are more biodegradable and more compatible with the ecologies of all environmental compartments with which they may come into contact.

1.3. Biotechnology in the pulp and paper industry

The pulp and paper industry is under steady, ever-increasing pressure from global competition, stringent environmental regulations and new market demands. In this scenario, the use of biotechnology has emerged as a very promising approach not only to developing cleaner processes, but also to obtaining novel, high-value products. Biotechnology in the pulp and paper sector has a strong focus on the use of biological products such as enzymes. The fact that the raw materials used to manufacture pulp and paper typically consist of natural fibres provides ample opportunity for applying enzyme technology in the process (Bajpai 1999; Kenealy and Jeffries 2003). The importance of enzyme technology is ascribed to its potential for enabling more specific reactions, affording less environmentally deleterious processes, decreasing resource consumption and, ultimately, reducing costs (Kenealy and Jeffries 2003). Specific enzymatic processes have developed for most of the unit operations in the pulp and paper industry and a number of them have been successfully transferred to industrial scale. Table 1-3 shows the main areas of application of enzymes in the pulp and paper industry.

Bleaching has been the subject of much research by the pulp and paper industry into the potential of enzymes for providing more environmentally friendly and profitable processes. Enzymes can be used to improve the bleaching process indirectly or directly. Thus, pulp bleachability can be indirectly improved through the action of enzymes of the hemicellulolytic type (particularly xylanases, which are xylan-attacking enzymes) to increase lignin extractability and reduce the consumption of bleaching chemicals as a result. Bleachability can also be improved in a direct manner, using oxidative enzymes from white-rot fungi, which can directly attack lignin and have a higher potential than xylanases. Whereas xylanases have been used on the industrial scale to enhance the bleachability of kraft pulp for about 20 years, the laccase–mediator concept is still under development at present (Bajpai 2004).

In recent years, pulp and paper research has increasingly switched to a different use of laccase such as the targeted modification of lignocellulose fibres with a view to improving intrinsic fibre properties or introducing novel ones.

Table 1-3. Main characteristics of selected enzymes used in various pulp and paper industrial processes.

Application	Enzyme type	Level of application	Function in P&P industry	References
Bleaching	Xylanase Laccase	Full scale Pilot scale	Boosts bleachability by degrading xylans Aids delignification	(Call and Mücke 1997, Fillat *et al.* 2010)
Mechanical pulping	Cellulase	Full scale	Saves energy by softening cellulose fibers	(Hoddenbagh *et al.* 2007)
Chemical pulping	Cellulase, hemicellulase, pectinase	Pilot scale	Increase the diffusivity of cooking liquors	(Jacobs *et al.* 1998)
Pitch control	Lipase Laccase	Full scale Lab-scale	Hydrolyzes pitch Oxidizes pitch	(Gutiérrez *et al.* 2006, Lee *et al.* 2011)
Deinking	Cellulase	Pilot scale	Aids ink release by degrading cellulose	(Lee *et al.* 2011)
Stickies control	Esterase	Full scale	Hydrolyzes PVAc	(Patrick 2004)
Refining of chemical pulps	Cellulase, xylanase	Pilot scale	Enhance beatability, improve drainage	(Dienes *et al.* 2004, Yang *et al.* 2011)
Fiber modification	Laccase	Lab-scale	Oxidative modification of fiber properties	(Fillat *et al.*, Garcia-Ubasart *et al.* 2011)

1.3.1. Use of xylanases

The most important application of enzymes in the pulp and paper industry at present is the pre-bleaching of chemical pulp with xylanase. Xylanases are hydrolytic enzymes that catalyse the hydrolysis of xylans. Xylans are complex heteropolysaccharides containing β-1,4 bonds of highly substituted d-xylopyranose units. Biodegradation of xylan, which is a naturally abundant polysaccharide, is a complex process that requires the coordinated action of several enzymes, β-1,4-endoxylanases playing the main role: breaking internal bonds such as those of β-1,4-xylose (Beg *et al.* 2001).

The ability of xylanases to facilitate pulp bleaching was first reported in 1986 by Viikari *et al.* (Viikari *et al.* 1986). They found xylanase treatments to provide substantial savings in bleaching chemicals or to raise pulp brightness. Since then, the use of

commercial xylanases has reached the industrial scale in just a few years. The primary factors explaining such a fast development can be summarized as follows (Bajpai 1999):

- Xylanase pre-bleaching is a soft technology that requires very little or no capital investment to operate.
- The process changes required are minimal in most cases.
- Xylanase helps reduce AOX emissions from bleaching plants.
- Savings from chemicals can pay for the process.
- The process can be easily combined with a number of bleaching sequences for ECF and TCF pulps.

Under alkaline conditions, much of the xylan in wood fibre is dissolved into the cooking liquor. As cooking proceeds and the alkali concentration decreases, dissolved xylan precipitates back onto crystalline cellulose fibres. It is believed that a loss of side groups from the xylan chains allows xylans to re-precipitate in a crystalline form which is less soluble than native xylan and it is this re-precipitated, insoluble xylan that hinders the solubilisation of residual lignin. Several mechanisms have been proposed to account for the action of xylanases (Pham *et al.* 1995, Wong *et al.* 1997). Their favourable effects are generally ascribed to the removal of xylans belonging to hemicelluloses in cellulose fibres, which are somehow present in between structured cellulose chains and the amorphous lignin fraction. Removing these xylans eliminates existing bonds between cellulose and lignin, thereby facilitating the removal of lignin, which will now be freer, more susceptible to attack by bleaching agents in subsequent stages (Paice *et al.* 1992, Pham *et al.* 1995, Roncero *et al.* 2000, Shatalov and Pereira 2007, Valls *et al.* 2010b).

A novel feature of xylanases was recently identified: their ability to reduce the content of hexenuronic acids in pulp (Aracri and Vidal 2011, Valls *et al.* 2010b). Because xylanases hydrolyse xylans on fibre surfaces and such xylans contain HexA, these compounds may be removed by enzymatic treatment with xylanases. A recent study (Valls *et al.* 2010a) examined the behaviour of hexenuronic acids in a bleaching sequence including a pretreatment with xylanase. The preliminary treatment was found to decrease the HexA content by 15% through the removal of xylans; also, it increased HexA removal in the subsequent oxidative stage from 10% to 35%.

1.3.2. Use of laccases

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a blue, copper-containing, glycoprotein oxidoreductase enzyme with an average molecular weight of 60–70 kDa. Yoshida first discovered laccases in 1883 after observing that latex from the Japanese lacquer tree (*Rhus vernicifera*) hardened in the presence of air. The enzyme was named laccase about 10 years later, following isolation and purification of the responsible catalyst (Bertrand 1894, Call and Mücke 1997). Since then, laccase activity has been detected in plants, some insects and a few bacteria. However, most biotechnologically useful laccases (*i.e.* those with a high redox potential) are of fungal origin (particularly, white-rot Basidiomycetes fungi, which are involved in lignin mineralization) (Thurston 1994).

Laccase-catalysed reactions involve the one-electron oxidation of a substrate molecule to the corresponding reactive radical and a subsequent four-electron reduction of molecular oxygen to two molecules of water (Figure 1-14).

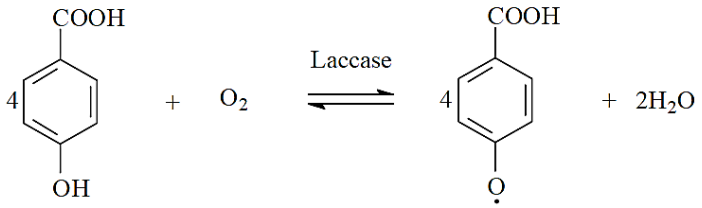


Figure 1-14. Example of a reaction of laccase with a phenolic compound.

The redox process takes place with the assistance of a cluster of four copper atoms that form the catalytic site of the enzyme (Figure 1-15). Laccases can use a wide range of substrates including phenols, polyphenols, anilines, aryl diamines, methoxy-substituted phenols, inorganic/organic metal compounds and many others, and this is the main reason for their attractiveness for a number of biotechnological applications (Riva 2006, Xu 2005).

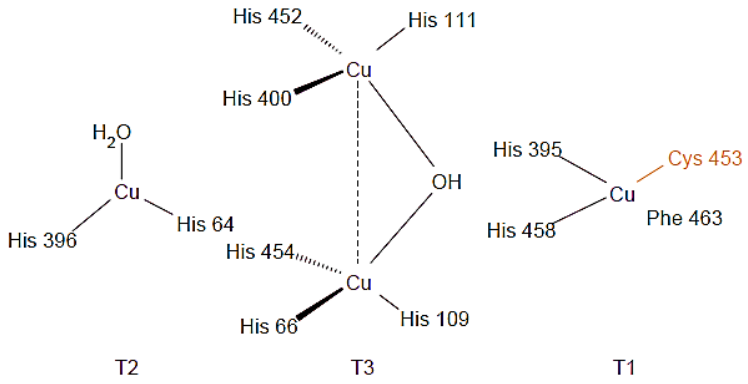


Figure 1-15. Model for the catalytic cluster of laccase from *T. versicolor* consisting of four copper atoms. Type 1 (T1) copper confers the typical blue colour to the protein and is the substrate oxidation site. Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place (Riva 2006).

Laccase plays a variety of roles including its participation in lignin biosynthesis (O'Malley *et al.* 1993), plant pathogenicity (Sbaghi *et al.* 1996) and the degradation of plant cell walls (Machuca and Durán 1993). Chemically, these functions are related to the oxidation of a wide range of aromatic substances. However, the net effect of such oxidations can vary widely and work in opposite directions. Thus, laccases from plants oxidize monolignols to polymeric lignins, whereas laccases form white-rot fungi degrade and depolymerize lignins (Mayer 2002). The latter function has enabled the use of laccases to bleach pulp. Although laccases play a central role in degrading lignin *in vivo*, they cause minor structural changes and further polymerization of lignin in the *in vitro* oxidation reactions they catalyse (Bajpai 1999). Therefore, these enzymes by themselves are unable to mimic the complete biological system and require the presence of a low-molecular weight compound known as a "redox mediator" for this purpose.

Basically, direct oxidation of a substrate by laccase may be impossible either because the substrate it is too large to penetrate into the enzyme active site or because its redox potential exceeds that of the enzyme (~0.5–0.8 V) (Gianfreda *et al.* 1999). Redox mediators are suitable intermediate substrates for laccase; thus, they are oxidized to radical forms by the enzyme and the resulting radicals are able to oxidize a bulky or high-redox potential substrate via a non-enzymatic mechanism (Figure 1-16) (Riva 2006). Ever since the discovery of chemical mediators capable of extending enzymatic oxidation to

non-phenolic compounds in lignin (redox potential > 1.5 V), research interests have focused on the potential of laccase–mediator systems (LMS) for aiding pulp bleaching (Barreca *et al.* 2003, Bourbonnais and Paice 1990, Fillat *et al.* 2010).

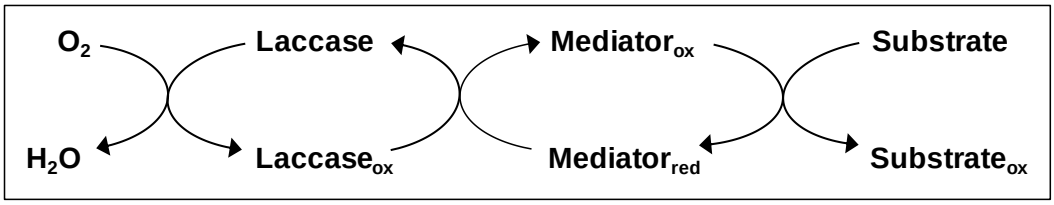
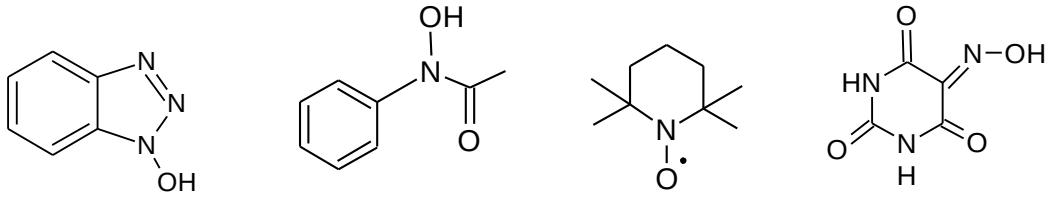


Figure 1-16. Expanding the role of laccase: oxidizing unusual substrates by the action of redox mediators.

1.3.2.1. Biobleaching

Biobleaching refers to the treatment of pulp with microorganisms such as fungi or their enzymes in order to delignify it and/or modify lignin to facilitate subsequent bleaching with chemicals. Laccase can directly oxidize accessible phenolic hydroxyl groups in pulp lignin to phenoxy radicals in the absence of an oxidative mediator. Transformation of the phenoxy radicals can result in lignin depolymerization or polymerization. The transformations promoting biobleaching via delignification (*i.e.* those contributing to lignin depolymerization and formation of hydrophilic functional groups, Leonowicz *et al.* 2001) include (1) side chain cleavage (C_1-C_α scissions) (Kawai *et al.* 1988), (2) α-OH oxidation of phenolic β-1 type lignin moieties (Kawai *et al.* 1988), and (3) degradation of phenolic β-O-4 structures by side-chain or β-O-4 fission (Higuchi 1990). However, no net depolymerization of lignin may occur if extensive repolymerization takes place via combination of phenoxy radicals to form condensed lignin structures (Crestini and Argyropoulos 2001, Shleev *et al.* 2006). Also, transformations of phenoxy radicals giving rise to chromophoric conjugated carbonyl structures (quinones and C_α carbonyl groups) (Chakar and Ragauskas 2001) reduce pulp brightness. Hence these chromophores must be removed from pulp lignin by alkaline extraction or oxidation (Chakar and Ragauskas 2000, Fillat and Roncero 2009). The ability of mediators to extend the effect of laccase to non-phenolic lignin units and to overcome accessibility restrictions of cell walls in pulp fibres, has promoted biobleaching with laccase–mediator systems (LMS).

Laccase-mediated oxidation of non-phenolic lignin units can follow an electron transfer, a radical hydrogen atom transfer or an ionic mechanism, depending on the particular mediator (Barreca *et al.* 2004). The first synthetic mediator known to oxidize high-redox non-phenolic lignin model compounds is 2,2′-azinobis-(3-ethylbenzenethiazoline-6-sulphonic acid) (ABTS) (Bourbonnais and Paice 1990). The synthetic laccase mediators most widely investigated for use by the pulp and paper sector are the N–OH compounds 1-hydroxybenzotriazole (HBT), violuric acid (VA), and *N*-hydroxyacetanilide (NHA), and the stable 2,2,6,6-tetramethylpiperidine-1-oxyl free radical (TEMPO) (Figure 1-17) (Fillat *et al.* 2011, Fillat and Roncero 2009, Moldes *et al.* 2008, Valls *et al.* 2010).



HBT **NHA** **TEMPO** **VA**

Figure 1-17. Chemical structure of selected synthetic laccase mediators.

While an LMS treatment followed by alkaline extraction is able to achieve significant delignification of chemical pulps (Bourbonnais and Paice 1996), LMS must be incorporated into a ECF or TCF-bleaching sequence to obtain fully bleached pulps. Research has shown that the laccase/HBT (Fillat and Roncero 2010, Ibarra *et al.* 2006), laccase/VA (Aracri and Vidal 2011, Fillat *et al.* 2011, Moldes and Vidal 2008) and laccase/NHA systems (Paice *et al.* 2002, Valls *et al.* 2010) can substantially reduce the requirements of bleaching chemicals for chemical pulp bleaching, or allow bleaching to smaller kappa numbers and higher brightness. LMS have recently proved efficient in removing sterols, the main culprits of pitch problems, from eucalyptus pulp (Gutierrez *et al.* 2009, Gutiérrez *et al.* 2006). The promising results obtained by Valls *et al.* (2009) demonstrate that sterols are better eliminated by an LMS stage than by a chlorine dioxide stage. Another recently found, beneficial feature of LMS relating to bleaching is their ability to reduce the HexA content of pulp via an as yet unclear mechanism. Valls *et al.* (2010b) found the HexA content of eucalyptus pulp to decrease upon treatment with a

laccase–HBT system, to an extent dependent on the enzyme and mediator doses, but not on the reaction time. The joint use of laccase and HBT on TCF eucalyptus pulp was found to remove hexenuronic acids by 23% and to significantly reduce brightness reversion (Cadena *et al.* 2010). Moreover, it has been demonstrated that the reduction of HexA can be consequence of the coupling of phenolic mediators to these structures (Cadena *et al.* 2011).

Despite the associated advantages of LMS, mediators are expensive and can generate toxic derivatives. Moreover, in some cases, laccase can be rendered inactive by the mediator radicals or the mediator be transformed into inactive compounds as regards mediation capabilities (e.g. oxidation of HBT by the enzyme can cause it to lose its hydroxyl group and form benzotriazol) (Fillat and Roncero 2010, Li *et al.* 1998). There have been recent trends to using eco-friendly, potentially cost-effective alternative mediators such as naturally occurring phenols, which can be readily obtained from plants and spent pulping liquors or, directly, from fungal metabolism (Aracri *et al.* 2009, Camarero *et al.* 2005, Eggert *et al.* 1996, Johannes and Majcherczyk 2000, Moldes *et al.* 2008, Nousiainen *et al.* 2009). Recently, phenolic compounds derived from lignin degradation (*e.g.* acetosyringone, syringaldehyde, ferulic acid, *p*-coumaric acid) have proved highly efficient natural laccase mediators for the removal of recalcitrant substances such as lignin (Camarero *et al.* 2007, Fillat *et al.* 2010) and lipids (Gutiérrez *et al.* 2007) from pulp.

1.3.2.2. Enzymatic functionalization

In the last decade, laccase has attracted considerable attention as a means for modifying fibre chemistry with a view to altering paper properties (particularly, strength-related properties). Thus, Felby *et al.* (1997) found laccase to increase auto-adhesion in wood fibres and the bonding mechanism to involve the enzymatic activation of lignin on fibre surfaces through the production of phenoxy radicals, which facilitated cross-linking of fibres during board making. Further studies showed combinations of laccase with a lignin-rich extractive or a mediator to substantially improve wet tensile strength in high-yield unbleached kraft pulp without the need for toxic synthetic adhesives (Lund and Felby 2000, Lund and Felby 2001). The improvement was ascribed to polymerization of lignin in the handsheets, and also to enhanced production of phenoxy radicals increasing cross-linking between fibres and facilitating water-resistant interfibre bonding as a result.

Radical coupling reactions involving phenolic compounds have also been used to enable bonding of low-molecular weight compounds to lignin-rich cellulose fibres. Radical coupling reactions competing with delignification represent an adverse, undesirable phenomenon in the biobleaching process (Camarero *et al.* 2007, Fillat *et al.* 2010). However, they have aroused increased interest as the key mechanisms behind the *biografting* 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 pulp and paper (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 (Viikari 2002). Laccase-catalysed biografting is a versatile functionalization method by virtue of the enzyme's non-specific substrate requirements, which allow bonding a wide range of phenolic compounds and thus incorporating several desired properties into the fibre matrix (Chandra *et al.* 2004, Elegir *et al.* 2008, Grönqvist *et al.* 2006). The feasibility of this approach has been demonstrated in a number studies; interest, however, has focused on wood materials and lignin-rich fibres, and little research has comparatively been carried out on non-wood fibres.

The laccase–TEMPO mediated system provides a potential approach to oxidatively modifying cellulose pulp fibres. TEMPO-mediated oxidation is a well-known procedure to introduce carboxyl and aldehyde functional groups into cellulose in aqueous media at room temperature (de Nooy *et al.* 1995b). Although the ability to use laccase to catalyse the regenerative oxidation of TEMPO has been demonstrated (Viikari *et al.* 1999), the reaction is commonly carried out in the presence of NaClO/NaBr as a co-oxidizer system (Bragd *et al.* 2001, Chang and Robyt 1996, de Nooy *et al.* 1995a, Isogai and Kato 1998). TEMPO-mediated oxidation has been successfully exploited to improve various physical properties of pulp fibres including interfibre bonding —and hence the strength-related properties of the resulting paper (Dang *et al.* 2007, Duarte *et al.* 2006, Lianshan *et al.* 2008, Marzorati *et al.* 2005). Improved wet strength in paper from TEMPO-oxidized fibres was recently reported in various studies and ascribed to the formation of large amounts of surface aldehyde groups as intermediate structures of the TEMPO-mediated oxidation process which can form interfibre covalent bonds through hemiacetal linkages with hydroxyl groups of adjacent fibre surfaces (Saito and Isogai 2005, Saito and Isogai 2006).

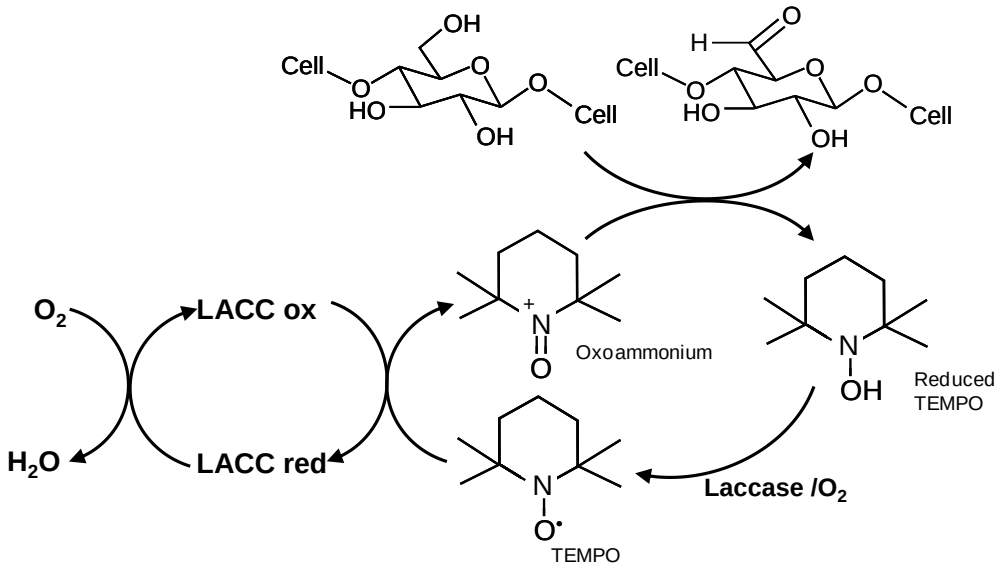


Figure 1-18. Proposed mechanism for the enzymatic production of oxoammonium ion.

Environmental concerns have recently driven research interests into the development of halide-free oxidative systems. One promising approach for this purpose is the use of laccase together with oxygen as primary oxidants (Arends *et al.* 2006, Viikari *et al.* 1999). Similarly to the NaClO/NaBr process, oxoammonium ion is regenerated *in situ*, so only oxygen is consumed in the course of the reaction (Figure 1-18). In addition to the environmental benefits associated with the use of an enzyme, this method provides the advantage of operating at near-neutral pH, which reduces the occurrence of β-elimination reactions and hence scission of the polysaccharide chain —a major drawback of the traditional method using pH 10–11 (Patel *et al.* 2011).

1.4. Objectives

Despite the strong efforts of the pulp and paper industry at increasing the efficiency and sustainability of their production processes, and complying with increasingly stringent environmental legislation, this sector is under steady pressure from global competition and growing market demands. The ability to incorporate environmentally

friendly technologies and develop novel, high-quality products is a key factor to ensure competitiveness.

High-priced non-wood fibres such as those from sisal, which are typically used to manufacture specialty paper, are also suitable for application of enzyme technologies thanks to the increased profit margins they provide. The operational flexibility and broad substrate specificity of laccase make it a powerful tool for developing cleaner processes and modifying lignocellulosic fibres to obtain novel, sustainable products. It would therefore be interesting to assess the potential of laccase for the biomodification of sisal pulp fibres.

The main objective of this doctoral work was

- **Modify sisal (*Agave sisalana*) pulp fibers by means of laccase-based systems for aiding bleaching and developing novel and/or improved fiber and paper properties.**

And its specific objectives were

- To explore the potential of four natural phenolic compounds for either aiding bleaching (as laccase mediators) or functionalizing sisal fibres (by radical coupling).
- To compare the efficiency of a well-known synthetic mediator and a natural phenolic compound in aiding lignin and HexA removal in a TCF bleaching sequence performed in the absence or presence of a xylanase stage.
- To develop new methods for exposing covalent binding of phenolic compounds to fibres and identifying the specific compound most likely to undergo grafting reactions upon laccase oxidation.
- To conduct grafting reactions under different conditions and use those ensuring the highest degree of phenol grafting in order to compare the effect obtained by applying the enzyme treatment to refined and unrefined pulp with a view to improving strength-related properties in the resulting paper.
- To assess a different approach to fibre functionalization based on the oxidation of cellulose by using the laccase-TEMPO system in order to

improve strength-related properties as an environmentally friendly alternative to established halide-based systems.

- To examine the influence of the operating conditions in a laccase-TEMPO treatment on the degree of functionalization and paper strength by using a sequential statistical plan involving three variables.

The following are novel, salient features of the proposed objectives:

- The use of an unconventional raw material such as sisal fibres to obtain bleached pulp of a high added value.
- The use of biotechnology for efficient TCF bleaching (specifically, of natural, environmentally safe compounds as substitutes for expensive, potentially toxic synthetic laccase mediators).
- The use of enzyme systems (xylanase and LMS) to reduce the HexA content of pulp.
- The application of biografting to low-lignin non-wood fibres to improve strength-related properties in paper.
- The search for an enzymatic modification approach alternative to biografting treatments capable of functionalizing cellulose and avoiding the deleterious effects of traditional systems.
- To compare the effects of the different laccase-based systems in terms of thermal degradation profile of the treated pulps to monitor the superficial changes induced in cellulosic microfibrils.

1.5. Thesis format

The experimental results of this thesis are presented as chapters based on papers either published as journal articles, or submitted to journals. Due to the use of this layout, there will be some repetition among the experimental and introduction sections. The publications are presented in their original or submitted form with subtle changes, and, in two cases, an addendum of some additional results was included in the chapter.

Publication 1:

Aracri, E., Colom, J. F. and Vidal, T. (2009). "Application of laccase-natural mediator systems to sisal pulp: An effective approach to biobleaching or functionalizing pulp fibres?". *Bioresource Technology.* 100, 5911-5916.

Addendum to Publication 1:

Bleaching assays for a comparative study of new synthetic and natural mediators.

Publication 2:

Aracri, E. and Vidal, T. (2011). "Xylanase- and laccase-aided hexenuronic acids and lignin removal from specialty sisal fibres". *Carbohydrate Polymers.* 83, 1355-1362.

Addendum to Publication 2:

Fate of lipophilic extractives of sisal pulp during TCF biobleaching sequences

Publication 3:

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Publication 4:

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Publication 7:

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Materials and methods

2.1. Raw material

The raw material used in this doctoral work was sisal pulp obtained by soda–anthraquinone cooking at the CELESA mill in Tortosa (Spain). Prior to use, unbleached pulp was conditioned with H_2SO_4 at pH 4 under stirring for 30 min at 2% consistency, which was followed by passage through a glass filter funnel and extensive washing with de-ionized water. This procedure was necessary to remove contaminants and metals, and also to bring the pulp pH closer to that for the enzyme treatments. The pulp was supplied in four different stocks, so the samples differed in their initial properties. Table 2-1 summarizes the properties of the starting pulp samples and states the chapters describing the studies conducted on them.

Table 2-1. Main properties of the initial pulp samples used in the different studies.

Initial pulp sample	Chapters	Kappa number	Brightness (% ISO)	Viscosity (ml/g)
Sisal a	3,5	7.8	47.3	784
Sisal b	4,10	7.9	37.9	733
Sisal c	6,7,10	7.4	52.1	750
Sisal d	8,9,10	7.1	51.1	716

2.1.1. Fibre composition and morphological properties

The initial pulp was classified into four fractions according to fibre length as measured with a Bauer–McNett instrument (T 233 cm-06 method). The initial pulp and each fraction obtained from it were analysed for morphological characteristics by using the TAPPI T 271 om-02 method on a Metso kajaaniFS300 instrument. Table 2-2 shows the proportions with respect to initial pulp weight of the fractions retained by the screens and their main morphological properties. The screens, which were used in series of three during measurements, had the following openings, in Mesh: 14 (1.190 mm), 30 (0.595 mm), 50 (0.297 mm) and 200 (0.074 mm).

As can be seen, sisal pulp consists mainly of fibres longer than 1.190 mm, a small fraction 1.19–0.297 mm in length and less than 5% of fines. The analysis on the kajaani

instrument revealed that the average fibre length, width and curl decreased with increasing Mesh —the P_{50-200} fraction excepted— and that the highest content in fines was that of the fraction retained by the 200 Mesh screen.

Table 2-2. Proportions with respect to initial pulp of the fractions retained on the different screens used. The average length, as numerical average length (L_n), length-weighted length (L_l) and weight-weigthed length (L_w); fibre width (W_n); fibre curl (C_n) and fiber content (F_n) for the initial pulp and each fraction.

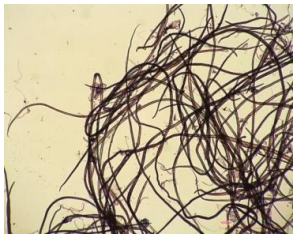
Fraction	Percentage	L_n (mm)	L_l (mm)	L_w (mm)	W_n (μm)	C_n (%)	F_n (%)
Initial**	100	1.43	2.04	2.55	17.05	32.06	8.84
$P_{>14}$	85.9	1.87	2.38	2.91	16.54	35.4	2.83
P_{14-30}	5.4	1.37	1.81	2.23	16.34	30.5	2.31
P_{30-50}	3.7	1.30	1.58	1.88	15.74	30.0	4.93
$P_{<50}$	5.1						2.03
P_{50-200}*	1.3	0.58	0.82	1.11	16.98	19.1	12.94

*Fraction obtained from the classification using the series of screens: 14-50-200.

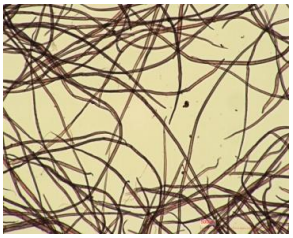
**Sisal b

Figure 2-1 shows light microscopy images at 40× and 100× magnification of the initial pulp and the fractions retained on the 30 and 50 Mesh screens. As can be seen, sisal pulp exhibits a high morphological uniformity and the conspicuous absence of other cell types. The isolated fractions differed from the initial pulp mainly in their reduced content of non-fibre particles and increased cleanliness.

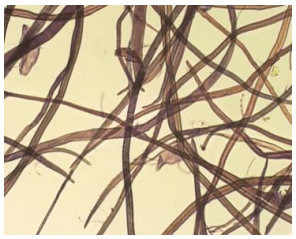
Table 2-3 lists the contents in glucan, xylan and Klason lignin of the initial pulp and the fractions retained on the 14, 30 and 50 Mesh screens. As can be seen, the long-fibre fraction exhibited the highest content in glucan and the lowest in Klason lignin, the latter significantly increasing with decreasing average fibre length.


Initial sisal pulp (x40)


Sisal pulp fraction P>30 (x40)


Sisal pulp fraction P>50 (x40)


Initial sisal pulp (x100)


Sisal pulp fraction P>30 (x100)


Sisal pulp fraction P>50 (x100)

Figure 2-1. Light microscopy images of the initial pulp and the fractions P>30 and P>50 isolated by the Bauer-McNett instrument.

Table 2-3. Glucan, xylan and Klason lignin contents (% odp) of the initial pulp and each fraction.

Fraction	Glucan (%)	Xylan (%)	Klason lignin (%)
Initial*	79.7	16.3	1.1
$P_{>14}$	82.2	15.9	1.0
P_{14-30}	77.3	14.7	1.2
P_{30-50}	78.7	15.1	1.6

*Sisal b

2.2. Enzyme systems

2.2.1. Laccase

The enzyme used here was laccase from *Trametes villosa* (*Tv*L) which was supplied by Novozymes (Bagsvaerd, Denmark); by exception, the tests described in Chapter 6 were conducted with laccase obtained from Novo Nordisk Biochem (Franklinton, North

Carolina). Some experiments reported in the Addendum to Publication 1 were conducted with laccase from *Myceliophthora thermophila* (*Mt*L), also supplied by Novozymes.

2.2.1.1. Laccase assays and mediators

Laccase activity was determined by oxidation of 2,2′-azino*bis*-(3-ethylbenzylthiozoline-6-sulphonate) (ABTS). One activity unit was defined as the amount of laccase transforming 1 μmol/min of ABTS to its cation radical at 436 nm (ε_{436} = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Three synthetic compounds and four natural *p*-hydroxycinnamic compounds, all purchased from Sigma–Aldrich, were used as laccase mediators. The synthetic mediators were 1-hydroxybenxotriazol (HBT), violuric acid (VA) and 2,2,6,6-tetramethyl-1-piperidinyloxy free radical (TEMPO); and the natural mediators included sinapic acid (SNC), ferulic acid (FRC), sinapyl aldehyde (SLD) and coniferyl aldehyde (CLD). In the Addendum to Publication 1, the natural compound methyl syringate (Novozymes) was assayed as *Mt*L mediator.

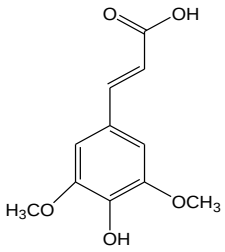
As described in Chapter 3, the oxidation of 50 μM HBT and various *p*-hydroxycinnamic compounds by laccase was studied by using a 300 mU/ml concentration of enzyme in 50 mM sodium tartrate buffer (pH 4) at 25 °C. UV–Vis spectra for the reaction mixtures were recorded at different times (0, 3, 5, 10, 15, 20 min) during the first 20 min of enzymatic oxidation, using a Thermo Scientific Evolution 600 spectrophotometer.

2.2.2. Xylanase

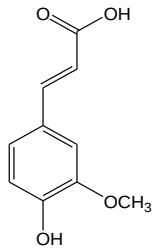
A commercial xylanase (Pulpzyme® HC) supplied by Novozyme was used. One activity unit was defined as the amount of enzyme converting 1 μmol of xylan reducing sugar (measured as xylose equivalents) per minute at pH 5 at 50 °C.

2.3. Bleaching assays

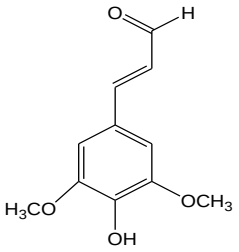
The tests described in Chapter 3 were performed with the *p*-hydroxycinnamic compounds SNC, FRC, SLD and CLD (Figure 2-2), which were used as laccase mediators and applied in combination with the enzyme to sisal pulp in order to assess their potential for either biobleaching or functionalizing pulp fibres. The enzyme treatment (an L stage) was followed by alkaline extraction with hydrogen peroxide in order to determine whether observable effects could be enhanced by removing LMS-modified lignin. The bleaching efficiency of the natural mediators was compared with that of the synthetic compound HBT. In Chapter 4, the bleaching efficiency of SLD is compared to that of the synthetic compound VA. These mediators were used in the L stage of an LQP_O bleaching sequence performed in the absence and presence of a xylanase pre-treatment.



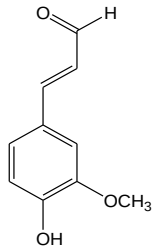
Sinapic acid (SNC)



Ferulic acid (FRC)



Sinapyl aldehyde (SLD)



Coniferyl aldehyde (CLD)

Figure 2-2. *p*-Hydroxycinnamic compounds used as laccase mediators.

2.3.1. Laccase–mediator treatments

Laccase-mediator treatments (L stages) were performed by using an amount of 40 g of sisal pulp at 5% consistency in 50 mM sodium tartrate buffer at pH 4, 20 U/g *Tv*L and a proportion of 1.5% (w/w) HBT, VA or natural mediator (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant. The treatments were carried out in a reactor (Figure 2-3) under O_2 pressure (0.6 MPa) at 30 rpm at 50 °C for 4 h. In the Addendum to Publication 1, the treatment with *Mt*L and MS was performed in 50 mM sodium dihydrogen phosphate buffer pH 6.5. In the tests of Chapter 4, SLD was used in combination with 40 U/g *Tv*L. Pulp samples treated under identical conditions in the absence of enzyme and mediator, or only the latter, were used as controls. Once treated, each pulp was filtered in a fritted glass funnel (porosity 40-100 μm), its residual liquor collected and the pulp extensively washed with de-ionized water.

Figure 2-3. Pressure reactor.

2.3.1.1. Residual laccase activity

Enzyme activity against ABTS as substrate was monitored for 4 h under the conditions of an L stage performed at a small scale in an O_2 atmosphere (continuous bubbling) in the absence or presence of mediator, and with or without pulp. Residual

activity values were expressed as percentages of initial activity, which was measured at the outset (time 0) of the incubation period.

2.3.2. Bleaching treatment

The laccase–mediator treatment was followed by an alkaline peroxide bleaching treatment (P stage) in an Ahiba Spectradye dyeing apparatus from Datacolor (Figure 2-4) equipped with closed vessels 150 ml in volume that were loaded with 5 g oven-dried pulp (odp) at 5% consistency, 3% odp H_2O_2, 1.5% odp NaOH, 1% odp diethylenetriaminepentaacetic acid (DTPA) and 0.2% odp $MgSO_4$ at 90 °C for 2 h. Then, each treated pulp sample was filtered in a fritted glass funnel and extensively washed with de-ionized water.

Figure 2-4. Easydye AHIBA reactor from Datacolor.

2.3.3. Bleaching sequences

Chapter 4 describes the two bleaching sequences studied, with and without a xylanase pre-treatment, and including an L stage performed as described in Section 2.3.1 in addition to a chelating stage and an oxygen-reinforced peroxide stage.

2.3.3.1. Xylanase treatment (X stage)

The X stage was performed in polyethylene bags that were immersed in a thermostatic water bath, using 3 U/g odp xylanase in Tris–HCl buffer (pH 7), at 10% pulp consistency at 50 °C for 2 h. The pulp was stirred by hand at 20 min intervals. At the end of the treatment, the pulp was filtered in a fritted glass funnel for thorough washing with de-ionized water and the effluents were collected (Valls *et al.* 2010).

2.3.3.2. Chelating treatment (Q stage)

The Q stage involved the use of chelating agents to reduce the contents in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) liable to degrade the bleaching agents and cellulose during the peroxide bleaching treatment (Heijnesson *et al.* 1995). The Q treatment was conducted in polyethylene bags, using 1% DTPA at 5% consistency, pH 5–6 and 85 °C for 1 h. The pulp was stirred by hand at 15 min intervals. At the end of the treatment, the pulp was filtered in a fritted glass funnel and thoroughly washed with de-ionized water, and the effluents were collected.

2.3.3.3. Oxygen-reinforced peroxide treatment (P_O stage)

The P_O stage was carried out at 5% consistency in the oxygen pressurized (0.6 MPa) reactor, using at a stirring rate of 30 rpm, 3% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$ 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 involving the addition of 1% odp H_2O_2 and withdrawal of a small amount of pulp from the reactor to monitor the main pulp properties. At the end of the treatment, the pulp was filtered in a fritted glass funnel and thoroughly washed with de-ionized water, and the effluents were collected.

2.4. Biografting assays

In the tests described in Chapter 5, which were intended to expose covalent binding of the *p*-hydroxycinnamic mediators to pulp fibres, laccase treatments were applied under the same conditions as in the bleaching assays. In those described in Chapter 6, which were used to examine laccase-induced grafting of FRC and the extent of phenol coupling under different reaction conditions, variable laccase and FRC doses, and reaction times, differing from those of the original treatment were used. Biografting treatments were performed in the presence of 20, 40 or 50 U/g laccase and a proportion of 1.5% or 3.5% (w/w) FRC for 1 or 4 h. Pulp samples treated under identical conditions in the absence of FRC were used as controls. After the enzyme treatment, the pulp samples were filtered in a fritted glass funnel and extensively washed with de-ionized water.

2.4.1. Soxhlet extraction

The pulp samples were subjected to Soxhlet extraction with acetone in order to remove extractives (or the fraction of extracted compounds failing to bind covalently to fibres from the samples treated with laccase and *p*-hydroxycinnamic compounds). To this end, an amount of about 2 g odp was added to a cellulose extraction thimble and placed in the extractor; 50 ml of acetone was then added to a flask which was also fitted to the extractor. Acetone was allowed to boil (heating temperature 140 °C) for 135 min. Finally acetone was recovered and residual solvent in the pulp let evaporate.

2.5. Laccase–TEMPO oxidation assays

The potential of the laccase–TEMPO system for oxidizing sisal pulp fibres and improving paper strength properties was first assessed at the Institute of Paper Science and Technology (IPST) of the Georgia Institute of Technology in Atlanta, GA (USA), and continued in the laboratories of the Textile and Paper Engineering Department (DETIP) in Terrassa. The work described in Chapter 7 was almost entirely conducted at IPST, and that of Chapters 8 and 9 at DETIP.

2.5.1. Laccase–TEMPO treatments

In the tests of Chapter 7, laccase (60 U/g) and TEMPO (1, 2, 4 or 8% by mass) were added with stirring to an aqueous suspension of sisal pulp at 1% consistency in a plastic jar and adjusted to pH 5 with 50 mM acetate buffer. The resulting slurry was stirred at room temperature (18–19 °C) under oxygen bubbling for 18 h. Pulp samples treated under identical conditions in the absence of enzyme, TEMPO or both were used as controls. After treatment, each pulp was filtered in a fritted glass funnel (porosity 40-100 μm) and washed with de-ionized water until a colourless, neutral filtrate was obtained.

Chapter 8 describes a series of preliminary tests performed on an amount of 30 g of pulp in a 5 L reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 in the presence of 60 U/g odp laccase and 8% odp TEMPO, and variable conditions of time, temperature, applied oxygen pressure and consistency (Table 2-4). Pulp samples treated in the absence of TEMPO, or both laccase and TEMPO, at room temperature (21–22°C) and 1% consistency under an oxygen pressure of 0.6 MPa for 18 h were used as controls.

Table 2-4. Operating conditions used in the laccase–TEMPO (LT) treatments of the preliminary study.

Sample ID	Time (h)	Temperature	Applied O_2 pressure (MPa)	Pulp consistency (% odp)
LT – 18 h	18	room	0.6	1
LT – 30 h	30	room	0.6	1
LT – 50 °C – 30 h	30	50 °C	0.6	1
LT – no P_{O2} – 30 h	30	room	-	1
LT – 4 h	4	room	0.6	1
LT – c 5% – 30 h	30	room	0.6	5

Subsequently, laccase–TEMPO treatments were performed according to a 2^3 experimental design in plastic containers, using 15 g of pulp at 1% consistency in 50 mM acetate buffer at pH 5 at room temperature under oxygen bubbling in a jar testing apparatus operating at a stirring speed of 60 rpm (Figure 2-5). The operating variables (factors) studied were the laccase dose, mediator dose and reaction time, which were normalized to three different values (–1, 0 and 1).

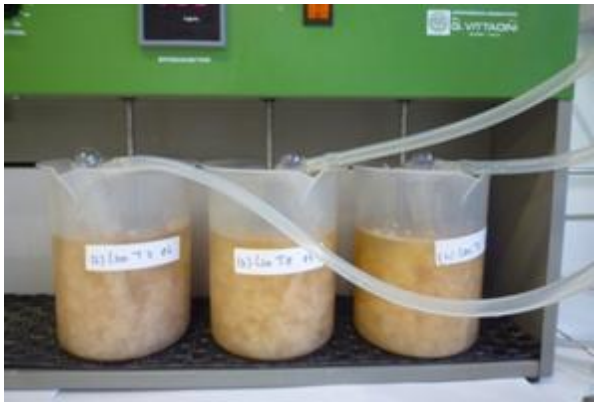


Figure 2-5. Laccase–TEMPO treatments conducted in the jar testing apparatus.

Table 2-5 shows the experimental range for each variable. After treatment, each pulp was filtered in a fritted glass funnel and washed with de-ionized water until a colourless, neutral filtrate was obtained.

Table 2-5. Variables distribution with the factor of each level.

	Variables	-1	0	+1
x_1	Laccase dose (U/g odp)	20	60	100
x_2	TEMPO dose (% odp)	2	5	8
x_3	Reaction time (h)	8	14	20

The laccase–TEMPO treatments of Chapter 9 were performed on an amount of 30 g of pulp at 5% consistency in 50 mM acetate buffer at pH 5 in the oxygen-pressurized reactor (0.6 MPa), which was stirred at 60 rpm. Tests were conducted under the conditions of laccase and TEMPO doses, and reaction time, resulting in maximum functionalization in the experimental plan.

2.5.2. Experimental design

In Chapter 8, a 2^3 experimental design was used to investigate the oxidation of sisal pulp fibres by the laccase–TEMPO system and the influence of process variables on various properties of the oxidized fibres and of handsheets made from them. The

experimental results were processed by using the analytical tool "regression" and the "stepwise backwards regression" method in the software Excel.

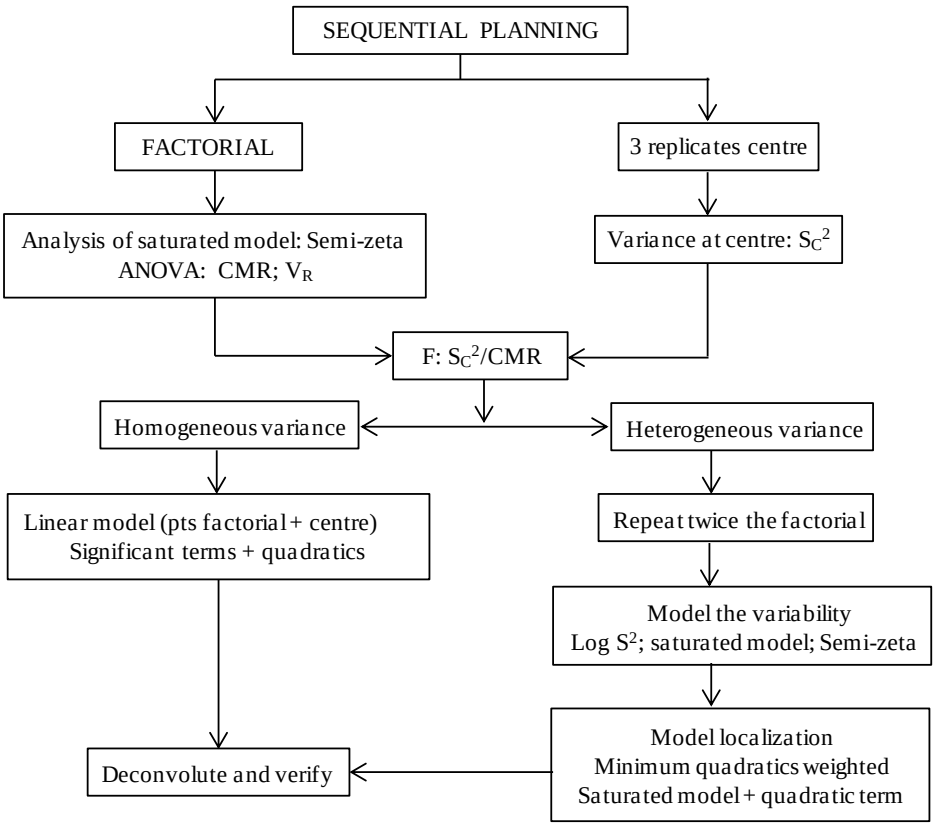


Figure 2-6. Scheme of the sequential testing plan (Pepió and Polo 2000).

The statistical analysis of pulp and handsheet properties was based on the results of a planned sequence of tests (Figure 2-6). The experimental design used to this end was conducted stepwise; thus, the results obtained in each step were used to decide whether the next was to be performed. The first step in a sequential plan is to establish a factorial design. The aim is to find a linear model accurately describing the principal variables and their mutual interactions (Step 1). To this end, the variance at the central point and the residual mean square of the factorial design are subjected to the homoscedasticity test by calculating Snedecor's *F*-value. If the variance is homogeneous, then the model is homoscedastic (i.e. the variance is constant throughout the experimental region examined

and the study can be extended to the detection of potential curvature in the fitted data, Step 2). This involves estimating a linear model from the terms deemed significant in the previous factorial design and performing replications at the central point in order to check whether any quadratic terms are significant (Step 3). If the representative quadratic term is significant, then the design is expanded with new experiments in order to deconvolute quadratic terms (Step 4). Finally, the resulting model is verified (Step 5).

2.6. Pulp characterization

2.6.1. Kappa number

Kappa number was determined in accordance with ISO 302:2004. This provides a measure of the lignin content of pulp; however, in this doctoral work, kappa number measurements were also used to assess the tendency of phenolic compounds to couple to sisal fibres. The kappa tests on handsheets described in Chapter 6 were used to assess the extent of FRC grafting in the final sheets. Each sample was analysed in duplicate.

2.6.2. Optical properties

Pulp brightness and colour were assessed in accordance with ISO 3688:1999 and ISO 5631:2000, respectively. Tests were performed in quadruplicate on a single specimen, using a Technidyne Colour Touch reflectance measurement instrument with the standard illuminant/observer combination C/2°. Samples were prepared following the traditional procedure based on the preparation of handsheets with grammage > 200 g/m^2 in a Büchner funnel furnished with a filter paper. Brightness was determined from the intrinsic reflectance factor. The reflectance factor, R, is the ratio of radiation reflected by a body to that reflected by a perfect reflecting diffuser. The intrinsic reflectance factor, R_∞, is the reflectance measured by using a thick enough stack of paper or a pad so that no transmission of light occurs through it. R_∞ was used to calculate the Kubelka–Munk coefficient, k/s, from the Kubelka–Munk equation [2-1], where k and s are measures of the ability of an infinitely thin layer to absorb and scatter light, respectively. Bleaching pulp diminishes its k value by removing light absorbing coloured compounds.

$$\frac{k}{s} = \frac{1-R_\infty{}^2}{2R_\infty} \qquad [2\text{-}1]$$

Sample colour was described according to the CIE L*a*b* colour system, where L*, a* and b* are the colour coordinates in the cylindrical colour space (Figure 2-7), based on the theory that colour is perceived as L* (Lightness, which ranges from 100 for perfect white to 0 for absolute black), a* (which ranges from greenness to redness), and b* (which ranges from blueness to yellowness, from negative to positive values) (Hunt 1998).

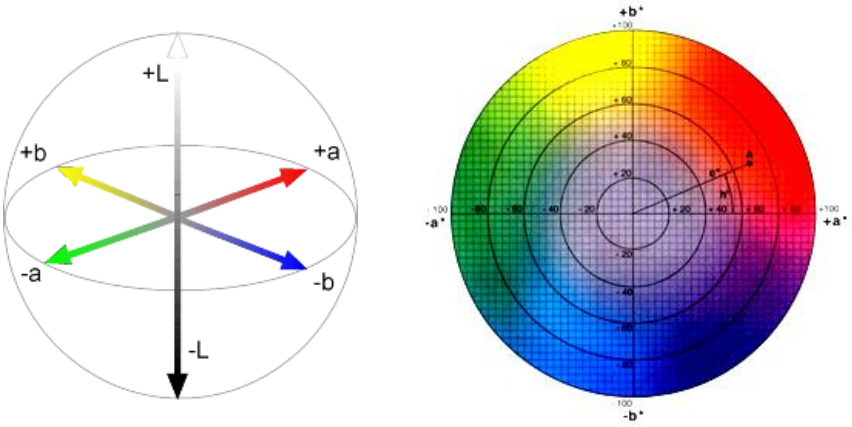


Figure 2-7. CIE L*a*b* colour space (left) and cross-section of the space (right).

The following optical parameters were also determined:

- *Chroma (C*):* A measure of colour saturation defined as the normal distance from the lightness axis:

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

- *Dye removal index (DRI)* (Fluet and Shepperd 1997): The proportion of original colour removed by a treatment [2-3],

$$\text{DRI} = -100[\Delta R^2/R_1{}^2] \qquad [2\text{-}3]$$

where,

$$R^2 = a^2 + b^2 + (100 - L)^2 \quad [2\text{-}4]$$

is the geometric distance from the pulp CIE L*a*b* location to the ideal bleach point where $a^* = b^* = 0$, and $L^* = 100$,

$$\Delta R^2 = R_2^2 - R_1^2 = R^2 \text{ (for treated pulp)} - R^2 \text{ (for reference pulp)} \quad [2\text{-}5]$$

2.6.3. Viscosity

Viscosity measurements were used to assess changes in the degree of cellulose polymerization during the bleaching assays and laccase–TEMPO treatments. Pulp viscosity (as intrinsic viscosity of a sample of cellulose dissolved in a dilute solution of cupriethylenediamine) was determined in accordance with ISO 5351:2001. In the tests described in Chapters 7-9, borohydride viscosity was measured in addition to standard viscosity. This involved treating oxidized pulp samples with sodium borohydride prior to measurement in order to inactivate carbonyl groups formed by reduction to hydroxyl groups in cellulose and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium. The treatment was performed in polyethylene bags, using pulp at 5% consistency in the presence of 2% $NaBH_4$ at room temperature for 30 min (Roncero *et al.* 2003), with manual stirring of the pulp at 5 min intervals. Viscosity measurements were made in duplicate.

2.6.4. Hexenuronic acid (HexA) content

The method used to quantify hexenuronic acids (HexA) in pulp is based on a procedure proposed by Li and co-workers (Chai 2001, Gellerstedt 1996, Valls 2008). We used a mercuric acetate–sodium acetate solution to ensure selective hydrolysis of HexA from pulp. HexA were determined with a sensitive colour test involving periodate oxidation to β-formyl pyruvic acid and coupling of the latter with thiobarbituric acid (TBA) to form a chromogen with a light-absorption maximum at 549 nm (Figure 2-8) (Gellerstedt and Li 1996). Two samples per pulp were hydrolysed and the filtrate from each reaction was analysed in quadruplicate.

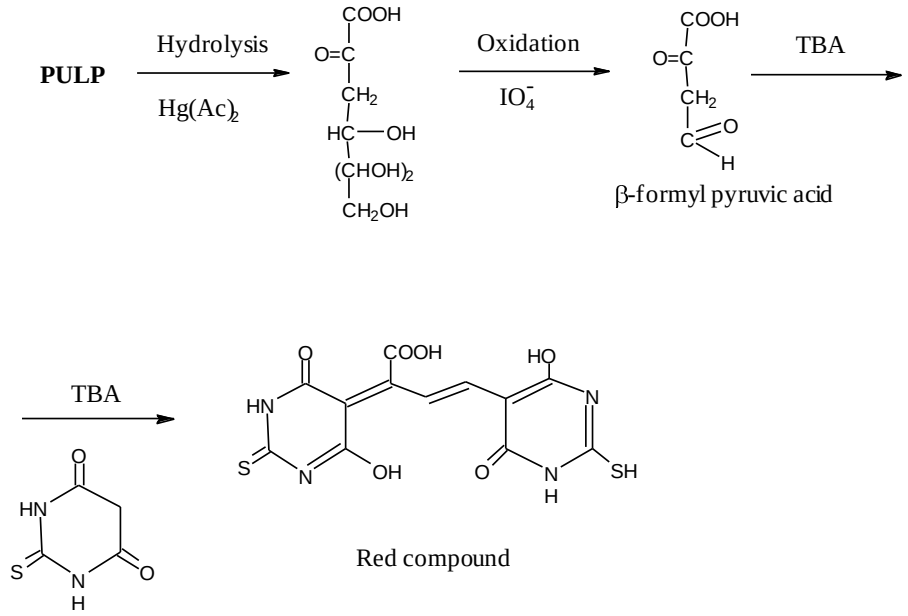


Figure 2-8. Formation of thiobarbituric acid–β -formyl pyruvate chromogen from 2-keto-3-deoxyheptanoic acid.

2.6.5. Carbohydrate composition and Klason lignin

The sugar composition of the pulp samples was determined by high performance liquid chromatography (HPLC) after Soxhlet extraction with acetone and grounding of the samples to a particle size < 0.5 mm. Two replicates of the resulting samples were hydrolysed by using a modified version of the TAPPI 249 cm-09 test method. The hydrolysis process involved the following two steps: (*a*) *Pre-hydrolysis with concentrated sulphuric acid.* Approximately 50 mg of sample was placed in a test tube and soaked with 5 ml of 72% sulphuric acid, after which the tube was placed in a water bath at 30 ± 0.5 °C for 1 h with occasional stirring. (*b*) *Final hydrolysis with dilute sulphuric acid.* The tube contents were washed in a 250-ml flask in order to obtain a final solution 4% in sulphuric acid and the flask was placed in an autoclave at 103 ± 7 kPa for 1 h. Once the reaction was complete, the specimen solution was cooled at room temperature and passed through a gooch filter No. 4 to remove lignin insoluble in sulphuric acid, which was taken to represent Klason lignin. Prior to HPLC analysis, the solution was filtered through a Whatman membrane of 0.45 μm pore size. The high performance liquid chromatograph

was equipped with a refractive index detector. The chromatographic determination was performed with an Agilent 1100 HPLC instrument furnished with column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 0.006 mol/l sulphuric acid; flow rate, 0.6 ml/min; column temperature, 60 °C. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from Sigma–Aldrich). Because the column failed to resolve xylose, mannose and galactose, their combined content was expressed as xylose (Garrote *et al.* 2001).

2.6.6. Surface anionic charge

Surface anionic charge was measured as described in Chapters 3 and 6, using the polyelectrolyte adsorption method as an additional tool to assess coupling of *p*-hydroxycinnamic acids to fibres. The method used in Chapter 3 was based on methylglycolchitosan (MGCh) and that employed in Chapter 6 on polydiallyldimethylammonium chloride (Poly-DADMAC). Pulp samples were analysed in duplicate with both.

Methylglycochitosan method

The cationic polyelectrolyte methylglycochitosan (MGCh) was purchased from Wako Pure Chemical Industries, Ltd. (Japan). The polymer molar mass and charge density were 1.5×10^5 and 4.04 meq/g, respectively. An amount of 0.25 g of pulp was diluted with 50 ml of 0.5×10^{-3} N MGCh and stirred for 30 min to facilitate the adsorption equilibrium. The resulting suspension was centrifuged and part of the clear supernatant (10 ml) collected for titration with the anionic polyelectrolyte (Cadena *et al.* 2009). The equivalence point was determined with the cationic dye indicator Toluidine Blue (Wako) or a Mütek PCD 03 particle charge detector (Germany) (Figure2-9). With the indicator, the supernatant was titrated with potassium polyvinyl sulphate (PVSK) (Wako); with PCD, titration was done with sodium polyethenesulphonate (PES-Na) (Oy G. W. Berg & CO Ab/BTG Mütek GmbH, Germany). Blank samples were titrated exactly in the same way as those brought into contact with pulp. The amount of fibre in each sample after titration was determined gravimetrically following filtering on pre-weighed filter paper and drying in an oven at 105 °C overnight.

Figure 2-9. PCD-03 Mütek™ particle charge detector.

Poly-DADMAC method

Polydiallyldimethylammonium chloride (poly-DADMAC) was purchased from BTG Mütek GmbH (Germany). The polymer molar mass and charge density were ~1.5×10^5 and 2.656 meq/g, respectively. Prior to charge measurements, each pulp sample was converted to its fully protonated form by soaking at 1% consistency in 0.01 M HCl for 30 min. This was followed by washing with de-ionized water until the conductivity of the filtrate fell below 5 μS/cm, and by conversion to the sodium form by soaking in 0.001 M $NaHCO_3$ at ~1% consistency for 10 min. The pH was then adjusted to 9 with NaOH and kept constant for 30 min. The pulp was subsequently washed with de-ionized water until the conductivity of the filtrate fell below 5 μS/cm. All adsorption measurements were made on pulp in its sodium form at 0.5% consistency in 0.001 N poly-DADMAC and 0.01 M NaCl. The pH was adjusted to 7.8 ± 0.1 in all adsorption runs. The suspension was stirred with a magnetic stirrer until anionic charge was completely neutralized by the cationic polyelectrolyte, which took about one hour. Fibres were separated from the solution by filtration in order to record dry weight. A filtrate volume of 10 mL was then pipetted into the cell of a Mütek PCD 03 titrator and titrated to endpoint (i.e. to a streaming potential of 0 mV) with 0.001 N sodium PES-Na. Blank samples were titrated in parallel exactly in the same way as those which had been in contact with pulp.

In both methods, fibre charge was calculated from the following equation:

$$q\ \mu eq\ g\ = \frac{V_o - V_{tit} * C_{p-D} * 1000}{m_{dry}} * \frac{V_{tot}}{V_{sample}} \quad [2\text{-}6]$$

where V_0 is the volume of anionic polyelectrolyte consumed by the blank sample, V_{tit} that consumed by the fibre samples, $C_{p\text{-}D}$ the concentration (N) of cationic polyelectrolyte, V_{tot} the total volume of filtrate, V_{sample} the volume of filtrate suspension used for titration and m_{dry} the sample dry mass.

2.6.7. Bulk acid group content

In the work described in Chapters 7-9, the bulk acid group content was measured by conductimetric titration according to Katz *et al.* (1984). An amount of 1.50 g o.d. pulp was stirred in 300 ml of 0.10 M HCl for 1 h, followed by rinsing with de-ionized water in a finely fritted funnel. The sample was then resuspended in 250 ml of 1.00 mM NaCl, spiked with 1.5 of 0.10 M HCl and titrated against 0.05 M NaOH in 0.25 ml increments, with conductivity measurement after each addition. The final data was plotted as conductance vs. volume in Microsoft Excel. The points at which conductance remained constant were quantified on the graph and divided by the weight of pulp sample in order to determine the milli-moles of acid groups. The carboxylic acid content (X) of pulp fibres was obtained from the following equation

$$X = \frac{B - A * C_{OH}}{m_{dry}} \quad [2\text{-}7]$$

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

The results were the averages of two measurements. By way of example, Figure 2-10 shows the resulting graph for a conductimentric titration.

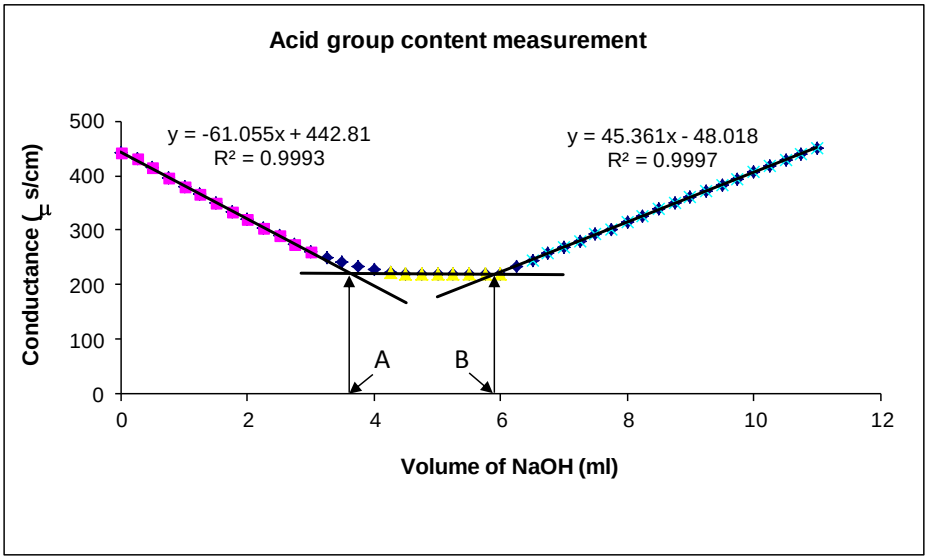


Figure 2-10. Conductimetric titration plot (conductance vs. NaOH volume) used to calculate the carboxyl group content.

2.6.8. Aldehyde group content

In the tests of Chapters 8 and 9, the aldehyde group content was measured in pulp samples treated with the laccase–TEMPO system. TEMPO-oxidized pulp samples (3.5 g odp) were treated with a 350 ml solution of 0.3 M $NaClO_2$ at pH 4–5 at room temperature for 48 h in order to selectively convert aldehyde groups into carboxyl groups (Roncero 2001). After treatment, the pulp was filtered and rinsed with de-ionized water in a finely fritted funnel. The carboxyl content was determined with the above-described conductimetric titration method. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally present in the pulp.

2.6.9. Pyrolysis analysis

In Chapter 5, Py–GC/MS analysis was used in order to determine whether the *p*-hydroxycinnamic compounds used in the laccase-treatments were covalently bound to fibres. Py–GC/MS analyses were performed by researchers from the Institute of Natural Resources and Agrobiology (IRNAS) in Seville, Spain. In each run, an amount of ca. 1 mg of pulp was pyrolysed on a 2020 micro-furnace pyrolyser from Frontier Laboratories,

Ltd. connected to an Agilent 6890 GC/MS system equipped with an Agilent J&W DB-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) and an Agilent 5973 mass selective detector (EI at 70 eV). The pyrolysis temperature was 500 ºC. The oven temperature was raised from 40 ºC (1 min) to 300 ºC at 6 ºC/min (10 min) and the carrier gas (He) was circulated at a rate of 1 ml/min. In addition, pulp samples were analysed by pyrolysis in the presence of tetramethylammonium hydroxide (TMAH) as a base and methylating reagent. For Py/TMAH, 1 mg of pulp was mixed with ca. 0.5 μl of TMAH (25%, w/w, aqueous solution) and pyrolysed as described above. Compounds were identified by comparing their mass spectra with those of the Wiley and NIST libraries, and previously reported values (Faix *et al.* 1990, Ralph and Hatfield 1991).

2.6.10. Morphological analyses

2.6.10.1. Fibre analysis

Morphological properties of fibres such as length, width, and curl, as well as the content of fines in a pulp specimen, were determined with the TAPPI T 271 om-02 method on a Metso kajaaniFS300 fibre analyser (Figure 2-11). Measurements were based on the ability of fibres to change the direction of polarized light.

An amount of 240 mg of pre-soaked sample was added to 2 L of de-ionized water and disintegrated for 30 000 revolutions to ensure complete individualization of fibres. The sample was then diluted to a final volume of 4 L in de-ionized water. A volume of 50 mL of diluted suspension was then taken by using a pipette with a tip opening at least 2 mm in diameter and placed in a beaker for analysis.

The instruments used provided different types of averages for each target property (numerical, length-weighted and weight-weighted).

Figure 2-11. Metso kajaaniFS300 fibre analyser.

2.6.10.2. Scanning electron micrograps

Handsheets were subjected to SEM analysis. The surface SEM pictures of Chapter 7 were obtained with a Hitachi S-800 FE-SEM instrument at IPST in Atlanta. Samples were placed on the SEM sample holding stub by means of conductive double side sticky carbon film and coated with Au/Pt alloy prior to analysis. Cross-sectional SEM images were obtained with a JEOL JSM-6400 microscope at DETIP (Terrassa), the handsheet samples being coated with Au/Pd alloy before analysis.

2.6.10.3. Light microscopy

Individual fibres were analysed by light microscopy according to ISO 9184-3:1990. Images were captured with a DeltaPix (Infinity X) digital camera fitted to a microscope and digitized with the software DeltaPix Viewer. The procedure was as follows: a tiny amount of pulp fibres was stained with 1–2 drops of Herzberg stain (a solution of zinc chloro-iodide) and stained fibres were individualized by means of awls for placement under a microscope and examination at ×40 and ×100 magnification.

2.6.11. Thermogravimetry

Thermogravimetric analysis was performed to compare the effects of the different laccase-based systems in terms of thermal degradation profile of the treated pulps, as described in Chapter 10. This analysis was performed at the Department of Chemical Engineering of the University of Huelva (UHU) in Huelva, Spain. Thermogravimetric runs were carried out with a Mettler Toledo model TGA/SDTA85e/LF1600 on samples of around 5 mg in a 4:1 N_2:O_2 synthetic air atmosphere, using three different heating rates (5, 10, and 20 ºC/min) from 25 to 900ºC. Pyrolysis runs were carried out under a nitrogen atmosphere (Barneto *et al.* 2011).

2.7. Effluent characterization

The effluents from the bleaching assays were analysed in terms of chemical oxygen demand (COD), colour and toxicity characterized as described in Chapters 3 and 4. Those from the biografting assays were analysed for toxicity and residual laccase activity as described in Chapter 6. The final pH of the effluent from each individual treatment was also measured.

2.7.1. COD and colour

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

2.7.2. Toxicity

Effluent toxicity was determined with the Microtox method, using the marine luminescent bacterium *Vibrio fischeri* in accordance with UNE-EN ISO 11348-3: 1999 on a Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental). The

difference between the amount of light emitted before and after addition of the sample was used to determine its toxicity. In order to prevent pH effects, samples were adjusted to pH 6–8 with an NaOH solution. Toxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed in toxicity units (TU). The reference toxicant ZnSO4·7H2O was used to control *V. fischeri* batch quality in accordance with the Basic Test procedure. Toxicity measurements were colour-corrected as per the recommendations of the equipment manufacturer. Toxicity tests were conducted in duplicate or triplicate.

2.7.3. Residual laccase activity

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

2.8. Pulp refining and handsheet formation

Pulp was refined and used for handsheet formation as described in Chapters 6 to 9. Biografting or a laccase–TEMPO treatment was used to improve the physical mechanical properties of the resulting paper. In Chapter 7, the treated pulp samples were disintegrated for 50 000 revolutions on a British Disintegrator, refined for 4500 revolutions according to TAPPI T-248 sp-08 in a PFI mill and used to form handsheets according to TAPPI T-205 sp-06 (Figure 2-12). In the work described in Chapters 6, 8 and 9, treated and untreated pulp samples were disintegrated for 30 000 revolutions, refined for 3000 or 4500 revolutions according to ISO 5264-2:2002 and, if previously treated, directly used to prepare handsheets. Untreated pulp samples were processed as described in Sections 2.4 and 2.5 and disintegrated for 10 000 revolutions prior to handsheet formation. Handsheets were prepared according to ISO 5269-2:2004 on a Rapid-Köthen laboratory former. The refined pulp samples were analysed for drainability (in Schopper–Riegler degrees, °SR) according to ISO 5267-1:1999, and for water retention value (WRV) according to ISO 23714:2007 (see Chapter 9).

Once formed, the handsheets were conditioned at 23 °C at 50% relative humidity for at least 24 h before physical testing.

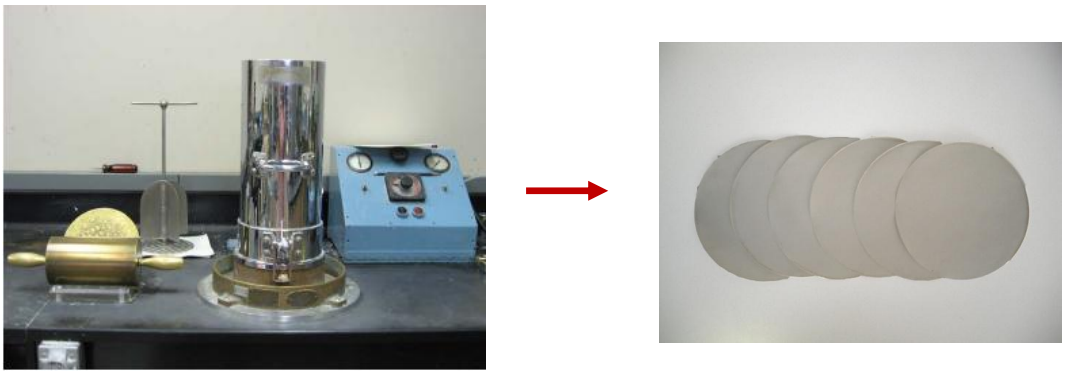


Figure 2-12. Conventional handsheet forming apparatus (left) and handsheet made from sisal pulp (right).

2.9. Physical testing

Physical tests on paper were performed on all samples treated as far as handsheet formation. Tensile, tear and burst strength values were expressed as indices relative to handsheet grammage.

2.9.1. Tensile strength

In the tensile strength tests, the specimen was subjected to uniaxial tension until failure. Tensile strength is the maximum tensile force developed in a test specimen before rupture, expressed as the force per unit width of the specimen. As described in Chapter 7, tensile testing was performed on 25 mm wide specimen strips, using an Instron tester according to TAPPI T-494 om-06. The instrument was connected to a data analysis system running Testworks software. Wet tensile testing was performed according to Tappi T-456 om-10, using a 5 s span between wetting of the sample and testing. The tensile tests reported in Chapters 6, 8 and 9 were conducted on 15 mm wide specimen strips, using a computer interfaced to a J. J. Lloyd universal testing machine (model T5K)

according to ISO 1924-3:1994. Wet tensile strength was measured according to ISO 3781:1983, using strips previously soaked in de-ionized water for 5 s.

2.9.2. Tear strength

This method measures the force normal to the plane of the paper required to tear multiple plies across a specified distance the tear is started by using the pendulum in an Elmendorf-type tearing tester. The work done in tearing is measured by the loss in potential energy of the pendulum. Tests were performed according to TAPPI T-414 om-04 (Chapter 7) or ISO 1974:1990 (Chapters 6, 8 and 9).

2.9.3. Burst strength

In this method, the test specimen, held between annular clamps, is subjected to an increasing pressure by a rubber diaphragm which is expanded by hydraulic pressure at a controlled rate until the specimen ruptures. The maximum pressure reading up to the rupture point is recorded as the burst strength. Tests were done according to TAPPI T-403 om-10 on a Mullen bursting tester (Chapter 7), or according to ISO 2758:2001 on a Lhomargy bursting tester (Chapters 6, 8 and 9).

2.9.4. Wet zero span tensile strength

The wet zero span tensile test measures the tensile load bearing capability of wet fibres in a test specimen clamped between two jaws in contact with each other ("zero-span") at the time of tensile failure. The wet zero span tensile strength value (WZSTS) is used to assess the average axial tensile strength of individual fibres and is independent of the relative bonded area in the measured sheets. In the tests of Chapters 7 and 9, WZST was measured according to TAPPI T-273 pm-95 and ISO 15361: 2000, respectively, on a Zero-Span 1000 Pulmac tester, using strips previously soaked in de-ionized water for 5 s. In Chapter 7, the results obtained were corrected for any discrepancy in strip grammage from the target value (60 g/m^2):

Corrected WZST value, N/cm: Instrument reading x (60/strip grammage)

In Chapter 9, wet zero span tensile index (WZSTI) was calculated, in relation to the handsheet grammage.

2.9.5. Air permeability

Air permeability was measured as the amount of time required for a certain volume of air to flow through a 1 cm^2 circular area of the test specimen under a prescribed differential pressure between the two surfaces of the handsheet. Permeability was determined on a K533 Messmer Büchel apparatus (see Chapter 9), and the results were expressed as Bekk seconds and were the average of ten measurements each.

2.9.6. Vertical wicking

Vertical wicking assays were performed as described in Chapter 7 in order to confirm whether the increase in carboxyl groups derived from the laccase–TEMPO treatments enhanced the water absorbing capabilities of the handsheets. Vertical wicking was measured on 10 cm × 1.5 cm strips of treated handsheets. The strips weighed 0.10 ± 0.01 g and had densities of 0.60 ± 0.02 g/cm^3. A Petri dish filled with de-ionized water was used as reservoir. The bottom 1 cm of each strip was inserted into the reservoir and the timer immediately started. The top of the strip was fixed with a movable clamp. Each time the water climbed 1 cm on a strip, the time was recorded. After 5 min, the total distance of liquid absorbed was also recorded. Each sample was analysed in duplicate.

2.10. References

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Application of laccase-natural mediator systems to sisal pulp: An effective approach to biobleaching or functionalizing pulp fibres?

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Abstract

The effects of laccase–natural mediator systems (LMS) on sisal pulp and their potential for either biobleaching or functionalizing (via radical coupling) its fibres were investigated. The enzyme treatment (L stage) was followed by extraction with hydrogen peroxide in order to determine whether observable effects could be enhanced by removing LMS-modified lignin. Four different plant phenols [*viz.* the *p*-hydroxycinnamic compounds sinapic acid (SNC), ferulic acid (FRC), coniferyl aldehyde (CLD) and sinapyl aldehyde (SLD)] were used as laccase redox mediators and their effects on pulp and effluents compared with those of the synthetic compound 1-hydroxybenzotriazole (HBT). During the L stage performed with HBT, laccase underwent a loss of 99% and 78% of the initial activity, in the absence and presence of pulp, respectively. With natural mediators inactivation was markedly reduced, being the residual activity between 65% and 100% of the initial one, in the presence of pulp. The pulp was found to protect the enzyme against inactivation: the activity was only reduced by 45% in its presence. Under the operating conditions used the natural mediators proved less efficient than HBT in facilitating pulp bleaching; rather, they tended to bind to pulp fibres. This effect could be used to functionalize fibres in order to improve intrinsic properties of pulp or introducing novel ones (e.g. antimicrobial, antioxidant, optical properties, etc.). This paper shows for the first time the application of laccase–mediator systems to sisal pulp.

3.1. Introduction

Wood continues to be the primary raw material for the pulp and paper industry; in fact, non-wood plants account for only 10% of the total amount of fibre used for papermaking worldwide. However, non-wood fibre plays a major role in the pulp and paper business inasmuch as it constitutes the mainstay of papermaking in many developing countries (particularly China and India) (López *et al.* 2004). Moreover, changes in agricultural policies, environmental concerns and wood supply issues are likely to raise the significance of non-wood fibre to the global pulp and paper industry in the near future (Ashori 2006). In developed countries, textile-type fibre obtained from non-wood plants is used mainly to obtain specialty paper. Sisal (*Agave sisalana*) is a monocotyledon plant endemic to Central America which provides fibre with a

papermaking potential. In fact, sisal, which has traditionally been used to make natural ropes, cordage and sacking, possesses some attractive properties for the production of a number of specialty paper varieties such as those used in tea bags, surgical gauze, filters or condensers (Hurter 2001). Moreover, new opportunities exist for sisal pulp to cost-effectively replace long-fibred chemical wood pulp for the reinforcement or basis weight reduction of many paper grades(Maddern and French 1994).

In recent years, the pulp and paper industry in Europe and North America has increasingly adopted enzyme technology, driven by the need to comply with stringent environmental legislation and improve competitiveness (Viikari 2002). By virtue of their high specificity and environmental friendliness, enzymes possess a high potential for improving a wide range of aspects of pulp and paper production processes (Bajpai 1999). For example, 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 pulp bleaching, where the passing of increasingly strong environmental restrictions has fostered a search for effective alternatives to chlorine-containing bleaching reagents and the development of elemental chlorine-free (ECF) and totally chlorine-free (TCF) sequences (Camarero *et al.* 2002, Rochefort *et al.* 2004). The high potential of laccase–mediator systems (LMS) for bleaching some pulp types has been widely demonstrated, and *N*-hydroxy based compounds were found to be especially prominent for this purpose such as the well known 1-hydroxybenzotriazole (HBT) (Camarero *et al.* 2004, Fillat and Roncero 2009, Poppius-Levlin *et al.* 1999, Valls and Roncero 2009a). However, the high cost of synthetic mediators and concerns about their potential toxicity have restricted industrial implementation of LMS and raised the need for alternative, easily available replacement substances for the pulp and paper industry such as natural phenols, which can be readily obtained from spent pulping liquors and plant materials or, directly, from fungal metabolism (Camarero *et al.* 2007, Eggert *et al.* 1996, Johannes and Majcherczyk 2000). Some potentially cost-effective lignin-derived phenols have recently been found to perform as mediators for the laccase oxidation of recalcitrant compounds (e.g. residual lignin, industrial dyes, polycyclic aromatic hydrocarbons, lipids) (Cañas *et al.* 2007, Gutiérrez *et al.* 2007).

Although the potential involvement of laccase in the bleaching process has been thoroughly investigated, interest has recently switched increasingly to a different use of the enzyme such as the targeted modification of lignocellulose fibre surfaces with a view

to improving intrinsic fibre properties or introducing novel ones (Buchert *et al.* 2005, Chandra and Ragauskas 2001, Schroeder *et al.* 2007). This use has been facilitated by the non-specific substrate requirements of laccase and the tendency of phenolic compounds to undergo coupling reactions following enzymatic oxidation to resonance-stabilized phenoxy radicals (Chandra and Ragauskas 2002, Milstein *et al.* 1994). Some authors have accomplished laccase-catalysed grafting of phenol compounds onto lignocellulose fibres; interest in this area, however, has focused mainly on wood materials and lignin-rich fibres (Chandra and Ragauskas 2002, Elegir *et al.* 2008, Suurnakki *et al.* 2006). In this study, we investigated the effects of various laccase–natural mediator systems on the pulp and effluents obtained in the processing of sisal pulp with a low lignin content. The primary aim was to examine the tendency of mediators to either couple onto pulp or promote delignification with a view to assessing their potential for either functionalizing or biobleaching sisal fibres.

3.2. Materials and methods

3.2.1. Laccase assays and mediators

Laccase (EC 1.10.3.2) from *Trametes villosa*, 588U/ml, was supplied by Novozymes (Denmark). One activity unit was defined as the amount of laccase transforming 1 µmol/min ABTS to its cation radical (ε_{436} nm = 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer (pH 5) at 25 °C. Sinapic acid (SNC), ferulic acid (FRC), coniferyl aldehyde (CLD), sinapyl aldehyde (SLD) and 1-hydroxybenzotriazole (HBT), all purchased from Sigma-Aldrich, were assayed as laccase mediators.

Oxidation of 50 µM HBT and p-hydroxycinnamic compounds by *T. villosa* laccase was assayed by using 300 mU/mL of enzyme in 50 mM sodium tartrate buffer (pH 4) at 25 °C. UV–Vis spectra for the reaction mixture were recorded at different times (0, 3, 5, 10, 15, 20 min) during the first 20 min of enzymatic oxidation, using a Thermo Scientific Evolution 600 spectrophotometer.

3.2.2. Pulp and laccase-mediator treatments

Sisal (*Agave sisalana*) alkaline pulps from a soda–anthraquinone cooking process were supplied by CELESA (Spain). Prior to initial characterization, the samples were washed with H_2SO_4 at 2% consistency at pH 4 for 30 min, which was followed by filtration and extensive washing with de-ionized water. This step ensured removal of contaminants and metals, and brought the pulp to the pH required for the enzyme treatment. The initial pulp samples had 47.3% ISO brightness, a kappa number of 7.8 and a viscosity of 784 ml/g.

Laccase-mediator treatments (L stage) were performed by using an amount of 40 g of sisal pulp at 5% consistency in 50 mM sodium tartrate buffer at pH 4, 20U/g of *T. villosa* laccase and a proportion of 1.5% (w/w) HBT or natural mediator (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant. The treatments were carried out in a reactor under pressurized O_2 (0.6 MPa) at 30 rpm at 50 °C for 4 h. Pulp samples treated under identical conditions in the absence of enzyme and mediator or only the latter were used as controls. Once treated, each pulp was filtered, its residual liquor collected and the pulp extensively washed with de-ionized water (Valls and Roncero 2009b).

3.2.3. Residual laccase activity

The enzyme activity, using ABTS as substrate, was monitored for 4 h under the conditions of an L stage performed at a small scale in an O_2 atmosphere (continuous bubbling) in the absence or presence of mediator, and with or without pulp. Residual activity values were expressed as percentages of initial activity, which was measured at the outset (time 0) of the incubation period.

3.2.4. Bleaching treatment

The laccase–mediator treatment was followed by an alkaline peroxide bleaching treatment (P stage) that was performed in an Ahiba Spectradye dyeing apparatus from Datacolor equipped with closed vessels 150 ml in volume that were loaded with 5 g oven-

dried pulp (odp) at 5% consistency, 3% odp H_2O_2, 1.5% odp NaOH, 1% odp DTPA (diethylenetriaminepentaacetic acid) and 0.2% odp $MgSO_4$ at 90 °C for 2 h. Then, each treated pulp sample was filtered and extensively washed with de-ionized water.

3.2.5. Analysis of pulp properties

Pulp brightness, kappa number (an estimation of lignin content) and viscosity (determined as the intrinsic viscosity of a sample of cellulose dissolved in a dilute solution of cupriethylenediamine) were determined in accordance with ISO 3688, ISO 302 and ISO 5351/1, respectively, at the different stages of the process.

3.2.6. Determination of anionic charge

The surface anionic charge of sisal fibres was determined by polyelectrolyte adsorption as described elsewhere (Wagberg *et al.* 1989). The cationic polyelectrolyte used for adsorption was methylglycochitosan (MGCh) from Wako Pure Chemical Industries, Ltd. (Japan). The polymer molar mass and charge density were 1.5×10^5 and 4.04 meq/g, respectively. An amount of 0.25 g of pulp sample was diluted with 50 ml of 0.5×10^{-3} N MGCh and stirred for 30 min to facilitate the adsorption equilibrium. The resulting suspension was centrifuged and part of the clear supernatant (10 ml) collected for titration with the anionic polyelectrolyte (Cadena *et al.* 2009). The point of equivalence was determined with the cationic dye indicator Toluidine Blue (Wako) or a Mütek PCD 03 particle charge detector (Germany). With the dye indicator, the supernatant was titrated with potassium polyvinyl sulphate (PVSK) (Wako); with the PCD, titration was done with sodium polyethensulphonate (PES-Na) (Oy G. W. Berg & CO Ab/BTG Mütek GmbH, Germany). Blank samples were titrated exactly in the same way as those brought into contact with pulp. The amount of fibre in each sample upon titration was determined gravimetrically following filtering on pre-weighed filter paper and drying in an oven at 105 °C overnight.

3.2.7. Effluent characterization

The effluents from the L and P stages were analysed for Chemical Oxygen Demand (COD) and colour following ASTM D1252-00 and ASTM D1209-00, respectively. Coefficient of variation was inferior to 5% in the case of COD and to 3% in the case of colour. A Thermo Scientific Evolution 600 spectrophotometer was used in both analyses.

Toxicity tests were conducted on triplicate samples of the L effluents, with a coefficient of variation between 5 and 10%, using the marine luminescent bacterium *Vibrio fischeri* in the Microtox M500 Analyzer (Strategic Diagnostic Inc., Azur Environmental) in accordance with a standard bioassay (UNE-EN ISO 11348-3: 1999). The difference between the amount of light emitted before and after addition of the sample was used to measure toxicity. In order to prevent pH effects, each sample was adjusted to pH 6–8 with NaOH solution. Toxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed as toxicity units (TU). The reference toxicant ($ZnSO_4 \cdot 7H_2O$) was used to control *V. fischeri* batch quality in accordance with the Basic Test procedure (two replicates) (Azur Environmental, 1998).

3.3. Results and Discussion

3.3.1. Laccase oxidation of potential mediators

UV–Vis spectra, reported in Figure 3-1, exposed a prompt structural change in the natural mediators during oxidation by laccase from *Trametes villosa*. The change occurred especially rapidly in FRC and CLD, the spectra for which were consistent with the formation of no new chemical species during the first 20 min of oxidative reaction. FRC oxidation was signalled by a decrease in the absorbance at 280 and 320 nm, and a simultaneous increase in that at 235 nm, while CLD oxidation reflected in a decrease in the absorbance at 340 nm and an increase in that at 230 nm. The spectra for SNC and SLD exposed the formation of new chemical species in the visible region. For SNC, the maximum at 315 nm decreased after 1 min and was replaced by two maxima at 340 and

520 nm; also, a maximum at 435 nm appeared after 20 min. For SLD, the peaks at 245 and 345 nm decreased and the shoulder at 220 nm disappeared, all simultaneously with the appearance of peaks at 280, 480 and 520 nm —the last of which accounted for the strong pink colour of the enzyme reaction mixture containing this mediator.

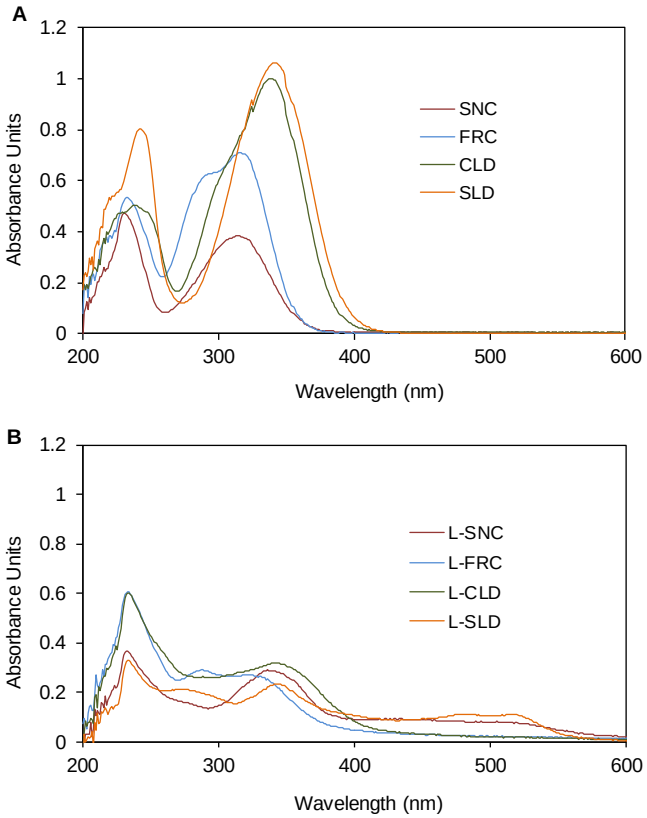

Figure 3-1. UV-Vis spectra of natural mediators before (A) and after (B) oxidation by laccase (collected 20 min after enzyme addition).

3.3.2. Residual laccase activity

Figure 3-2 shows the variation of the residual laccase activity during the L stage as performed at a small scale. In the absence of pulp, the enzyme alone underwent 74%

inactivation during the incubation period, which was 30 percentage units lower in the presence of pulp. HBT was found to strongly reduce laccase activity (to 1% after only 1 h of incubation in the absence of pulp). In the presence of pulp, inactivation by HBT was less marked, but only 12% activity was recovered after 4 h. The natural mediators were found to exert a stabilizing effect on the enzyme in the absence and presence of pulp; in contrast, with FRC in the absence of pulp the enzyme exhibited only 8% residual activity after 4 h. The addition of pulp significantly increased residual activity with FRC and CLD, which was 58 and 38 percentage units higher, respectively, than in the absence of pulp; however, it had virtually no effect with SNC and SLD in which case activity kept similar to that obtained in the absence of pulp.

The significant loss of activity observed with HBT can be ascribed to the mediator's nitroxy radicals, which inactivate laccase by oxidation of aromatic amino acids on the protein surface (Amann 1997). Previous studies (Fillat *et al.* 2007, Ibarra *et al.* 2006) exposed a protective action of pulp against laccase inactivation by the mediator; thus, pulp may act as a reducing substrate for free radicals. Ibarra *et al.* (2006) found laccase inactivation to be more pronounced in the presence of oxygen-delignified pulp than in that of unbleached pulp; the authors ascribed this result to a lower lignin content contributing to the consumption of HBT free radicals. The strongly inactivating effect of HBT in the presence of pulp observed in this work can also be ascribed to the low lignin content of sisal pulp.

The different variation of laccase activity in the presence of natural mediators suggests a disparate behaviour of these compounds in relation to HBT once they are oxidized to phenoxy radicals. Also, it is suggestive of a structure–effect relationship for the natural mediators. Thus, SNC and SLD (two 4-hydroxy-3,5-dimethoxycinnamic compounds) and/or their secondary oxidation products seemingly stabilize the enzyme, both in the absence of pulp and in its presence. By contrast, FRC and CLD (two 4-hydroxy-3-methoxycinnamic compounds) are more likely to induce denaturation of the enzyme in the absence of pulp —probably by an increased reactivity of their radicals— and undergo coupling reactions with fibre lignin in the presence of pulp —which may account for the substantially increased activity observed upon addition of the pulp. Monitoring laccase activity during the treatments of flax pulp with *p*-coumaric acid exposed a similar effect in a recent study (Fillat *et al.* 2008): the acid exhibited a strong

tendency to couple to the pulp and the enzyme inactivation was nearly 80% lower in the presence of pulp than in its absence.

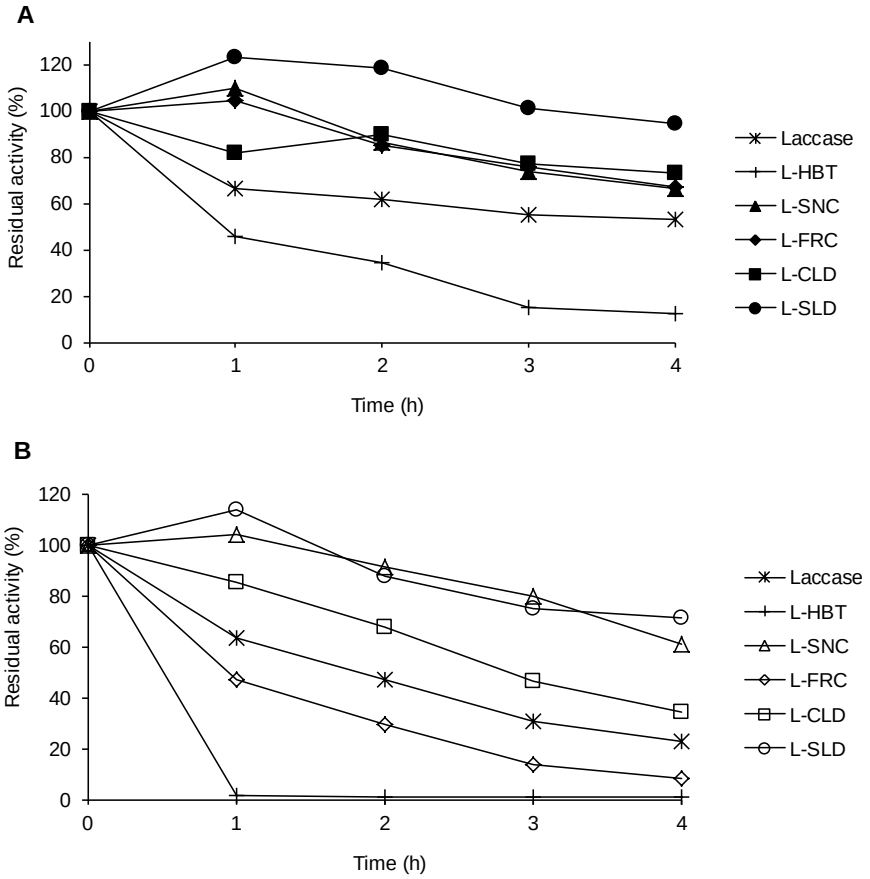

Figure 3-2. Variation of the activity of *T. villosa* laccase during the incubation period (4 h) in the presence (A) and absence (B) of pulp, both without (Laccase) and with a natural mediator or HBT (L-MEDIATOR).

3.3.3. Analysis of pulp properties

Pulp samples from the L and P stages were analysed in order to assess the involvement of the natural mediators in grafting or oxidative degradation reactions during the enzyme treatment, as well as their ability to enhance lignin removal in the subsequent

bleaching treatment. Figure 3-3 shows the kappa number and brightness values obtained in the tests.

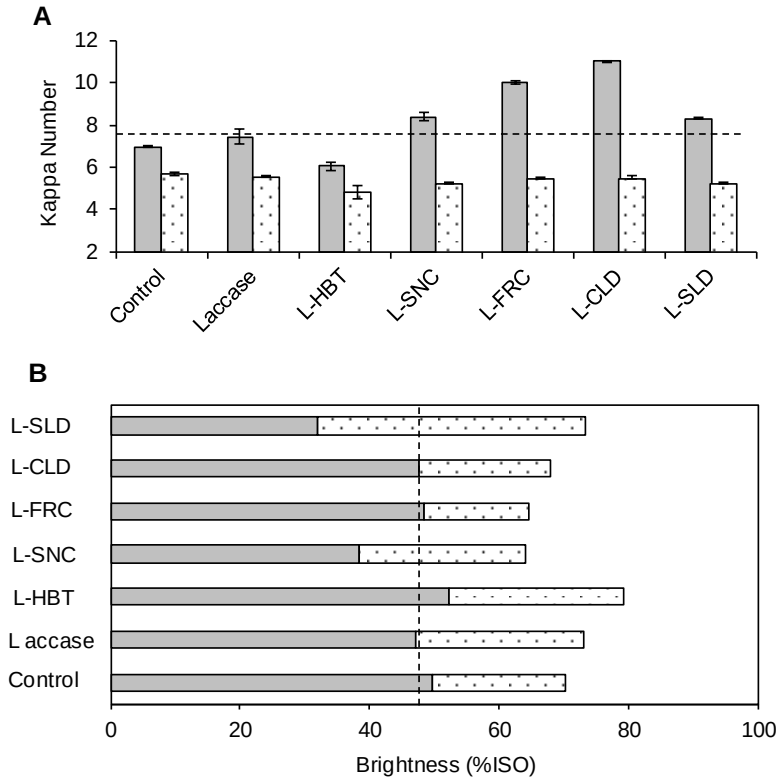

Figure 3-3. Changes in kappa number (A) and brightness (B) of sisal pulp treated with laccase in the presence of mediators in the L stage (grey bars), followed by hydrogen peroxide bleaching (dotted bars). The laccase and control pulp samples were treated in the absence of mediator, and both the mediator and enzyme, respectively. The dashed line corresponds to the kappa number of the initial pulp.

After the L stage, HBT was the only mediator capable of decreasing the kappa number with respect to the control pulp (13%); by contrast, all natural mediators resulted in substantially increased kappa numbers, which is consistent with their partial condensation on the pulp via radical-coupling reactions. Also, consistent with the previous assumption of their stronger tendency to couple to pulp fibres, the increase was especially marked for CLD and FRC (58% and 43% respectively). In order to completely

suppress the contribution to kappa number of phenolic compounds simply adsorbed onto pulp, samples from the L stage were extensively washed with acetone in a Soxhlet apparatus. This led to a decreased kappa number in the samples treated with the natural mediators (results not shown) which, however, was still significantly higher than that for the control pulp, thus testifying to the covalent binding of these compounds to fibre surfaces. After Soxhlet washing, the CLD and FRC treated samples still had a kappa number ~15% greater than the SNC and SLD treated samples. The less marked grafting observed in the dimethoxylated compounds may have reflected increased steric hindrance and stability in their phenoxy radicals precluding condensation of C_5 in the aromatic ring with residual lignin in the pulp fibres (Camarero *et al.* 2007, Chandra *et al.* 2004, Moldes *et al.* 2008). After the P stage, the samples treated with HBT exhibited the smallest kappa numbers (15% less than the control sample) and those treated with the natural mediators showed a comparatively small decrease (6–7% with respect to the control pulp). These results underline the potential of HBT for bleaching sisal pulp and suggest that the bleaching efficiency can be improved to a certain extent by using a natural mediator despite the undesirable coupling reactions it may undergo in the L stage. Therefore, natural mediators may be involved simultaneously in oxidative degradation and grafting reactions; also, the ultimate effect of laccase–natural mediator systems must be strongly dependent on the balance between these two types of reactions.

Brightness was only improved by HBT, which raised it by 3.6% in the L stage and 13% in the P stage. By contrast, all natural mediators diminished brightness in the L stage (especially SLD, with a 36% decrease and much less markedly FRC, with 3%). The loss of brightness in the SLD and SNC L-treated samples was accompanied by a markedly increased *k*/*s* ratio (Jordan 1996) and changes in the CIE $L^*a^*b^*$ colour coordinates (Hunt 1998) (results not shown), which suggest the formation of a substantial amount of chromophoric groups as a result of the enzyme treatment (Fillat and Roncero 2009, Moldes *et al.* 2008). After the P stage, SLD treated pulp was the only sample exhibiting (slightly) increased brightness with respect to the control pulp (4%). It also exhibited the greatest brightness gain from L to P stage as a result of the oxidation and dissolution of chromophoric species, and lignin degradation products, in the alkaline bleaching medium used.

The pulp samples were subjected to viscosity measurements in order to assess the effect of each treatment on cellulose integrity (see Figure 3-4). After the L stage, the

enhanced bleaching obtained with HBT was accompanied by a marked drop in viscosity; on the other hand, the pulp samples treated with the natural mediators retained their viscosity. These results are indicative of a disparate reaction mechanism for the natural mediators and HBT; in fact, the synthetic mediator probably caused degradation and/or oxidation of carbohydrate chains in cellulose. After the P stage, the samples treated with SLD and CLD exhibited no viscosity loss relative to the control, whereas those treated with HBT, SNC and FRC exhibited a decrease of 10-12%. Probably, phenolic acids oxidize carbohydrate chains in cellulose to carbonyl groups during the L stage, thus making the pulp vulnerable to degradation by the strong alkaline medium used in the bleaching stage.

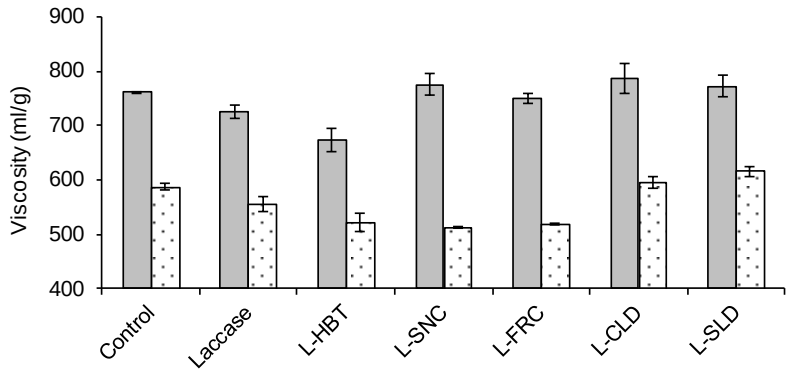

Figure 3-4. Viscosity changes in sisal pulp after the L stage (grey bars) and P stage (dotted bars) relative to the initial pulp (dashed line).

The tendency of natural mediators to graft onto fibre surface can be assessed by analysing fibres for functional groups typically present in them. The presence of carboxylic groups in phenolic acids can be used to estimate their contents in pulp after an enzyme treatment (Chandra and Ragauskas 2002, Elegir *et al.* 2008). Figure 3-5 shows the results of polyelectrolyte titrations of pulp samples after the L stage performed by using the colorimetric and streaming current end-point detection methods. Although the latter method provided higher charge values, the results of both clearly followed a similar trend; thus, as expected, the samples treated with the phenolic acids (especially FRC) contained the highest amount of anionic charge. The increase in charge with respect to the

control samples echoes that in kappa number and confirms that FRC tends to graft onto fibre surfaces more markedly than does SNC.

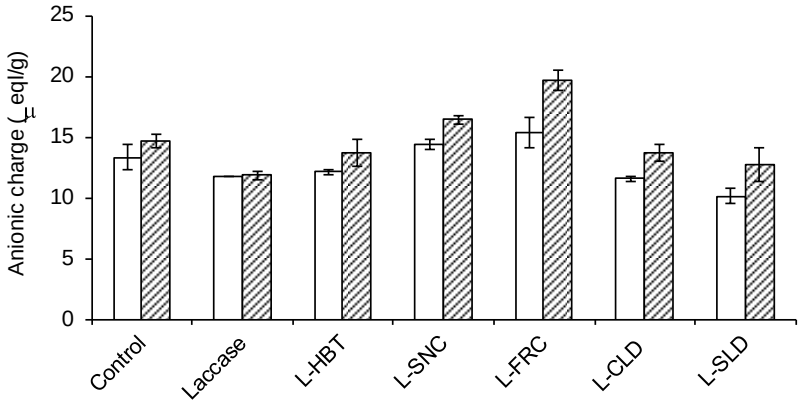

Figure 3-5. Surface anionic charge of sisal fibres after the L stage as determined with the colorimetric (white bars) and streaming current (hatched bars) end-point detection methods.

3.3.4. Effluents characterization

Effluent analyses provide further information about the overall LMS-based process. Determining COD, colour and toxicity is important with a view for assessing the environmental impact of the effluents and the feasibility of closing circuits in a future industrial implementation. Moreover, such analyses, which have never to date been conducted on effluents from laccase–natural mediators treatments, provide an additional tool for interpreting the effects on pulp. The COD and colour data obtained are shown in Table 3-1, and the results of the toxicity assays in Table 3-2.

None of the LMS treatments increased COD in the effluents significantly with respect to the control; among natural mediators, SNC and SLD resulted in greater COD than did FRC and CLD. The effluents from the P stage had much lower COD values than those from the L stage as a result of the lower concentrations and the oxidized form of the chemical species dissolved during the former. However, the effluents resulting from the bleaching (P stage) of the FRC and CLD treated pulp samples had increased COD values relative to the SNC and SLD treated samples. The observed differences in COD may be

related to differences in the degree of fibre grafting of the natural mediators. Thus, SNC and SLD were those least markedly contributing to the increase in kappa number during the L stage; therefore, they should be present at higher concentrations in the resulting effluents and raise their COD as a result. In contrast, FRC and CLD, which tend to couple onto pulp, can be expected to be less concentrated in the effluents from the L stage and contribute more markedly to COD in those from the subsequent bleaching stage.

Like COD, colour depends on the nature and concentration of the particular chemical species present in the effluent. The enzyme treatments involving HBT, SNC and SLD produced effluents with markedly strong colour relative to FRC and CLD due to the presence of an increased amount of chromophoric species resulting from the oxidation and/or degradation of lignin and the mediators (Fillat and Roncero 2009). The effluents from the bleaching of HBT, SNC and SLD treated samples exhibited dramatically reduced colour which, however, was similar to that of the effluents from the FRC and CLD treated samples after the L stage. Colour data may provide further evidence of the disparate degree of grafting in the two types of natural mediators after the L stage.

Table 3-1. COD and Colour values of effluents obtained from treating pulps in L and P stages.

	L stage effluent		P stage effluent	
	COD (kg O_2/t_{pulp})	Colour (kg Pt/t_{pulp})	COD (kg O_2/t_{pulp})	Colour (kg Pt/t_{pulp})
Control	95	2.8	17	2.0
Laccase	103	2.3	19	1.7
L-HBT	102	66.0	17	0.9
L-SNC	110	41.1	27	6.4
L-FRC	103	7.6	34	5.4
L-CLD	90	3.4	32	4.6
L-SLD	132	87.6	21	0.9

Table 3-2. Toxicity values of solutions of mediators at the same concentration as in the enzymatic reaction treatment and of effluents obtained from L stage.

	Toxicity (T.U.)	
	Mediator solution	L stage effluent
Control	-	1
Laccase	-	11
L-HBT	1	53
L-SNC	10	158
L-FRC	7	30
L-CLD	125	33
L-SLD	20	147

Toxicity tests constitute an important tool for assessing the environmental risk of industrial wastewater; however, no research on effluents from LMS-based processes in this respect appears to have been conducted previously. The bioassay with *Vibrio fischeri* is of technical interest according to Spanish regulations. The Community of Catalonia (Spain) has passed a specific regulation (Decree 130/2003) to establish emission limit values (ELV) for wastewater, the maximum limit allowed being 25 equitox/m^3 [1 equitox/m^3 = $(1/EC_{50})$*100].

Bioassays were performed on effluents from the L stage and on solutions containing the mediators at the same concentration as in the reaction medium with a view to comparing their toxicity, both by themselves and in the presence of pulp and enzyme. As can be seen from Table 3-2, the presence of laccase resulted in slightly increased toxicity relative to the control value. Application of the LMS treatment further raised it above the Catalonian ELV, to a different extent depending on the type of mediator used. Thus, FRC and CLD resulted in the smallest increase, followed by HBT with a twice greater effect, and, at an even greater extent, by SNC and SLD. The solutions containing the mediators in their reduced forms exhibited toxicity values lower than the ELV; by exception, that of CLD unexpectedly resulted in very high toxicity relative to the other studied compounds. The increased toxicity observed in the enzyme treatment can be ascribed to various

factors including the formation of oxidized/radical species and degradation products from both lignin and the mediators. Toxicity can be expected to rise with increasing concentration of these species in the effluents. Therefore, the results can help to better understand the reaction pathway followed by the mediators in the presence of laccase and pulp; thus, the low toxicity observed with FRC and CLD may have resulted from the low concentration of lignin-derived compounds, radical species and by-products of the mediators in the effluent by effect of their having stronger tendency to couple onto pulp than to promote delignification. On the other hand, the less marked tendency of SNC and SLD to undergo grafting reactions, the increased stability of their radicals, the formation of by-products by the mediators and also, possibly, from degraded lignin, may account for the much higher toxicity observed with these mediators.

3.4. Conclusions

The aim of this study was to explore the potential of some natural mediators for either biobleaching or functionalizing sisal fibres. Based on the pulp and effluent properties obtained, and on the variation of laccase activity during the L stage, natural mediators follow a different reaction pathway in the presence of laccase and pulp than does HBT. The synthetic mediator was the best performer with regards to delignification and brightness; this, however, was at the expense of a slight loss of pulp viscosity, and of a strong, rapid inactivation of the enzyme during the L stage. Under the operating conditions used here, the selected natural mediators proved ineffective for bleaching of sisal pulp; in fact, they exhibited a tendency to couple onto fibres, which can be useful with a view to functionalizing lignocellulosic fibres by laccase-aided biografting.

3.5. Addendum to Publication 1: Bleaching assays for a comparative study of new synthetic and natural mediators

Summary

This addendum shows the results, in terms of kappa number and brightness, of further bleaching assays, consisting of an L and a P stage, performed with laccase from *Trametes villosa* (*Tv*L) and the synthetic mediator violuric acid (VA), and laccase from *Myceliophthora thermophila* (*Mt*L) and the natural mediator methyl syringate (MS). The results were compared with those obtained in Publication 1 with *Tv*L and 1-hydroxybenzotriazole (HBT) or sinapyl aldehyde (SLD). The latter was assayed at different doses and in the presence of two different enzyme doses, to better understand its potential for delignification.

Materials and methods

L and P stages were performed the same as in Publication 1; however, the treatment with *Mt*L and MS (both supplied by Novozymes) was performed in 50 mM sodium dihydrogen phosphate buffer pH 6.5. L treatments were carried out using 1.5% (w/w) of mediator and 20 U/g odp of laccase, whereas SLD was assayed at three different doses (0.1, 0.8 and 1.5% w/w) and in the presence of two doses of laccase.

Results and discussion

Table 3-3 reports the results of kappa number and brightness determinations of pulps from L and P stages treated with *Tv*L+VA and *Mt*L+MS systems and their corresponding laccase controls, in comparison to those obtained with *Tv*L+HBT and *Tv*L+SLD. The interest in assaying *Tv*L+VA derived from the good bleaching performance and acceptable effluent characteristics it was reported to show (Moldes *et al.* 2008, Monje *et*

al. 2010). *Mt*L+MS was interesting to assay due to the characteristics of stability of the enzyme (to pH and temperature) (Berka *et al.* 1999, Xu *et al.* 1996) and its advantage of being commercially-available and cheap, as well as to the cost-effectiveness of the natural mediator (Babot *et al.* 2011). It was observed that *Tv*L+HBT and *Tv*L+VA systems resulted in similar delignification effect, but the latter showed higher bleaching efficiency, providing a brightness of 4% ISO points higher in the final stage. The application of *Mt*L+MS system resulted in higher kappa number and lower brightness than those provided by *Tv*L+HBT and *Tv*L+VA systems, although, interestingly, control treatment with *Mt*L resulted in better values of brightness than treatment with *Tv*L, after both L and P stages.

Table 3-3. Kappa number (± standard deviation) and brightness (standard deviation=0) determinations of pulps from L and P stages treated with *Tv*L+VA and *Mt*L+MS systems and their corresponding laccase controls, in comparison to those obtained with *Tv*L+HBT and *Tv*L+SLD, the latter applied at different doses of SLD.

	Kappa number		**Brightness (% ISO)**	
	L stage	**P stage**	**L stage**	**P stage**
Control TvL	7.4 ± 0.3	5.6 ± 0.0	47.3	73.3
TvL + HBT	6.1 ± 0.2	4.8 ± 0.3	51.6	77.0
TvL + VA	6.7 ± 0.4	4.5 ± 0.3	51.7	80.7
Control MtL	7.6 ± 0.3	5.8 ± 0.0	53.2	75.7
MtL + MS	7.0 ± 0.1	5.5 ± 0.0	48.7	76.3
TvL20 + SLD0.1	7.6 ± 0.2	5.7 ± 0.3	47.3	73.3
TvL20 + SLD0.8	7.7 ± 0.3	5.5 ± 0.3	36.0	69.4
TvL20 + SLD1.5	8.3 ± 0.1	5.3 ± 0.1	31.9	73.4
TvL40 + SLD1.5	8.8 ± 0.1	4.8 ± 0.2	29.9	74.6

Compared to *Tv*L+SLD at the same conditions of laccase and mediator doses, *Mt*L+MS system provided better results of brightness (especially after L stage), but slightly less delignification after the P stage. Raising the dose of SLD resulted in increasing kappa number and decreasing brightness after the L stage, due to partial retention and/or coupling of the mediator onto pulp, and formation of chromophores, but an opposite effect was observed after the P stage. The combination of SLD with 40U/g

odp of laccase yielded a final kappa number similar to those obtained with synthetic mediators, although brightness was somewhat lower. These results suggested that *Tv*L+SLD modified lignin in such a way that the delignification effect could be visible only after application of the P stage.

Conclusions

For comparative purposes, further bleaching assays with new LMS were performed in order to select those with the highest bleaching potential and apply them in the L stage of a TCF bleaching sequence. VA was selected for its better bleaching performance compared to HBT, and SLD was considered worthy of further study, despite the loss of brightness it caused, due to its ability to facilitate delignification after P stage.

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Xylanase- and laccase-aided hexenuronic acids and lignin removal from specialty sisal fibres

Based on **Publication 2**: Elisabetta Aracri and Teresa Vidal. *Carbohydrate Polymers*, Volume 83, No. 3, pp. 1355-1362.

Abstract

This work was conceived to investigate for the first time the effectiveness of the combined use of xylanase and laccase for the removal of hexenuronic acids (HexA) and lignin from sisal pulp fibres. To this end, xylanase (X) and laccase (L) treatments were used in an $XLQP_O$ sequence (where Q denotes a chelating stage and P_O an oxygen-reinforced peroxide multi-step treatment) that was applied to pulp in order to obtain sisal fibres with a high cellulose content. The results of the $XLQP_O$ sequence were compared with those of an LQP_O sequence. The L stage of both sequences was performed in the presence of either the natural compound sinapyl aldehyde (SLD) or the synthetic compound violuric acid (VA), employed as mediators, in order to compare their efficiency in aiding pulp bleaching and HexA removal. Changes in HexA content and the contributions of lignin and HexA to kappa number during each sequence were examined. The xylanase treatment was found to remove 47% of lignin, 15% of xylan and 27% of HexA from the initial pulp, whereas the laccase–VA system removed 28% of HexA and exhibited higher efficiency than the laccase–SLD system in reducing kappa number and increasing brightness. In any case, when the X treatment was applied, the sequence including laccase-SLD treatment resulted in the strongest delignification effect. The effluents from each stage of the bleaching sequences were analysed for COD, colour and toxicity, which peaked after the L stage and were significantly higher with SLD than with VA.

4.1. Introduction

Increasing pressure from environmental legislation has led the pulp and paper industry to seek cleaner production methods aimed at minimizing the use of polluting chemicals in the bleaching process (Roncero and Vidal 2007). 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. In fact, xylanases have already proved effective for boosting pulp bleachability and saving chemical bleaching reagents at the mill scale (Roncero *et al.* 2002, Viikari *et al.* 1994). Xylanases are hydrolytic enzymes that catalyse xylan degradation. Their beneficial effect has been ascribed to their selectively hydrolysing xylans re-precipitated on fibre surfaces, thereby contributing to

releasing lignin and facilitating reagent penetration in subsequent bleaching stages (Roncero *et al.* 2003, Torres *et al.* 2000). Recently, an innovative aspect of xylanases has been identified: their ability to reduce the content of hexenuronic acids (HexA) in pulp (Valls *et al.* 2010c). HexA are known to form during alkaline cooking of wood by elimination of methanol from the 4-*O*-methylglucuronic acid group bonded as a side group to xylans (Daniel *et al.* 2003). The significance of HexA relies on their adverse effects on pulp bleaching; thus, they contribute to kappa number, increase the consumption of bleaching agents, retain metal ions by chelation, cause brightness reversion and facilitate the formation of oxalic acid and the scaling of process circuits by calcium oxalate (Cadena *et al.* 2010, Valls and Roncero 2009). Because xylanases hydrolyse xylans on fibre surfaces and such xylans contain HexA, these compounds may be removed by enzymatic treatment with xylanases (Valls and Roncero 2009, Valls *et al.* 2010b).

The combination of a fungal laccase with a chemical a mediator, which is called a "laccase–mediator system" (LMS), has proved more effective than the use of xylanases alone for the biobleaching of various types of pulp by virtue of their direct action on lignin; however, their effectiveness at the mill scale remains to be assessed (Barreca *et al.* 2003, Bourbonnais and Paice 1990, Camarero *et al.* 2004, Fillat and Roncero 2010, Ibarra *et al.* 2006). Some synthetic mediators such as 1-hydroxybenzotriazole (HBT) and violuric acid (VA) have proved highly effective in promoting pulp delignification; however, concerns about their possible toxicity and high cost have so far hindered broad industrial use (Bourbonnais *et al.* 1997, Moldes *et al.* 2008, Shleev *et al.* 2006, Valls *et al.* 2010). This has promoted a search for natural, potentially cost-effective mediators such as lignin-derived phenols, which can be readily obtained from plants and spent pulping liquors or, directly, from fungal metabolism (Aracri *et al.* 2009, Camarero *et al.* 2005, Eggert *et al.* 1996, Johannes and Majcherczyk 2000, Moldes *et al.* 2008). Recent studies have shown the effectiveness of some natural phenols to mediate laccase catalysis towards recalcitrant substrates such as pulp lignin (Camarero *et al.* 2007, Fillat *et al.* 2010) and lipids (Gutiérrez *et al.* 2007). Valls *et al.* (2010c) examined the effect of HexA content reduction in eucalyptus kraft pulp by a laccase–HBT system and found it to be strongly boosted by a xylanase pre-treatment. However, no study has so far examined the removal of HexA by LMS from non-wood pulp, the potential for which of natural mediators remains unknown. The aim of this work was to investigate for the first time changes in HexA and lignin in sisal pulp during two different TCF bleaching sequences

performed with and without a xylanase pre-treatment (an X stage) and including an LMS treatment (an L stage) where either the natural compound sinapyl aldehyde (SLD) or the synthetic compound violuric acid (VA) was used as laccase mediator. The resulting effluents were characterized with a view to assessing the environmental impact of the bleaching treatments and the feasibility of closing circuits in a future industrial implementation, a scarcely explored aspect of bleaching sequences (Aracri *et al.* 2009, Fillat *et al.* 2010, Fillat and Roncero 2010).

4.2. Materials and methods

4.2.1. Raw material

The raw material was sisal (*Agave sisalana*) alkaline pulp obtained by soda–anthraquinone cooking at the CELESA mill (Tortosa, Spain). Prior to characterization, the pulp was washed with acidified water at pH 4 at 2% pulp consistency for 30 min, followed by filtration and thorough washing with de-ionized water. This procedure was needed to remove contaminants and metals, and also to bring the pulp pH closer to that for the enzyme treatments. The main properties of the initial pulp were as follows: 37.9 ± 0.1% ISO brightness, 7.9 ± 0.05 kappa number, 733 ± 1 ml/g viscosity and a hexenuronic acid (HexA) content of 41.4 ± 1.1 μmol/g oven dried pulp (odp).

4.2.2. Bleaching sequences

Two different TCF bleaching sequences were applied to the pulp, namely: LQP_O and $XLQP_O$, where L denotes the laccase-mediator treatment, Q a chelating treatment, P_O an oxygen-reinforced hydrogen peroxide multi-step treatment and X an enzyme pretreatment with xylanase. Control sequences were performed without addition of the mediator in the L stage for comparison. At the end of each stage, the pulp was filtered and thoroughly washed with de-ionized water.

The enzyme used in the X stage was a commercial xylanase (Pulpzyme® HC) supplied by Novozymes (Bagsvaerd, Denmark). The X treatment was performed in

polyethylene bags, using 3 U/g odp xylanase in Tris–HCl buffer (pH 7), at 10% pulp consistency, at 50 °C for 2 h.

Laccase from *Trametes villosa* (NS-51002) was supplied by Novozymes. One activity unit was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical ($\varepsilon_{436\ nm}$ = 29300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Sinapyl aldehyde (SLD) and violuric acid (VA) purchased from Sigma–Aldrich were used as laccase mediators. Laccase treatments were carried out at 5% pulp consistency in an oxygen pressurized (0.6 MPa) reactor at 50 °C, using a stirring rate of 30 rpm for 4 h; the reactor was supplied with 50 mM sodium tartrate buffer at pH 4, 20 or 40 U/g odp laccase and 1.5% (w/w) VA or SLD in addition to Tween 80 (0.05% w/v) as surfactant.

The L treatment was followed by a Q stage involving the use of chelating agents to reduce the contents in metal ions (Fe^{2+}, Cu^{2+}, Mn^{2+}) liable to degrade the bleaching agents and cellulose during the peroxide bleaching treatment (Heijnesson *et al.* 1995). The Q treatment was conducted in the presence of 1% DTPA (diethylenetriaminepentaacetic acid) at 5% consistency, pH 5–6 and 85 °C for 1 h.

The last step of the TCF sequence was a P_O stage carried out at 5% consistency in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm, using 3% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$, 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 involving the addition of 1% odp H_2O_2 and withdrawal of a small amount of pulp from the reactor to determine brightness and kappa number at the end.

4.2.3. Pulp properties

Pulp properties were determined after each stage in all sequences. Treated pulp samples were characterized in terms of kappa number, brightness and viscosity according to ISO 302, 3688 and 5351-1, respectively. The hexenuronic acid (HexA) content was determined by UV detection (Chai *et al.* 2001). An estimate of the actual lignin content of the pulp was obtained by determining the kappa number due to lignin (KN_{lig}) (Li *et al.* 2002, Valls *et al.* 2010c). This involved measuring kappa number following removal of HexA by acid hydrolysis with mercury acetate and efficient washing with de-ionized water. The KN values thus obtained were used to calculate the actual degree of pulp

delignification. Analyses were performed in duplicate for kappa number, viscosity and HexA content, and in quadruplicate for brightness.

4.2.4. Carbohydrate analysis by HPLC

The sugar composition of the initial pulp and the enzymatically treated pulp (X and L) from all sequences was determined by high performance liquid chromatography (HPLC) of samples previously Soxhlet-extracted with acetone and ground to a particle size < 0.5 mm. Two replicates of the resulting samples were hydrolysed by using a modified version of the TAPPI T 249 cm-09 test method. The hydrolysis process involved the following two steps: (*a*) *pre-hydrolysis with concentrated sulphuric acid.* Approximately 50 mg of sample was placed in a test tube and impregnated with 5 ml of 72% sulphuric acid, after which the tube was placed in a water bath at 30 ± 0.5 °C for 1 h with occasional stirring. (*b*) *Final hydrolysis with dilute sulphuric acid.* The tube contents were washed in a 250-ml flask in order to obtain a final solution 4% in sulphuric acid and the flask was placed in an autoclave at 103 ± 7 kPa for 1 h. Once the reaction was complete the specimen solution was cooled at room temperature and passed through a glass filter to remove lignin insoluble in sulphuric acid. Prior to HPLC analysis, the solution was filtered through a Whatman membrane of 0.45 μm pore size. The high performance liquid chromatograph was fitted with a refractive index detector. The chromatographic determination was performed with an Agilent 1100 HPLC instrument furnished with column packed with Aminex HPX-87H ion-exchange resin under the following operating conditions: mobile phase, 0.006 mol/l sulphuric acid; flow rate, 0.6 ml/min; column temperature, 60 °C. Measurements were interpolated into calibration curves run from standards of glucose, rhamnose, arabinose and xylose (all from Sigma–Aldrich). Because the column failed to resolve xylose, mannose and galactose, their combined content was expressed as xylose (Garrote *et al.* 2001).

4.2.5. Effluent properties

The effluents from each stage in the bleaching sequences were analysed for chemical oxygen demand (COD) and colour following ASTM D1252-00 and ASTM D1209-00,

respectively. Absorbance data were taken at 600 nm for COD and 465 nm for colour. A Thermo Scientific Evolution 600 spectrophotometer was used in both cases.

Effluent toxicity was 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: 1999 standard. The difference between the amount of light emitted before and after addition of the sample was used to measure toxicity. In order to prevent pH effects, each sample was adjusted to pH 6–8 with an NaOH solution. Toxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed in toxicity units (TU). The reference toxicant $ZnSO_4 \cdot 7H_2O$ was used to control *V. fischeri* batch quality in accordance with the Basic Test procedure. Toxicity measurements were colour-corrected as per the recommendations of the equipment manufacturer. COD, colour and toxicity tests were conducted on duplicate samples.

4.3. Results and discussion

4.3.1. Pulp properties

This work was designed to assess the potential of a xylanase pre-treatment on sisal pulp for boosting bleaching and HexA removal during a bleaching sequence, as well as to compare the effectiveness of VA and SLD as laccase mediators in aiding lignin and HexA removal. Six different bleaching sequences were used for this purpose, namely: L20 and L40, two control sequences where the L stage was performed with 20 and 40 U/g odp laccase, respectively, in the absence of mediator (VA and SLD, respectively); $L20_{VA}$ and $L40_{SLD}$, where L was performed in the presence of VA and SLD, respectively; and $XL20_{VA}$ and $XL40_{SLD}$, both of which included a xylanase pre-treatment as additional stage. A previous study (Aracri *et al.* 2009) revealed SLD to be the best choice for aiding pulp delignification among various *p*-hydroxycinnamic compounds assayed as natural mediators of laccase. This led us to select it for further testing here, where it was used in the L treatment under the optimum conditions established in previous tests —this is why we used a different laccase dose with each mediator. Figure 4-1 shows kappa number of

the pulp obtained after each stage of the bleaching sequences including the L treatments with VA and SLD and their corresponding controls. P1, P2 and P3 represent the three steps of H_2O_2 addition during the Po stage. As can be seen, the xylanase reduced the kappa number of the initial pulp from 7.9 to 6.8, which can be ascribed to a delignifying effect resulting from the removal of lignin trapped between xylan chains (Roncero *et al.* 2005), as well as to the removal of HexA bonded as side groups to xylans (Valls *et al.* 2010a). The $L20_{VA}$ sequence proved efficient in decreasing kappa number with respect to the control sequence, particularly in the L stage (1.5 points decrease); thus, it provided a kappa number 15% smaller than that yielded by the control sequence. The reduction in kappa number caused by the L stage was higher in the xylanase pre-treated pulp than in the initial pulp. This suggests that the X stage increased the accessibility of the laccase–VA system into pulp fibres, thereby facilitating removal of lignin and hexenuronic acids contributing to kappa number, which is consistent with the bleach boosting effect of xylanase treatments previously observed by other authors. The beneficial effect of xylanase resulted in a 22% decrease in final kappa number with respect to the sequence excluding the X stage. In contrast to the effect observed with the laccase–VA system, the L stage carried out in the presence of the laccase–SLD system resulted in a significant increase in kappa number —which exceeded the values for the control sequence after the L and Q stages. This suggests a disparate behaviour of the natural compound in relation to VA after it is oxidized to a phenoxy radical by the enzyme. When phenolic compounds are used as laccase mediators for pulp bleaching, the delignification effect may be hindered by adverse reactions involving the phenoxy radicals generated by enzymatic oxidation of the mediator; such is the case with depleting reactions (*e.g.* homopolymerization and cross-coupling in the lignin structure) or fragmentations (Aracri *et al.* 2009, Moldes *et al.* 2008). The increase in kappa number observed upon treatment with the laccase–SLD system may have resulted from adsorption or partial condensation of the phenol on the pulp via radical-coupling reactions. The increase caused by the L stage was more marked in the initial pulp (from 7.9 to 9.3) than in the xylanase-treated pulp (from 6.8 to 7.3), probably as a result of the higher content of reactive sites (*e.g.* lignin, hexenuronic acids) promoting binding of SLD in the former.

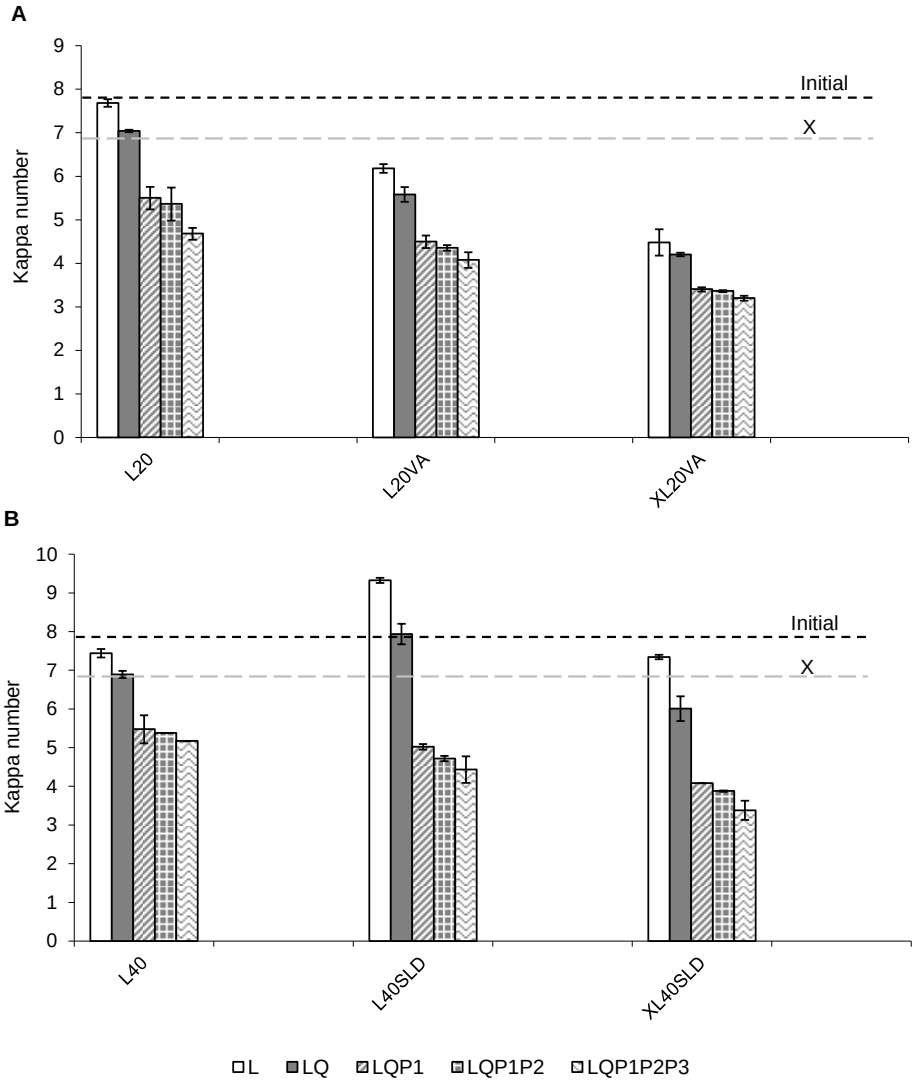

Figure 4-1. Kappa number of pulps obtained from each stage of the sequences involving VA (A) and SLD (B), including the three steps of H_2O_2 addition in the P_O stage. Dashed lines indicate the values of the initial pulp and the xylanase treated pulp (X).

Despite the adverse effect on the enzyme treatment, the final bleaching stage yielded a kappa number smaller than the control value (14%), which suggests that the natural mediator may be simultaneously involved in coupling and oxidative degradation reactions during the L stage, the effect of the latter only being observed at the end of the bleaching

sequence. Similarly to VA, application of the X stage prior to the laccase–SLD treatment led to a final kappa number 24% smaller than in the absence of an X stage and similar to that for the $XL20_{VA}$ sequence.

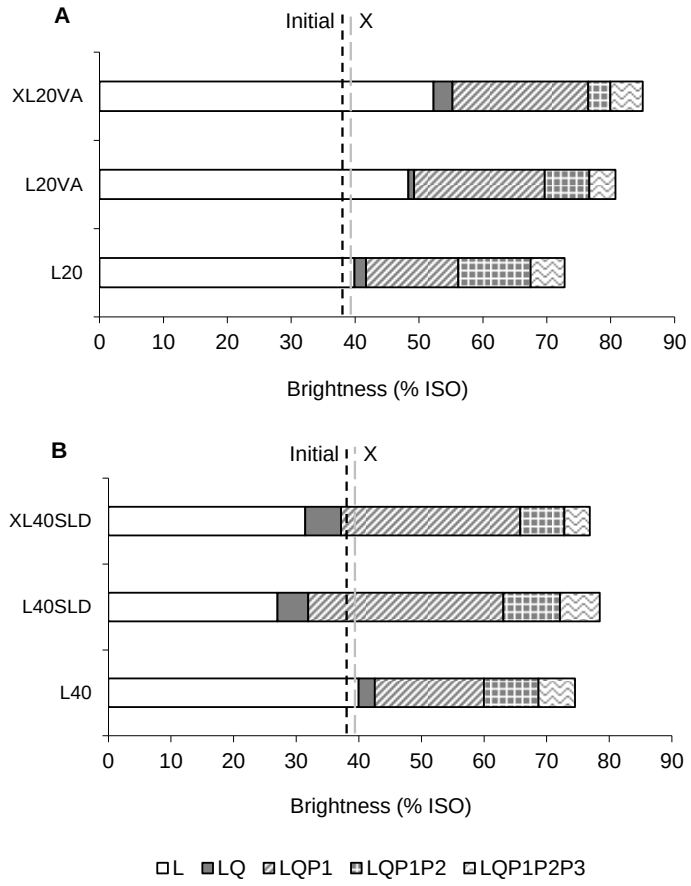

Figure 4-2. Brightness of pulps obtained from each stage of the sequences involving VA (A) and SLD (B), including the 3 steps of H_2O_2 addition in the P_O stage. Dashed lines indicate the values of the initial pulp and the xylanase treated pulp (X). Standard deviation= 0.1.

Figure 4-2 shows the brightness values obtained with the sequences involving VA and SLD —and their corresponding controls. As can be seen, the laccase–VA system exhibited a high efficiency in raising pulp brightness in all stages, especially when a xylanase pre-treatment was applied; thus, the $L20_{VA}$ and $XL20_{VA}$ sequences raised

brightness by 11% and 17%, respectively, with respect to the control sequence. Similarly to kappa number, pulp brightness was adversely affected by the laccase–SLD system in the L and Q stages, especially in the absence of an X stage. The loss of brightness in the laccase–SLD treated samples was accompanied by a markedly increased k/s ratio (Jordan 1996) and by changes in the CIE L*a*b* colour coordinates (Hunt 1998) (results not shown), which suggests the formation of a substantial amount of chromophoric groups as a result of the enzyme treatment (Fillat and Roncero 2009a, Moldes *et al.* 2008). After the Po stage, however, the laccase–SLD treated pulp samples from both sequences (with and without an X stage) exhibited increased brightness with respect to the control pulp (3% and 5% higher, respectively) as a result of the oxidation and dissolution of chromophoric species, and lignin degradation products, in the alkaline bleaching medium used. Using the LMS afforded a reduction in hydrogen peroxide dose. Using laccase-VA system with and without xylanase pre-treatment allowed reaching the same brightness as the control sequence with 56% and 82% less hydrogen peroxide, respectively. The use of laccase-SLD system could provide 14% and 10% hydrogen peroxide reductions in the absence and in the presence of the X stage, respectively.

Pulp samples from each stage of the six bleaching sequences were subjected to viscosity measurements in order to assess the effect of each treatment on cellulose integrity (Table 4-1). The L and Q stages in all bleaching sequences led to similar pulp viscosity values, with no appreciable losses from the initial pulp. The viscosity reduction caused by the Po stage was more marked with VA than with SLD; probably, the mediators oxidized carbohydrate chains in cellulose to carbonyl groups during the L stage, thus making the pulp vulnerable to degradation by the strong alkaline medium used in the bleaching stage. The increased viscosity values obtained at the end of the sequences including an X stage may have resulted from partial removal of xylans from the pulp increasing the average degree of polymerization of carbohydrates in the fibres (Roncero *et al.* 2002).

Table 4-1. Viscosity (± standard deviation) of initial and xylanase-treated pulps, and pulps obtained from each stage of each bleaching sequence.

Viscosity (ml/g)			
X		733 ± 1	
Initial		756 ±16	
	L	Q	P_O
L20	774 ± 13	745 ± 13	620 ± 8
L40	729 ± 3	758 ± 35	663 ± 37
$L20_{VA}$	721 ± 26	750 ± 15	600 ± 33
$L40_{SLD}$	739 ± 39	748 ± 10	592 ± 25
$XL20_{VA}$	765 ± 21	801 ± 9	691 ± 41
$XL40_{SLD}$	703 ± 30	788 ± 32	691 ± 10

The xylan contents of the initial and enzymatically (X and L) treated pulp samples as calculated after acid hydrolysis and HPLC analysis are reported in Table 4-2. As can be seen, the L-VA and L-SLD treatments reduced xylan contents only slightly in comparison with the initial pulp; by contrast, their application after the X stage reduced the xylan content by 20% and 25%, respectively, the decrease occurring largely (66% and 54%, respectively) during the xylanase treatment. This result confirms that xylanase acts by hydrolysing xylans present in the pulp and shows that xylan removal is boosted by the subsequent L stage with both mediators.

Pulp samples from the L and Po stages in all sequences were analysed for HexA. As can be seen from Table 4-2, the xylanase treatment successfully removed a substantial fraction of HexA in the initial pulp (27%) by releasing xylans chains from fibre surfaces. Interestingly, laccase reduced the HexA content in the control treatment, especially at the higher enzyme dose. HexA reduction was more marked in the laccase–VA treated pulp, to the same extent as that produced by xylanase, which suggests the laccase–VA system can destroy HexA by oxidizing their double bonds similarly to electrophilic bleaching agents (Ventorim *et al.* 2008). The pulp samples treated with the laccase–SLD system exhibited no reduction in HexA content, which confirms the disparate behaviour of the enzymatically oxidized natural mediator towards fibres. Finally, the pulp samples from

the sequences including an X stage exhibited a reduction in HexA with respect to those subject to no X pre-treatment, which can be ascribed to the xylanase treatment since no synergistic effect between the xylanase and the laccase–mediator systems was observed.

Table 4-2. Xylan content (% odp) (± standard deviation) of initial and enzymatically (X and L) treated pulps from the bleaching sequences. HexA content (± standard deviation) of initial and xylanase-treated pulps, and pulps after L and P_O stages.

	% XYL	HexA (μmol/g)	
Initial	16.3 ± 0.4	41.4 ± 1.1	
X	14.1 ± 0.3	30.1 ± 0.7	
	L	L	P_O
L20	16.2 ± 0.4	36.4 ± 4.8	36.2 ± 0.3
L40	15.7 ± 0.5	31.5 ± 0.1	30.6 ± 1.5
$L20_{VA}$	15.8 ± 0.3	26.6 ± 0.4	25.2 ± 0.7
$L40_{SLD}$	15.2 ± 0.3	32.7 ± 0.4	31.8 ± 3.3
$XL20_{VA}$	13.0 ± 0.4	23.7 ± 1.9	23.8 ± 2.7
$XL40_{SLD}$	12.1 ± 0.5	26.7 ± 0.7	25.8 ± 0.8

Residual lignin and HexA are known to be the main substances contributing to kappa number in pulp fibres (Costa and Colodette 2007, Li *et al.* 2002, Valls *et al.* 2010c). In this work, their contribution was estimated in pulp samples from the L and Po stages in all bleaching sequences, as well as in the initial and xylanase pre-treated pulp (Figure 4-3), in order to assess the actual delignifying effect of the different treatments. The xylanase treatment decreased KN_{lig} for the initial pulp from 3.9 to 2.1, which indicates substantial delignification by effect of the enzyme. This reflected in the decreased KN_{lig} value obtained in both the L and Po stage after the X treatment. Interestingly, the final KN_{lig} value for the $XL40_{SLD}$ sequence was slightly smaller than that for the $XL20_{VA}$ sequence in spite of the significant increase due to the laccase–SLD system in the L stage. This indicates that oxidative degradation of lignin by the laccase–natural mediator system is enhanced after the xylanase treatment and results in the best delignifying performance at the end of the bleaching sequence.

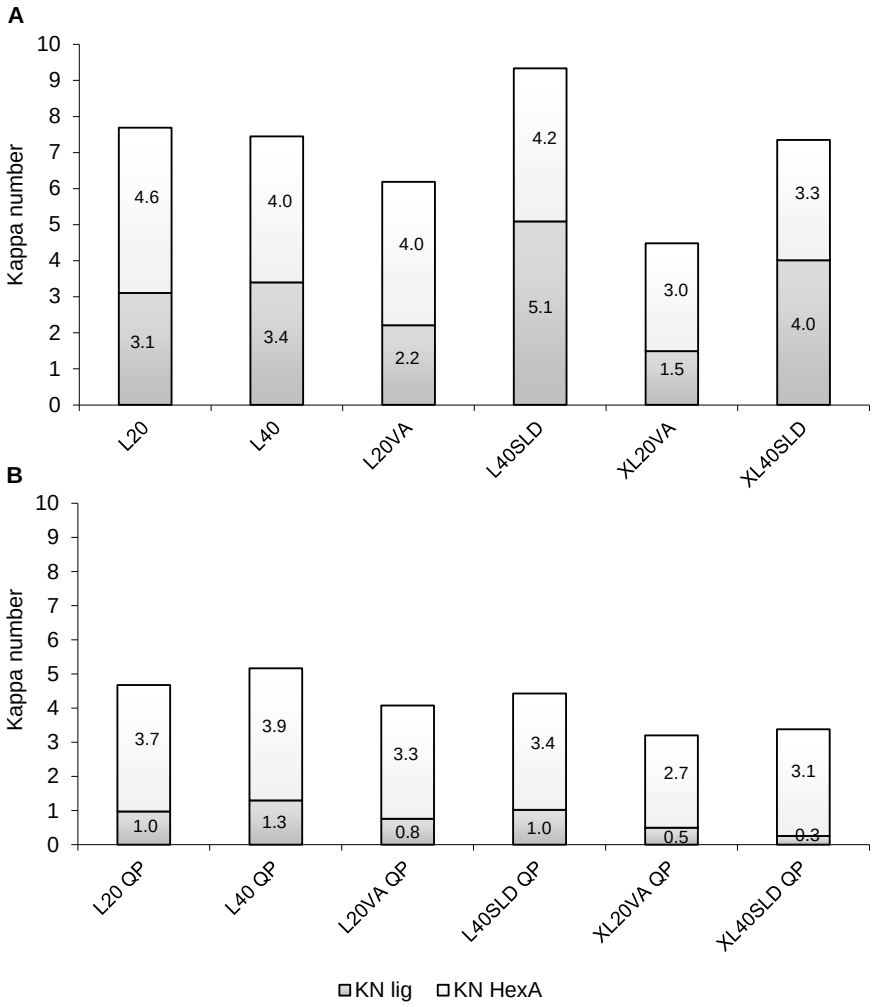

Figure 4-3. Contributions to kappa number of pulps obtained from the L (A) and P_O (B) stages of each bleaching sequence due to lignin and to HexA.

4.3.2. Effluent properties

Table 4-3 shows the COD and colour values, respectively, as determined in the effluents from each stage of the studied bleaching sequences. As can be seen, the xylanase treatment produced a high COD accounting for more than 40% of the total value for the sequences including an X stage. This can be ascribed to substantial carbohydrate

degradation and lignin removal at this stage, which testifies to the efficiency of the enzyme (Fillat and Roncero 2009). The substantial contribution of X to the total COD for the bleaching sequence is consistent with previously reported results (Siles *et al.* 1996) and is the greatest disadvantage of using xylanases in bleaching processes. In all instances, COD after the L stage was markedly higher than it was after Q and P_O; this was mainly a result of the presence of sodium tartrate buffer and the use of commercial laccase (Fillat *et al.* 2010, Fillat and Roncero 2009), as suggested by the high values obtained in the control treatments. The effluents from the L stage carried out in the presence of the mediator exhibited higher COD values than the control process by effect of the presence of the mediator and of degradation products of the mediator and lignin (Aracri *et al.* 2009, Fillat and Roncero 2009a). When the laccase–mediator treatments were inserted after the X stage, the resulting effluents had higher COD levels than those obtained in the absence of an X pre-treatment. This increase can be ascribed to further dissolution of xylans during the L stage when performed after the xylanase treatment, as confirmed by the carbohydrate analysis of the pulp samples. COD for the effluents from the Q and Po stages performed after the laccase–mediator treatments were lower in the sequence including an X stage; however, the combined COD for all stages was significantly higher when a xylanase treatment was applied.

Table 4-3. COD and colour values (± standard deviation) of effluents obtained from xylanase treatment, and L, Q and Po stages of each bleaching sequence.

	COD (kg O_2/t_{pulp})			Colour (kg Pt/t_{pulp})		
X	100 ± 3			4.4 ± 0.1		
	L	Q	P_O	L	Q	P_O
L20	94 ± 2	10 ± 1	11 ± 2	3.3 ± 0.1	2.6 ± 0.1	2.1 ± 0.1
L40	117 ± 13	3 ± 1	8 ± 1	4.2 ± 0.1	1.8 ± 0.1	1.4 ± 0.0
$L20_{VA}$	99 ± 4	6 ± 3	13 ± 5	15.3 ± 0.5	3.0 ± 0.1	1.4 ± 0.0
$L40_{SLD}$	131 ± 4	15 ± 3	11 ± 2	78.2 ± 2.3	13.7 ± 0.4	2.0 ± 0.1
$XL20_{VA}$	103 ± 4	4 ± 1	8 ± 1	16.3 ± 0.5	3.3 ± 0.1	0.8 ± 0.0
$XL40_{SLD}$	136 ± 1	7 ± 1	8 ± 1	90.9 ± 2.7	10.7 ± 0.3	2.0 ± 0.1

Similarly to COD, the colour of the effluents produced in the L stage was higher than the values measured after the Q and P_O stages in all cases, and decreased in the sequence L > Q > P_O. The effluents from the L stage performed in the presence of a laccase–mediator system exhibited a marked increase in colour relative to the control effluents by effect of the increased amount of chromophoric species resulting from oxidation and/or degradation of lignin and the mediators. This effect was particularly strong in the presence of the laccase–SLD system, which was also responsible for the marked drop in brightness and increase in k/s ratio in the treated pulp (Aracri *et al.* 2009). The effluents from the Q and P_O stages involving SLD-treated pulp samples continued to exhibit some effect of the presence of chromophoric species produced by the natural mediator in the L stage, their colour being significantly higher than in the effluents from the VA treatments. Similarly to COD, the colour of the effluents from the L stage performed after xylanase treatment was higher than in the absence of an X stage.

One of the major hurdles for industrial implementation of LMS-based processes is concern about the potential toxicity of the synthetic mediator, which recently promoted a search for alternative, environmentally safe natural compounds (Camarero *et al.* 2005, Camarero *et al.* 2007, Gutiérrez *et al.* 2007, Moldes *et al.* 2008). However, little research has previously been conducted to evaluate the toxicity of effluents from LMS-based bleaching processes and compare natural and synthetic mediators in this respect (Aracri *et al.* 2009, Fillat *et al.* 2010, Fillat and Roncero 2009b). Toxicity tests provide a valuable tool for assessing the environmental hazards of industrial wastewater. Spanish legislation has imposed a limit on the composition of discharged inhibitory matter (IM); thus, the Catalonian regional government has passed a specific regulation (Decree 130/2003) on public sewage services to establish an emission limit value (ELV) for IM discharges at 25 equitox/m^3 [1 equitox/m^3 = 1 T.U. = (1/EC_{50})*100]. The bioassay with *V. fisheri* is of technical interest according to Spanish regulations. In this work, we used it to assess toxicity in the effluents produced during the different bleaching sequences with emphasis on the contribution of the mediators used in the L stage. Toxicity data are reported in Table 4-4. The effluents analysed were those from the X and L stages, which had the strongest impact on COD and colour of the bleaching sequences. As can be seen, the treatments with xylanase and laccase in the absence of a mediator led to low toxicity values, which were raised above the Catalonian ELV by the LMS treatment —to a similar extent in the presence and absence of a xylanase pre-treatment. The increase was especially marked with the laccase–SLD treatment, the resulting effluent being roughly 6

times more toxic than that from the laccase–VA treatment —the latter being only slightly higher than the ELV. In order to determine whether the impact on effluent toxicity was due to the laccase-oxidized or reduced form of the mediator, and to evaluate the influence of pulp, bioassays were performed on effluents from L stages without pulp and on solutions of the mediators at the same concentrations as in the reaction medium . The results showed that VA, whether in its reduced or oxidized form, introduced no toxicity in the effluents; by contrast, reduced SLD led to a toxicity level slightly below the ELV that was dramatically increased after laccase oxidation, probably through the formation of stable radicals and by-products from the mediator (Aracri *et al.* 2009). The addition of pulp to the reaction medium at the L stage increased toxicity in the effluents, particularly with the laccase–VA treatment; this can be ascribed to the dissolution of degradation products from lignin. The effluents from the stages following the L treatment were expected to exhibit very low toxicity as previously found by Fillat *et al.* (2010) (Fillat *et al.* 2010). In fact, only the effluents from the Q and P_O stages involving LMS-treated pulp were analysed to assess the effect of the mediators throughout the bleaching sequences, and they showed very low toxicity levels with both mediators (*ca.* 2 T.U.). The previous results are all consistent with those reported by other authors for effluents from LMS-based processes (Fillat *et al.* 2010, Fillat and Roncero 2009b).

Table 4-4. Toxicity values (± standard deviation) of effluents obtained from X and L stages of each bleaching sequence, effluents obtained from L treatments performed without pulp of the sequences $L20_{VA}QP_O$ and $L40_{SLD}QP_O$, and of solutions of mediators at the same concentration as in the L treatment.

	Toxicity (T.U.)
Enzymatic stages	
X	4 ± 0
L20	11 ± 3
L40	7 ± 0
$L20_{VA}$	33 ± 4
$L40_{SLD}$	208 ± 28
$XL20_{VA}$	38 ± 2
$XL40_{SLD}$	165 ± 5
L stage without pulp	
$L20_{VA}$ (no pulp)	1 ± 0
$L40_{SLD}$ (no pulp)	195 ± 57
Mediator solution	
VA	2 ± 0
SLD	20 ± 10

4.4. Conclusions

This work was designed to assess the potential of a xylanase pre-treatment of sisal pulp for boosting bleaching and HexA removal during a bleaching sequence, as well as to compare the effectiveness of VA and SLD as laccase mediators for aiding lignin and HexA removal. The pre-treatment with xylanase exhibited substantial delignification and HexA removal effects on the initial sisal pulp. The laccase–VA system was efficient in decreasing kappa number and raising brightness in all stages, as well as in significantly reducing the HexA content. On the other hand, the laccase–SLD system increased kappa number and decreased brightness in the L stage through adsorption and/or covalent binding to fibres, thereby failing to reduce the HexA content; if an X pre-treatment was applied, however, the laccase–SLD treatment exhibited the strongest delignification effect

at the end of the bleaching sequence and provided sisal fibres with a high cellulose content.

Analysis of the effluents showed the enzymatic stages to provide the most significant contribution to total COD and colour in the bleaching sequences. The effluents from the LMS stages exhibited the highest toxicity, which clearly exceeded ELV in the presence of SLD. Toxicity was due mainly to the presence of pulp in the laccase–VA treatment and to oxidation of the mediator by laccase in the laccase–SLD treatment.

4.5. Addendum to Publication 2: Fate of lipophilic extractives of sisal pulp during TCF biobleaching sequences

Summary

This addendum provides a chemical characterization of the main lipophilic extractives from sisal pulp and information into the extent of their removal along the bleaching sequences performed in Publication 2. Lipophilic compounds may play an important role during the pulp and paper manufacturing processes since they are the origin of "pitch" deposits, causing drastic decreases in the final product quality and affecting negatively the runnability of the paper machine.

Materials and methods

The pulp samples from the TCF biobleaching sequences studied in Publication 2 were analysed by GC/MS at the IRNAS in Seville, Spain. The lipophilic extractives were determined after the X, L and Po stages of the different sequences.

Pulps were air dried until constant weight and the samples were Soxhlet-extracted with acetone for 8 h. All the extracts were evaporated to dryness and redissolved in chloroform for chromatographic analyses of the lipophilic extractives. The GC and GC/MS analyses were performed following the methodology previously developed by Gutiérrez *et al.* (1998).

The GC analyses were performed 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.

Results and discussion

It has been demonstrated that laccase-mediator system is capable to reduce the content of sterols from eucalyptus pulp, not only using HBT (Gutiérrez *et al.* 2006, Valls *et al.* 2009), but also using natural mediators (Babot *et al.* 2011, Gutiérrez *et al.* 2007). For this reason, the content of lipophilic extractives in sisal pulp samples from the different bleaching stages was monitored. Marques *et al.* (2010) followed the fate of lipophilic extractives from several non-wood species (bast and leaf fibres) during soda/AQ pulping and TCF and ECF bleaching processes, and identified for crude sisal pulp the more predominant lipids: free sterols, fatty alcohols, fatty acids, sterol glycosides and alkanes.

Table 4-5 reports the content of the main lipophilic compounds present in the unbleached sisal pulp employed in the TCF biobleaching sequences.

Laccase-VA treatment and the whole sequence involving the use of the synthetic mediator resulted in a reduced content of alkanes, free sterols and sterol glycosides from initial pulp, as shown in Table 4-6. The sequence including the X stage provided enhanced elimination of most of the lipophilic compounds compared to the same sequence performed in its absence, although the xylanase pre-treatment itself did not result in the removal of any lipophilic compound. In contrast to the effect of the laccase-VA system, no reduction in the content of lipophilic compounds was observed when laccase-SLD system was applied. A significant reduction of free sterols and sterol glycosides was observed at the end of the control sequence performed with the higher laccase dose.

Table 4-5. Content of the main lipophilic extractive compounds present in unbleached sisal pulp.

Lipophilic extractives	mg/100 g odp
Alkanes	
n-Pentacosane	3.26
Fatty acids	
n-Hexadecanoic	16.94
Fatty alcohols	
n-Octacosanol	1.84
Free sterols	
Sitosterol	1.22
Sterol glycosides	
Sitosterol 3β-D-Glcp	0.94

Table 4-6. Removal (percentage reduction) of the main lipophilic extractives from sisal pulp after L and P_O stages of the two control sequences and the sequences involving VA in the L stage, as well as after the xylanase pre-treatment (X stage).

	X	L20		L40		$L20_{VA}$		$XL20_{VA}$	
		L	P_O	L	P_O	L	P_O	L	P_O
Alkanes n-Pentacosane	0	0	0	0	0	51	23	43	60
Fatty acids n-Hexadecanoic	0	0	0	0	0	0	0	0	8
Fatty alcohols n-Octacosanol	0	0	0	0	10	0	0	0	27
Free sterols Sitosterol	0	0	2	14	47	45	63	33	56
Sterol glycosides Sitosterol 3β-D-Glcp	0	0	0	0	43	14	36	15	76

Figure 4-4 shows the chromatograms of pulps obtained from the L stage and the whole sequences involving VA, compared to that of L-treated control pulp. The chromatogram of pulp after the L stage performed with laccase-VA system clearly showed decreased intensity of the signals corresponding to sterol glycosides, in particular sitosterol 3β-D-Glcp, and to free sterols, in particular sitosterol.

When no xylanase pre-treatment was applied, the chromatogram of pulp resulting from the bleaching stage did not differ significantly from that exhibited by pulp treated in the previous stage with laccase-VA system. When an X stage was included in the sequence, the chromatogram of the pulp resulting from the P_O stage showed considerably reduced intensity in almost all the signals detected, compared to that of pulp bleached in the absence of the X pre-treatment. This indicates that the xylanase pre-treatment boosted the removal of lipophilic extractives in the subsequent bleaching stage by providing an increased accessibility to the bleaching agents.

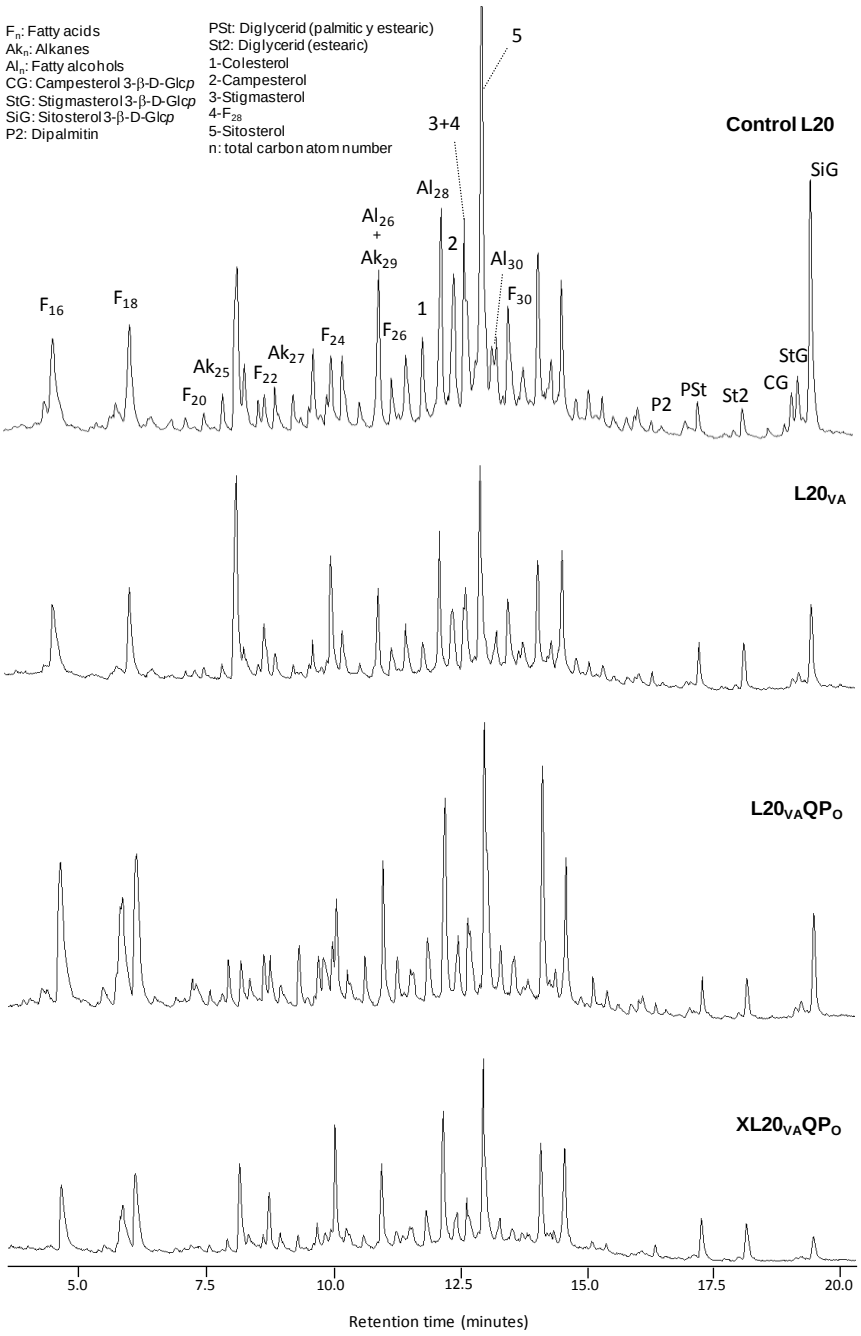

Figure 4-4. Chromatographic analysis of lipophilic extractives in sisal pulp samples after the L stages performed in the absence (control) and in the presence of VA, and the whole bleaching sequences involving VA, with and without xylanase pre-treatment ($XL20_{VA}QP_O$ and $L20_{VA}QP_O$, respectively).

Conclusions

The content of lipophilic extractives in sisal pulp was monitored along the TCF biobleaching sequences in order to evaluate the potential of the enzymatic systems for aiding the removal of these compounds and thus reducing the formation of pitch deposits.

It was found that the application of laccase alone at higher dose reduced the content of free sterols in the L stage and led to a decreased amount of sterol glycosides in the subsequent bleaching stage. Laccase-SLD system proved ineffective for further reducing the amount of lipophilic compounds compared to the corresponding control. In contrast, laccase-VA system enabled a significant reduction of alkanes, sterol glycosides and free sterols, being the latter the main responsible for pitch deposits. The xylanase stage did not reduce the content of lipophilic extractives, but did enhance their removal in the subsequent bleaching stage.

The combination of substantial savings of bleaching agents and effective removal of pitch compounds shows the high potential of the laccase-VA system for providing economic advantages in the development of enzymatic processes alternative to current industrial process.

4.6. References

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Enzymatic grafting of simple phenols on sisal pulp fibres using laccase

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Abstract

Sisal pulp was treated with laccase from *Trametes villosa* (*Tv*L) in the presence of different phenolic compounds (viz. coniferyl aldehyde, sinapyl aldehyde, ferulic acid and sinapic acid). In most cases the enzymatic treatments resulted in increased kappa number of pulp suggesting the incorporation of the phenols into fibres. The covalent binding of these compounds to fibre lignin was evidenced by the analysis of the treated pulps, after acetone extraction, by pyrolysis coupled with gas chromatography/mass spectrometry in the absence and in the presence of tetramethylammonium hydroxide (TMAH) as methylating agent. The highest extents of phenol incorporation were observed with ferulic acid. The present work shows for the first time the use of analytical pyrolysis as an effective approach to study fibre functionalization by laccase-induced grafting of phenols.

5.1. Introduction

The pulp and paper industry is under steady and ever-increasing pressure from global competition, stringent environmental regulations and new market demands. Enzymes are the most promising examples of new technologies designed to help the sector meet these challenges for their potential to supply specific reactions, to provide less environmentally deleterious processes, to reduce resources consumption and, ultimately, to decrease costs (Kenealy and Jeffries 2003, Ragauskas 2002). Since the raw materials employed for the manufacture of pulp and paper are natural fibres, the possibilities for introducing biotechnologies in this process are numerous (Bajpai 1999).

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). Their high availability at a reasonable price and broad oxidative capabilities make them suitable for a wide range of applications (Riva 2006, Widsten and Kandelbauer 2008).

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 *et al.* 2010, Valls and Roncero 2009). In previous works (Aracri *et al.* 2009, Fillat *et al.* 2010) 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 *et al.* 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 *et al.* 2003).

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

In this work, a novel approach of analysis was adopted to gain an insight on the mechanism of the laccase-induced coupling of natural phenols onto sisal pulp. In

particular, the treated pulps were analysed by analytical pyrolysis, a powerful and sensitive tool for the "in situ" analysis of residual lignin in pulps without the need of prior isolation (del Río *et al.* 2001). In addition, in order to avoid some of the analytical limitations of the pyrolysis technique, such as decarboxylation of carboxyl groups, the pyrolysis was also performed in the presence of tetramethylammonium hydroxide (TMAH) as methylating reagent (del Río *et al.* 1996), that allowed the detection of intact carboxylic acids as their methyl derivatives.

5.2. Materials and methods

5.2.1. Raw material, laccase and natural phenols

Sisal (*Agave sisalana*) alkaline pulp was obtained by soda-anthraquinone cooking and supplied by CELESA pulp mill (Tortosa, Spain). Initial pulp had a kappa number of 7.8, a viscosity of 784 ml/g, and an ISO brightness of 47.3 %. Pulp was treated with laccase and different phenols, as reflected in Table 1. 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. This procedure was necessary to remove contaminants and metals and to bring the pulp to the pH of the enzymatic treatments. *Trametes villosa* laccase (*Tv*L) was provided by Novozymes (Bagsvaerd, Denmark). Activity was followed by measuring the 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 that transforms 1 μmol/min of ABTS at 25 ºC. All measurements were carried out using a Shimadzu UV-Vis 1603. The phenolic compounds coniferyl aldehyde, sinapyl aldehyde, ferulic acid and sinapic acid were purchased from Sigma-Aldrich.

5.2.2. Laccase treatments and Soxhlet extraction

Laccase treatments were carried out in an oxygen pressurized (0.6 MPa) reactor at 30 rpm, 50 °C, for 4 h, in 50 mM sodium tartrate buffer (pH 4) using an amount of 40 g of sisal pulp at 5% consistency, 20U/g of *T. villosa* laccase and a proportion of 1.5% (w/w)

phenolic compound (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant.

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.

5.2.3. Evaluation of pulp properties

Brightness and kappa number of pulp before and after acetone extraction were assessed according to ISO 3688 and ISO 302, 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 1g of totally bleached pulp was supposed, were measured for different phenol concentrations. Thus, a calibration line was originated, providing the amount of grafted phenol in correspondence to the increase of kappa number produced by this compound with respect to laccase control (samples coming from the acetone extraction). Calibration lines were:

$$y = 150.75x + 0.0404 \quad \text{(SLD)}, \qquad [5\text{-}1]$$

$$y = 153.72x + 0.0336 \quad \text{(SNC)}, \qquad [5\text{-}2]$$

$$y = 147.36x + 0.1416 \quad \text{(CLD)}, \qquad [5\text{-}3]$$

$$y = 150.68x + 0.2774 \quad \text{(FRC)}, \qquad [5\text{-}4]$$

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

The optical properties of pulp were analysed using a reflectance measuring Technidyne Colour Touch apparatus at a standard illuminant/observer combination C/2°.

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 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).

Other optical parameters used were:

- *Chroma* (*C**):

Perpendicular distance from lightness axis, measure of colour saturation,

$$C^* = (a^{*2} + b^{*2})^{1/2} \qquad [5\text{-}5]$$

- *Dye removal index* (*DRI*) (Fluet and Shepperd 1997):

The percentage of original colour removed by the treatment,

$$DRI = -100[\Delta R^2/R_1^2] \qquad [5\text{-}6]$$

where,

$$R^2 = a^2 + b^2 + (100 - L)^2 \qquad [5\text{-}7]$$

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

$$\Delta R^2 = R_2^2 - R_1^2 = R^2 \text{ (for treated pulps)} - R^2 \text{ (for reference pulp)} \qquad [5\text{-}8]$$

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

5.2.4. 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 500 ºC. The oven temperature was programmed from 40 ºC (1 min) to 300 ºC at 6 ºC/min (10 min) and the carrier gas was set at (1 ml/min). 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 were 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).

5.3. Results and discussion

5.3.1. Pulp properties

All the phenolic compounds employed in the laccase treatments of sisal pulps resulted in increased kappa numbers, with respect to the control pulp (Figure 5-1), suggesting the occurrence of grafting copolymerization reactions of the phenolic compounds with the fibres lignin. After acetone extraction, a slight decrease of kappa number was observed in all pulps; however, all the laccase treated pulps still presented a kappa number higher than the control pulp, thus indicating that a binding of these simple phenols onto fibres effectively occurred.

The kappa numbers of the acetone-extracted pulps indicated a higher degree of grafting for the guaiacyl-type phenolic compounds, FRC and CLD (causing 42 % and 36 % kappa number increases, respectively) than for the syringyl-type phenolic compounds, SNC (23 % increase) and SLD (16 % increase). This difference may be due to the steric

effects of the structure of the phenols. SNC and SLD are of syringyl-type, bearing two methoxy groups, and present more steric hindrance than FRC and CLD, of guaiacyl-type (monomethoxylated), which increases the long-living of the phenoxy radicals and prevents coupling reactions, thus resulting in less grafting onto the fibres (Astolfi *et al.* 2005). The minimum amount of grafted phenol for the laccase treated sisal pulps was estimated to be 6.6, 9.6, 17.3 and 16.9 μmol/ g of pulp for SLD, SNC, CLD and FRC, respectively.

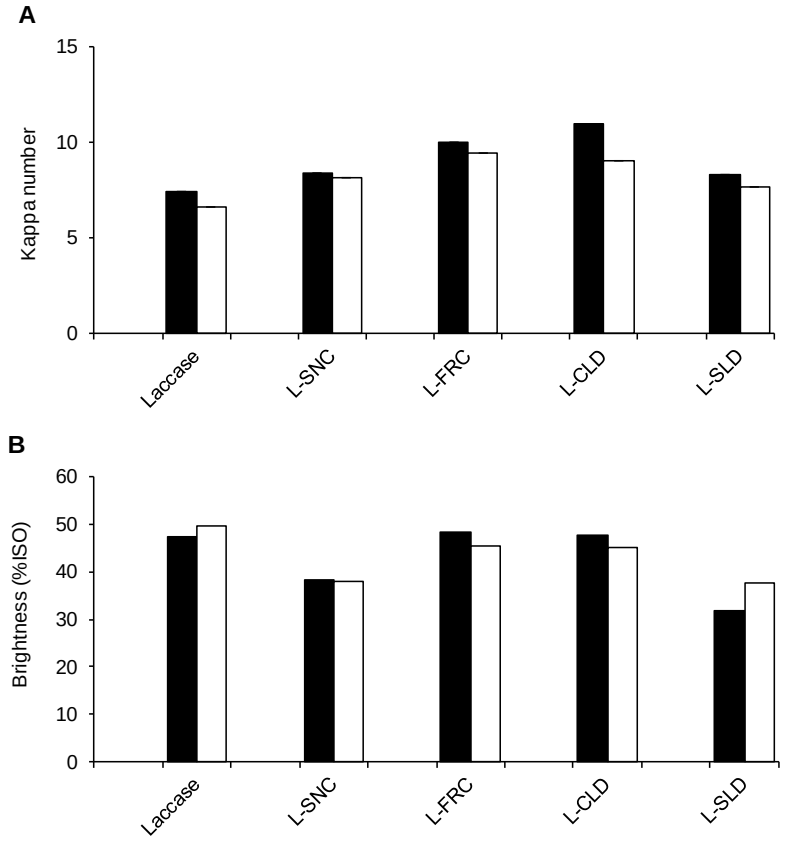

Figure 5-1. Kappa number and brightness of sisal pulps after the enzymatic treatment (black bars) and the subsequent Soxhlet extraction with acetone (white bars). Laccase control sample was treated in the absence of phenolic compounds.

A marked decrease of brightness, respect to the control pulp, was observed in the pulps treated with the syringyl-type phenols, SNC (18 % decrease) and SLD (32 % decrease), while this parameter was barely modified after treatment with the guaiacyl-type phenols, FRC and CLD. After acetone extraction, no improvement of brightness was obtained in the treated pulps, by exception of that treated with SLD, in which the removal of the adsorbed products of oxidation resulted in 18.5 % increase of brightness compared to the non-extracted sample.

The optical properties corresponding to L* and C* data of the laccase treated sisal pulps are shown in Figure 5-2. Similarly to brightness, Chroma and Lightness properties of FRC and CLD treated pulps did not result in significant difference with respect to control pulp, whilst a marked increase of colour saturation was induced by SNC and SLD (26.6 % and 25.4 % respectively), the latter being accompanied by a pronounced loss of L* (16 %). The removal of the adsorbed phenol from pulps by acetone extraction resulted in enhanced Lightness in all enzymatically treated pulps. However, no Chroma decrease was observed in any case, indicating that the quinone-like oxidized compounds were still present in the extracted pulps. DRI values (Table 5-2) followed the same trend as C* data, being more negative after acetone extraction than after the L stage, by exception of SLD-treated pulp, which also experimented the most important Lightness gain after the extraction.

Table 5-1. Dye Removal Index of sisal pulp after treatment with laccase and natural phenols, and after Soxhlet extraction.

	SNC	FRC	CLD	SLD
L-phenol treatment	-58.72	5.07	-0.64	-155.26
Soxhlet extraction	-85.13	-29.33	-32.73	-80.27

Laccase control pulp used as reference (DRI = 0%).

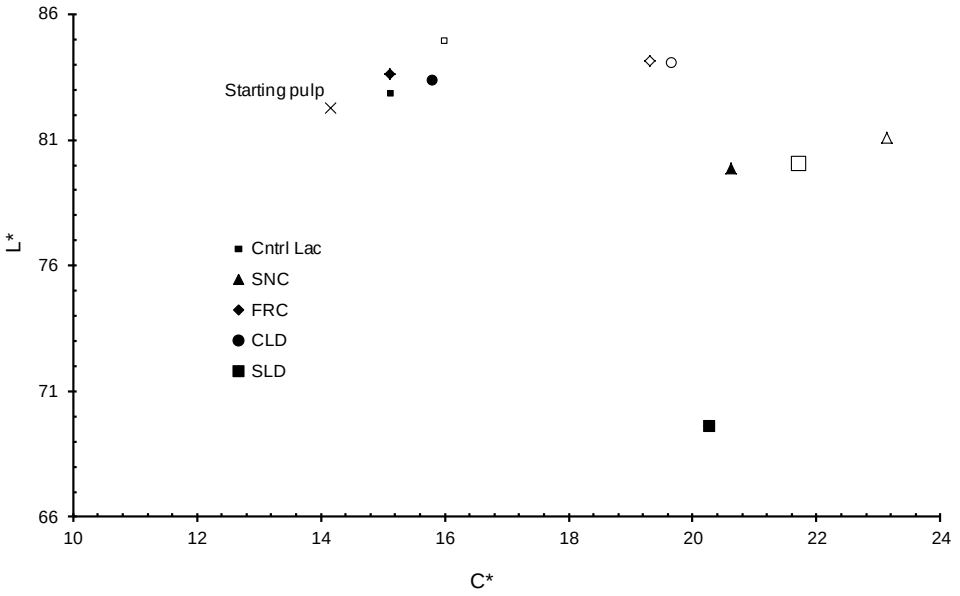

Figure 5-2. Chromatic coordinates (L* and C*) of sisal pulp treated with different enzymatic systems (black spots) and after a subsequent Soxhlet extraction with acetone (white spots). Laccase represents the control pulp sample: pulp treated only with laccase.

5.3.2. Py-GC/MS analysis

The analysis of the laccase treated pulps (after acetone extraction) by Py-GC/MS and Py/TMAH demonstrated the incorporation of some of these phenolic compounds into the fibres. Py-GC/MS of pulp treated with FRC released high amounts of 4-vinylguaiacol, a compound arising from the decarboxylation of the FRC during pyrolysis (del Río *et al.* 1996), and which was completely absent in the control pulps. This data was further confirmed by Py/TMAH of this pulp that released intact FRC as its methyl derivative (i.e. the methyl ester of 3,4-dimethoxycinnamic acid), which was absent in the control pulp (Figure 5-3), indicating that FRC was covalently bound to the pulp. In the case of sisal pulp treated with CLD, Py-GC/MS released some structurally related compounds, such as 4-vinylguaiacol, but not CLD as such. The analysis of this pulp by Py/TMAH also released other structurally related compounds (i.e. vanillin and ferulic acid, as their methyl derivatives) but not intact CLD. This seems to indicate that CLD did not incorporate as such into the sisal pulps but in a different or modified form, probably as vanillin and/or ferulic acid. In the case of SNC, Py-GC/MS and Py/TMAH did not release

any compound that could clearly indicate its incorporation as such into the pulps, although a significative increase of syringic acid (as its methyl derivative) was observed by Py/TMAH. This could indicate that SNC was probably incorporated into the pulps as syringic acid. In the case of SLD, neither Py-GC/MS nor Py/TMAH could detect any phenolic marker indicative of its incorporation into the pulp, and therefore, in this particular case, we could not firmly demonstrate the incorporation of SLD to the pulp fibres.

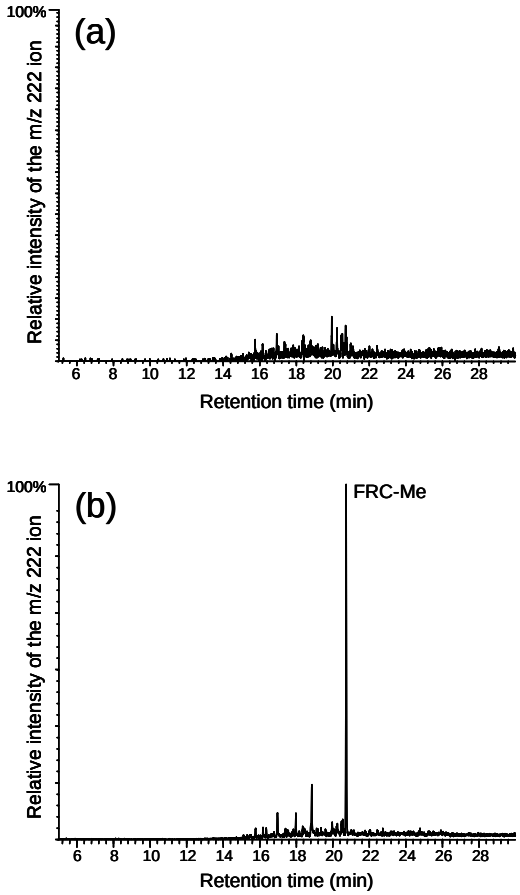

Figure 5-3. Analysis by Py-GC/MS in the presence of TMAH (Py/TMAH) of (a) sisal pulp control (treated with laccase alone), after acetone extraction, and (b) sisal pulp treated with laccase and FRC, after acetone extraction. Single ion chromatograms of the fragment at *m/z* 222, characteristic of ferulic acid methyl derivative (FRC-Me) are shown.

5.4. Conclusions

The enzymatic treatment of sisal pulp with laccase in the presence of simple phenols resulted in their incorporation into the pulps. This assertion is based on the pulp properties obtained, and on the Py-GC/MS and Py/TMAH results. This enzymatic system

could be regarded as a method for the grafting of phenols onto the pulp fibres, which may give them improved or novel properties.

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Studying the effects of laccase-catalysed grafting of ferulic acid on sisal fibres

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Abstract

Functionalization of sisal specialty pulp fibres by laccase-catalysed grafting of ferulic acid (FRC) was investigated. To this end, the extent of phenol coupling to fibres under different reaction conditions (laccase and FRC rates, and time) was evaluated in terms of pulp properties including kappa number (expressed as the combined contributions of lignin and hexenuronic acids), brightness, Klason lignin and surface anionic charge after Soxhlet extraction of acetone-treated pulp. The specific treatment resulting in the highest degree of grafting was then used in a comparative study of the effects of applying the laccase–FRC system to refined and unrefined pulp with a view to confirming whether the increased surface area obtained by effect of fibrillation would lead to enhanced grafting. Based on the results, refining the pulp prior to the enzyme treatment resulted in increased grafting which in turn led to handsheets with improved strength-related properties (particularly wet tensile strength) relative to control samples.

6.1. Introduction

Progress in pulp and paper research has led to an increasing adoption of enzyme technology on the grounds of its potential as an effective, environmentally friendly aid to improving pulp and paper production in a number of respects (Bajpai 1999). Among the most widely investigated enzymes for this purpose are laccases (EC. 1.10.3.1). These are multi-copper oxidases produced by microorganisms and plants which catalyse the monoelectronic oxidation of phenols and aromatic or aliphatic amines to reactive radicals with concomitant reduction of oxygen to water (Claus 2004, Riva 2006). The last decade has witnessed a remarkable rise in the number of publications reporting the use of laccases to functionalize lignocellulosic materials with a view to imparting improved or novel properties to fibre-based products (Widsten and Kandelbauer 2008). This use has been facilitated by the non-specific substrate requirements of laccase and the tendency of phenolic compounds to undergo coupling reactions with lignin following enzymatic oxidation to resonance-stabilized phenoxy radicals (Chandra and Ragauskas 2002, Milstein *et al.* 1994). Laccase-induced coupling of phenols or certain other types of low-molecular weight compounds, known as “laccase biografting”, provides a flexible method

of fibre functionalization; thus, depending on the choice of the laccase substrate, lignocellulosic materials can be imparted with various desirable properties.

Some authors found laccase-catalysed grafting of phenolic acids to kraft pulp fibres to increase tensile and burst strength in the resulting paper (Chandra and Ragauskas 2002, Chandra *et al.* 2004); the improvement was ascribed to the ability of carboxyl groups to promote inter-fibre hydrogen bonding and fibre swelling (Barzyk *et al.* 1997, Scallan 1983), as well as cross-linking of phenoxy radicals in paper sheets. Other studies have shown substantially improved wet tensile strength in kraft pulp after treatment with a combination of laccase and a lignin-rich extractive or mediator; this effect was ascribed to enhanced production of phenoxy radicals increasing cross-linking between fibres and facilitating water resistant inter-fibre bonding as a result (Lund and Felby 2000, 2001). Laccase biografting has been successfully used to develop antibacterial properties in various types of lignocellulosic substrates. Widsten *et al.* (2010) reported an important improvement in antibacterial resistance against *S. Aureus* in wood veneers and kraft pulp upon treatment with laccase and hydrolysable tannins. Fillat *et al.* (2011) showed laccase-induced coupling of different lignin-derived phenols to flax fibres to significantly increase their antibacterial activity towards *K. pneumoniae*, *P. aeruginosa* and *S. Aureus*. In a study by Elegir *et al.* (2008), the antibacterial properties of unbleached kraft paper towards *S. Aureus* and *E. Coli* were boosted by their laccase-assisted modification with natural phenols including caffeic acid and isoeugenol, or their oligomeric coupling products.

Recent examples of the use of laccase biografting to tailor lignocellulosic materials have been reported by Kudanga *et al.* (2010) and Garcia-Ubasart *et al.* (2011a, 2011b), who found enhanced hydrophobicity on surface veneers and in kraft pulp, respectively, by effect of oxidative coupling of hydrophobic phenols. Kudanga *et al.* provided mechanistic evidence of a covalent attachment of fluorophenolics onto wood surfaces, which increased hydrophobicity up to 65.5% relative to the same treatments in the absence of laccase. Garcia-Ubasart *et al.* proposed an innovative method for internal sizing in the papermaking process based on an enzyme treatment of pulp using laccase in combination with various hydrophobic substrates among which lauryl gallate proved the most effective towards developing internal sizing in the resulting paper. Recently, our group (Aracri *et al.* 2010) investigated the laccase biografting of simple phenols on flax and sisal specialty pulp fibres, and exposed covalent binding of these compounds to fibres

by using a novel analytical approach. Specifically, treated pulp samples were subjected to analytical pyrolysis, a powerful, sensitive tool for the *in situ* analysis of residual lignin in pulp requiring no prior isolation (del Río *et al.* 2001) that was coupled with gas chromatography/mass spectrometry in the absence and/or presence of tetramethylammonium hydroxide as methylating agent. *p*-Coumaric and ferulic acids exhibited the greatest extent of incorporation into flax and sisal fibres, respectively, among the assayed phenolic compounds. In the present work, we further investigated laccase-catalysed grafting of ferulic acid on unbleached sisal pulp fibres and evaluated the extent of phenol coupling under different reaction conditions via selected pulp properties. Also, residual laccase activity and effluent toxicity were assessed after each treatment. The particular conditions ensuring the highest degree of ferulic acid grafting were subsequently used to study the enzymatic functionalization of refined pulp in order to confirm whether the increased surface area obtained by effect of fibrillation during the mechanical treatment resulted in enhanced grafting.

6.2. Materials and methods

6.2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Sigma–Aldrich and used as received. Laccase from *Trametes villosa* was supplied by Novozymes (Bagsvaerd, Denmark) and frozen until use. Unbleached sisal (*Agave sisalana*) pulp from a soda–anthraquinone cooking process was supplied by CELESA pulp mill (Tortosa, Spain). The pulp, at 2% consistency, was conditioned with H_2SO_4 at pH 4 under stirring for 30 min, which was followed by passage through a glass filter funnel and extensive washing with de-ionized water. This step was needed to remove contaminants and metals, and also to bring the pulp to the pH required for the enzyme treatment. The most salient properties of the initial pulp were as follows: 7.4 ± 0.3 kappa number, 52.1 ± 0.1% ISO brightness, 1.1 ± 0.1% Klason lignin, a hexenuronic acid (HexA) content of 36.0 ± 3.0 μmol/g odp (oven dried pulp) and a surface anionic charge of 60.3 ± 5.0 μeq/g odp.

6.2.2. Enzyme assay

Laccase activity was determined by oxidation of 2,2'-azino*bis*-(3-ethylbenzylthiozoline-6-sulfonate) (ABTS). One activity unit (U) was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical (ε_{436} nm = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C.

6.2.3. Pulp treatments

Pulp treatments were performed by using an amount of 40 g odp sisal pulp at 5% consistency in 50 mM sodium tartrate buffer at pH 4 in the presence of a 20, 40 or 50 U/g laccase concentration and a proportion of 1.5% or 3.5% (w/w) ferulic acid (FRC) (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant. Treatment runs were conducted in a reactor under pressurized O_2 (0.6 MPa) at 30 rpm at 50 °C for 1 or 4 h. Pulp samples treated under identical conditions in the absence of FRC were used as controls. After the enzyme treatment, the pulp samples were passed through a glass filter funnel and extensively washed with de-ionized water.

6.2.4. Pulp properties

Pulp properties were analysed after Soxhlet extraction of each pulp with acetone (Aracri *et al.* 2010) in order to remove the fraction of FRC that failed to couple with the pulp. Kappa number and brightness were determined according to ISO 302 and ISO 3688, respectively. The hexenuronic acid (HexA) content was determined by UV spectroscopy (Chai *et al.* 2001). An estimate of the actual lignin and lignin-bound ferulic acid content of the pulp was obtained by determining the kappa number due to lignin (KN_{lig}) (Li *et al.* 2002, Valls *et al.* 2010). This involved measuring kappa number following removal of HexA by acid hydrolysis with mercury acetate and efficient washing with de-ionized water.

The surface anionic charge of the fibres was determined by polyelectrolyte titration, using a particle charge detector (Mütek PCD 03, Germany). The cationic polyelectrolyte used for adsorption was polydiallyldimethylammonium chloride (poly-DADMAC) (BTG

Mütek GmbH, Germany). The polymer molar mass and charge density were ~1.5 × 10^5 and 2.656 meq/g, respectively. Prior to charge measurements, each pulp sample was converted to its fully protonated form by soaking at 1% consistency in 0.01 M HCl for 30 min. This was followed by washing with de-ionized water until the conductivity of the filtrate fell below 5 μS/cm, and by conversion to the sodium form by soaking in 0.001 M $NaHCO_3$ at ~1% consistency for 10 min. The pH was then adjusted to 9 with NaOH and held constant for 30 min. The pulp was subsequently washed with de-ionized water until the conductivity of the filtrate fell below 5 μS/cm. All adsorption measurements were carried out with pulp in its sodium form at 0.5% consistency in 0.001 N poly-DADMAC and 0.01 M NaCl. The pH was adjusted to 7.8 ± 0.1 in all adsorption runs. The suspension was stirred with a magnetic stirrer until anionic charge was completely neutralized by the cationic polyelectrolyte, which took about one hour. Fibres were separated from the solution by filtration in order to record dry weight. A filtrate volume of 10 mL was then pipetted into the cell of a PCD-03 titrator from (BTG Mütek GmbH, Germany) and titrated to endpoint (i.e. to a streaming potential of 0 mV) with 0.001 N sodium polyethylenesulphate (PES-Na). Blank samples were titrated in parallel exactly in the same way as those which had been in contact with pulp. Fibre charge was calculated from the following equation [6-1]:

$$q\ \mu eq\ \ g\ = \frac{V_o - V_{tit}\ * C_{p-D} * 1000}{m_{dry}} * \frac{V_{tot}}{V_{sample}} \qquad [6\text{-}1]$$

where V_0 is the volume of PES-Na consumed by the blank sample, V_{tit} that consumed by the fibre samples, $C_{p\text{-}D}$ the concentration (N) of cationic polyelectrolyte, V_{tot} the total volume of filtrate, V_{sample} the volume of filtrate suspension used for titration and m_{dry} the sample dry mass.

Klason lignin in each pulp was determined as the fraction of lignin insoluble in sulphuric acid resulting from acid hydrolysis as described elsewhere (Aracri and Vidal 2011). All pulp analyses were conducted in duplicate.

6.2.5. Effluent toxicity

Effluent toxicity was 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: 1999 standard. The difference between the amount of light emitted before and after addition of the sample was used to determine toxicity. In order to prevent pH effects, each sample was adjusted to pH 6–8 with an NaOH solution. Toxicity was quantified as EC_{50}, which is defined as the effective concentration of sample reducing the light emission intensity by 50% after 15 min of contact. EC_{50} is inversely proportional to biological toxicity, expressed in toxicity units (TU). The reference toxicant $ZnSO_4 \cdot 7H_2O$ was used to control *V. fischeri* batch quality in accordance with the Basic Test procedure. Toxicity measurements were colour-corrected as per the recommendations of the equipment manufacturer. All toxicity tests were conducted in duplicate.

6.2.6. Handsheet formation and physical testing

Handsheets were formed from the pulp samples provided by the treatment resulting in the highest extent of FRC coupling. Treated and untreated pulp samples were disintegrated for 30 000 revolutions (ISO 5263) and filtered through a Buchner funnel with the aid of a laboratory vacuum pump in order to form a wet fibre pad, the filtrate being recirculated twice to avoid losses of fines. Then, the samples were refined for 4500 revolutions according to ISO 5264-2 and, if previously treated, directly used to prepare handsheets. Untreated pulp samples were processed as described in Section 2.3 prior handsheet formation. Handsheets were prepared on a Rapid-Köthen laboratory former according to ISO 5269-2.

Once formed, the handsheets were conditioned at 23 °C at 50% relative humidity for at least 24 h before physical testing. Apparent density, dry tensile strength, tearing resistance, bursting strength and wet tensile strength were determined according to ISO 438, ISO 1924-3, ISO 1974, ISO 2758 and ISO 3781, respectively, and wet tensile index was measured in 15 mm wide specimen strips soaked in de-ionized water for 5 s.

6.3. Results and discussion

6.3.1. Effect of the treatment conditions on grafting

6.3.1.1. Pulp properties

Sisal pulp was treated with laccase from *T. villosa* and FRC under different reaction conditions in order to evaluate the influence of the laccase and FRC rates, and reaction time, on the extent of grafting. In previous work (Aracri *et al.* 2010), we demonstrated the covalent coupling of FRC to sisal fibres in the presence of laccase by Py-GC/MS and Py/TMAH: the former released large amounts of 4-vinylguaicol and the latter intact FRC as its methyl derivative, both of which were absent from the control pulp. These results were consistent with the marked increase in kappa number observed in treated fibres. In the present work, we investigated the laccase-catalysed grafting of FRC in pulp subjected to other treatments where the enzyme rate, FRC rate or time was changed with respect to the conditions used in the previous study (viz. 20 U laccase/g, 1.5 wt% FRC and 4 h, respectively). Pulp treated with 20 U laccase/g alone for 4 h was used as control. Grafting reflects in a change in various fibre properties depending on the nature of the particular grafted compound. In this work, we evaluated grafting in differently treated pulp samples via changes in kappa number, brightness, Klason lignin and surface anionic charge. Since sisal pulp fibres were recently found to contain a substantial amount of hexenuronic acids (HexA) contributing to kappa number (Aracri and Vidal 2011), we chose to remove them in order to determine the contribution to kappa number of lignin and lignin-derived compounds such as FRC. Measuring kappa number prior to and after HexA removal allowed us to quantify the contributions of both types of substances. As can be seen from Figure 6-1A, the pulp sample treated with the lowest enzyme rate exhibited the highest contribution of HexA to kappa number; such a contribution was significantly decreased by the presence of 40 or 50 U laccase/g.

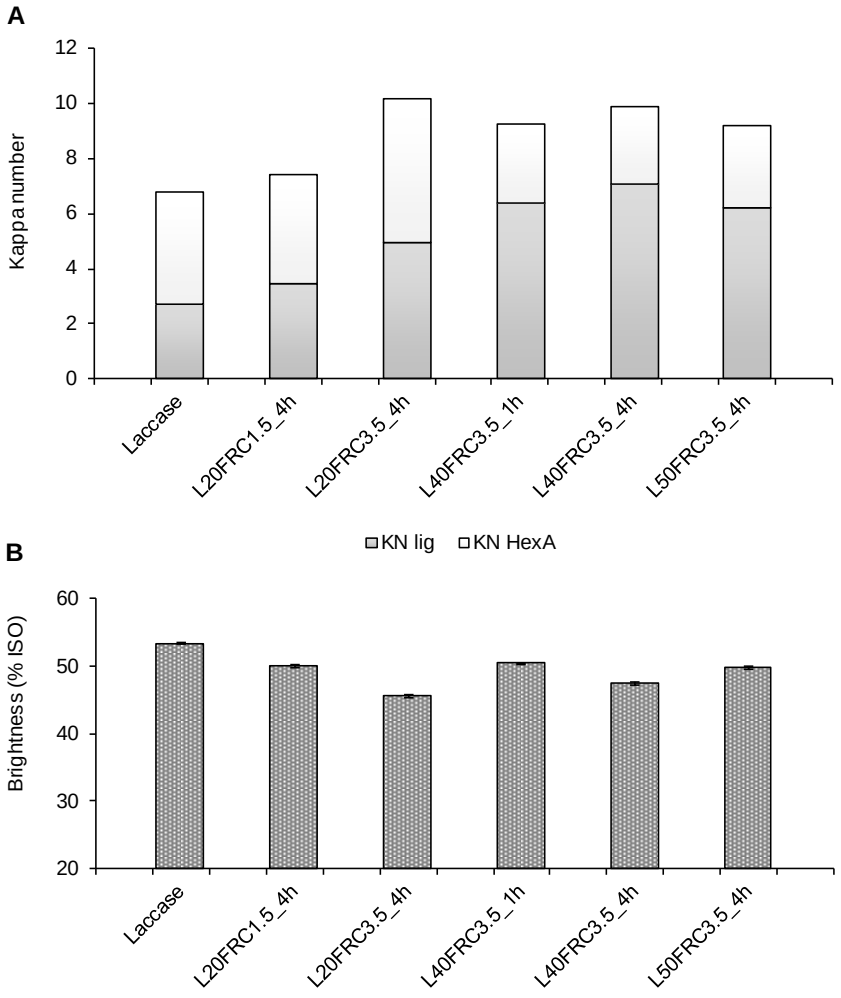

Figure 6-1. Kappa number, expressed as the combined contribution of lignin and HexA (A), and brightness (B) of pulp samples obtained with various laccase–FRC treatments. A treatment of 4h with laccase alone (20 U/g) was used as control. The errors associated with kappa number measurements were inferior to 3%.

Accordingly, the results of the HexA analysis (Table 6-1) showed a significant reduction in their content after treatment of the initial pulp with laccase alone (16% reduction) and also by effect of raising the laccase rate from 20 to 40 or 50U/g. This reduction in HexA content through oxidative modification by laccase is consistent with previously reported results (Aracri and Vidal 2011, Cadena *et al.* 2011). The addition of 1.5% of FRC resulted in decreased HexA content relative to the control pulp, probably as

a consequence of a coupling reaction between HexA double bonds and FRC in its phenoxy radical form (Cadena *et al.* 2011); on the other hand, the addition of 3.5% FRC in the presence of the enzyme at a low rate resulted in a slightly increased HexA content with respect to the control pulp which was probably a consequence of the higher reactivity of laccase towards the phenolic structure of FRC and a prevalence of polymerization reactions of FRC over the reaction with the isolated double bonds in HexA. Raising the FRC rate from 1.5% to 3.5% increased the kappa number due to lignin by 42% with 20 U laccase/g, and by 85% and 105% with 40 U laccase/g for 1 h and 4 h, respectively. Further raising the laccase rate in the treatment with 3.5% FRC for 4 h resulted in a slightly reduction of the kappa number due to lignin with respect to 40 U laccase/g.

Table 6-1. HexA and Klason lignin contents (± standard deviation) of treated pulps.

Pulp sample	HexA (μmol/g)	Klason lignin (%)
L20_4h	30.9 ± 0.8	1.2 ± 0.2
L20FRC1.5_4h	26.9 ± 0.8	1.2 ± 0.2
L20FRC3.5_4h	31.8 ± 0.5	1.2 ± 0.1
L40FRC3.5_1h	27.9 ± 2.3	1.6 ± 0.3
L40FRC3.5_4h	26.1 ± 1.0	1.7 ± 0.2
L50FRC3.5_4h	25.0 ± 0.6	1.6 ± 0.2

As can be seen from Figure 6-1B, the enzyme treatment not only increased kappa number, but also decreased pulp brightness as consequence of the incorporation of phenolic compounds into the fibres. Using a high FRC rate resulted in more marked brightness losses than with 1.5% FRC (particularly, the combination of a high FRC rate and a low laccase rate resulted in the greatest brightness reduction, but failed to maximize grafting). Similarly, the 1 h treatment with 40 U laccase/g and 3.5% FRC provided a brightness value close to that obtained with low rates of both the enzyme and phenolic compound, but a 85% higher KN_{lig} value. This suggests that brightness loss was affected not only by coupling of the aromatic compound, but also by the formation of chromophores absorbing light at 457 nm —the reflectance wavelength used for ISO

brightness measurement (Moldes *et al.* 2008)— via structural changes in FRC and/or lignin. Further evidence of FRC grafting was provided by the increased amount of Klason lignin (Table 6-1) and surface anionic charge (Figure 6-2) of the fibres. The increased amount of Klason lignin in the treated pulp samples reflects covalent coupling of FRC to the lignin component of the fibres.

Similarly to kappa number, titration of acid groups in fibres has also previously been used to estimate the amount of phenolic acids attached to pulp, and hence to assess grafting efficiency (Chandra and Ragauskas 2002, Elegir *et al.* 2008). The increase in surface anionic charge and Klason lignin of the treated pulp samples with respect to the control sample echoes that in the kappa number due to lignin and clearly shows that the treatment using 40 U laccase/g and 3.5% FRC for 4 h was that leading to the greatest extent of grafting.

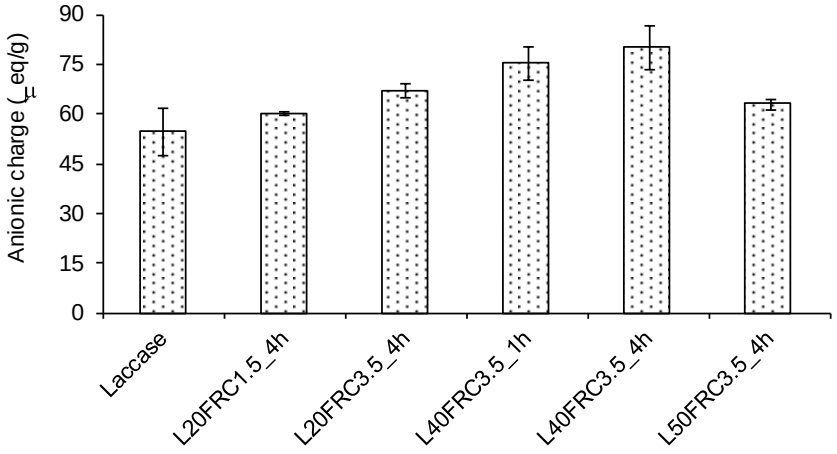

Figure 6-2. Surface anionic charge content of treated pulp fibres.

6.3.1.2. Effluents

Effluent analyses have scarcely been conducted to date (Aracri *et al.* 2009) in studies of laccase-based processes for functionalizing pulp fibres, even though such analyses are important for assessing the environmental impact of the effluents. Moreover, they provide an additional tool for interpreting the effects on pulp. In this work, we analysed the

effluents from the different enzyme treatments in terms of residual laccase activity and toxicity (Table 6-2). In previous work (Aracri *et al.* 2009), we monitored changes in enzyme activity during 4 h treatments at a small scale with 20 U laccase/g and various natural phenols including FRC at a rate of 1.5% in the absence and presence of pulp, and found the addition of pulp to the reaction medium to significantly increase residual activity and have virtually no effect in the presence of phenols with a less marked tendency to coupling to fibres than FRC. This result was ascribed to consumption of FRC phenoxy-radicals causing enzyme denaturation through coupling with fibres in the presence of pulp. Residual laccase activity values, measured at the end of the enzyme treatments, were expressed as percentages of the initial laccase rate. As can be seen, the enzyme residual activity was not adversely affected by the presence of FRC in the treatments; in fact, it remained at levels close to those of the control treatment when used at a rate of 20 U/g in the presence of FRC at both high and low rates, and increased only slightly when the enzyme was used at higher rates. The last effect can be ascribed to the use of increased enzyme rates as previously found by Fillat and Roncero (2009) in a study of effluents from a laccase–mediator delignification process. Residual laccase activity was found to steadily decrease with time in the treatment with a 40 U laccase/g and 3.5% FRC carried out for 24 h in a trial aimed at checking whether grafting was improved by effect of a prolonged treatment. A bioassay with *V. fisheri* was used to assess effluent toxicity as described elsewhere (Aracri *et al.* 2009, Fillat *et al.* 2010, Fillat and Roncero 2009). A solution of FRC in its reduced form at the same concentration as in the enzyme treatment using a 1.5% rate was found to exhibit a toxicity value of 7 TU (Aracri *et al.* 2009). The increased toxicity observed in the enzyme treatment can be ascribed to various factors including the formation of oxidized/radical species and degradation by-products derived from FRC and lignin. Laccase–FRC treatments raised the toxicity levels of the effluents above the emission limit value (ELV = 25 TU) for wastewater established by the Community of Catalonia. As expected, toxicity increased as the laccase and FRC rates were further raised. Interestingly, however, the effluent from the treatment with 40 U enzyme/g and 3.5% FRC exhibited a dramatic reduction in toxicity after the sixth hour of treatment. Since no enhanced FRC coupling was observed in the pulp (results not shown), this effect was ascribed to a reduction of toxic species by reactions in solution (e.g. polymerization and/or structural changes in the phenoxy-radical yielding less toxic products) during the treatment time.

Table 6-2. Residual laccase activity and toxicity values (± standard deviation) measured in effluents obtained from the enzymatic treatments.

Pulp sample	Residual laccase activity (%)	Toxicity (T.U.)
L20_4h	21 ± 1	11 ± 1
L20FRC1.5_4h	23 ± 2	30 ± 3
L20FRC3.5_4h	22 ± 1	79 ± 8
L40FRC3.5_1h	39 ± 2	75 ± 1
L40FRC3.5_4h	28 ± 3	73 ± 5
L50FRC3.5_4h	32 ± 3	78 ± 8
L40FRC3.5_6h	22 ± 1	29 ± 3
L40FRC3.5_8h	18 ± 1	3 ± 0
L40FRC3.5_24h	3 ± 0	2 ± 1

6.3.2. Refining before and after the laccase–FRC treatment

The treatment previously found to provide the highest degree of FRC grafting was selected to compare the extent of grafting achieved by applying the laccase–FRC treatment to refined and unrefined pulp with a view to improving the physical strength of the resulting handsheets.

Handsheets were formed from pulp samples refined before and after laccase–FRC treatment. Leftover handsheets from the physical tests were Soxhlet-extracted with acetone, resuspended in water, disintegrated at 10 000 revolutions and filtered on a Buchner funnel with recirculation of the filtrate twice to avoid losses of fines. The resulting pulp samples were analysed in terms of anionic charge, kappa number and brightness. The effects of the laccase–FRC treatment were compared with those of treatments performed under the same conditions but in the absence of FRC, laccase or both. Figure 6-3 shows the results of the comparative analysis of pulp properties resulting from the treatments applied before and after pulp refining.

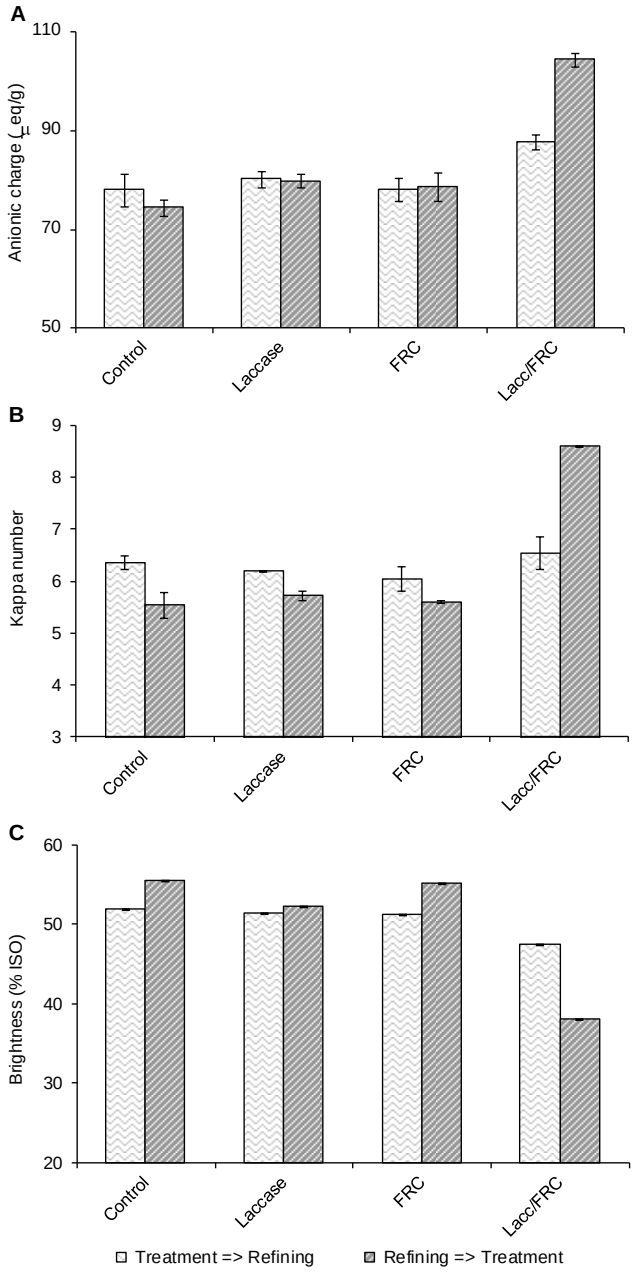

Figure 6-3. Surface anionic charge (A), kappa number (B) and brightness (C) of pulp samples treated with 40 U laccase/g and 3.5% FRC for 4 h before and after refining. The control pulp was treated with neither enzyme nor FRC.

It was hypothesized that the increased exposure of fibre surfaces to laccase and FRC resulting from refining would increase the number of potential sites for coupling of

phenoxy-radicals produced during the reaction. As can be seen, the changes in pulp properties caused by the laccase–FRC treatment relative to the control was significantly more marked in the samples refined before treatment (RT) than in those refined after treatment (TR). The increased surface anionic charge of these samples (Figure 6-3A) as compared to those discussed in Section 3.1.1 was a result of the increased fibre surface area exposed to the neutralizing polycation used in the charge measurements (Bhardwaj *et al.* 2004). The increase in anionic charge in the RT and TR pulp samples with respect to the control sample was 41% and 13%, respectively. The kappa number for RT pulp was 2.1 units higher and brightness 9 % ISO lower than the corresponding values for TR pulp. Similarly, the more marked loss of brightness in RT was accompanied by a more marked increase in k/s ratio (Jordan 1996) than in TR (results not shown), which suggests the presence of greater amounts of chromophoric groups in the former. These results clearly show that grafting can be increased by refining the pulp prior to the enzyme treatment as this increases its fibre surface area and the accessibility of the functionalizing reactant.

Handsheets formed from the different pulp samples treated before and after refining were tested for physical strength properties (viz. dry tensile, wet tensile, burst and tear strength); the results are shown in Figure 6-4 in terms of relative increases in strength index relative to the control sample, which was treated in the absence of both laccase and FRC. An increase in surface acid groups in pulp fibres is known to lead to increased strength in the resulting paper (Barzyk *et al.* 1997). We expected the increase in carboxylic groups at the pulp surface after the laccase–FRC treatment to increase paper strength through fibre swelling and enhanced inter-fibre hydrogen bonding (Barzyk *et al.* 1997, Lund and Felby 2000).

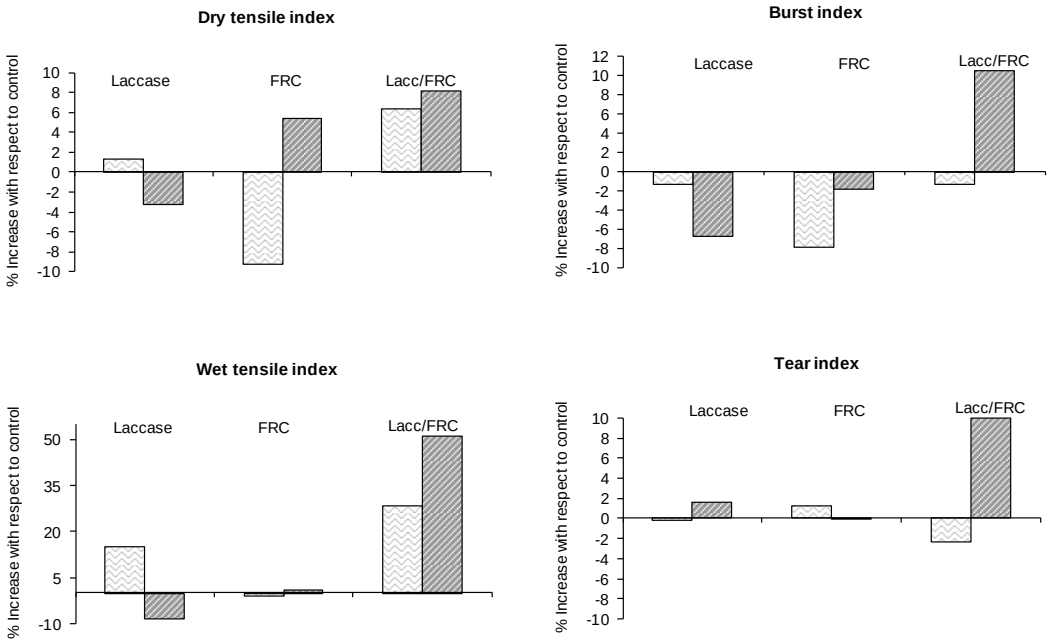

Figure 6-4. Strength-related properties of handsheets obtained from pulp samples treated with 40 U laccase/g and 3.5% FRC for 4 h before (▭) and after (▨) refining. The control sample was treated with neither enzyme nor FRC. The errors associated with these measurements were between 5% and 10%.

Handsheets obtained from treatments with laccase or FRC alone applied to both refined and unrefined pulp showed a loss or insignificant improvement in strength-related properties relative to the control sample; by exception, laccase TR and FRC RT handsheets exhibited a 15% increase in wet tensile strength and a 5.5% increase in dry tensile strength, respectively. As can be seen, the laccase–FRC treatment increased dry tensile strength by 6.5% and 8.3% in TR and RT handsheets, respectively. Based on the results, the laccase–FRC treatment provided a substantial gain in wet tensile strength (28.7% in TR sheets and 51.2% in RT sheets); this is suggestive of the potential formation of water-resistant covalent bonds between fibres by coupling of phenoxy-radicals in the sheets (Lund and Felby 2001). Application of the laccase–FRC treatment to refined pulp significantly improved burst and tear strength in the handsheets (up to 10.5%), but had a slightly detrimental effect on these properties when applied prior to refining. In all cases, the laccase–FRC treatment resulted in more marked improvements in paper strength when applied after refining. This provides further evidence for increased FRC coupling in RT pulp and suggests the need to refine pulp prior to grafting-based

functionalization of fibres in order to maximize its effect. The loss of brightness induced by the enzymatic grafting makes the resulting pulp not adequate for the manufacture of high-brightness paper grades, such as printing-writing qualities. However, unbleached sisal pulp with good mechanical performance is suitable for the manufacture of numerous packaging products able to resist different types of mechanical stresses.

6.4. Conclusions

The enzymatic treatment of sisal pulp with laccase in the presence of FRC was investigated and the degree of phenol coupling under different reaction conditions evaluated in terms of the laccase and FRC rates, and time. The kappa number due to lignin exhibited a similar trend as surface anionic charge and Klason lignin, whose changes were suggestive of phenolic acid coupling to pulp fibres. A comparison of the results obtained by applying the laccase–FRC treatment before and after refining the pulp showed that increasing the fibre surface area in a previous step enhanced grafting and led to further improved strength-related properties (particularly wet tensile strength) in the resulting paper.

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Wet strength development in sisal cellulose fibres by effect of a laccase-TEMPO treatment

Based on **Publication 5**: Elisabetta Aracri, Teresa Vidal, Arthur J. Ragauskas. *Carbohydrate Polymers*, Volume 84, No. 4, pp. 1384-1390.

Abstract

The aim of this study was to investigate the oxidation of sisal pulp fibres by laccase-TEMPO system which had never previously been examined. High cellulose content sisal fibres were treated with laccase from *Trametes villosa* and a varying amount of TEMPO in order to evaluate the potential of laccase-TEMPO system to improve the paper physical properties of pulp fibres. The effect of the oxidative treatment on the carboxyl group content and viscosity of pulp fibres was examined. The resulting papers were analysed in terms of strength-related properties, water absorbency capabilities and fibre morphology. Oxidation of sisal pulp by laccase-TEMPO system resulted in a very important improvement of wet strength of the resulting papers, suggesting the formation of a substantial amount of aldehyde groups in cellulose chains providing inter-fibre bonding through hemiacetal linkages.

7.1. Introduction

Over the last few decades, laccase has played an increasingly important role in pulp and paper research by virtue of its broad oxidative capabilities and flexibility of use in the production of paper and its derivative (Widsten and Kandelbauer 2008). Ever since the discovery of chemical mediators capable of extending enzymatic oxidation to non-phenolic compounds, research interests have focused mainly on the potential of laccase–mediator systems for aiding pulp bleaching (Barreca *et al.* 2003, Bourbonnais and Paice 1990, Fillat *et al.* 2010). Recently, laccase has attracted considerable attention as a means for modifying fibre chemistry with a view to altering paper properties (particularly, strength-related properties). Thus, Felby *et al.* (1997) found laccase to increase auto-adhesion in wood fibres and the bonding mechanism to involve the enzymatic activation of lignin on fibre surfaces through the production of phenoxy radicals, which facilitated cross-linking of fibres during board making. Further studies showed combinations of laccase with a lignin-rich extractive or a mediator to substantially improve wet tensile strength in high-yield unbleached kraft pulp (Lund and Felby 2000, 2001). The improvement was ascribed to polymerization of lignin in the handsheets, and also to enhanced production of phenoxy radicals increasing cross-linking between fibres and facilitating water-resistant inter-fibre bonding as a result. Radical-based activation has

also been used to enable bonding of low-molecular weight compounds to lignin-rich cellulose fibres. The reactions involved introduce new functional groups and provide a means for altering the physico–chemical properties of fibres as desired (Chandra *et al.* 2003, Elegir *et al.* 2008, Grönqvist *et al.* 2006, Lund *et al.* 2001). For example, laccase-catalysed grafting of phenolic acids to kraft pulp fibres was found to increase tensile and burst strength in the resulting paper (Chandra *et al.* 2004, Chandra and Ragauskas 2002); the improvement was ascribed to the ability of carboxyl groups to promote inter-fibre bonding and fibre swelling (Barzyk *et al.* 1997, Scallan 1983). Laccase-based approaches to improving paper properties have been preferentially applied to lignin-rich pulp and aimed specifically at lignin modification.

The selective catalytic oxidation of primary hydroxyl groups in carbohydrates by stable, water-soluble nitroxyl radicals such as 2,2,6,6-tetramethyl-1-piperidinyloxy, TEMPO), has opened up new prospects for the modification of cellulose materials (de Nooy *et al.* 1995). A number of studies have shown TEMPO-mediated oxidation to provide an efficient method for introducing carboxyl and aldehyde functional groups into cellulose in aqueous media at room temperature (Gert *et al.* 2005, Saito *et al.* 2006, Tahiri and Vignon 2000). The reaction is commonly carried out in the presence of the co-oxidizer system NaOCl/NaBr, which affords the regeneration of TEMPO and hence its use as a catalyst. The application of TEMPO-mediated oxidation to cellulose pulp has been widely investigated over the last decade. Kitaoka *et al.* (1999) studied the chemical modification of bleached hardwood kraft pulp fibres with this method and found it to result in an increase in carboxyl content from 60 to 470 mmol/kg. Dang *et al.* (2007) demonstrated the potential of the TEMPO–KBr–NaClO system for oxidizing ECF bleached softwood kraft pulp fibres; the system provided a 480% increase in carboxyl groups, a 62.9% greater water retention value (WRV) and a 13.8% increase in tensile index. In recent work, TEMPO-mediated oxidation was successfully used to improve inter-fibre bonding, and hence strength-related properties, in thermomechanical pulp (Le Roux *et al.* 2006, Lianshan *et al.* 2008a). Saito and Isogai (2005, 2006) found the wet tensile strength of sheets prepared from TEMPO-oxidized cellulose fibres to be considerably improved. Development of this property was ascribed to the formation of substantial amounts of aldehyde groups on the surfaces of cellulose fibres as intermediate structures during the course of the TEMPO-mediated oxidation; once formed, the aldehyde groups seemingly established covalent inter-fibre bonds through hemiacetal linkages with sterically close hydroxyl groups in cellulose. This hypothesis was supported

by the facts that no improvement in wet tensile strength was obtained when handsheets were prepared from TEMPO-oxidized pulp reduced with $NaBH_4$ or oxidized with NaOCl (Saito *et al.* 2005). The contribution of hemiacetal linkages to wet strength improvement in paper has also been proposed for aldehyde-containing wet strength resins (Chen *et al.* 2002, Dunlop-Jones 1991).

In 1999, Viikari *et al.* (1999) reported on the ability of laccase to catalyse the regenerative oxidation of TEMPO in the presence of oxygen, and hence on its potential for replacing the environmentally harmful halide-containing compounds commonly used as co-oxidizer systems in the TEMPO-mediated oxidation of cellulose materials. Laccase–TEMPO mediated oxidation was proposed as a method for oxidizing various types of cellulose pulp for the production of paper or board with improved properties (particularly, technical properties, flexibility, WRV and strength-related properties). In addition to the environmental benefits associated with the use of an enzyme, this method provides the advantage of operating at a near-neutral pH —the traditional method uses pH 10–11. This reduces the occurrence of β-elimination reactions causing depolymerization of cellulose chains, thus a loss in mechanical properties of the oxidized pulp (Isogai and Kato 1998).

The purpose of this work study was to study the laccase–TEMPO mediated oxidation of low-lignin-content sisal pulp, which had never previously been examined. To this end, the effect of the oxidative treatment on the carboxyl group content and viscosity of pulp fibres was examined. The resulting papers were analysed in terms of strength-related properties, water absorbency capabilities and fibre morphology.

7.2. Materials and methods

7.2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Aldrich and used as received. Laccase (EC. 1.10.3.2) from *Trametes villosa* was kindly supplied by Novo Nordisk Biochem, North Carolina and frozen until use. Sisal (*Agave sisalana*) pulp (1% lignin content) from a soda–anthraquinone cooking process was supplied by a Spanish manufacturer

(CELESA). The pulp was conditioned at pH 4 with H_2SO_4, under stirring for 30 min at 2% consistency, followed by filtration and extensive washing with de-ionized water. This step was necessary to remove contaminants and metals, and also to bring the pulp to the pH required for the enzymatic treatment.

7.2.2. Enzyme assay

Laccase activity was determined by oxidation of 2,2'-azino*bis*-(3-ethylbenzylthiozoline-6-sulfonate) (ABTS). One activity unit (U) was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical (ε_{436} nm = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C.

7.2.3. Pulp treatment

Laccase (60 U/g odp) and TEMPO (1, 2, 4 or 8% by mass) were added with stirring to a 1% consistency aqueous suspension of sisal pulp buffered at pH 5 with 50 mM acetate buffer. The resulting slurry was stirred at room temperature under oxygen bubbling for 18 h. At the end of each treatment, the effluent pH was checked and only slight changes (<0.2) were observed with respect to the initial value. Pulp samples treated under identical conditions in the absence of enzyme, TEMPO or both were used as controls. After treatment, each pulp was filtered and washed with de-ionized water until a colourless, neutral filtrate was obtained.

7.2.4. Analysis of pulp properties

Pulp viscosity (as intrinsic viscosity for a sample of cellulose dissolved in a dilute solution of cupriethylenediamine) was determined in accordance with ISO 5351/1. Borohydride viscosity, measured after treatment with 2% $NaBH_4$, at 5% consistency at room temperature for 30 min, was also determined (Roncero *et al.* 2003). Pulp brightness was analysed according to ISO 3688. The bulk acid group content was determined by conductimetric titration as described elsewhere (Katz *et al.* 1984). In brief, an amount of 1.50 g o.d. pulp was stirred in 300 ml of 0.10 M HCl for 1 h, followed by rinsing with de-

ionized water in a finely fritted funnel. The sample was then resuspended in 250 ml of 1.00 mM NaCl, spiked with 1.5 of 0.10 M HCl and titrated against 0.05 M NaOH in 0.25 ml increments, with conductivity measurement after each addition. Titration data were plotted in conductance vs. volume graphs in order to determine the millimoles of acid groups present in each gram of pulp. All reported results were the averages of two measurements which typically differed by less than 5%.

7.2.5. Paper testing

The treated pulp samples were disintegrated for 50 000 revolutions and then refined for 4500 revolutions according to TAPPI T 248. Handsheets were formed according to TAPPI T 205 and TAPPI conditioned (23 °C, 50% relative humidity) for 48 h before physical testing. Apparent density, dry tensile strength, tearing resistance, bursting strength, wet zero span tensile strength and wet tensile strength were determined according to TAPPI T 210, T 494, T 414, T 403, T 273 and T 456, respectively. Wet tensile index was measured in 25 mm wide specimen strips soaked in deionized water for 5 s.

Vertical wicking was performed on 10 cm x 1.5 cm strips of the treated handsheets. The strips weighed 0.10 ± 0.01 g and had densities of 0.60 ± 0.02 g/cm^3. A Petri dish filled with nanopure deionized water was used as reservoir. The bottom 1 cm of each strip was inserted into the reservoir and the timer immediately started. The top of the strip was fixed with a movable clamp. Each time the water climbed 1 cm on a strip, the time was recorded. After 5 min, the total distance of liquid absorbed was also recorded. Each sample was analysed in duplicate.

Surface SEM pictures of the handsheets were taken on a Hitachi S-800 FE-SEM instrument. Samples were placed on the SEM sample holding stub by the conductive double side sticky carbon film and coated with Au/Pt alloy prior to analysis. Cross-sectional SEM images were obtained with a JEOL JSM-6400 microscope, the handsheet samples being coated with Au/Pd alloy before analysis.

7.3. Results and discussion

7.3.1. TEMPO-mediated oxidation of sisal pulp: effect on carboxyl groups content in pulp fibres and on physical properties of paper

A preliminary study was conducted by using 1% TEMPO as oxidant in order to assess its impact on the carboxyl group content of pulp fibres and strength-related properties of the resulting handsheets. The results are shown in Table 7-1. The laccase treatment slightly increased the content in acid groups relative to the control pulp as a result of lignin being oxidized by the enzyme (Konishi *et al.* 1974). The TEMPO-treated pulp had an acid content similar to that of the control pulp. The laccase–TEMPO treated pulp exhibited the highest yield in carboxyl groups (a 30% increase relative to the control pulp) as a result of the conversion of C6 primary hydroxyl groups in cellulose via an aldehyde group. Under the reaction conditions used, however, the laccase–TEMPO system increased the carboxyl group content to a much smaller extent than did others in the presence of NaOCl/NaBr (Kitaoka *et al.* 1999, Lianshan *et al.* 2008b). Several studies (Barzyk *et al.* 1997, Chandra *et al.* 2004, Lianshan *et al.* 2008a) have shown carboxyl groups to promote hydrogen bonding between fibres and improve dry-strength properties in paper as a result. Our tests showed the laccase treatment to bring about a slight increase in dry tensile and burst strength in the resulting paper relative to the control treatment. The laccase–TEMPO treatment resulted in a slight loss of all dry-strength properties despite the higher increased yield in acid groups obtained; however, the treatment had a significant beneficial effect on wet strength in the modified paper, which was 88% higher than in the control sample. Depolymerization by some active species such as hydroxyl radicals formed *in situ* in side reactions of the hydroxylamine structure with oxygen during the oxidative treatment (Shibata and Isogai 2003) may have degraded strength-related properties in the pulp. The gain in wet tensile strength suggests the formation of inter-fibre covalent bonds in the handsheets through hemiacetal linkages between aldehyde groups on fibre surfaces formed as intermediates in the TEMPO-mediated oxidation and hydroxyl groups in adjacent fibre surfaces (Saito and Isogai 2006).

Table 7-1. Bulk acid group content of control pulp, laccase-treated pulp, TEMPO-treated pulp and laccase/TEMPO-treated pulp (Lac/T), and physical properties of the resulting handsheets.

Sample ID	Bulk acid groups (μmol/g)	Dry tensile index (N·m/g ± 3%)	Wet tensile Index (N·m/g ± 3%)	Tear index ($mN \cdot m^2/g \pm 3\%$)	Burst index ($kPa \cdot m^2/g \pm 3\%$)
Control	83 ± 1	79.9	3.3	16.1	5.82
Laccase	91 ± 3	82.2	3.2	16.3	6.15
TEMPO	85 ± 1	79.0	3.1	16.0	6.04
Lac/TEMPO	108 ± 1	76.9	6.2	15.9	5.64

7.3.2. Effect of the TEMPO dose on intrinsic viscosity, strength and carboxyl groups content of oxidized pulp fibres

The next step in this study was to examine the effect of the TEMPO dose on various properties of oxidized fibres. As noted earlier, the oxidative treatment may cause depolymerization reactions involving radical species and leading to a diminished fibre strength. Changes in cellulose polymerization were assessed via intrinsic viscosity measurements. Because the cupriethylenediamine solution is highly alkaline, depolymerization through β-elimination reactions promoted by carbonyl groups may take place on oxidized fibres during viscosity measurements and provide underestimated viscosity values. This problem was avoided by measuring viscosity after the pulp was treated with sodium borohydride (borohydride viscosity) in order to inactivate carbonyl groups by reduction to hydroxyl groups (Cadena *et al.* 2010, Roncero *et al.* 2002). The difference between the viscosity values determined with and without the reductive treatment provided an indication of the depolymerizing effect of carbonyl groups produced in the TEMPO-mediated oxidation.

As can be seen from Table 7-2, a loss of borohydride viscosity of up to 22% with respect to the control value was observed as the TEMPO load was increased from 1 to 8%; this reflects the occurrence of degradation reactions in cellulose chains during the oxidative treatment, especially at high TEMPO doses. When no prior reductive treatment was applied, control and laccase-treated pulp had viscosity values similar to those obtained after borohydride treatment; by contrast, TEMPO-treated pulp exhibited a dramatic drop in viscosity (up to 64%) compared to the reduced samples, the difference increasing with increase in TEMPO dose from 1% to 8%. This suggests that substantial

amounts of carbonyl groups are generated during a laccase–TEMPO treatment (particularly at high mediator doses).

Table 7-2. Borohydride viscosity and standard viscosity values (± standard deviation) of control pulp, laccase-treated pulp and pulps treated with laccase and different amounts of TEMPO.

Sample ID	Borohydride viscosity (ml/g)	Viscosity (ml/g)
Control	736 ± 20	736 ± 33
Laccase	736 ± 13	727 ± 7
Lac/T 1%	710 ± 12	442 ± 19
Lac/T 2%	649 ± 23	348 ± 21
Lac/T 4%	626 ± 21	238 ± 3
Lac/T 8%	576 ± 30	207 ± 10

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

$$\boldsymbol{CS} = \frac{\boldsymbol{DP_o - DP}}{\boldsymbol{DP}} \qquad [7\text{-}1]$$

where DP_O is the degree of polymerization of the initial pulp and DP that after the oxidative treatment. The degree of polymerization is calculated from the intrinsic viscosity value [η], using the equation of Evans and Wallis (1987) (SCAN-CM 15:88):

$$DP^{0.85} = 1.1 * [\eta] \qquad [7\text{-}2]$$

In pulp subjected to no borohydride post-treatment, CS is the combination of scissions caused by the oxidative treatment (CS_{GT}) and the viscosity measurement procedure when carbonyl groups are present ($CS_{C=O}$):

$$CS_T = CS_{GT} + CS_{C=O} \qquad [7\text{-}3]$$

On the other hand, the CS value obtained after the reductive treatment (CS_{TR}) represents depolymerization exclusively due to the oxidative treatment, with exclusion of the contribution of β-elimination reactions occurring around carbonyl groups:

$$\boldsymbol{CS_{TR} = CS_{GT}} \quad [7\text{-}4]$$

Subtracting Eq. [7-4] from Eq. [7-3] gives the number of chain scissions due to the presence of carbonyl groups ($CS_{C=O}$):

$$CS_{C=O} = CS_T - CS_{TR} \quad [7\text{-}5]$$

The intrinsic viscosity values obtained in the presence and absence of a reductive treatment allowed us to calculate the number of scissions due to the oxidative treatment itself and to β-elimination reactions promoted by carbonyl groups. Figure 7-1 shows the CS_T, CS_{GT} and $CS_{C=O}$ values for each pulp provided by the oxidative treatments. As can be seen, the control and laccase samples exhibited very low total chain scission values that were virtually the exclusive result of the oxidative treatment. Application of the laccase–TEMPO system at a TEMPO dose of 1%, 2%, 4% and 8% increased CS_T to 0.92, 1.54, 2.97 and 3.68, respectively. As can be seen, the number of chain scissions caused by the oxidative treatment increased as the TEMPO dose was increased; however, it accounted for only ~10% of the total number of scissions (CS_T), most of which were due to carbonyl groups produced by the laccase–TEMPO system.

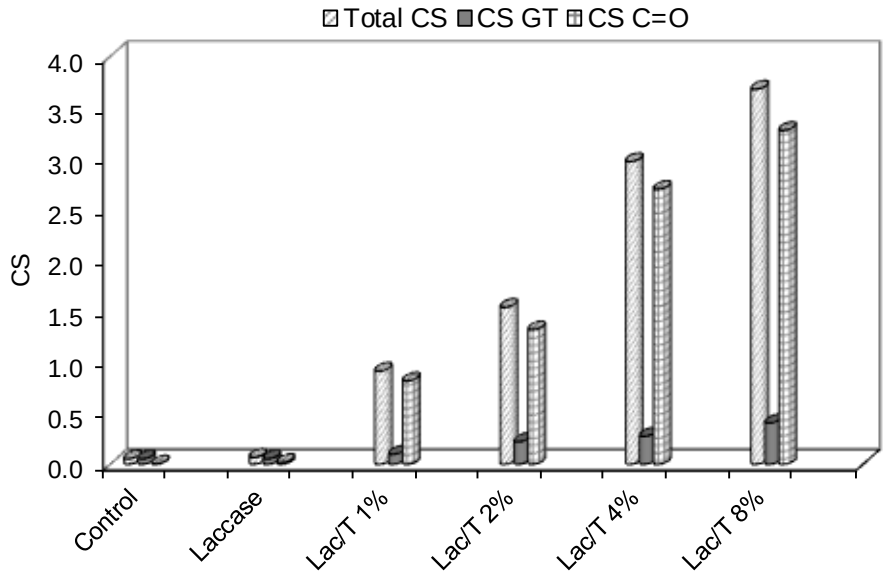

Figure 7-1. CS_T, CS_{GT} and $CS_{C=O}$ of control pulp, laccase-treated pulp and pulps treated with laccase and different amounts of TEMPO.

Modifications induced in lignin by the laccase-TEMPO system have not been investigated here; however, it is known from studies performed with lignin models (Barreca *et al.* 2003, Fabbrini *et al.* 2001) that TEMPO selectively interacts with benzyl alcohol (or ethers) groups of lignin oxidizing them to α-carbonyl derivatives. Pulp brightness was determined after each treatment in order to evaluate whether the introduction of new functional groups in fibres by the oxidative system led to a loss of this property. The results obtained (data not shown) indicate that no significant change of brightness was produced by laccase-TEMPO treatments with respect to control pulp.

The mean strength of fibres was estimated from the wet zero span tensile strength (WZSTS) of the handsheets. In theory, tensile tests on wetted samples with zero span between instrument jaws measures the work required to rupture individual fibres without altering inter-fibre bonding (Clark *et al.* 1997).

Figure 7-2 shows the borohydride viscosity values for the oxidized pulp samples together with the variation of WZSTS in the resulting handsheets. As can be seen, pulp viscosity was reduced by the oxidative treatments, and so was WZSTS. This confirms the correlation between cellulose depolymerization and a decreased fibre strength.

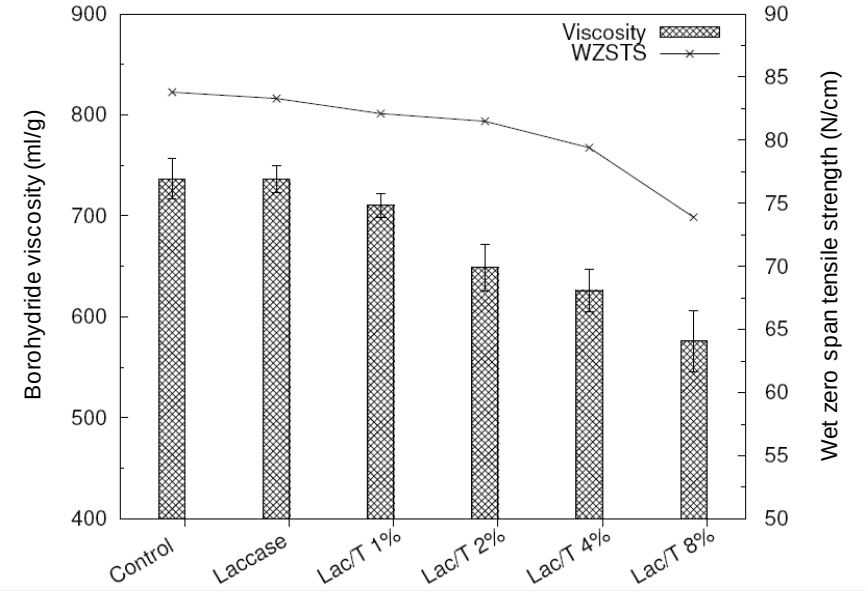

Figure 7-2. Borohydride viscosity values of control pulp, laccase-treated pulp and pulps treated with laccase and different amounts of TEMPO, and wet zero span tensile strength of the resulting handsheets.

Figure 7-3 shows the carboxyl group content of pulp fibres after treatment with TEMPO at a variable dose. The results indicate that the addition of 2% TEMPO failed to increase the carboxyl group content in relation to the initial dose. On the other hand, the addition of 4% and 8% TEMPO resulted in a 42% and 63% increase, respectively, in carboxyl groups with respect to the control value.

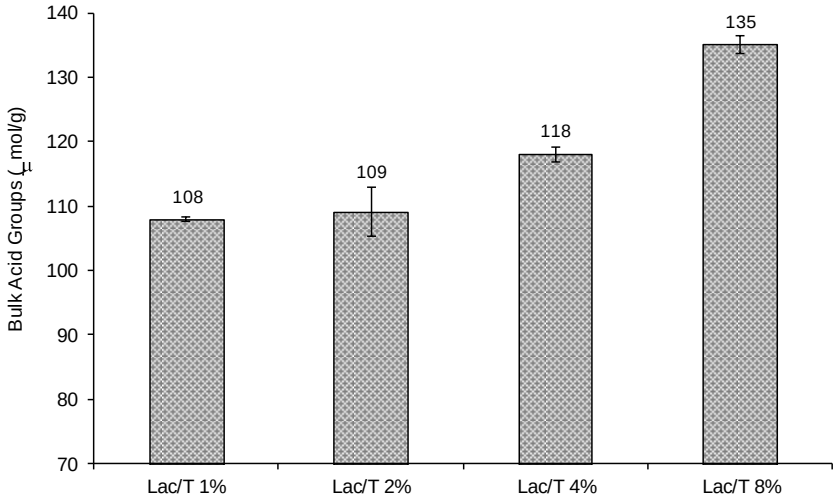

Figure 7-3. Bulk acid group content of pulps that were treated with laccase and different amounts of TEMPO.

7.3.3. Effect of TEMPO dose on water absorbency capability of the resulting handsheets

An increased concentration of acid groups in pulp fibres is widely believed to increase fibre hydrophilicity and swelling (Dang *et al.* 2006, Scallan 1983). In this work, we measured vertical wicking in order to confirm whether the increase in carboxyl groups derived from the laccase–TEMPO treatments enhanced the water absorbing capabilities of the handsheets. Wicking flow (i.e. spontaneous liquid flow in porous media) is strongly influenced by interfacial forces between the wetting fluid and the porous solid. Wicking flow in randomly oriented handsheets has been the subject of little study (Back 1965, Hodgson and Berg 1988b). Wicking flow in vertical tubes is governed by Eq. [7-6], proposed by Lucas and Washburn (1921):

$$\frac{dh}{dt} = \frac{\gamma r \cos\theta}{4\eta h} - \frac{r^2 \rho g}{8\eta} \qquad [7\text{-}6]$$

where h denotes height of liquid rise, t time, γ surface tension, η viscosity, r pore radius, θ liquid contact angle, ρ liquid density, and g the gravitational constant.

Based on the integrated form of Eq. [7-6] in the paper by Hodgson and Berg (1988a), the square-root of time should be directly proportional to the distance travelled by the wetting front. This has been shown to be the case with random oriented handsheets (Hodgson and Berg 1988a). Indeed, our distance vs. $t^{1/2}$ plots (Figure 7-4) were virtually linear. As can be seen, the samples treated with laccase and TEMPO wicked at a faster rate than did the control samples, which indicates that the oxidative treatments increase pulp hydrophilicity. Although the higher TEMPO doses increased the carboxyl content, no further increase in wicking rate was observed in the samples treated with TEMPO at doses above 2%. As shown below, this result can be ascribed to the formation of an increased amount of hemiacetal linkages implying the reduction of free hydroxyl groups in fibres.

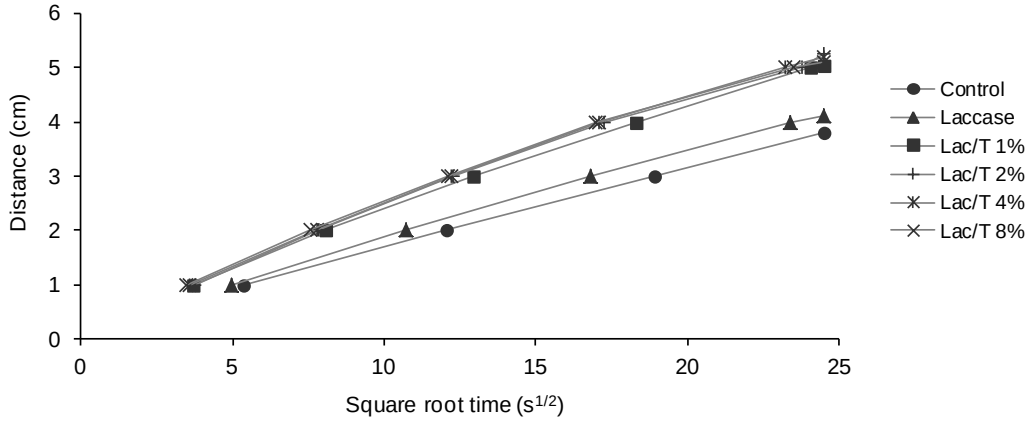

Figure 7-4. Plot of distance wicked vs. square-root of time for samples treated with laccase and TEMPO.

7.3.4. Effect of TEMPO dose on wet strength improvement in handsheets from oxidized pulp fibres

The preliminary paper tests performed in this study showed the use of 1% of TEMPO and laccase in the oxidative treatment of sisal pulp to have no beneficial effect on the dry-strength properties of the resulting paper sheets despite their increased carboxyl group

content. On the other hand, the laccase–TEMPO treatment doubled wet strength, which suggests the formation of water-resistant inter-fibre bonds in the paper. Since increasing the TEMPO dose from 1% to 8% caused no significant further increase in carboxyl groups and detracted somewhat from viscosity and fibre strength, we believed it of interest to focus on the development of wet strength in the treated pulp samples, which was affected mainly by the presence of covalent inter-fibre bonds. Figure 7-5 shows the wet tensile strength and wet burst strength —the latter was measured according to TAPPI T 403 on water-soaked strips as for wet tensile strength measurements— for handsheets obtained from pulp treated with increasing doses of TEMPO. As can be seen, both properties were dramatically improved as the TEMPO dose was raised from 1% to 8%; thus, the wet tensile index and wet burst index were increased by up to 660% and 510%, respectively, relative to the control value. These results suggest the formation of increasingly large amounts of inter-fibre hemiacetal linkages during sheet-making as the TEMPO dose used in the oxidative treatment of pulp was increased. It is generally agreed that paper with a wet-to-dry (W/D) strength ratio (*i.e.* the ratio of wet tensile strength to dry tensile strength) of more than 15% should be considered wet-strength paper (Scott 1996). W/D in our paper sheets increased from 4% in the control sample to 35% at the highest TEMPO dose. Wet strength development was achieved by using 2% TEMPO in the oxidative treatment, which provided W/D = 16%.

In previous studies, significantly improving wet strength with a TEMPO-mediated oxidation treatment of bleached pulp fibres was found to require the addition of aluminium sulfate or a cationic polymer in the handsheet-making process (Saito and Isogai 2005, 2007). However, the degree of wet strength improvement obtained with these methods was not as high as in the present work, were the NaOCl/NaBr system was replaced with laccase and no further chemical agent was used to obtain the handsheets after pulp oxidation.

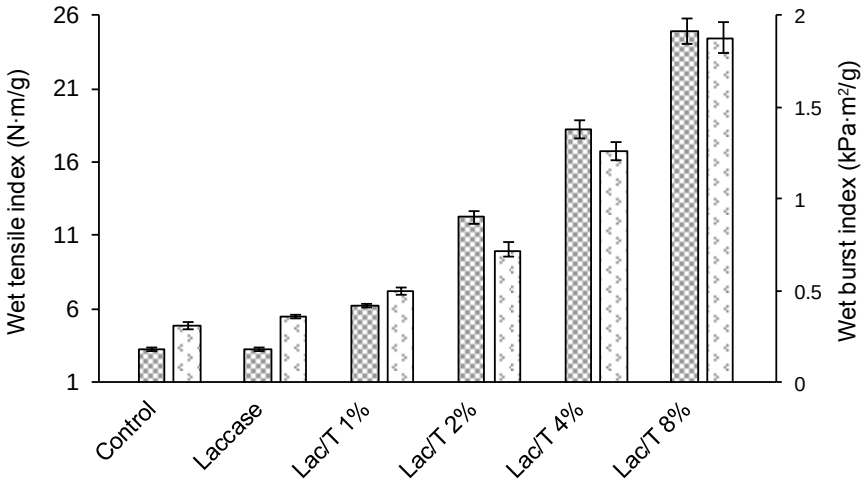

Figure 7-5. Wet tensile (▩) and wet burst (▨) strengths of handsheets made from control pulp, laccase-treated pulp and pulps treated with laccase and different amounts of TEMPO.

7.3.5. SEM analysis of handsheets

Figure 7-6 shows surface and cross-sectional scanning electron micrographs for handsheets made from the control and laccase-treated pulp samples, as well as from pulp treated with the enzyme and 8% TEMPO. The surface images are consistent with a significant increase in fibre roughness and inter-fibre adhesion by effect of the laccase–TEMPO treatment. The effect of the oxidative treatment on inter-fibre bonding is more apparent in the cross-sectional images, which reveal strong agglutination of fibres leading to highly compacted handsheets. Based on these SEM images, the extent of fibre adhesion and sheet compaction is consistent with the increase in wet strength observed with the laccase–TEMPO treatment.

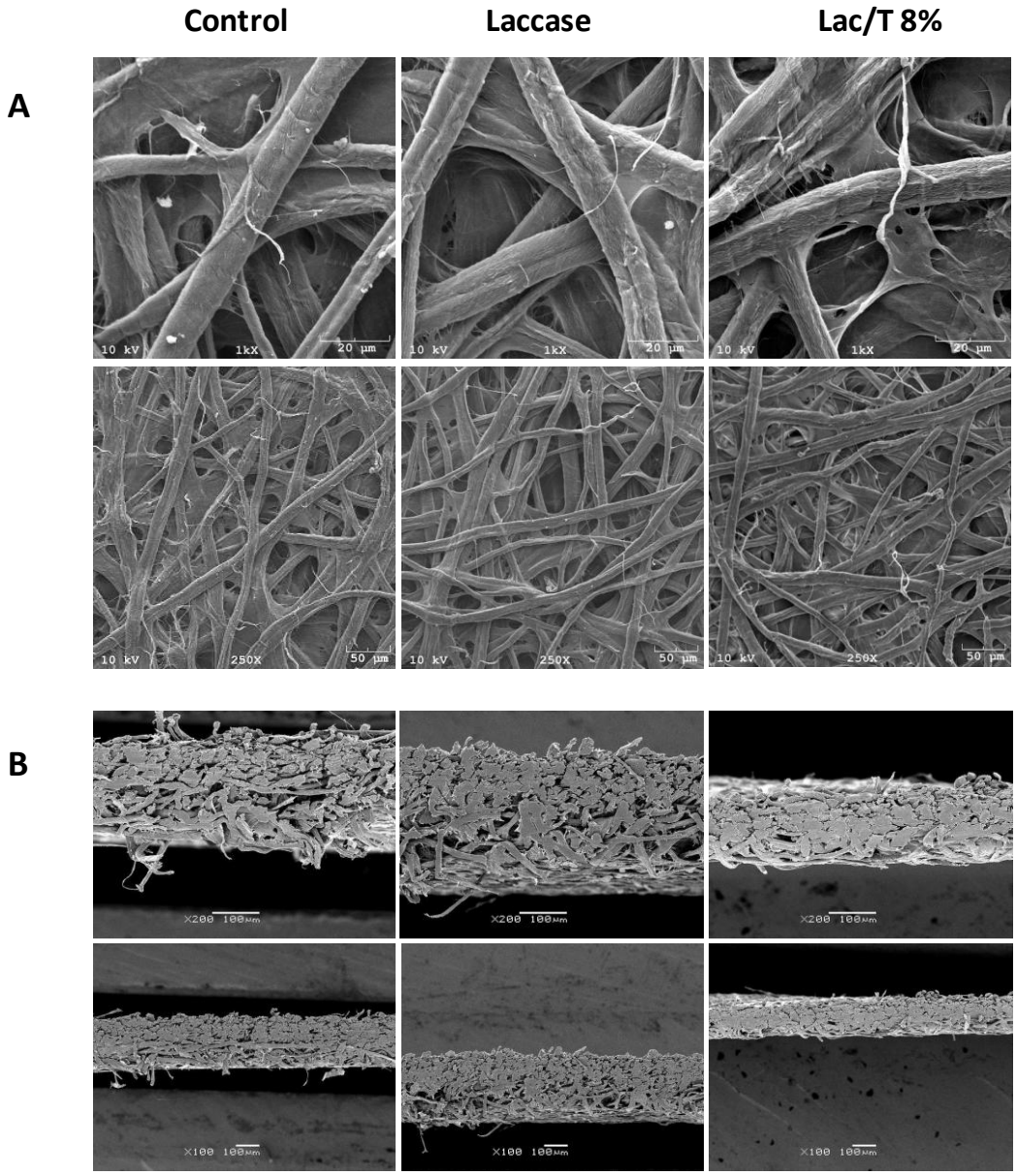

Figure 7-6. Scanning electron microscope (SEM) images of surface (A) and cross-section (B) of handsheets made from control pulp, laccase-treated pulp and laccase/TEMPO 8%-treated pulp.

7.4. Conclusions

As shown for the first time in this work, a laccase–TEMPO treatment effectively improves wet strength in sisal pulp. The ability of the enzyme to catalyse the oxidation of TEMPO allows one to use a near-neutral pH to ensure the selective oxidation of cellulose fibres and avoid the need for environmentally harmful halide-containing chemicals. TEMPO-mediated oxidation causes the formation of aldehyde and carboxyl groups in proportions dependent on the particular reaction conditions. Under those used in this work, the laccase–TEMPO system resulted in a modest increase in carboxyl groups in cellulose fibres and, as suggested by the viscosity results and wet strength improvement obtained, the formation of a substantial amount of aldehyde groups that provide inter-fibre bonding through hemiacetal linkages. Further study is required to optimize the oxidation process and examine the effect of the operating conditions on the ratio and distribution of carboxyl and aldehyde groups in fibres.

Oxidation by the laccase–TEMPO system provides a novel, effective, environmentally friendly approach to the production of paper with excellent strength performance under high moisture conditions.

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Paper strength improvement by oxidative modification of sisal cellulose fibres with laccase-TEMPO system: influence of the process variables

Based on **Publication 6**: Elisabetta Aracri, Cristina Valls, Teresa Vidal. Accepetd for publication in *Carbohydrate Polymers*.

Abstract

The oxidation of sisal pulp fibres by the laccase–TEMPO system was investigated and the influence of process variables including the laccase and TEMPO doses, and reaction time, on various properties of the oxidized fibres and of handsheets made from them was for the first time assessed by using a three-variable statistical plan. The laccase–TEMPO system was found to oxidatively modify cellulose fibres, largely by introducing aldehyde groups and, to a much less extent, by introducing carboxyl groups. Based on the mathematical models used, increasing the TEMPO dose and reaction time increases the aldehyde content of the fibres, thereby also increasing their wet strength by effect of inter-fibre covalent bonding via hemiacetal linkages. Although no accurate model for the carboxyl content could be established, this property was found to peak under the specific conditions yielding the highest response in the dry tensile index model. The fact that the oxidative treatment diminished pulp viscosity is indicative of partial depolymerization of cellulose. This was especially marked under the conditions providing the highest contents in aldehyde and carboxyl groups, and the greatest improvements in the dry and wet tensile indices.

8.1. Introduction

Laccase (EC.1.10.3.2.) is a family of blue multi-copper oxidases produced by microorganisms and plants which catalyze the one-electron oxidation of phenols and aromatic or aliphatic amines to reactive radicals with the concomitant reduction of oxygen to water. The broad substrate range for this enzyme makes it attractive for a number of biotechnological applications (Kunamneni *et al.* 2008, Riva 2006). Moreover, the range can be expanded by using the enzymes in combination with a chemical mediator enabling the oxidative transformation of compounds with a redox potential higher than that of the enzyme (Bourbonnais and Paice 1990). In the last few decades, laccase has played an increasingly important role in pulp and paper research by virtue of its wide applicability along the entire production chain of paper products (Bajpai 1999, Widsten and Kandelbauer 2008). Special emphasis has been placed on the potential of laccase and laccase–mediator systems (LMS) for use in biobleaching and mill water treatments (Font *et al.* 2003, Valls and Roncero 2009). One attractive, fast-growing field

of research at present is the enzymatic modification of fibres with a view to improving the chemical or physical properties of fibre products or developing novel alternatives (Chandra and Ragauskas 2001, Viikari 2002). One well-known procedure for functionalizing polysaccharides is the catalytic oxidation of primary hydroxyl groups into aldehyde and/or carboxyl groups by using the stable nitroxyl radical 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) in aqueous media at room temperature (de Nooy *et al.* 1995b). In virtually all studies concerning carbohydrate oxidation, the established NaClO/NaBr system has been used as a primary oxidant (Bragd *et al.* 2004, Chang and Robyt 1996, de Nooy *et al.* 1995a, Isogai and Kato 1998). In this process, TEMPO and its oxoammonium cation (the actual oxidant) are reduced to an N-hydroxyderivative that is continuously reoxidized by sodium hypochlorite, thus affording the use of TEMPO in catalytic amounts. Environmental concerns have shifted research interest towards halide-free oxidative systems. One promising approach for this purpose is the use of oxidative enzymes such as laccases in combination with oxygen as primary oxidants (Arends *et al.* 2006, Viikari *et al.* 1999b). Similarly to the NaClO/NaBr system, oxoammonium ion is regenerated in situ, so only oxygen is consumed during the reaction. In addition to the environmental benefits associated with the use of an enzyme, this method has the advantage that it operates at near-neutral pH, which reduces the occurrence of β-elimination reactions causing cleavage of polysaccharide chains —a major drawback of the traditional method using pH 10–11 (Isogai and Kato 1998).

Although the laccase–TEMPO system has been shown to catalyze the oxidation —and consequent functionalization— of various types of polysaccharides (Jetten *et al.* 2000, Marzorati *et al.* 2005, Viikari *et al.* 1999a, Viikari *et al.* 1999b), the influence of the experimental conditions and the characteristics of the resulting products have scarcely been examined. Much research aimed at developing specific applications for cellulose pulp has focused on oxidation reactions mediated by the TEMPO–NaBr–NaClO system on the grounds of its efficiency in functionalizing fibre surfaces with large amounts of aldehyde and/or carboxyl groups. This oxidative modification has been exploited to improve or modulate several physical properties of various types of pulp fibres including inter-fibre bonding capacity —and hence strength-related properties in the resulting paper (Dang *et al.* 2007, Duarte *et al.* 2006, Lianshan *et al.* 2008). Paper from TEMPO-oxidized fibres was recently shown to have an increased wet strength; this was ascribed to the ability of aldehyde groups to form inter-fibre covalent bonds through hemiacetal linkages with hydroxyl groups in adjacent fibre surfaces (Saito and Isogai 2005, 2006).

The potential of laccase for replacing halide-based co-oxidizer systems in the TEMPO-mediated oxidation of cellulose fibres has scarcely been explored to date, however (Viikari *et al.* 1999b). In recent work (Aracri *et al.* 2011), we studied the oxidation of sisal pulp fibres by the laccase–TEMPO system and found it to slightly increase the carboxyl group content of the fibres and to considerably improve the wet strength of the resulting paper, the latter suggesting the formation of a substantial amount of aldehyde groups providing inter-fibre bonding through hemiacetal linkages. In this work, we further investigated the process and examined the influence of the operating conditions (viz. the laccase and mediator doses, and treatment time) on the distribution of carboxyl and aldehyde groups in fibres, pulp borohydride viscosity, and dry and wet strength properties of the handsheets for the first time by using a three-variable sequential statistical plan.

8.2. Materials and methods

8.2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Aldrich and used as received. Laccase from *Trametes villosa* was supplied by Novozymes (Bagsvaerd, Denmark). One activity unit was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical (ε_{436nm} = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Sisal (*Agave sisalana*) pulp (1% lignin content) from a soda–anthraquinone cooking process was supplied by CELESA (Tortosa, Spain). Following conditioning at 2% consistency at pH 4 (H_2SO_4) under stirring for 30 min, the pulp was filtered and extensively washed with de-ionized water. This step was needed to remove contaminants and metals, and also to bring the pulp to a suitable pH for the enzyme treatment.

8.2.2. Pulp treatments

A series of preliminary tests was performed by using an amount of 30 g of pulp in a 5 l reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5 in the presence of 60 U/g odp laccase and 8% odp TEMPO, and variable conditions of time, temperature, applied

oxygen pressure and consistency (Table 7-1). Pulp samples treated in the absence of TEMPO, or both laccase and TEMPO, at room temperature and 1% consistency under an oxygen pressure of 0.6 MPa for 18 h were used as controls.

Table 8-1. Operating conditions used in the laccase–TEMPO (LT) treatments of the preliminary study.

Sample ID	Time (h)	Temperature	Applied O_2 pressure (MPa)	Pulp consistency (% odp)
LT – 18 h	18	room	0.6	1
LT – 30 h	30	room	0.6	1
LT – 50 °C – 30 h	30	50 °C	0.6	1
LT – no P_{O2} – 30 h	30	room	-	1
LT – 4 h	4	room	0.6	1
LT – c 5% – 30 h	30	room	0.6	5

Subsequently, laccase-TEMPO treatments were performed according to the established experimental design in plastic containers, using a jar testing apparatus at a stirring speed of 60 rpm, 15 g of pulp at 1% consistency in 50 mM acetate buffer at pH 5 at room temperature under oxygen bubbling. The operating variables (factors) studied were the laccase dose, mediator dose and reaction time. After treatment, each pulp was filtered and washed with de-ionized water until a colourless, neutral filtrate was obtained.

8.2.3. Experimental design

Laccase–TEMPO treatments were conducted in accordance with a 2^3 experimental design involving three variables at two levels each and three replicates at the central point, which required a total of 11 tests. The ranges examined for the independent variables were 20–100 U/g odp (laccase dose, x_1), 2–8% odp (TEMPO dose, x_2) and 8–20 h (reaction time, x_3), and the results were coded as −1 or +1, both for direct comparison of coefficients and for easier understanding of the effect of the variables on the responses. The independent variables were zeroed at the central point. The results of the three replicates at the central point, and their variance, were used in combination with the variance of the saturated model to calculate Snedecor's *F*-value in order to determine whether the variance was homogeneous or heterogeneous. The variance was homogeneous in all cases, so a linear model was constructed, its significant terms

identified and its potential curvature detected. Linear multiple regression was applied by means of an Excel spreadsheet in order to implement the stepwise backward regression method and discard all terms with a probability $p < 0.05$.

8.2.4. Analysis of pulp properties

Pulp viscosity was determined in accordance with ISO 5351/1. Borohydride viscosity was measured after treatment with 2% $NaBH_4$, at 5% consistency at room temperature for 30 min (Roncero *et al.* 2003). The bulk acid groups content was determined by conductimetric titration as described elsewhere (Aracri *et al.* 2011). TEMPO-oxidized pulp samples were further oxidized with $NaClO_2$ for selective conversion of aldehyde groups into carboxyl groups at room temperature for 48 h. The carboxyl content was determined with the above-described conductimetric titration method. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally present in the pulp (Saito and Isogai 2005).

8.2.5. Paper testing

Prior to oxidative treatment, each pulp was disintegrated for 30 000 revolutions and then refined for 4500 revolutions according to ISO 5263. Once treated, the samples were disintegrated for 10 000 revolutions and used to prepare handsheets on a Rapid-Köthen laboratory former according to ISO 5269-2. The handsheets were then conditioned at 23 °C at 50% relative humidity for at least 24 h before physical testing. Dry tensile strength and wet tensile strength were determined according to ISO 1924-3 and ISO 3781, respectively, the latter being measured in 15 mm wide specimen strips soaked in de-ionized water for 5 s.

8.3. Results and discussion

8.3.1. Preliminary pulp treatments

Preliminary tests were conducted by using 60 U/g odp laccase and 8% odp TEMPO under different operating conditions (reaction time, temperature, applied oxygen pressure and pulp consistency). In treatment LT-30 h, refining was applied both before and after treatment. As can be seen from Table 8-2, all LT treatments introduced aldehyde groups, especially at long treatment times and high consistency levels. The wet tensile index increased with increasing aldehyde content, which confirmed their mutual correlation. Both raising the temperature to 50 °C and removing applied O_2 pressure resulted in low yields of aldehyde and carboxyl groups relative to the initial treatment applied for 30 h. This can be ascribed to thermal decomposition of oxoammonium ion via a ring-opening mechanism (Ma *et al.* 2011) and to the enzyme requiring O_2 to regenerate TEMPO from its reduced form. Application of treatment LT-30 h to unrefined pulp resulted in lower yields of aldehyde and carboxyl groups relative to refined pulp; this was probably the result of an increased fibre surface area available for the oxidant after refining. The most salient effect was that obtained by increasing pulp consistency, which increased the amount of carboxyl and aldehyde groups by 150 and 50%, respectively, with respect to the same treatment at 1% consistency by effect of the improved oxidant–fibre interactions. Treatment 4h provided only a slight increase in aldehyde groups, and hence a modest improvement in wet tensile index, thus reflecting the significant influence of the reaction time on these properties. As a result of enhanced inter-fibre hydrogen bonding (Barzyk *et al.* 1997), the pulp samples with an increased carboxyl content exhibited an also increased dry tensile index. The tear index of the LT-treated samples was very low in relation to the control samples, especially at high consistency levels and, to a less extent, short reaction times; this was ascribed to the loss of fibre strength as a consequence of depolymerization of cellulose chains during the oxidative treatment. Finally, the burst index was seemingly influenced by both the carboxyl group content and fibre strength.

Table 8-2. Carboxyl and aldehyde group bulk contents of the control pulp, and of samples treated with laccase alone or the laccase–TEMPO system (LT) under variable operating conditions, and physical properties of the resulting handsheets. TR denotes pulp treated prior to refining.

Sample ID	Carboxylic Groups (μmol/g)	Aldehyde Groups (μmol/g)	Dry Tensile Index (N·m/g)	Wet Tensile Index (N·m/g)	Burst Index ($kPa \cdot m^2/g$)	Tear Index ($mN \cdot m^2/g$)
Control - 18 h	99 ± 2	1 ± 0	55.6 ± 4.0	1.4 ± 0.2	4.16 ± 0.44	24.4 ± 0.5
Laccase - 18 h	94 ± 2	1 ± 1	56.7 ± 4.6	1.5 ± 0.1	3.83 ± 0.46	21.4 ± 0.7
LT - 18 h	129 ± 2	73 ± 6	61.4 ± 5.7	7.6 ± 1.3	3.82 ± 0.52	11.9 ± 0.7
LT - 30 h	126 ± 2	107 ± 13	61.7 ± 4.3	9.9 ± 1.4	3.75 ± 0.59	11.4 ± 0.3
TR LT - 30 h	106 ± 6	86 ± 15	58.7 ± 4.1	6.6 ± 0.7	3.16 ± 0.49	11.0 ± 0.7
LT - 50°C - 30 h	93 ± 3	49 ± 3	54.5 ± 5.7	3.6 ± 0.7	2.87 ± 0.62	11.7 ± 0.5
LT - no P_{O2} - 30 h	96 ± 11	53 ± 10	54.4 ± 4.5	3.6 ± 0.6	3.23 ± 0.46	11.2 ± 0.7
LT - 4 h	93 ± 5	17 ± 4	58.7 ± 4.9	2.6 ± 0.2	4.04 ± 0.46	15.5 ± 0.5
LT - c 5% - 30 h	312 ± 11	159 ± 11	68.7 ± 4.0	13.1 ± 1.6	4.40 ± 0.29	9.1 ± 0.5

8.3.2. Experimental design

8.3.2.1. Modelling

Experimental data (Table 8-3) were fitted to a second-order polynomial equation with wet tensile index (Y_{WTI}), dry tensile index (Y_{DTI}), aldehyde content (Y_{CHO}) and borohydride viscosity (Y_{BV}) as response variables. Available data allowed no accurate model for the carboxyl content to be constructed from the process variables. A preliminary test with the models for aldehyde content, dry tensile index and borohydride viscosity revealed that the quadratic term was significant ($p < 0.05$). Two additional tests were therefore required in order to identify the specific variables possessing a significant term and ensure their accurate discrimination. A second analysis of the modelling equations provided responses where all significant terms had $p < 0.05$. The quadratic term in the model for wet tensile index was not significant.

Wet tensile index model:

$$Y_{WTI} = 3.3 + 1.1x_2 + 0.7x_3 \quad [8\text{-}1]$$

with $R^2 = 0.79$

Dry tensile index model:

$$Y_{DTI} = 50.3 - 0.7x_1 - 1.1x_1x_3 - 1.1x_1x_2x_3 + 3.7x_1^2 + 2.1x_2^2 \quad [8\text{-}2]$$

with $R^2 = 0.98$

Aldehyde content model:

$$Y_{CHO} = 42 + 23x_2 + 9x_3 - 8x_1x_3 + 3x_2x_3 - 6x_1x_2x_3 - 24x_1^2 - 12x_2^2 + 29x_3^2 \quad [8\text{-}3]$$

with $R^2 = 0.99$

Borohydride viscosity model:

$$Y_{BV} = 403 - 1x_1 - 67x_2 - 13x_3 - 6x_1x_3 + 6x_2x_3 - 10x_1x_2x_3 - 61x_1^2 + 27x_2^2 + 76x_3^2 \quad [8\text{-}4]$$

with $R^2 = 0.99$

Table 8-3 Applied conditions of the LT experiences and results of wet tensile index, dry tensile index, carboxyl content, aldehyde content and borohydride viscosity.

Exp.	Laccase dose (U/g)	TEMPO dose (%)	Time (h)	**WTI (Nm/g)**	**DTI (Nm/g)**	**COOH (µmol/g)**	**CHO (µmol/g)**	**Borohydride viscosity (ml/g)**
1	20	2	8	1.8	57.3	105	6	536
2	100	2	8	1.9	55.4	118	7	527
3	20	8	8	2.9	53.8	101	31	370
4	100	8	8	3.7	57.4	110	60	400
5	20	2	20	2.5	56.9	119	21	491
6	100	2	20	2.5	55.1	114	15	495
7	20	8	20	6.6	59.0	137	83	389
8	100	8	20	4.6	53.5	113	57	353
9	60	5	14	2.9	50.6	110	43	402
10	60	5	14	3.6	49.6	99	43	403
11	60	5	14	3.0	50.4	110	41	404
12	100	5	14	2.5	53.5	126	18	341
13	60	2	14	1.9	52.5	121	8	498

8.3.2.2. Wet tensile index model

The model for wet tensile index (Eq. [8-1] and Figure 8-1) predicted responses from 1.4 to 5.1 Nm/g. Based on it, the specific variables affecting this property were x_2 (TEMPO dose) and x_3 (reaction time), both in a linear manner. By contrast, the laccase dose had no effect on the wet tensile index over the studied range. This suggests that the lowest enzyme dose used sufficed to effect the conversion of primary hydroxyl groups to aldehyde groups promoting the formation of hemiacetal linkages between fibres, and hence the development of wet strength in the resulting handsheets.

8.3.2.3. Dry tensile index model

The model relating dry tensile index and process variables conformed to Eq. [8-2] and predicted property responses from 50.1 to 59.0 Nm/g. Based on it, the laccase dose (x_2) had a weak linear influence on the dry tensile index, with a coefficient of –0.7. In addition, a quadratic influence on the response was exhibited by the laccase dose, x_1^2, and TEMPO dose, x_2^2. As can be seen from Figure 8-2, the dry tensile index exhibited a constant value of 56.8 Nm/g at a low laccase dose ($x_1 = –1$) and an also low TEMPO dose ($x_2 = –1$), throughout the time range studied. Increasing the laccase dose from 20 to 52 U/g at a low TEMPO dose reduced the index to 52.7 Nm/g; also, further increasing the enzyme dose increased it up to 55.4 Nm/g irrespective of the particular reaction time. Using a low laccase dose ($x_1 = –1$) in combination with a medium ($x_2 = 0$) or high ($x_2 = 1$) TEMPO dose caused the dry tensile index to increase linearly with time (from 53.6 to 55.8 Nm/g and from 54.6 to 59.0 Nm/g, respectively). The opposite trend was observed at a high laccase dose ($x_1 = 1$), with dry tensile index decreasing from 54.4 to 52.2 Nm/g at a medium TEMPO dose and from 57.6 to 53.2 Nm/g at a high TEMPO dose. The index value obtained at a medium laccase dose was lower than those at high and low laccase doses throughout the TEMPO dose and reaction time ranges studied.

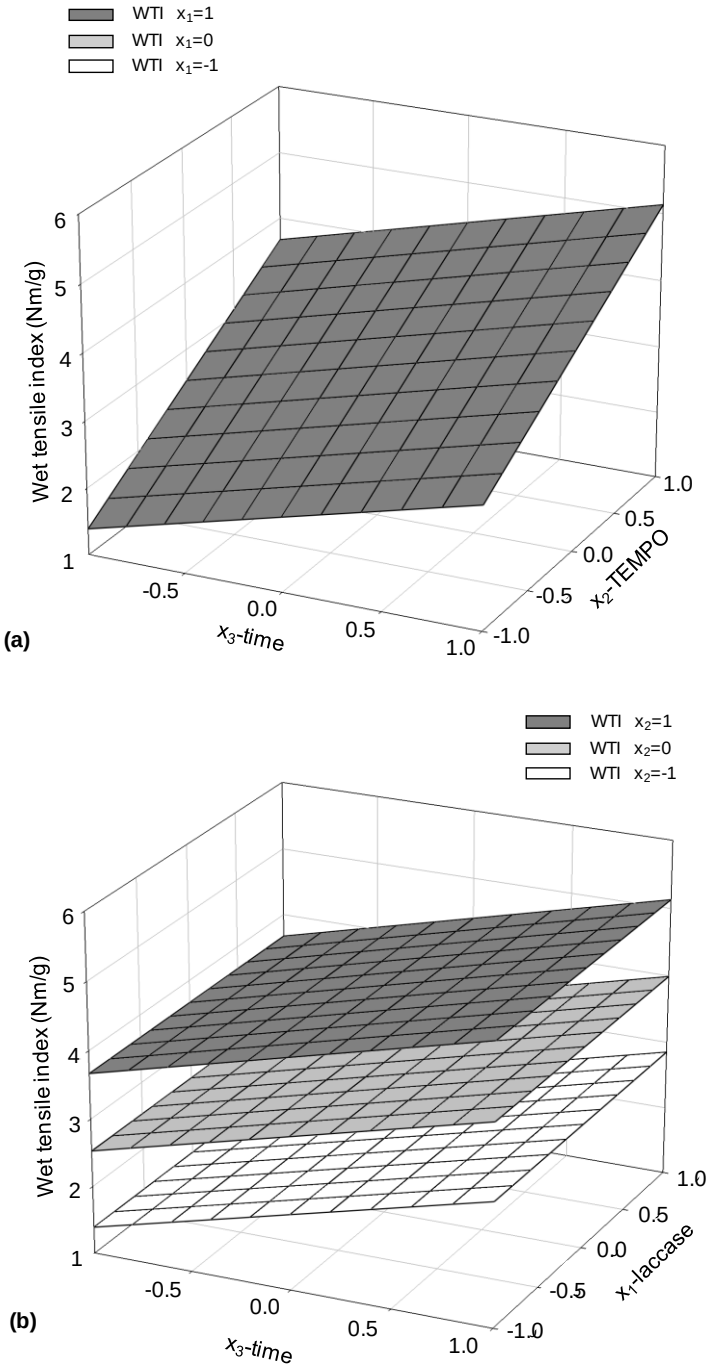

Figure 8-1. Variation of the wet tensile index as a function of the factors of the statistical plan, with the laccase dose, x_1 (a), and TEMPO dose, x_2 (b), at low, medium and high levels.

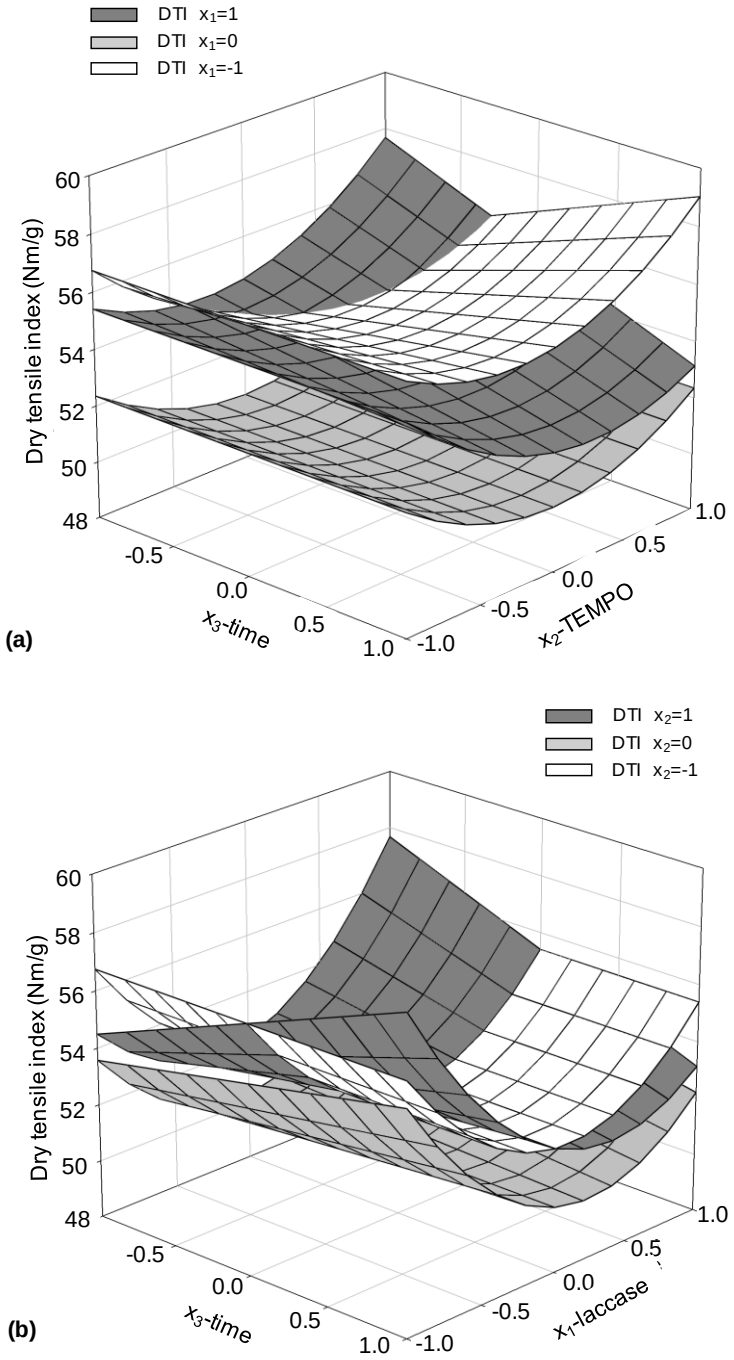

Figure 8-2. Variation of the dry tensile index as a function of the factors of the statistical plan, with the laccase dose, x_1, (a), and TEMPO dose, x_2, (b) at low, medium or high levels.

8.3.2.4. Aldehyde content model

Figure 8-3 shows the surface graphs for the aldehyde content model, based on Eq. [8-3]. As can be seen, the model predicted aldehyde contents from –17 to 96 μmol/g. The negative predictions were taken to be 0 μmol/g. Based on the model, the variables influencing the aldehyde content were the TEMPO dose (x_2) and reaction time (x_3). Similarly to the model for the wet tensile index, the laccase dose (x_1) had no influence on the aldehyde content and the TEMPO dose was the variable most strongly influencing this property, with a coefficient of 23. All variables exerted a quadratic influence on the response. With high (x_1 = +1) and low (x_1 = –1) laccase doses combined with a low TEMPO dose (x_2 = –1), the model predicted a decrease in aldehyde content from *ca.* 10 to –17 μmol/g between the eighth and twelfth hour of reaction, and an increase up to *ca.* 20 μmol/g after the twelfth. The decrease during the former time may have resulted from the oxidation rate of aldehyde groups to carboxyl groups exceeding that of primary alcohols to aldehyde groups. With a medium laccase dose and a low TEMPO dose, the aldehyde response decreased from 30 to 18 μmol/g as the reaction time was increased from 8 to 17 h, respectively, after which the response increased up to 42 μmol/g. Whichever the laccase dose, raising the TEMPO dose increased the aldehyde content —especially from the low mediator level to its medium level. Based on the model, the highest aldehyde content can be expected from the use of a high TEMPO dose in combination with a medium laccase dose and a high reaction time.

8.3.2.5. Borohydride viscosity model

Borohydride viscosity measurements were performed to assess changes in the degree of cellulose polymerization during the laccase–TEMPO treatments. Prior to measurement, oxidized pulp samples were treated with sodium borohydride to inactivate carbonyl groups (by reduction to hydroxyl groups) and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium (Aracri *et al.* 2011). The model for borohydride viscosity (Eq. [8-4] and Figure 8-4) predicted property responses from 330 to 593 ml/g. Based on it, all process variables influenced borohydride viscosity, the laccase dose (x_1) exhibiting a weak effect (a coefficient of 1) and the mediator dose (x_2) the strongest influence (a coefficient of 67). In addition, all variables exerted a quadratic influence on the response. As can be seen from

Figure 8-4, borohydride viscosity was decreased as the time was increased from 8 to 14, 16.5 and 17.5 h with a low, medium and high TEMPO dose, respectively, used in combination with low laccase dose. Although, based on the mathematical model, increasing the time beyond these points resulted in slightly increased viscosity, the increase was experimentally unlikely and the property was instead assumed to remain constant over the second time range. Borohydride viscosity dropped as the TEMPO dose was raised, especially from a low dose ($x_2 = -1$) to medium one ($x_2 = 0$). The response peaked as the laccase dose was raised from 20 to 60, 79 and 72 U/g at a low, medium and high TEMPO dose, respectively, and decreased slightly to values similar to those obtained at a low laccase dose as the time was increased to 20 h. The highest borohydride viscosity in the studied plan (593 ml/g) was obtained at a low reaction time and an also low TEMPO dose in combination with a medium laccase dose, which however provided low degree of aldehyde and carboxyl functionalization and almost no improvement of dry and wet tensile index.

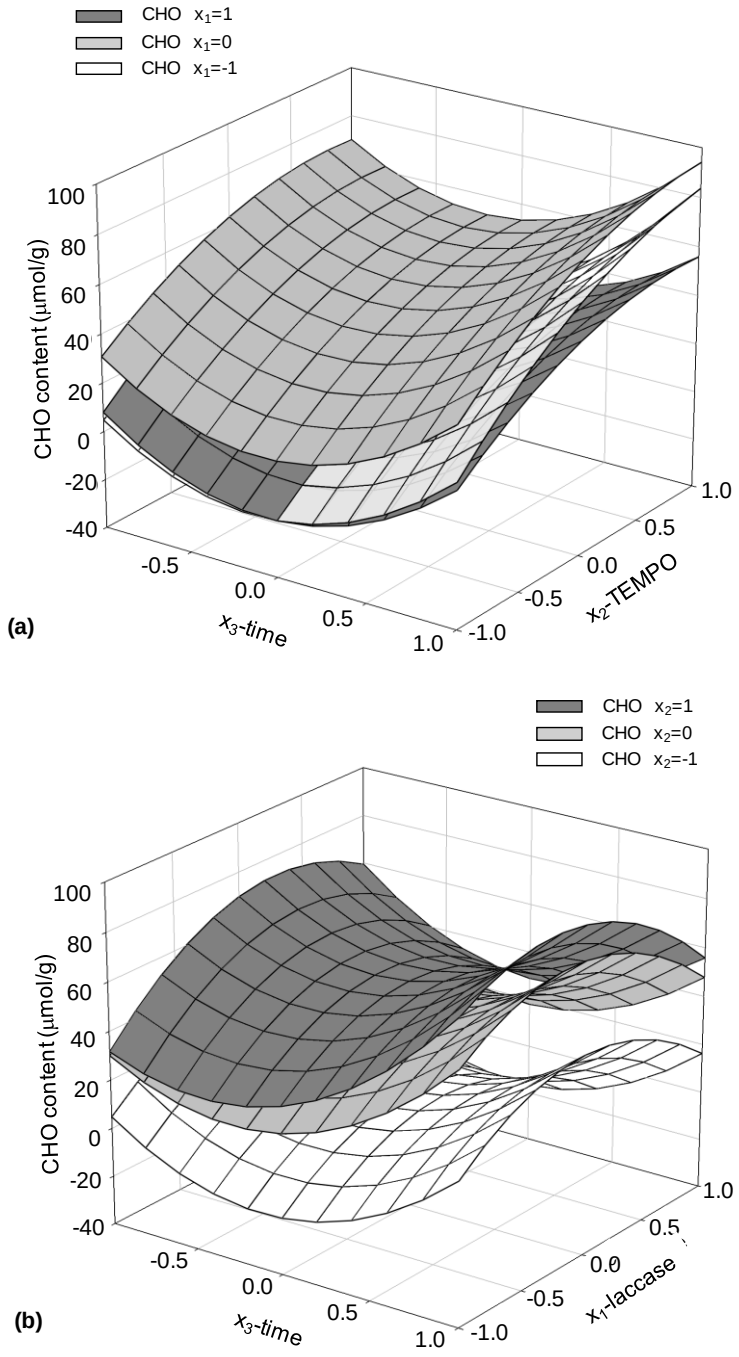

Figure 8-3. Variation of the aldehyde content as a function of the factors of the statistical plan, with the laccase dose, x_1, (a), and TEMPO dose, x_2, (b) at low, medium or high levels.

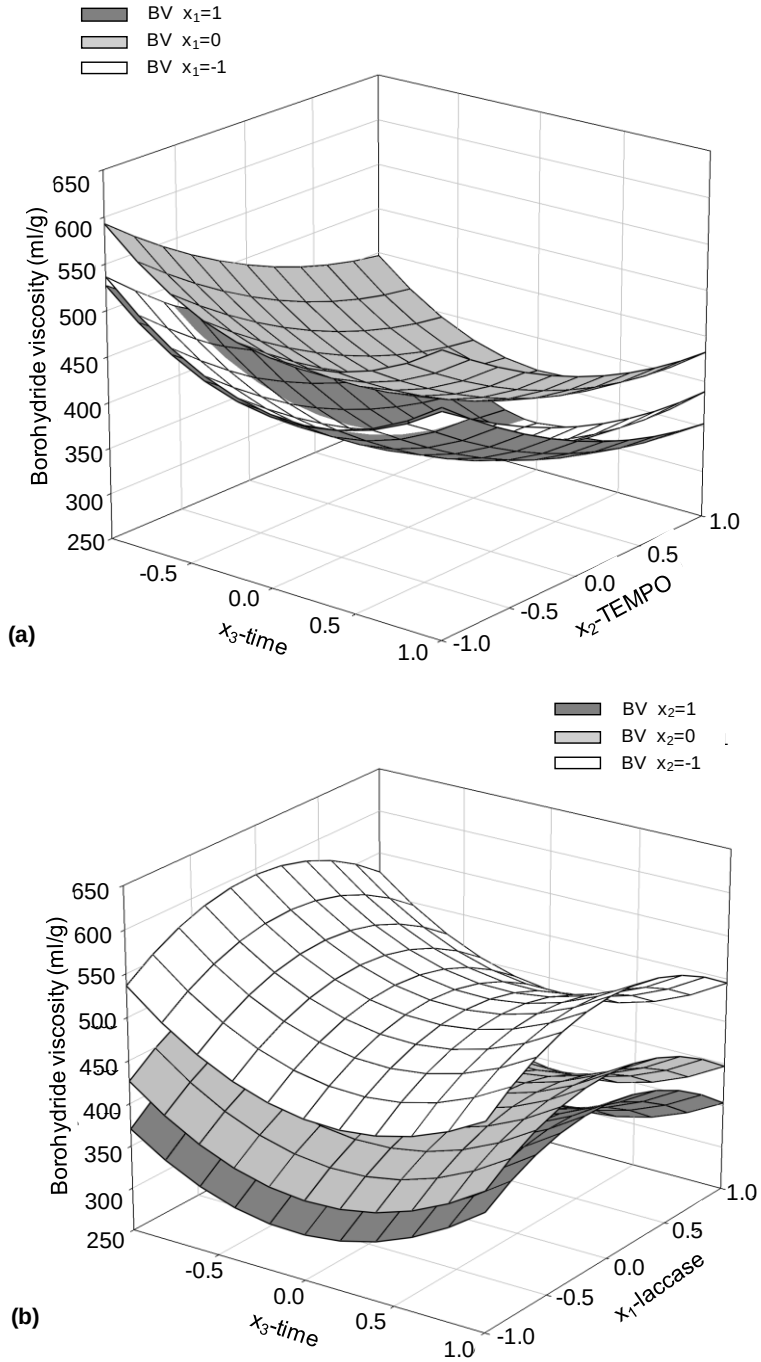

Figure 8-4. Variation of the borohydride viscosity as a function of the factors of the statistical plan, with the laccase dose, x_1, (a), and TEMPO dose, x_2, (b) at low, medium or high levels.

8.3.2.6. Statistical analysis of carboxyl groups

The 2^3 factorial design used allowed us no accurate model for predicting the carboxyl content of the fibres to be constructed. In fact, the points in the corresponding semi-zeta graph for the saturated model obtained clustered in no definite manner (results not shown). This may have been the result of (a) heteroscedasticity (i.e. a non-constant variance), (b) the factor having little influence on the response or (c) excessive experimental noise. Although the carboxyl content could be fitted to no specific model, we reported the results of the experiments prescribed by the statistical plan in graphs such as those shown in Figure 8-5 in order to assess the influence of changing a variable between its minimum and maximum levels at four different combinations of maximum and minimum levels of the other two variables. Figure 8-5a illustrates the effect of the laccase dose on the carboxyl content. The most marked changes as the laccase dose was increased from 20 to 100 U/g were observed with both the mediator dose (x_2) and treatment time (x_3) at their lowest (A) or highest levels (D), with an increase from 105 to 118 μmol/g in the former case, and a decrease from 137 to 113 μmol/g in the latter. Based on the graph of Figure 8-5b, increasing the TEMPO dose from 2 to 8% odp had significant, opposite effects when a high laccase dose was used in combination with a low reaction time (B) (a decrease from 118 to 110 μmol/g) or a low enzyme dose was used together with a high reaction time (C) (an increase from 118 to 137 μmol/g). As can be seen from Figure 8-5c, increasing the reaction time increased the carboxyl content in all cases except B (where x_1 was at the highest level and x_2 at the lowest), especially with a low laccase rate and a high TEMPO rate.

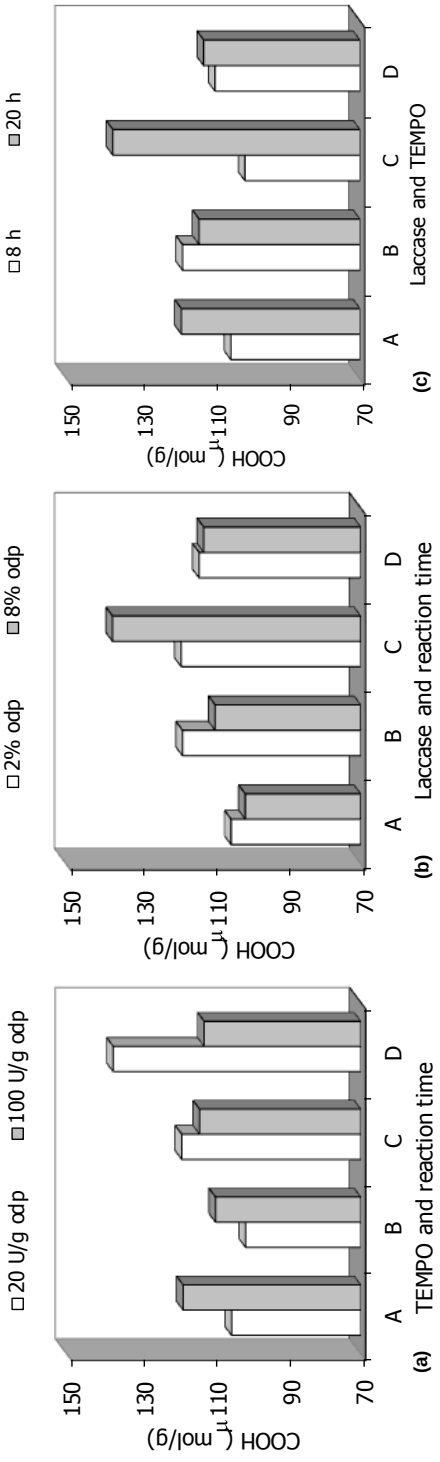

Figure 8-5. Effect of increasing the variables from their lowest level to the highest on the content of carboxyl groups. (a) Effect of increasing the laccase dose from 20 to 100 U/g odp: (A) 2% TEMPO, 8 h; (B) 8% TEMPO, 8 h; (C) 2% TEMPO, 20 h; (D) 8% TEMPO, 20 h. (b) Effect of increasing the TEMPO dose from 2 to 8% odp: (A) 20 U/g laccase, 8 h; (B) 100 U/g laccase, 8 h; (C) 20 U/g laccase, 20 h; (D) 100 U/g laccase, 20 h. (c) Effect of increasing the reaction time from 8 to 20 h: (A) 20 U/g laccase, 2% TEMPO; (B) 100 U/g laccase, 2% TEMPO; (C) 20 U/g laccase, 8% TEMPO; (D) 100 U/g laccase, 8% TEMPO.

An increased concentration of acid groups in pulp fibres is known to increase the dry tensile strength of the resulting paper by facilitating inter-fibre hydrogen bonding. In a recent study (Patel *et al.* 2011) reporting the effect of the laccase–TEMPO system on cotton linter pulp, the oxidative modification was found to involve the introduction of carbonyl groups and, to a much less extent, that of carboxyl groups. The latter result was ascribed to aldehyde autoxidation promoted by the oxygen-saturated medium since both isolated laccase and the TEMPO-derived oxoammonium ion proved unable to oxidize aldehyde groups to the corresponding carboxyl groups. In this study, we could not establish the exact influence of process variables on the carboxyl content; also, the mechanism by which aldehyde groups are further oxidized to carboxyl groups by the laccase–TEMPO system remains unclear. This may explain why the model for the dry tensile index was more complex than that for the wet tensile index —development of which depends mainly on the formation of inter-fibre covalent bonds such as those provided by hemiacetal linkages involving aldehyde groups. However, the highest carboxyl content was obtained under those conditions providing the highest response of dry tensile index in the model for this property, which confirms the contribution of carboxyl groups to improving the dry tensile index in handsheets from sisal fibres.

8.3.3. Assessing the effects of aldehyde groups on pulp properties

8.3.3.1. Intrinsic viscosity

The effect of the oxidative treatments on the degree of cellulose polymerization in the fibres was assessed from intrinsic viscosity measurements. Because a cupriethylenediamine solution is highly alkaline and depolymerization by β-elimination promoted by carbonyl groups may occur in oxidized fibres during viscosity measurements, the pulp samples were treated with sodium borohydride (borohydride viscosity) in order to inactivate carbonyl groups by reduction to hydroxyl groups (Cadena *et al.* 2010, Roncero *et al.* 2002).

The difference between the viscosity values obtained with and without the reductive treatment provided an indication of the depolymerizing effect of carbonyl groups formed by TEMPO-mediated oxidation.

Oxidized pulp samples exhibited a marked loss in viscosity (up to 53%) compared to reduced samples, the difference increasing with increase in TEMPO dose and reaction time (results not shown).

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

$$\mathbf{CS} = \frac{\mathbf{DP_o - DP}}{\mathbf{DP}} \quad [8\text{-}5]$$

where DP_O is the degree of polymerization of the initial pulp and DP that after the oxidative treatment. The degree of polymerization is calculated from the intrinsic viscosity [η], using the equation of Evans and Wallis (1987) (SCAN-CM 15:88):

$$\boldsymbol{DP^{0.85} = 1.1 * [\eta]} \quad [8\text{-}6]$$

The intrinsic viscosity values obtained in the presence and absence of a reductive treatment allowed us to calculate the number of scissions due to the oxidative treatment itself (CS_{GT}) and to β-elimination promoted by carbonyl groups ($CS_{C=O}$)(Aracri *et al.* 2011). Figure 8-6 shows CS_{GT}, $CS_{C=O}$ and their combination (CS_T) for each pulp obtained in the experiments of the statistical plan. As can be seen, all oxidative treatments caused cellulose depolymerization and the formation of carbonyl groups responsible for further chain scissions in the alkaline measurement medium. TEMPO-mediated oxidation is highly selective for primary hydroxyl groups, which are converted into aldehydes/(hemi)acetals; however, secondary hydroxyl groups can take part in side reactions leading to the formation of keto groups (Fabbrini *et al.* 2001).

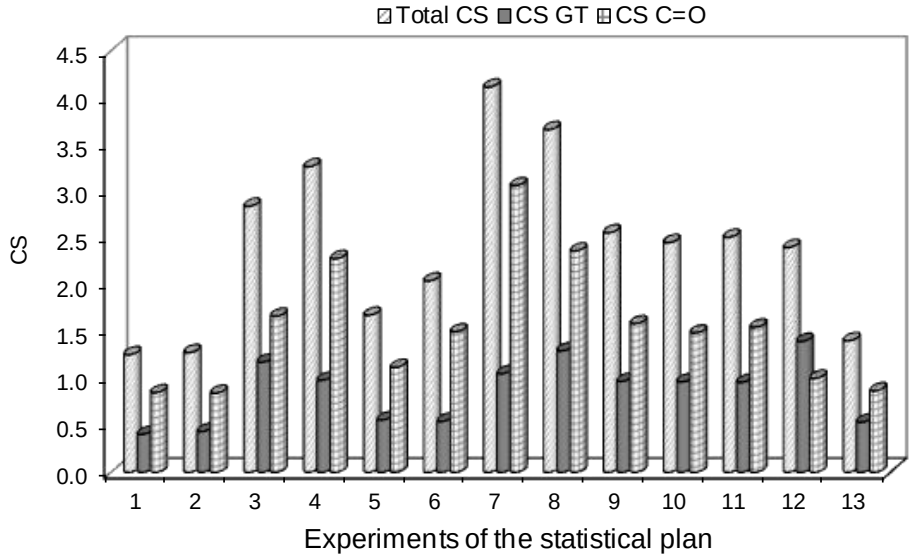

Figure 8-6. CS_T, CS_{GT} and $CS_{C=O}$ for each pulp obtained in the experiments of the statistical plan.

We found a linear relationship between the aldehyde content and the number of chain scissions due to carbonyl groups as determined in the experimental plan (Figure 8-7); this is suggestive of preferential conversion of hydroxyl groups into aldehyde groups. The classical oxidation method with alkaline hypohalite as the actual oxidant results in severe molecular weight losses in cellulose that are mainly due to alkali-induced β- elimination reactions starting from carbonyl groups (Potthast *et al.* 2009).

The slightly acidic medium used by the laccase–TEMPO system hinders β-elimination, so cellulose degradation is most probably a result of homolytic processes involving some active radical species formed *in situ* as by-products during the oxidation treatment (Patel *et al.* 2011, Tamura *et al.* 2010). Since cellulose degradation decreases fibre strength, the loss of pulp viscosity is a major drawback in processes intended to have paper develop strength-related properties. The improved dry and wet tensile index values obtained under certain conditions of the experimental plan despite the decreased pulp viscosity can be ascribed to the formation of hydrogen and covalent inter-fibre bonds offsetting the loss of fibre strength.

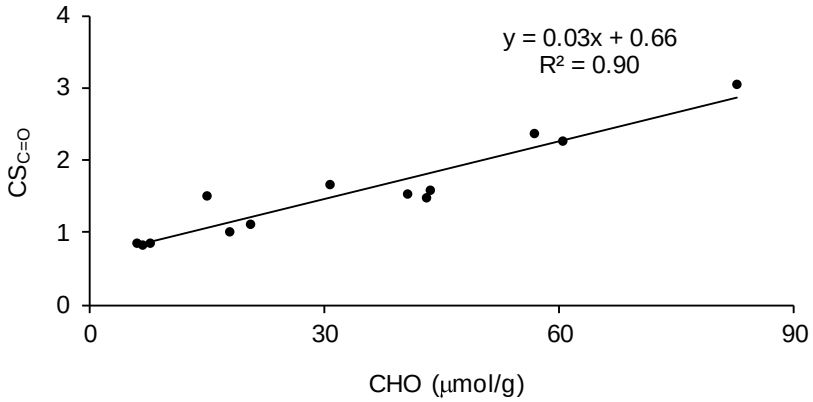

Figure 8-7. Variation of the number of chain scissions in the cellulose chain with the content in aldehyde groups.

8.3.3.2. Wet tensile index

In recent years, TEMPO-mediated oxidation in the presence of NaClO/NaBr has proved an efficient method to improve wet strength in cellulose materials including pulp fibres by effect of the introduction of aldehyde functionalities promoting the formation of inter-fibre covalent bonding through hemiacetal linkages with sterically close hydroxyl groups in cellulose (Saito and Isogai 2006). In previous work (Aracri *et al.* 2011), we demonstrated the ability of the enzymatic method (viz. the laccase–TEMPO system) to significantly improve the wet strength of sisal pulp fibres; this suggested the formation of a substantial amount of aldehyde groups during the oxidative treatment. In this work, we found the wet tensile index to be influenced similarly to the aldehyde content by process variables; in addition, a graphical comparison of these properties revealed that the wet tensile index and aldehyde content followed a clear-cut trend that fitted a straight line with a coefficient of determination $R^2 = 0.88$ (Figure 8-8).

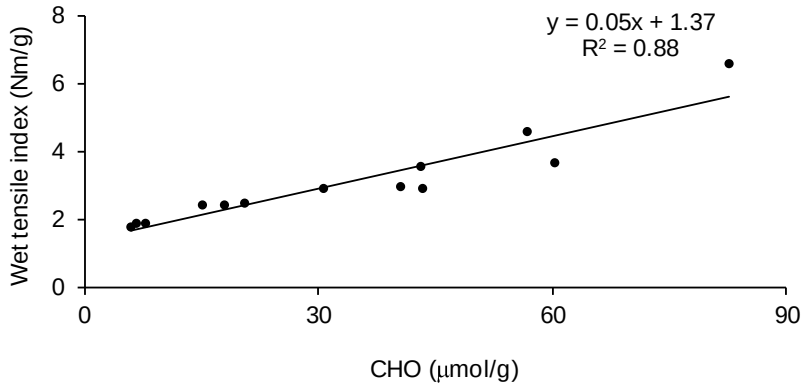

Figure 8-8. Variation of the wet tensile index with the content in aldehyde groups.

The formation of hemiacetal linkages is the mechanism by which aldehyde-containing resins develop temporary wet-strength in paper (Chen *et al.* 2002). These resins improve wet strength of paper by forming inter-fibre covalent bonds through hemiacetal/or acetal linkages between hydroxyl groups of cellulose/hemicellulose and the aldehyde groups of the resins. Laccase-TEMPO oxidation generates aldehyde functionalities in cellulose fibres and these are able to form hemiacetal linkages with hydroxyl groups in adjacent fibre surfaces, with the advantages of developing wet strength without the addition of any synthetic resin and providing easily repulpable papers.

The use of laccase for improving wet strength of lignocellulosic pulps has been already reported (Lund and Felby 2000, 2001), although limited to lignin-rich pulps. In these studies combinations of the enzyme with a lignin-rich extractive or a mediator were applied to high-yield unbleached kraft pulp. The improvements obtained were ascribed to polymerization of lignin in the handsheets, and also to enhanced production of phenoxy-radical increasing cross-lignin between fibres. However, the gain in wet strength showed by the authors was not as high as in the present work unless it was combined with a heat treatment.

8.4. Conclusions

The influence of laccase–TEMPO process variables on the properties of a cellulose pulp was for the first time examined by using a statistical plan. Preliminary results showed applying oxygen pressure and using room temperature, a long reaction time and, especially, a high consistency, to boost functionalization and improve the wet and dry tensile indices of sisal pulp as a result. Based on the mathematical models derived, using a high TEMPO dose and a long reaction time favours the formation of aldehyde groups and leads to improved wet strength in the resulting handsheets. Based on the model for dry tensile index, the responses can be expected to peak at high TEMPO doses used in combination with either a low laccase dose and long reaction time or a high laccase dose and short reaction time. Although no accurate model for carboxyl content could be established, the improvement peaked under those conditions yielding the highest dry tensile index, which suggests that carboxyl groups promote inter-fibre hydrogen bonding in the resulting handsheets. Besides the environmental benefits associated to the replacement of halide-containing compounds with an enzyme as co-oxidant, laccase–TEMPO oxidation has the advantage over the classical method that it operates at near-neutral pH —which reduces cellulose depolymerization by β-elimination. As shown here, however, the enzyme treatment reduces pulp viscosity somewhat, probably by effect of side reactions involving active radical species formed during the oxidation process. Further study will be required to confirm this assumption and acquire a better understanding of the mechanisms behind cellulose degradation in the enzymatic process with a view to preventing fibre strength losses and maximizing the benefits derived from the oxidative effects in terms of paper strength.

8.5. References

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Enhancing the effectiveness of a laccase–TEMPO treatment has a biorefining effect on sisal cellulose fibres

Based on **Publication 7**: Elisabetta Aracri and Teresa Vidal. Submitted to *Cellulose*.

Abstract

Enhancing the effectiveness of a laccase–TEMPO treatment on sisal pulp by increasing pulp consistency was for the first time found to increase the biorefining potential of this enzyme–mediator system. The operating conditions used were those previously found to maximize oxidative functionalization and paper strength. Prior to the enzyme treatment, the pulp was refined at a variable intensity (0, 3000 and 4500 revolutions) in order to ascertain whether the increased surface area would lead to enhanced functionalization and boost the refining effect as a result. Increasing pulp consistency increased the contents in aldehyde and carboxyl groups by 130% and 94%, respectively. Also, it resulted in more marked reduction of pulp viscosity during the enzyme treatment, especially at a high refining intensity; this had a detrimental effect on fibre strength and significantly reduced tear strength in the refined pulp. Oxidized pulp exhibited a considerably increased water retention value (WRV) with respect to the initial pulp, particularly after refining. Dry tensile index was increased by 21%, 18% and 12%, and burst index by 23%, 16% and 13% at 0, 3000 and 4500 rev, respectively, by the laccase–TEMPO treatment as a result of increased inter-fibre hydrogen bonding offsetting the loss of fibre strength, an effect that can provide substantial savings in refining energy. Based on the results, a laccase–TEMPO treatment is an enzymatic booster of mechanical refining with the added advantages of providing unaltered drainability and increased air permeability. The most salient effect of the laccase–TEMPO treatment was an increase in wet tensile strength (by 160%, 553% and 588% at 0, 3000 and 4500 rev, respectively) that can be ascribed to inter-fibre covalent bonding through hemiacetal linkages promoted by aldehyde groups. The improvement was much greater than that obtained at a lower consistency under identical conditions.

9.1. Introduction

Currently, wood is by far the major raw material for the global pulp and paper industry; in fact, non-wood plants account for less than 10% of the total amount of fibre used for papermaking worldwide (Leponiemi 2008). However, many countries abound with non-woods plants and some with inadequate forest supplies (particularly China and India) used them as their primary source of fibre for papermaking (López *et al.* 2004). In

addition, environmental pressure, restrictions on forest use, significant increases in wood and recycled fibre costs and the increasing world demand for paper are leading many paper companies in the traditionally forest-rich countries to take a renewed look at non-woods (Kissinger *et al.* 2007). In developed countries, the modern use of non-wood fibres in papermaking has largely been applied in the production of specialty papers. That is, those papers which require properties which could not easily be achieved solely through the use of wood fibre furnish. Fibres strength, length, the ratio of fibre length to diameter are primary factors in the consideration of uses (Martínez 1998). Sisal fibres are extracted from the leaves of sisal (*Agave sisalana*), a monocotyledonous plant endemic to Central America, and are among the most widely used natural fibres by virtue of the ease of cultivation of sisal plants and their short renewal times (Mukherjee and Satyanarayana 1984, Samal *et al.* 1994). Sisal fibres have traditionally been used to manufacture natural ropes, cordage and sacking. In recent years, stiff competition from synthetic materials and the lack of technological development has eroded the traditional sisal markets (Hurter 1997). However, sisal pulp has some features including a high tear resistance, alpha cellulose content, porosity, bulk, absorbency and folding endurance which make it an excellent raw material for a variety of specialty papers. Moreover, the fact that sisal pulp surpasses softwood kraft pulp in physical properties facilitates its use as reinforcing fibre in high recycled content paper or for reducing basis weight while maintaining product quality (Maddern and French 1994).

Substantial research efforts have been made in the last few decades at introducing biotechnology in the pulp and paper industry with a view to innovating and making it more eco-friendly. Biotechnology in the pulp and paper sector has a strong focus on the application of biological products such as enzymes (Bajpai 1999, Kenealy and Jeffries 2003). The importance of enzyme technology lies in its potential for supplying more specific reactions, avoiding deleterious effects on the environment, reducing resource consumption and, ultimately, decreasing costs (Kenealy and Jeffries 2003). Among the most widely investigated enzymes in the pulp and paper industry are the multi-copper oxidases laccases (EC. 1.10.3.1), which possess a wide range of oxidative capabilities and flexibility of use for the production of paper and its derivatives (Widsten and Kandelbauer 2008). Ever since the discovery of chemical mediators capable of extending enzymatic oxidation to non-phenolic compounds, research interests have focused mainly on the potential of laccase–mediator systems for aiding pulp bleaching (Barreca *et al.* 2003, Bourbonnais and Paice 1990, Fillat *et al.* 2010). In recent years, pulp and paper

research has increasingly switched to a different use of laccases such as the targeted modification of lignocellulose fibres with a view to improving intrinsic fibre properties or developing novel ones. The laccase-TEMPO mediated system provides a potentially effective approach to the oxidative modification of cellulose pulp fibres. TEMPO-mediated oxidation is a well-known procedure for introducing carboxyl and aldehyde functional groups into cellulose in an aqueous medium at room temperature (de Nooy *et al.* 1995a). This approach has been exploited to improve various physical properties of pulp fibres including inter-fibre bonding and hence strength-related properties in the resulting paper (Dang *et al.* 2007, Duarte *et al.* 2006, Lianshan *et al.* 2008, Marzorati *et al.* 2005). Introducing aldehyde groups into pulp has been shown to increase paper wet strength by effect of inter-fibre covalent bonding through hemiacetal linkages with hydroxyl groups on adjacent fibre surfaces (Saito and Isogai 2005, Saito and Isogai 2006). Although laccase has been shown to catalyse the regenerative oxidation of TEMPO (Viikari *et al.* 1999), the reaction requires the presence of NaClO/NaBr as a co-oxidizer system (Bragd *et al.* 2001, Chang and Robyt 1996, de Nooy *et al.* 1995b, Isogai and Kato 1998). Environmental concern has raised interest in replacing halide-based oxidative systems with laccase and oxygen as primary oxidants (Arends *et al.* 2006, Viikari *et al.* 1999). Similarly to the NaClO/NaBr system, oxoammonium ion is regenerated *in situ*, so only oxygen is consumed in the course of the reaction.

In recent work, we demonstrated the ability of the laccase–TEMPO system to significantly improve wet strength by facilitating the formation of a substantial amount of aldehyde groups in sisal pulp (Aracri *et al.* 2001b, 2011c). The influence of process variables including the laccase and TEMPO doses, and reaction time, on various properties of oxidized fibres and of handsheets made from them was for the first time assessed by using a three-variable statistical plan. In this work, the most effective laccase–TEMPO treatment for improving paper strength found with the statistical plan was applied at an increased pulp consistency and following refining at a variable intensity in order to ascertain whether the improved interaction between fibres and oxidant, and the increased surface area, would lead to enhanced functionalization and boost the refining effect.

9.2. Materials and methods

9.2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Aldrich and used as received. Laccase from *Trametes villosa* was supplied by Novozymes (Bagsvaerd, Denmark). One activity unit was defined as the amount of enzyme transforming 1 μmol ABTS/min to its cation radical (ε_{436nm} = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. Sisal (*Agave sisalana*) pulp (1% lignin content) from a soda–anthraquinone cooking process was supplied by CELESA (Tortosa, Spain). Following conditioning at 2% consistency at pH 4 (H_2SO_4) under stirring for 30 min, the pulp was filtered and extensively washed with de-ionized water. This step was needed to remove contaminants and metals, and also to bring the pulp to a suitable pH for the enzyme treatment.

9.2.2. Pulp treatment

Laccase–TEMPO oxidation treatments were performed at room temperature on an amount of 30 g of oven-dried pulp (odp) at 5% consistency, in a 5 l reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5, under an oxygen pressure of 0.6 MPa, and conditions of laccase and TEMPO doses and reaction time which were found to provide the greatest increases in carboxyl and aldehyde contents, and improvements of wet and dry tensile strength, in an experimental plan developed in previous work. The specific conditions used were as follows: 20 U/g odp laccase, 8% odp TEMPO and a reaction time of 20 h. After the enzyme treatment, the pulp samples were filtered through a fritted glass funnel of 40–100 μm pore size and extensively washed with de-ionized water.

9.2.3. Pulp refining and handsheet formation

Prior to oxidative treatment, pulp samples were disintegrated for 30 000 revolutions and filtered through a Buchner funnel in order to form a wet fibre pad and recirculate the filtrate twice to avoid losses of fines. Then, the samples were refined at a variable intensity (0, 3000 and 4500 rev) according to ISO 5264 and disintegrated for 10 000 rev

to determine drainability (expressed in Schopper–Riegler degrees, °SR) according to ISO 5267-1 and the water retention value (WRV) according to ISO 23714. Finally, the samples were used to obtain handsheets on a Rapid–Köthen laboratory former according to ISO 5269-2 and the handsheets conditioned at 23 °C at 50% relative humidity for at least 24 h before physical testing.

9.2.4. Analysis of pulp properties

The enzymatically treated pulp samples were compared to the initial pulp at each refining point in terms of drainability, water retention value, carboxyl and aldehyde contents, viscosity and morphological characteristics of the fibres. The bulk acid groups content was determined by conductimetric titration as described elsewhere (Aracri *et al.* 2011c). The samples were oxidized with $NaClO_2$ at room temperature for 48 h for selective conversion of aldehyde groups into carboxyl groups. The carboxyl content was determined by conductimetric titration. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally present in the pulp (Saito and Isogai 2005). Pulp viscosity was determined in accordance with ISO 5351-1. Borohydride viscosity was measured after treatment with 2% $NaBH_4$, at 5% consistency at room temperature for 30 min (Roncero *et al.* 2003). The morphological properties of the fibres (viz. length, width and curl), and the content in fines of the pulp samples were determined in accordance with TAPPI T 271 on a Metso kajaaniFS300 fibre analyser. All samples were analysed in duplicate.

9.2.5. Physical properties and SEM images of handsheets

Apparent density, dry tensile strength, tearing resistance, burst strength, wet zero span tensile strength and wet tensile strength were determined according to ISO 438, ISO 1924-3, ISO 1974, ISO 2758, ISO 15361 and ISO 3781, respectively, and wet tensile index was measured in 15 mm wide specimen strips soaked in de-ionized water for 5 s. Air permeability was measured on a Bekk tester as the amount of time required for a certain volume of air to flow through a 1 cm^2 circular area of the test specimen under a prescribed pressure difference between the two handsheet surfaces. The results thus obtained were the averages of ten measurements and expressed in Bekk seconds. Surface

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

9.3. Results and discussion

9.3.1. Pulp properties

Pulp samples refined at a variable refining intensity were analysed for drainability (°SR) and water retention value (WRV) prior to and after laccase–TEMPO treatment (Table 9-1). As a rule, drainage resistance increased with increasing refining intensity. Specifically, enzymatically treated samples exhibited no change in drainability when refined, whereas unrefined samples exhibited an increase in this property by 25%. Possibly, a certain amount of fines produced during refining and contributing to drainage resistance was lost through filtering of the pulp after the oxidative treatment, thus avoiding the effect observed in unrefined pulp. Similarly to drainage resistance, WRV, which is a measure of the water absorption capacity of fibres, increased with increasing refining intensity by effect of structural changes in the cell walls of pulp fibres. Interestingly, the laccase–TEMPO treatment substantially increased WRV with respect to the initial pulp, particularly after refining (more than 30%).

Table 9-1. Water retention (WRV) and drainability values (± standard deviation) of initial and laccase-TEMPO (LT) – treated pulps at each refining intensity (PFI revolutions).

	Initial pulp			LT-treated pulp		
PFI revolutions	**0**	**3000**	**4500**	**0**	**3000**	**4500**
Schopper-Riegler (°SR)	8 ± 1	20 ± 1	27 ± 0	10 ± 0	19 ± 0	27 ± 1
WRV (g/g)	1.10 ± 0.02	1.51 ± 0.00	1.71 ± 0.06	1.24 ± 0.10	2.06 ± 0.05	2.28 ± 0.20

Figure 9-1 compares enzymatically treated pulp with the initial pulp at each refining intensity in terms of the contents in carboxyl and aldehyde groups. In previous work (Aracri *et al.* 2011b), we found the laccase–TEMPO system to oxidatively modify sisal cellulose fibres, largely by introducing aldehyde groups and, to a much less extent,

carboxyl groups. In a trial aimed at checking whether the degree of functionalization was increased by effect of prior refining, we obtained a 20% increase in carboxyl groups and a 25% increase in aldehyde groups in pulp treated after refining with respect to pulp treated prior to refining, and ascribed the effect to the availability of an increased fibre surface area for the oxidant. A similar result was obtained in a study where sisal fibres were functionalized by laccase-catalysed grafting of ferulic acid (Aracri *et al.* 2011a): refining the pulp prior to the enzyme treatment resulted in increased grafting of the phenolic compound. As can be seen from Figure 9-1, the contents in carboxyl and aldehyde groups of the initial pulp were not altered by refining; on the other hand, the laccase–TEMPO treatment introduced both types of functional groups, to an increasing extent as the refining intensity was raised.

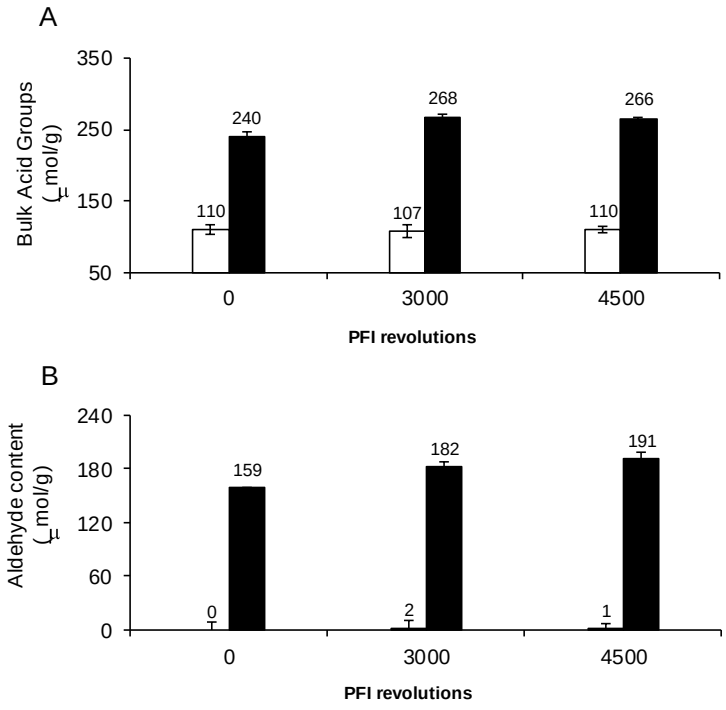

Figure 9-1. Bulk acid groups (A) and aldehyde (B) contents of initial pulp (white bars) and Laccase-TEMPO – treated pulp (black bars) at each refining intensity.

The increased carboxyl content accounts for the increased WRV values of oxidized pulp relative to the initial pulp as carboxyl groups make fibres hydrophilic. The content in

carboxyl groups was increased by 118%, 150% and 142%, and that of aldehyde groups by 160%, 180% and 190%, after treatment of pulp refined at 0, 3000 and 4500 rev, respectively. Therefore, increasing the refining intensity rendered fibre walls more accessible to the oxidative system. The increase in carboxyl groups with refining was only observed from 0 to 3000 revolutions (12%); in fact, stronger refining failed to raise the carboxyl content. By contrast, the aldehyde content increased linearly with increasing refining intensity. This may have been the result of carboxyl groups and aldehyde groups forming via different mechanisms. Thus, aldehyde groups formed by TEMPO-mediated catalytic conversion of primary hydroxyl groups in cellulose or hemicellulose, which was boosted by an increased fibre wall accessibility; on the other hand, carboxyl groups probably formed by aldehyde autoxidation promoted by the oxygen-saturated medium since both isolated laccase and the TEMPO-derived oxoammonium ion are unable to oxidize aldehyde groups to carboxyl groups (Patel *et al.* 2011). One other variable with a substantial effect on oxidative functionalization was pulp consistency. In this work, we applied the conditions found to maximize functionalization in a statistical plan of laccase–TEMPO tests performed in previous work (Aracri *et al.* 2011b) to pulp at an increased consistency (5% instead of 1%). The higher consistency used resulted in considerably higher aldehyde and carboxyl group contents (130% and 94%, respectively) with respect to pulp treated under the same conditions but 1% consistency. This testifies to the boosting effect of improved oxidant–fibre interaction through the use of an increased consistency in laccase–TEMPO treatments.

Viscosity measurements were used to assess changes in the degree of cellulose polymerization during the laccase–TEMPO treatment. Prior to measurement, oxidized pulp samples were treated with sodium borohydride to inactivate carbonyl groups by reduction to hydroxyl groups and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium (Aracri *et al.* 2011c, Roncero *et al.* 2003). The difference between the viscosity values obtained with and without the reductive treatment provided an indication of the depolymerizing effect of carbonyl groups formed by TEMPO-mediated oxidation. As shown in Table 9-2, non-reduced pulp samples exhibited a marked viscosity loss (up to 54%) relative to reduced samples. Borohydride viscosity allows one to assess molecular weight losses in cellulose due to the oxidative treatment itself. As can be seen, the laccase–TEMPO treatment caused a significant drop in pulp viscosity with respect to the initial pulp —especially at a high refining intensity, which boosted functionalization.

Table 9-2. Viscosity and borohydride viscosity values (± standard deviation) of initial and laccase-TEMPO (LT) – treated pulps at each refining intensity. Wet zero span tensile index values (WZSTI) (± standard deviation) of the resulting handsheets.

	Initial pulp			LT-treated pulp		
PFI revolutions	**0**	**3000**	**4500**	**0**	**3000**	**4500**
Viscosity (ml/g)	716 ± 27	727 ± 24	731 ± 9	146 ± 12	131 ± 21	122 ± 0
Borohydride viscosity (ml/g)	736 ± 0	756 ± 5	717 ± 21	294 ± 18	285 ± 29	253 ± 35
WZSTI (Nm/g)	107 ± 6	115 ± 9	115 ± 9	50 ± 5	54 ± 2	45 ± 6

The intrinsic viscosity values obtained in the presence and absence of a reductive treatment allowed us to calculate the number of cellulose chain scissions due to the oxidative treatment itself (CS_{GT}) and to β-elimination promoted by carbonyl groups ($CS_{C=O}$) (Aracri *et al.* 2011c, Roncero 2003). Figure 9-2 shows CS_{GT}, $CS_{C=O}$ and their combination (CS_T) for enzymatically treated pulp after refining at a variable intensity. For comparison, the graph also shows the CS values for control samples treated in the absence of laccase, and both TEMPO and laccase, obtained in previous work (Aracri *et al.* 2011b). As can be seen, all oxidative treatments caused cellulose depolymerization and the formation of carbonyl groups responsible for further chain scissions in the alkaline measurement medium. The control and laccase samples exhibited very low total chain scission values that were virtually the exclusive result of the treatment itself. Refined pulp exhibited higher $CS_{C=O}$ values than unrefined pulp as a consequence of the increased content in aldehyde groups of the former (Aracri *et al.* 2011b). Moreover, CS_{GT} in the treated samples increased with increasing refining intensity, probably as a result of fibre walls being more accessible to the species effecting cellulose depolymerization.

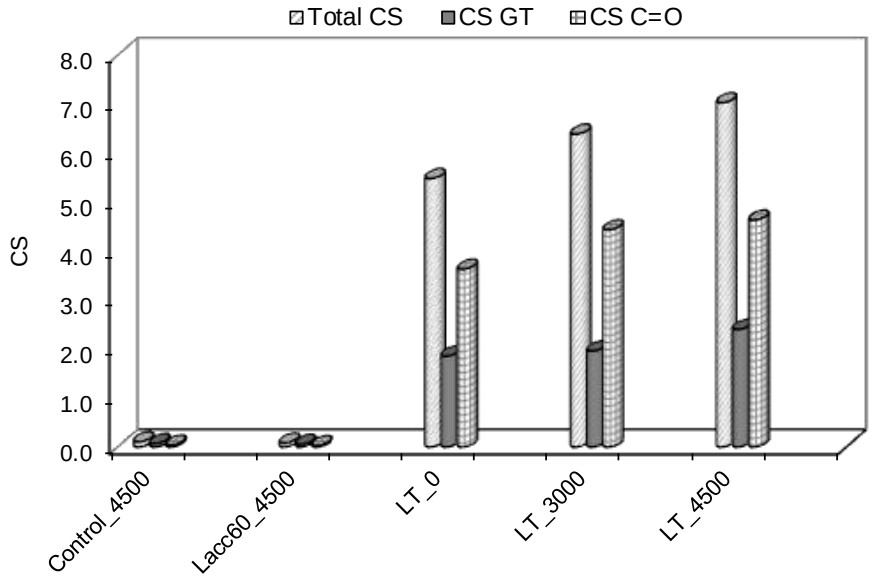

Figure 9-2. Total CS, CS_{GT} and $CS_{C=O}$ of laccase-TEMPO – treated pulps at each refining intensity and of control pulps.

The classical oxidation method with alkaline hypohalite as the actual oxidant results in severe molecular weight losses in cellulose that are mainly due to alkali-induced β-alkoxy elimination reactions starting from carbonyl groups (Potthast *et al.* 2009). The slightly acidic medium used with the laccase–TEMPO system hinders β-elimination, so cellulose degradation is most probably a result of homolytic processes involving some active radical species formed *in situ* as by-products during the oxidation treatment (Patel *et al.* 2011, Tamura *et al.* 2010). In order to ascertain whether the laccase–TEMPO treatment influenced fibre morphology, the initial and treated pulp samples were subjected to kajaani fibre analysis. Table 9-3 shows the average fibre length, curl, width and percentage of fines in the initial and treated pulp samples on refining for 0, 3000 and 4500 rev. As expected, increasing the refining intensity resulted in decreased fibre length and fibre curl, and in increased contents of fines and fibre width, the latter being consistent with the increase in WRV. Pulp treatments were shown to have no adverse effect on fibre length and to slightly increase fibre width. Moreover, treated pulp fibres exhibited decreased curl values, which was taken to be a favourable effect since the degree of curl in fibres has been shown to adversely affect the tensile strength of pulp (Page *et al.* 1984). Laccase–TEMPO treated samples additionally exhibited a decreased

content of fines at all refining intensities with respect to the initial pulp, which was ascribed to the loss of fines during filtering after the oxidative treatment.

Table 9-3. Average values of fibre length (L), fibre width (W), fibre curl (C) and fines content (F) for the initial pulp and laccase-TEMPO – treated pulps at each refining intensity. Numerical average (n) values are provided for all properties; length-weighted average (l) values are provided for length and fines content; weight-weighted average (w) value is provided for length.

	Initial pulp			LT-treated pulp		
PFI revolutions	**0**	**3000**	**4500**	**0**	**3000**	**4500**
L(n) mm	1.43	1.28	1.31	1.47	1.41	1.31
L(l) mm	2.04	1.87	1.93	2.04	1.98	1.87
L(w) mm	2.55	2.32	2.41	2.54	2.45	2.29
Fines(n) %	8.84	9.71	10.80	6.63	6.93	8.14
Fines(l) %	0.51	0.74	0.82	0.41	0.50	0.65
Fiber curl (n) %	32.60	26.70	24.40	32.00	23.10	22.10
Fiber width (n) μm	17.05	17.35	17.62	17.22	17.50	17.87

9.3.2. Physical properties and SEM images of handsheets

As can be seen from the wet zero span tensile (WZST) values of Table 9-2, depolymerization of cellulose had an adverse impact on the intrinsic strength of fibres. WZST is a reliable measure of the mean strength of fibres in paper sheets (Page 1989). The laccase–TEMPO treatment caused a marked drop in borohydride viscosity, as reflected in a loss of fibre strength by more than 50% with respect to the initial pulp, especially at the higher refining intensities.

Figure 9-3 shows the dry tensile, wet tensile, burst and tear indices of handsheets obtained from the initial and treated pulp samples as a function of refining intensity. Tensile and burst strength behave differently from tear strength. Thus, the latter shows a refining curve, as visible for the initial pulp in Figure 9-3d, with the characteristic maximum. The position and height of the maximum depends mainly on fibre length and strength. In poorly bonded sheets, tear strength depends heavily on fibre length and, less markedly, also on fibre strength, well-bonded sheets exhibiting the opposite trend (Seth and Page 1988). In a poorly bonded sheet, the tear strength depends on fibre pull-out rather than fibre strength; therefore, sheets with longer fibres are more resistant to tearing.

In well-bonded sheets, fibre strength becomes significant since fibres break in the tear zone. As can be seen from Figure 9-3d, the curve for the laccase–TEMPO treated samples had no maximum for tear index; also, refining the pulp at 3000 or 4500 rev decreased this property. Since, as suggested by the kajaani analysis, the laccase–TEMPO treatment had no adverse effect on fibre length, the loss of tear index can be ascribed to that of fibre strength during oxidation, which exerts a predominant effect at a high bonding level.

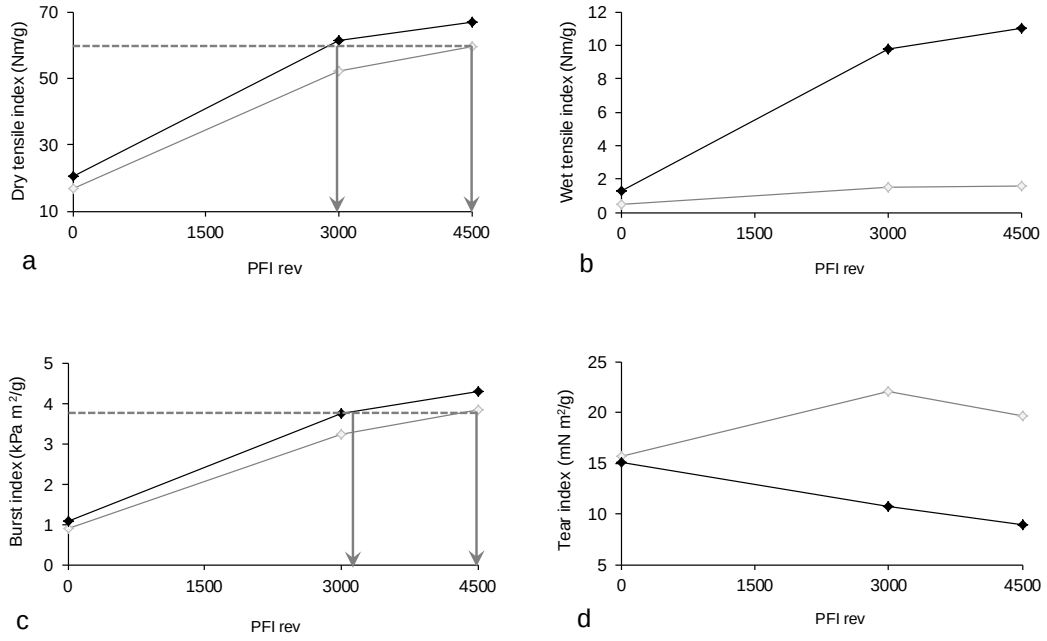

Figure 9-3. Dry tensile (a), wet tensile (b), burst (c) and tear (d) index values of handsheets obtained from initial (gray lines) and laccase-TEMPO – treated (black lines) pulps at each refining intensity. The errors associated with these measurements were lower than 5%.

Tensile strength depends mainly on the degree of bonding between fibres and also, to a less extent, on fibre strength, the latter affecting the property at high relative bonded areas (Retulainen 1996). An increase in fibre width and length, and a decrease in fibre curl, are known to have a favourable effect on tensile strength as well. Burst strength is not clearly defined in physical terms, but somehow relates to tensile strength. As can be seen, dry tensile index and burst index were increased by the oxidative treatment at all refining levels by effect of the increased inter-fibre hydrogen bonding provided by carboxyl groups introduced in enzymatically treated pulp (Barzyk *et al.* 1997).

Specifically, dry tensile index was increased by 21%, 18% and 12%, and burst index by 23%, 16% and 13%, at 0, 3000 and 4500 rev, respectively, in laccase–TEMPO treated pulp. The comparatively smaller increase in strength at a high refining intensity may have resulted from fibre weakening in highly bonded sheets. Handsheets obtained from pulp refined for 3000 rev and then treated exhibited higher dry tensile strength than paper from the initial pulp refined for 4500 rev. A similar effect was observed in burst strength. Based on these results, treating sisal fibres with the laccase–TEMPO system facilitates development of their properties and allows a target level of tensile and burst strengths to be obtained by using less energy at the refining stage. These results, together with the increase in WRV and the changes in morphological properties, testify to the potential of laccase–TEMPO oxidation for biorefining sisal pulp. The most salient effect of the laccase–TEMPO treatment, already observed in previous studies (Aracri *et al.* 2011b, 2011c), was the increase in wet tensile strength (160%, 553% and 588% at 0, 3000 and 4500 rev, respectively). Sisal pulp possesses a very low wet tensile strength that can be increased by using an eco-friendly approach such as an enzyme treatment. Wet tensile strength is mainly dependent on the presence of water-resistant inter-fibre bonds. The development of wet strength was ascribed to the introduction of aldehyde functionalities promoting the formation of inter-fibre covalent bonding through hemiacetal linkages with sterically close hydroxyl groups in cellulose (Saito and Isogai 2006). Similarly to aldehyde-containing resins (Chen *et al.* 2002), the mechanism followed by oxidized fibres allows paper to develop temporary wet strength. In a recent study on the effect of process variables in the laccase–TEMPO oxidation of sisal pulp, we found the wet tensile index to be influenced similarly to the aldehyde content by process variables; in addition, the two properties were closely aligned. In this work, using an increased pulp consistency resulted in the formation of considerably greater amounts of aldehyde and carboxyl groups, and hence in better dry and wet tensile strengths.

Figure 9-4 is a graph of air permeability as a function of density for handsheets obtained from the initial pulp and laccase–TEMPO treated samples. The denser is paper, the more closed is its structure and the more difficult the passage of air through it as a result. Refining pulp fibres increases their bonding capacity and collapsibility, which leads to a more closed structure. Certain paper grades must retain some porosity while developing strength-related properties. Figure 9-4 shows some interesting results. Thus, air permeability, expressed as the time required for air to pass through a handsheet, was significantly higher in laccase–TEMPO treated samples than in the initial pulp after

refining (37% and 35% at 3000 and 4500 rev, respectively), although density was slightly higher in the former. Such an unusual result of mechanical refining and biorefining with a cellulase (García *et al.* 2002) suggests that increased density levels can be obtained at a target degree of porosity.

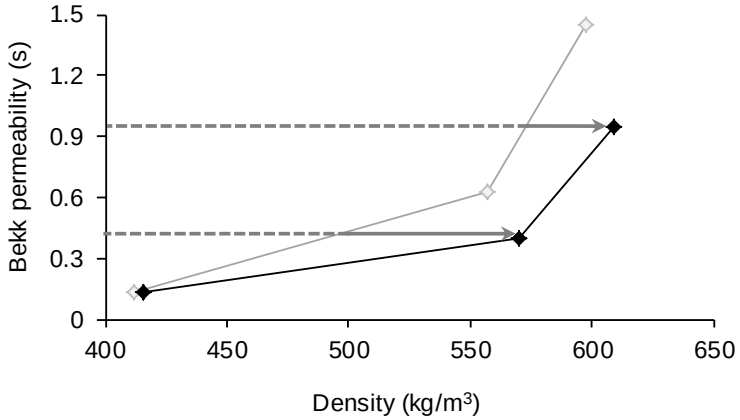

Figure 9-4. Air permeability, expressed as Bekk seconds, of handsheets obtained from initial (gray line) and laccase-TEMPO – treated (black line) pulps at each refining intensity. Increment in refining goes from left to right. The errors associated with these measurements were lower than 2%.

Figure 9-5 shows surface and cross-sectional scanning electron micrographs for handsheets made from the initial and laccase-TEMPO treated pulp samples at each refining intensity. The biorefining effect exerted by the laccase-TEMPO system was observed in an increased fibrillation degree of fibres after treatment, clearly visible in handsheets from unrefined pulps (Figure 9-5a), accounting for their increased bonding properties. The effect of mechanical refining is apparent from the increased compaction of the handsheets due to the increased collapsibility and bonding capacity of the fibres. As can be seen in Figure 9-5b, especially in the refined samples, handsheets from oxidized pulp exhibited stronger compaction than those from the initial pulp, consistent with the increase in tensile strength. However, compaction was seemingly not uniform throughout the cross-section and left zones with a more open inner structure, which probably accounts for the increased air permeability of the handsheets from laccase–TEMPO treated pulp.

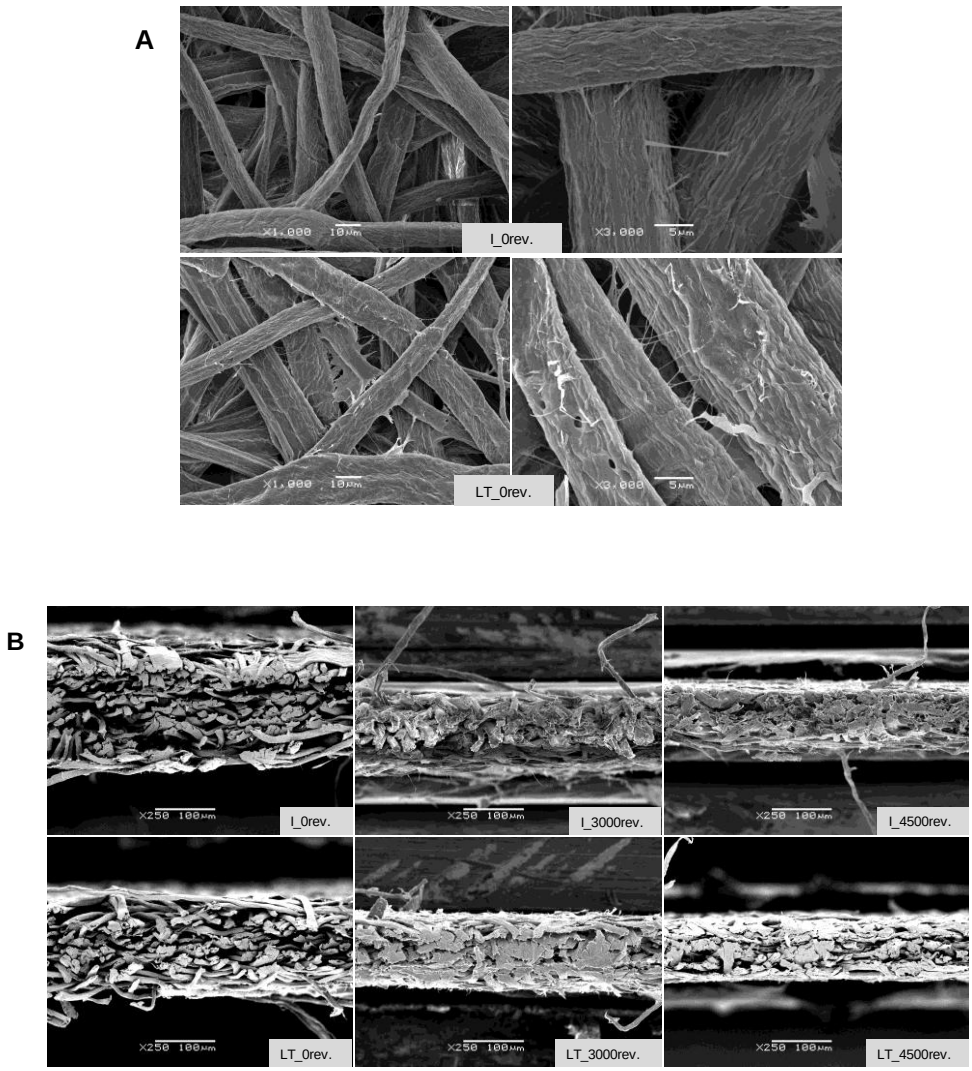

Figure 9-5. Surface (A) and cross-sectional (B) SEM images of handsheets obtained from initial (upper images) and laccase-TEMPO – treated (lower images) pulps, at 0 rev. in (A) and at each refining intensity in (B).

9.4. Conclusions

The effectiveness of a laccase–TEMPO treatment for sisal pulp found to maximize functionalization in tests of a statistical plan performed in previous work was boosted by using a higher pulp consistency, which for the first time has exposed the biorefining potential of this system. Prior to treatment, the pulp was refined at a variable intensity in order to ascertain whether the resulting increased surface area would lead to enhanced functionalization, and hence to a boosted refining effect. The use of an increased pulp consistency resulted in the formation of greater amounts of aldehyde and carboxyl groups, which were found to significantly increase with increasing refining intensity. Oxidized pulp exhibited increased WRVs as a result of its increased hydrophilicity. Increasing pulp consistency additionally reduced pulp viscosity more markedly during the enzyme treatment, especially at a high refining intensity, as the likely result of the increased accessibility of fibre walls to the species effecting cellulose depolymerization. The drop in pulp viscosity had a detrimental effect on fibre strength, and hence on tear strength, which was considerably reduced in the refined pulp samples. The dry tensile strength and burst strength of oxidized pulp were significantly better despite the loss of viscosity through the formation of inter-fibre hydrogen bonds offsetting the loss of fibre strength. This may allow a target level of tensile strength to be obtained by using less energy at the refining stage and reveals the potential of laccase–TEMPO oxidation for biorefining sisal pulp. The wet tensile index was dramatically increased by the oxidative treatment as a result of the formation of inter-fibre covalent bonds through hemiacetal linkages promoted by aldehyde groups. The improvement was considerably greater than that obtained at a lower consistency under identical conditions. Finally, one interesting feature of the handsheets from oxidized pulp was their increased porosity relative to the initial pulp despite their slightly higher density —an uncommon result of mechanical refining or biorefining with cellulase, where the increase in sheet density is usually accompanied by a decrease in porosity and an increase in drainage resistance. The enhanced effectiveness of the laccase–TEMPO oxidative system observed in this work can help reduce the TEMPO dose or treatment time, but further work will be needed to confirm this assumption.

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Comparative study of the effects induced by different laccase-based systems on sisal cellulose fibres

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Submitted to *Industrial & Engineering Chemistry Research*.

Abstract

This paper reports a comparative study of the effects induced on sisal pulp fibres by three different laccase-based systems, namely, laccase-sinapyl aldehyde, laccase-ferulic acid and laccase-TEMPO systems, applied to perform biobleaching, biografting and cellulose oxidation, respectively. A novel aspect of this study was the use of thermogravimetry to monitor superficial changes in cellulosic microfibrils during the enzyme treatments and gain a greater understanding of the mechanisms of action of the laccase-based systems. The natural compound sinapyl aldehyde (SLD) was applied as laccase mediator in the L stage of a LQP_O bleaching sequence, performed with and without a xylanase pre-treatment (X stage). Ferulic acid (FRC) was applied in biografting treatments using high and low doses of enzyme and FRC, and the extent of phenol coupling was evaluated via changes in selected pulp properties. Laccase-TEMPO oxidation of sisal fibres was investigated under different conditions of enzyme and mediator doses, reaction time and pulp consistency. The different modes of action of the studied laccase-based systems reflected in the different degradation profiles of pulps after treatment. Thermogravimetric analysis showed laccase to modify the thermal degradation path of the initial pulp, increasing the proportion of cellulose degrading at low temperature. The addition of SLD resulted in virtually no change of the thermal degradation path of the initial pulp, indicating that laccase-SLD system basically exerted its action on the lignin component of fibres. In contrast to SLD, FRC was found to significantly increase the amount of the paracrystalline fraction of cellulose, probably as a consequence of its incorporation into fibres. The presence of TEMPO, especially at those conditions boosting the oxidative functionalization, was found to cause an intense degradation of cellulose and the formation of a substantial amount of amorphous cellulose degrading at low temperature. A novel aspect of laccase-TEMPO system was identified in this work: its ability to reduce the HexA content of pulp under specific reaction conditions.

10.1. Introduction

In the last decades, laccase (EC 1.10.3.2) has attracted increasing attention in the pulp and paper research as a potential tool for developing cleaner processes and modifying

lignocellulosic fibres to obtain novel, sustainable products, by virtue of its operational flexibility and broad substrate specificity (Widsten and Kandelbauer 2008). On the other hand, high-priced non-wood fibres such as those from sisal, which are typically used to manufacture specialty paper, are specially suitable for application of enzyme technologies thanks to the increased profit margins they provide.

Ever since the discovery of chemical mediators capable of extending enzymatic oxidation to non-phenolic compounds, research interest has mainly focused on the potential of laccase-mediator systems for aiding pulp bleaching. Research has shown that the laccase-mediator systems (LMS) can substantially reduce the requirements of bleaching chemicals for chemical pulp bleaching, or allow bleaching to smaller kappa numbers and higher brightness (Fillat and Roncero 2010, Ibarra *et al.* 2006). Despite the associated advantages of LMS, mediators are expensive and can generate toxic derivatives. There have been recent trends to using eco-friendly, potentially cost-effective alternative mediators such as naturally occurring phenols, which can be readily obtained from plants and spent pulping liquors or, directly, from fungal metabolism (Camarero *et al.* 2007, Eggert *et al.* 1996, Johannes and Majcherczyk 2000, Moldes *et al.* 2008).

In the last decade, laccase has attracted considerable attention as a means for modifying fibre chemistry with a view to altering paper properties (particularly, strength-related properties). Radical coupling reactions involving phenolic compounds have been used to enable bonding of low-molecular weight compounds to lignin-rich cellulose fibres (Chandra *et al.* 2004). Radical coupling reactions competing with delignification represent an adverse, undesirable phenomenon in the biobleaching process (Camarero *et al.* 2007, Fillat *et al.* 2010). However, they have aroused increased interest as the key mechanisms behind the *biografting* of low-molecular weight phenols onto pulp fibres. This is a versatile functionalization method by virtue of the enzyme's non-specific substrate requirements, which allow bonding a wide range of phenolic compounds and thus incorporating several desired properties into the fibre matrix (Chandra *et al.* 2004, Fillat *et al.* 2012, Grönqvist *et al.* 2006). The feasibility of this approach has been demonstrated in a number studies; interest, however, has focused on wood materials and lignin-rich fibres, and little research has comparatively been carried out on non-wood fibres.

The laccase–TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl free radical) mediated system provides a potential approach to oxidatively modifying cellulose pulp fibres.

TEMPO-mediated oxidation is a well-known procedure to introduce carboxyl and aldehyde functional groups into cellulose in aqueous media at room temperature (de Nooy *et al.* 1995b). Although the ability to use laccase to catalyse the regenerative oxidation of TEMPO has been demonstrated (Viikari *et al.* 1999), the reaction is commonly carried out in the presence of NaClO/NaBr as a co-oxidizer system (Bragd *et al.* 2001, Chang and Robyt 1996, Isogai and Kato 1998). TEMPO-mediated oxidation has been successfully exploited to improve various physical properties of pulp fibres including inter-fibre bonding —and hence the strength-related properties of the resulting paper (Dang *et al.* 2007, Duarte *et al.* 2006). Environmental concerns have recently driven research interests into the development of halide-free oxidative systems. One promising approach for this purpose is the use of laccase together with oxygen as primary oxidants (Arends *et al.* 2006, Viikari *et al.* 1999). Similarly to the NaClO/NaBr process, oxoammonium ion is regenerated *in situ*, so only oxygen is consumed in the course of the reaction.

In laccase-based treatments of pulp fibres, the effects produced on the cellulose microfibril surface has not or has scarcely (Barneto *et al.* 2011) been investigated up to date. Thermogravimetric analysis is a powerful tool to detect the chemical changes on the microfibril surface, and determine the crystalline and amorphous cellulose contents. In this work, we compared the effects of the above-described laccase-based approaches, applied in previous studies (Aracri *et al.* 2011a, Aracri *et al.* 2011b, Aracri and Vidal 2011), in terms of pulp properties and thermal degradation profiles. The studied laccase-based treatments were: biobleaching, with and without a xylanase pre-treatment, using the natural mediator sinapyl aldehyde (SLD), biografting using ferulic acid (FRC), and cellulose oxidation mediated by TEMPO.

10.2. Materials and methods

10.2.1. Chemicals, enzyme and pulp

All chemicals were purchased from Sigma–Aldrich and used as received. Laccase from *Trametes villosa* was supplied by Novozymes® (Bagsvaerd, Denmark) and frozen until use. Laccase activity was determined by oxidation of 2,2'-azino*bis*-(3-

ethylbenzylthiozoline-6-sulfonate) (ABTS). One activity unit (U) was defined as the amount of laccase transforming 1 μmol/min ABTS to its cation radical (ε_{436} nm = 29 300 M^{-1} cm^{-1}) in 0.1 M sodium acetate buffer at pH 5 at 25 °C. A commercial xylanase (Pulpzyme® HC) supplied by Novozymes® was used in the xylanase pre-treatment of the bleaching sequence, which was performed in polyethylene bags, using 3 U/g odp xylanase in Tris–HCl buffer (pH 7), at 10% pulp consistency, at 50 °C for 2 h.

Unbleached sisal (*Agave sisalana*) pulp from a soda–anthraquinone cooking process was supplied by CELESA pulp mill (Tortosa, Spain). The pulp, at 2% consistency, was conditioned with H_2SO_4 at pH 4 under stirring for 30 min, which was followed by passage through a glass filter funnel and extensive washing with de-ionized water. This step was needed to remove contaminants and metals, and also to bring the pulp to the pH required for the enzyme treatment.

10.2.2. Pulp treatments

Sisal pulp was bleached by means of two different TCF biobleaching sequences, namely: LQP_O and $XLQP_O$, where L denotes the laccase-mediator treatment, Q a chelating treatment, Po an oxygen-reinforced hydrogen peroxide treatment and X an enzyme pre-treatment with xylanase. A control sequence was performed without addition of the mediator in the L stage for comparison. At the end of each stage, the pulp was filtered and thoroughly washed with de-ionized water. Laccase treatments were carried out at 5% pulp consistency in an oxygen pressurized (0.6 MPa) reactor at 50 °C, using a stirring rate of 30 rpm for 4 h; the reactor was supplied with 50 mM sodium tartrate buffer at pH 4, 40 U/g odp laccase and 1.5% (w/w) sinapyl aldehyde (SLD) in addition to Tween 80 (0.05% w/v) as surfactant. The L treatment was followed by a Q stage conducted in the presence of 1% DTPA (diethylenetriaminepentaacetic acid) at 5% consistency, pH 5–6 and 85 °C for 1 h. The last step of the TCF sequence was a P_O stage carried out at 5% consistency in an oxygen pressurized (0.6 MPa) reactor at a stirring rate of 30 rpm, using 3% odp H_2O_2, 1.5% odp NaOH, 0.3% odp DTPA and 0.2% odp $MgSO_4$, at 90 °C for 4 h.

Biografting treatments were performed by using an amount of 40 g odp sisal pulp at 5% consistency in 50 mM sodium tartrate buffer at pH 4 in the presence of a 20, or 40

U/g laccase concentration and a proportion of 1.5% or 3.5% (w/w) ferulic acid (FRC) (all relative to pulp dry weight). Tween 80 (0.05% w/v) was added as surfactant. Treatment runs were conducted in a reactor under pressurized O_2 (6 bar) at 30 rpm at 50 °C for 4 h. Pulp samples treated under identical conditions with 20 U/g laccase, in the absence of FRC were used as controls. After the enzyme treatment, the pulp samples were passed through a glass filter funnel and extensively washed with de-ionized water.

Three laccase-TEMPO treatments were performed in a jar testing apparatus at a stirring speed of 60 rpm, 15 g of pulp at 1% consistency in 50 mM acetate buffer at pH 5 at room temperature under oxygen bubbling, using the following conditions of laccase dose, TEMPO dose and reaction time: 100 U/g laccase, 2% w/w TEMPO, 20 h ($L_{100}T_2t_{20}$ treatment); 20 U/g laccase, 8% w/w TEMPO, 20 h ($L_{20}T_8t_{20}$ treatment); 60 U/g laccase, 5% w/w TEMPO, 14 h ($L_{60}T_5t_{14}$ treatment). Prior to treatments, pulp samples were disintegrated for 30 000 revolutions, filtered through a Buchner funnel and refined at 4500 revolutions according to ISO 5264. Two laccase-TEMPO oxidation treatments were performed on unrefined and refined pulp at room temperature on an amount of 30 g oven dried pulp (odp) at 5% consistency, in a 5 l reactor stirred at 60 rpm, using 50 mM acetate buffer at pH 5, under an oxygen pressure of 0.6 MPa, and conditions of laccase and TEMPO doses and reaction time of treatment $L_{20}T_8t_{20}$. Pulp treated with 60 U/g laccase, in the absence of TEMPO, at room temperature and 1% consistency under an oxygen pressure of 0.6 MPa for 18 h was used as control. After treatment, each pulp was filtered and washed with de-ionized water until a colourless, neutral filtrate was obtained.

10.2.3. Pulp properties

Pulps obtained from L and P_O stages of the biobleaching sequences were analysed in terms of kappa number due to lignin (KN_{lig}), brightness, hexenuronic acids (HexA) content and viscosity. Pulps coming from the biografting treatments were analysed for KN_{lig}, brightness, Klason lignin, surface anionic charge and HexA content after Soxhlet extraction with acetone (Aracri *et al.* 2010) in order to remove the fraction of FRC that failed to couple to fibres. KN_{lig}, HexA, carboxyl and aldehyde groups contents and borohydride viscosity was determined in pulps treated with laccase-TEMPO system.

HexA content was determine by UV spectroscopy (Chai *et al.* 2001, Valls *et al.* 2010). KN_{lig}, providing an estimate of the actual lignin and lignin-bound ferulic acid content of the pulp (Li *et al.* 2002, Valls *et al.* 2010), involved measuring kappa number following removal of HexA by acid hydrolysis with mercury acetate and efficient washing with de-ionized water. Kappa number and brightness were determined according to ISO 302 and ISO 3688, respectively. Pulp viscosity was determined in accordance with ISO 5351-1. Borohydride viscosity was measured after treatment with 2% $NaBH_4$, at 5% consistency at room temperature for 30 min (Roncero *et al.* 2002). The surface anionic charge of the fibres was determined by polyelectrolyte titration, using a particle charge detector (Mütek PCD 03, Germany) as described elsewhere (Aracri *et al.* 2011a). Klason lignin in each pulp was determined as the fraction of lignin insoluble in sulphuric acid resulting from acid hydrolysis as described in Aracri and Vidal (2011). The bulk acid group content was determined by conductimetric titration as described in Aracri *et al.* (2011b). Pulp samples were oxidized with $NaClO_2$ for selective conversion of aldehyde groups into carboxyl groups at room temperature for 48 h. The carboxyl content was determined with the conductimetric titration method. The carboxyl groups formed by effect of $NaClO_2$ oxidation were assumed to derive from aldehyde groups originally present in the pulp (Saito and Isogai 2005). All pulp analyses were carried out in duplicate.

10.2.4. Thermogravimetric analysis

TG runs were carried out with a Mettler Toledo model TGA/SDTA851e/LF1600 on samples of around 5 mg. Pyrolysis and combustion runs were carried out in nitrogen and synthetic air (N_2:O_2 4:1), respectively. Three heating rates (5, 10, and 20 ºC/min) from 25 ºC to 900 ºC were applied.

10.3. Results and discussion

10.3.1. Biobleaching

Three TCF biobleaching sequences were applied to sisal pulp, namely LQP_O, the control sequence where the L stage was performed with 40 U/g odp laccase; $L_{SLD}QP_O$, where L was performed in the presence of SLD, and $XL_{SLD}QP_O$, which included a xylanase pre-treatment as additional stage.

Due to the high content of hexenuronic acids (HexA) in soda-AQ sisal pulp and their important contribution to kappa number, kappa number due to lignin, KN_{lig}, was determined after removal of HexA, in order to assess the actual delignifying effect of the different treatments. Table 10-1 reports the values of KN_{lig}, brightness, HexA content and viscosity of pulps obtained from L and Po stages of the three bleaching sequences. As can be seen, the L stage carried out in the presence of laccase-SLD system resulted in a significant increase of KN_{lig}, which may have resulted from adsorption or partial incorporation of the phenol in pulp fibres via radical-coupling reactions. The increase in KN_{lig} was more marked in the initial pulp (from 3.4 to 5.1) than in the xylanase-treated pulp (from 3.4 to 4.0), probably as a result of the higher content of reactive sites (*e.g.* lignin, HexA) promoting binding of SLD in the former. Despite the adverse effect on the enzyme treatment, the final bleaching stage yielded a KN_{lig} smaller than control value, which suggests that natural mediator may be simultaneously involved in coupling and oxidative degradation reactions during the L stage, the effect of the latter being observed at the end of the bleaching sequence. The application of the X stage prior to the laccase-SLD treatment led to a final KN_{lig} 70% smaller than in the absence of an X stage, which was ascribed to the delignifying effect of xylanase treatment resulting from the removal of lignin trapped between xylan chains (Roncero *et al.* 2003). Similarly to KN_{lig}, pulp brightness was adversely affected by the laccase-SLD system in the L stage, especially in the absence of an X stage. After the Po stage, however, the laccase-SLD – treated pulp samples from both sequences exhibited increased brightness with respect to control pulp, as a result of the oxidation and dissolution of chromophoric species and lignin degradation products, in the alkaline bleaching medium used.

It is known that HexA groups in pulp can be more or less efficiently removed depending on the particular bleaching agent (Li *et al.* 2002). 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) (Costa and Colodette 2002, Roncero *et al.* 2003). Recently the use of laccase, both alone and in combination with mediators, has proved efficient in reducing HexA content of pulp. The best results have been obtained with 1-hydroxybenzotriazole (HBT) and violuric acid (VA) (Aracri and Vidal 2011, Cadena *et al.* 2010, Fillat *et al.* 2011, Valls *et al.* 2010). The potential of natural mediators for aiding HexA removal has been scarcely investigated up to date (Fillat *et al.* 2011). The results of HexA analysis showed a significant reduction in their content after treatment of the initial pulp with laccase alone (24% reduction), consistent with previously reported results (Cadena *et al.* 2011, Fillat *et al.* 2011). The pulp samples treated with laccase-SLD system exhibited no reduction in HexA content. Finally, the xylanase pre-treated pulps exhibited a reduction in HexA with respect to those subject to no X pre-treatment, due to the ability of xylanase to remove these substances by releasing xylans chains from fibre surface (Valls and Roncero 2009, Valls *et al.* 2010).

The effect of each treatment on cellulose integrity was evaluated by measurements of viscosity. Laccase-SLD resulted in no appreciable losses of viscosity with respect to control pulp, in contrast to what commonly observed with synthetic mediators (Fillat *et al.* 2010). Pulps treated in the P_O stages showed decreased viscosity due to the degrading effect the hot, strongly alkaline medium used in this stage has on cellulose.

Table 10-1. KN_{lig}, brightness, HexA content and viscosity of initial pulp and pulps obtained from L and P_O stages of the three bleaching sequences.

	KN_{lig}	Brightness (% ISO)	HexA (μmol/g)	Viscosity (ml/g)
Initial	3.9	37.9	41.4 ± 1.1	733 ± 1
L	3.4	40.0	31.5 ± 0.0	729 ± 3
L_{SLD}	5.1	27.0	32.7 ± 0.4	739 ± 39
XL_{SLD}	4.0	31.5	26.7 ± 0.7	703 ± 30
LQP	1.3	74.5	30.6 ± 1.5	663 ± 37
$L_{SLD}QP$	1.0	78.5	31.8 ± 3.0	592 ± 25
$XL_{SLD}QP$	0.3	76.9	25.8 ± 0.8	691 ± 10

10.3.2. Biografting

Previous studies (Aracri *et al.* 2009, 2010), reporting the effects of various *p*-hydroxycinnamic compounds combined with laccase on sisal pulp properties, revealed FRC to be the best choice for investigating biografting due to the most pronounced tendency it showed to couple with fibres. Grafting reflects in a change in various fibre properties depending on the nature of the particular grafted compound. In this work, it was evaluated via changes in KN_{lig}, brightness, Klason lignin and surface anionic charge. Treated pulps were analysed for HexA content as well.

Two laccase-FRC treatments were applied to sisal pulp, using two doses of laccase and FRC, and the resulting pulp properties (Table 10-2) were compared with those if control pulp treated with laccase alone (20 U/g odp). KN_{lig} was used to assess the actual lignin and lignin-bound FRC content of the pulp. Due to the phenolic structure of FRC, this should be prone to oxidation by acidic permanganate, thus contributing to the kappa number (Li and Gellerstedt 1998). As can be seen from Table 10-2, laccase-FRC treatment at low laccase and FRC doses resulted in 23% higher KN_{lig} compared to control, to which corresponded a 6% lower brightness. Further raising the laccase and FRC dose from 20 to 40 U/g odp, and from 1.5 to 3.5%, respectively, increased KN_{lig} by 163% and decreased pulp brightness by 11% with respect to control treatment. Further evidence of the different extent of FRC grafting obtained in the two treatments was provided by the increased amount of Klason lignin and surface anionic charge of the

fibres. The increased amount of Klason lignin in the treatment performed at high doses of laccase and FRC reflects the higher degree of covalent coupling of FRC to the lignin component of the fibres.

Similarly to the results obtained in the biobleaching sequences, the laccase treatment resulted in a lower content of HexA compared to initial pulp. The addition of FRC further decreased HexA content, probably as a consequence of a coupling reaction between HexA double bonds and FRC in its phenoxy radical form (Cadena *et al.* 2011) which would provide further evidence for the major tendency of FRC to couple to fibres compared to SLD.

Table 10-2. KN_{lig}, brightness, Klason lignin, surface anionic charge and HexA content of initial, control and laccase-FRC – treated pulps. $L_{20}FRC_{1.5}$ denotes pulp treated with 20 U/g laccase and 1.5% (w/w) FRC. $L_{40}FRC_{3.5}$ denotes pulp treated with 40 U/g laccase and 3.5% (w/w) FRC.

	KN_{lig}	Brightness (% ISO)	Klason lignin	Surface anionic charge (μeq/g)	HexA (μmol/g)
Initial	3.2	52.1	1.1 ± 0.1	60 ± 5	36.0 ± 3.0
Laccase	2.7	53.4	1.2 ± 0.2	54 ± 7	30.9 ± 0.8
$L_{20}FRC_{1.5}$	3.5	50.0	1.2 ± 0.2	60 ± 0	26.9 ± 0.8
$L_{40}FRC_{3.5}$	7.1	47.5	1.7 ± 0.2	80 ± 7	26.1± 0.1

10.3.3. Laccase-TEMPO oxidation

Three preliminary laccase-TEMPO treatments were performed in a jar testing apparatus under variable conditions of laccase and TEMPO doses, and reaction time, and were compared in terms of aldehyde and carboxyl groups generated in pulps (Table 10-3). The most effective for oxidatively functionalizing sisal fibres was $L_{20}T_8t_{20}$ treatment, whose conditions were then selected to perform two treatments in reactor at increased pulp consistency, on both refined and unrefined pulps, with a view to determining whether the increased fibre surface area and consistency would lead to enhanced functionalization.

The increase of pulp consistency resulted in the formation of 130% and 94% higher amounts of aldehyde and carboxyl groups contents, respectively. Moreover, the treatment

performed on refined pulp yielded 20% and 11% higher contents of aldehyde and carboxyl groups, respectively, with respect to that applied to unrefined pulp. Viscosity measurements were performed to assess changes in the degree of cellulose polymerization during the laccase-TEMPO treatments. Prior to measurement, oxidized pulp samples were treated with sodium borohydride to inactivate carbonyl groups (by reduction to hydroxyl groups) and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium (Aracri *et al.* 2011b, Roncero *et al.* 2002). As can be observed from Table 10-3, laccase-TEMPO treatments caused a significant loss of borohydride viscosity with respect to control pulp, more markedly at those conditions resulting in the highest functionalization degree. The slightly acidic medium used by the laccase–TEMPO system hinders β-elimination, so cellulose degradation is most probably a result of homolytic processes involving some active radical species formed *in situ* as by-products during the oxidation treatment (Patel *et al.* 2011).

Two additional properties examined in laccase-TEMPO – treated pulps were KN_{lig} and HexA content, in order to evaluate whether this oxidative system could exert a delignification effect or reduce the content of HexA groups in sisal pulp. Since it is known from studies performed with lignin models (Fabbrini *et al.* 2001) that TEMPO selectively interacts with benzyl alcohol (or ethers) groups of lignin oxidizing them to α-carbonyl derivatives, it was of interest to evaluate whether these modifications led to a delignification effect in pulps treated with laccase-TEMPO system at different operating conditions. As shown in Table 10-3, no delignification effect was observed in pulps treated at high consistency, compared to control pulp. KN_{lig} was decreased by 11% in pulps treated at low consistency, which indicates that a slight delignification occurred at those conditions.

The potential of TEMPO for HexA removal has not been investigated up to date, therefore it was of interest to determine whether laccase-TEMPO treatments of sisal pulp resulted in any change of HexA content. As reported in Table 10-3, laccase alone was able to eliminate 22% of HexA from initial pulp. Laccase-TEMPO treatments performed at low consistency did not result in any decrease of HexA, while an important reduction was obtained from treatments performed at high consistency, especially after refining (44%). These results indicates that TEMPO used in combination with laccase is able to reduce the content of HexA from sisal pulp, and the effect is considerably enhanced in

conditions providing good interaction between fibres and oxidant (high consistency) and accessibility in the fibre wall (refining).

Table 10-3. KN_{lig}, brightness, HexA, carboxyl and aldehyde groups contents, and borohydride viscosity of initial, control and laccase-TEMPO – treated pulps. LT5%_0rev denotes unrefined pulp treated at high consistency, LT5%_0rev denotes pulp treated at high consistency after refining for 4500 rev.

	KN_{lig}	HexA (μmol/g)	COOH (μmol/g)	CHO (μmol/g)	Borohydride viscosity (ml/g)
Initial	3.8 ± 0.2	38.9 ± 1.3	110 ± 7	0 ± 9	736 ± 0
Laccase	3.2 ± 0.2	30.2 ± 0.7	94 ± 2	1 ± 0	686 ± 8
$L_{100}T_{2}t_{20}$	2.8 ± 0.1	29.1 ± 2.4	114 ± 5	15 ± 2	495 ± 15
$L_{20}T_{8}t_{20}$	2.8 ± 0.0	30.2 ± 2.7	137 ± 4	83 ± 8	389 ± 32
$L_{60}T_{5}t_{14}$	2.9 ± 0.0	32.6 ± 1.4	106 ± 5	43 ± 5	403 ± 10
LT5%_0rev	3.2 ± 0.1	26.7 ± 2.1	240 ± 7	159 ± 0	294 ± 18
LT5%_4500rev	3.1 ± 0.0	21.7 ± 1.5	266 ± 2	191 ± 7	253 ± 35

10.3.4. Thermogravimetric analysis

The described enzymatic approaches of bleaching and functionalization are usually studied via pulp properties (functional groups content, chemical composition, optical properties, etc.). However, this approach of analysis excludes the detection of potential structural changes on cellulose microfibril surface, which can provide further useful information concerning the mechanisms of action of the different laccase-based systems on pulp fibres. As a complementary study, and for the first time on sisal pulp, thermogravimetry was applied in this work to analyse from a different perspective the effects induced by the enzyme treatments on sisal cellulose fibres.

10.3.4.1. Biobleaching

As expected from their oxidative nature, laccase-based treatment not only affected lignin or hemicellulose components, but it also acted on cellulose. As shown in Figure 9-1, laccase modified the thermal degradation path of the initial pulp, increasing the proportion of cellulose that degrades at low temperature (between 280-300 ºC). As lignin

underwent an oxidative degradation (which explains the KN_{lig} reductions), the surface of the cellulosic microfibril was also affected by the enzyme. According to Nishiyama *et al.* (2003), cellulose microfibril is a thin and long crystalline entity which consists of bundles of cellulose polymer chains with alternate crystalline (cellulose crystallites) and amorphous zones. The crystallite surface is ordered, and a network of hydrogen bonds protects it from external attacks (i.e. oxygen), including thermal degradation (Barneto *et al.* 2011). Crystalline cellulose degrades at higher temperature than amorphous cellulose or hemicellulose. However, superficial adsorptions (enzymes, xylans, lignin, etc.) or oxidations, can deteriorate cellulose surface and partially remove the hydrogen bonds shield. Under this circumstance the surface (or a part of surface) of the crystallite degrades in a similar way than amorphous cellulose (paracrystalline cellulose) (Ioelovich *et al.* 2010), that is, at lower temperature (Barneto *et al.* 2011), yielding a broader peak in TG analysis. This fact is observed after laccase application (see Figure 10-1). These changes in TG curves depend on the environment used during analysis, being more evident when the heating is performed in air atmosphere. If oxygen is present, the thermal degradation of the crystalline structure is accompanied by oxidations in the superficial zones where the hydrogen bond network was previously weakened. Therefore, the presence of oxygen during TG analysis increases the differences between samples.

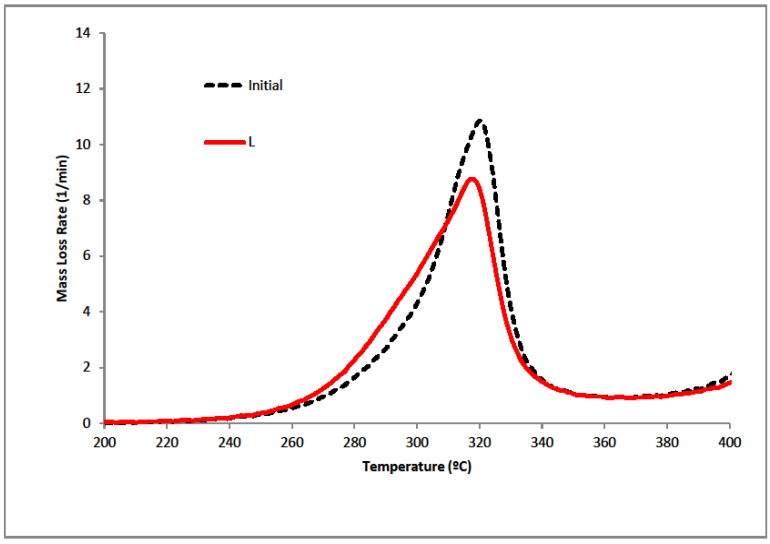

Figure 10-1. Effect of the laccase (L) treatment on the thermal degradation profile of sisal pulp. TG run performed in air environment at 5 °C/min.

When SLD was used as laccase mediator, an increased KN_{lig} and a reduced brightness were observed. However, despite these adverse effects, the thermal degradation path of initial pulp almost did not change. In the presence of SLD, the laccase treatment induced chemical modifications (coupling or oxidative degradation of lignin) with small effects on cellulose surface (see Figure 10-2). Experimental conditions during the bleaching P_O stage (alkaline medium, oxygen and hydrogen peroxide) reduced KN_{lig} and increased brightness but had unfavourable effects on cellulose fibres. As shown in Table 10-1, after bleaching, pulp viscosities were significantly reduced, and, at the same time, TG analysis showed an increased amount of cellulose degrading at low temperature (paracrystalline cellulose) (see Figure 10-2).

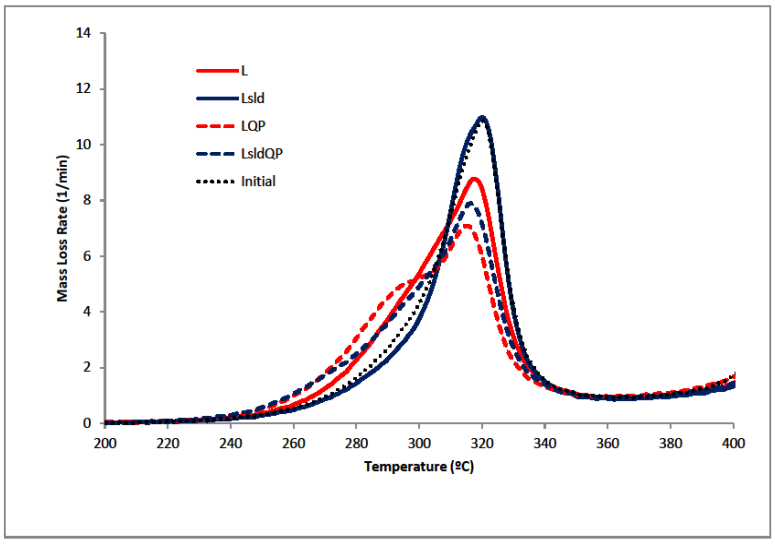

Figure 10-2. Effect of the bleaching P_O stage on the thermal degradation profile of pulp. TG run performed in air environment at 5 °C/min.

Both L- and L_{SLD}-treated pulps showed broader TG peaks after the bleaching stage (LQP_O and $L_{SLD}QP_O$), which exposed damages on the fibre surface and loss of superficial crystallinity. However, as shown in Figure 10-3, xylanase pre-treatment prevented these effects. Comparing the thermal degradation paths of pulps after XL_{SLD} and $XL_{SLD}QP_O$ sequences, it can be seen that the bleached pulp exhibited a TG peak sharper than that exhibited by the unbleached pulp, which indicated the existence of a more crystalline cellulosic surface in the former. This result is consistent with viscosity measurements, which showed that the X pre-treatment yielded higher viscosity values at the end of the bleaching sequence.

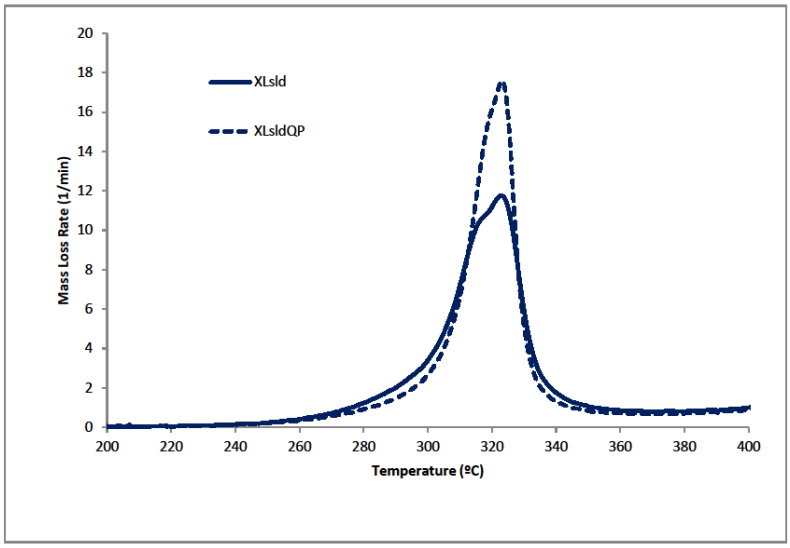

Figure 10-3. Comparison of the thermal degradation paths of pulps treated with L-SLD system after the L stage and at the end of the bleaching sequence, in the presence of a xylanase pre-treatment. TG run performed in air environment at 5 °C/min.

10.3.4.2. Biografting

As can be seen in Figure 10-4, the laccase-FRC system significantly modifies the thermal degradation path of initial pulp. Similarly to that observed during the biobleaching assay, laccase degraded the superficial cellulose, increasing the paracrystalline cellulose content and, consequently, making the TG peak broader. However, in contrast to the effect observed with SLD, the presence of similar concentration of FRC boosted the superficial modification of cellulosic fibres, leading to the formation of a higher amount of paracrystalline cellulose. Whereas the effect of laccase-SLD system was limited to lignin, the presence of FRC boosted the superficial modification of cellulosic fibres. When the concentrations of laccase and FRC increased to 40 U/g and 3.5%, respectively, the phenomenon was intensified, and the thermal degradation profile of cellulose showed two clearly separated peaks (paracrystalline and crystalline celluloses) (see Figure 10-4). Under these experimental conditions the whole fibre surface was converted to paracrystalline cellulose, being the remaining core crystalline cellulose.

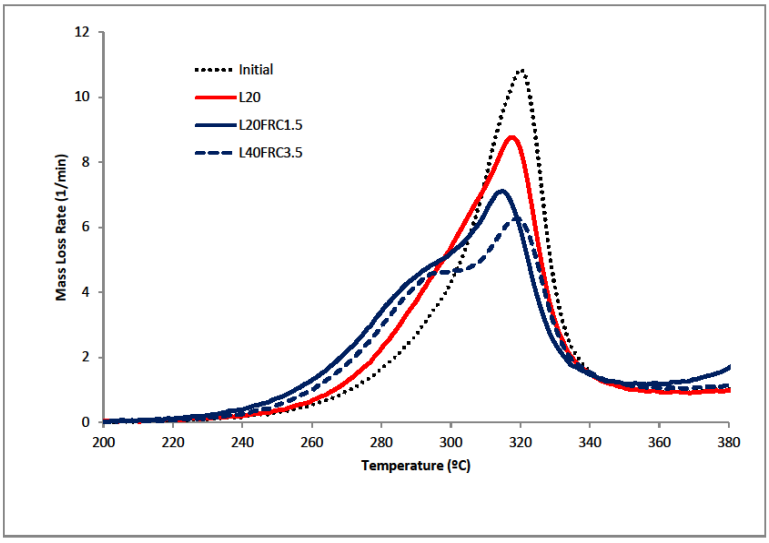

Figure 10-4. Effect of laccase-FRC treatments on the thermal degradation path of sisal pulp. TG run performed in air environment at 5 °C/min.

10.3.4.3. Laccase-TEMPO oxidation

As can be seen in Figure 10-5a, despite simultaneous laccase concentration reduction, as TEMPO dose was increased, the thermal degradation path of pulps showed a progressively higher secondary peak at low temperature. This aggressive behaviour is specific of TEMPO. Under similar conditions, other mediators like SLD or FRC did not show comparable levels of cellulose degradation (see Figure 10-5b). TEMPO intensely degraded cellulosic chains, causing a severe depolymerization which yielded a significant viscosity reduction. As a result, a fraction of crystalline cellulose was transformed in amorphous cellulose which thermally degrades at very low temperature.

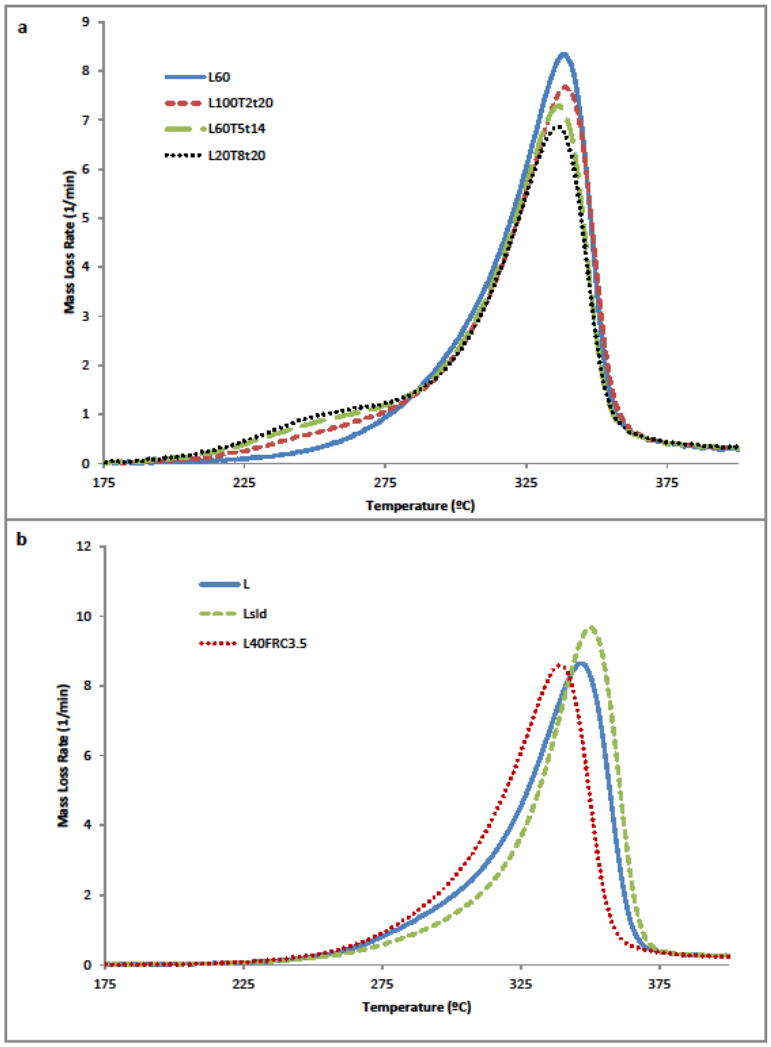

Figure 10-5. Thermal degradation profiles of sisal pulps treated with laccase-TEMPO (a), laccase-SLD and laccase-FRC systems (b). TG run performed in nitrogen environment at 5 °C/min.

On the other hand, in consistence with the pulp properties analysis, thermogravimetry analysis showed that applying refining prior to the laccase-TEMPO treatment significantly increased the effect of the latter. As depicted in Fig. 10-6, after the enzyme treatment, refined pulp degraded at lower temperature with respect to unrefined pulp, and showed a more intense degradation at low temperature. According to these data, applying a previous refining step provided more damaged fibres due to both the mechanical action

of refining and the boosted oxidation effect of laccase-TEMPO system, which reflected in their easier thermal degradation.

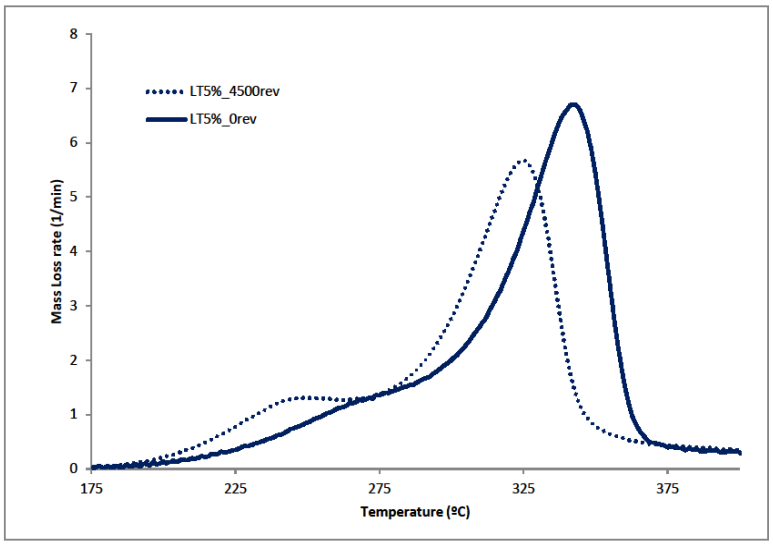

Figure 10-6. Comparison of the thermal degradation profiles of sisal pulps treated with laccase-TEMPO system in the presence and in the absence of a previous refining step. TG run performed in nitrogen environment at 5 °C/min.

10.3.4.4. Thermogravimetric analysis under oxidative environment

In order to obtain a whole vision of the mediator effects and to increase differences between the thermal degradations profiles of the cellulosic fibres, the volatilization steps of studied sisal pulps were compared under oxidative environment (Figure 10-7). Under this atmosphere the thermal degradation of pulp showed two mass loss steps. The former, between 200 – 350 ºC, was the consequence of pulp volatilization and yielded volatiles and char. The second, between 400 – 450 ºC, led to char oxidation.

As can be seen in Fig. 10-7, the mediator had a significant influence on the thermal degradation profiles of pulps. While SLD-treated pulp volatilized in a narrow temperature interval (sharp peak) at high temperature, the TEMPO-treated pulp volatilized in a very broad temperature interval showing an important proportion of cellulose degrading at low temperature (broad peak). Finally, ferulic acid was in an intermediate position. As mass

losses during volatilization step depend on the cellulose crystallinity, it can be concluded that ferulic acid and, mainly, TEMPO affected the cellulose chains located on the microfibril surface increasing the amorphous or paracrystalline cellulose proportion. The more damages underwent cellulose, the more aggressive was the mediator. In this sense it is known that TEMPO oxidizes cellulose and increases the content of carbonyl and carboxyl groups in pulp.

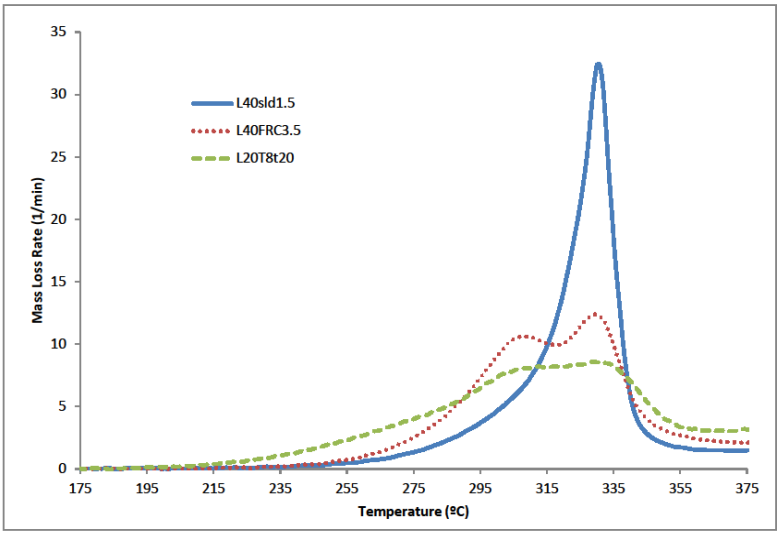

Figure 10-7. Thermal degradation profiles of sisal pulps treated with different laccase-based systems (laccase-sinapyl aldehyde, laccase-ferulic acid and laccase-TEMPO).

10.4. Conclusions

Three different laccase-based systems were applied to sisal cellulose fibres in order to perform biobleaching, biografting and cellulose oxidation. The effects of these treatments were compared in terms of pulp properties and thermal degradation profile of the treated pulps. Laccase-SLD system was found to oxidatively modify lignin in the L stage of the bleaching sequence, which resulted in easier delignification in the subsequent bleaching stage, particularly when a xylanase pre-treatment was included. Thermogravimetric analysis showed laccase to modify the thermal degradation path of the initial pulp,

increasing the proportion of cellulose degrading at low temperature. The addition of SLD resulted in virtually no change of the thermal degradation path of the initial pulp, confirming that laccase-SLD system basically exerted its action on the lignin component. The xylanase effect of removing xylans deposited on fibres surface and providing a cleaner microfibril surface was visible in the thermal degradation profile of the X-treated pulp showing a sharper peak than that observed in the absence of an X stage. Biografting treatments, performed with laccase-FRC systems, resulted in different extents of phenol coupling according to the conditions of enzyme and FRC doses used. In consistence to the changes observed in pulp properties, the thermal degradation paths of pulps treated with laccase-FRC system were significantly modified with respect to that of laccase-treated pulp, and to a higher extent at those conditions providing a higher degree of grafting. Thermogravimetric results showed that laccase-FRC treatment caused a deterioration of cellulose surface, leading to the formation of a higher amount of paracrystalline cellulose, probably as a consequence of the incorporation of FRC in fibres. Laccase-TEMPO oxidation was investigated in terms of aldehyde and carboxyl functional groups introduced in the cellulose chains of sisal fibres, under different operating conditions. The increasing degree of functionalization obtained with increasing doses of TEMPO was accompanied by an increasing degradation of cellulose, as shown by thermogravimetric analysis. A boosted oxidative functionalization of fibres was obtained by applying laccase-TEMPO treatment at increased pulp consistency and after refining, which resulted in strongly degraded cellulosic chains and the formation of a substantial amount of amorphous cellulose degrading at low temperature. A novel aspect of laccase-TEMPO system was identified in this work: its ability to reduce the HexA content of pulp under specific reaction conditions.

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General summary and main conclusions

11.1. General summary

This chapter presents a general summary of the work carried out and the results obtained along this thesis. Various plant phenols were assayed as laccase redox mediators and their potential for either biobleaching or functionalizing sisal pulp fibres was evaluated. TCF biobleaching sequences were performed in order to compare the effectiveness of a selected natural mediator and a well-known synthetic mediator, as well as to evaluate the effect of a xylanase pre-treatment. The phenolic compound showing the highest tendency to coupling to fibres was selected to investigate functionalization by biografting reactions. The use of a novel analytical approach exposed the covalent binding of this compound and the extent of grafting degree was evaluated under different reaction conditions to achieve an improvement of the strength properties in the resulting papers. A different approach of fibre functionalization aiming at cellulose oxidation by means of laccase-TEMPO system was studied. The first part of this study showed the high potential of this system for improving wet strength of sisal pulp. Subsequently, studying the effect of process variables allowed enhancing the effectiveness of laccase-TEMPO system for obtaining a biorefining effect. By using several analytical methods, an in-depth study on the mechanisms of action of the different enzyme systems on sisal pulp was carried out.

- **Assessing the potential of natural mediators for either bleaching or functionalizing sisal fibres**

Four different plant phenols [viz. the *p*-hydroxycinnamic compounds sinapic acid (SNC), ferulic acid (FRC), coniferyl aldehyde (CLD) and sinapyl aldehyde (SLD)] were assayed as laccase redox mediators and their tendency to either promote delignification or couple onto pulp was examined with a view to assessing their potential for either bleaching or functionalizing sisal fibres. The enzyme treatment (L stage) was followed by an alkaline extraction with hydrogen peroxide (P stage) in order to determine whether observable effects could be enhanced by removing L-modified lignin.

Residual laccase activity, monitored during the L stage as performed at small scale, was found to be strongly and rapidly reduced when HBT was used, especially in the

absence of pulp. This effect was ascribed to the mediator's nitroxy radicals, which inactivate laccase by oxidation of aromatic acids on the protein surface. Pulp was found to protect the enzyme against inactivation by acting as a reducing substrate for free radicals. The natural mediators were found to exert a stabilizing effect on the enzyme in the absence and presence of pulp, with the exception of FRC in the absence of pulp. The addition of pulp significantly increased residual activity with FRC and CLD, while it had virtually no effect with SNC and SLD. This was probably due to the higher reactivity of the former in their radical form, which induce denaturation of the enzyme in the absence of pulp and undergo coupling reactions with fibre lignin in the presence of pulp.

Pulp samples from L and P stages were analysed in order to assess the involvement of the natural mediators in grafting or oxidative degradation reactions during the enzyme treatment, as well as their ability to enhance lignin removal in the subsequent bleaching treatment. After the L stage, HBT was the only mediator capable of decreasing kappa number and increasing brightness with respect to control pulp. By contrast, all natural mediators resulted in substantially increased kappa numbers, which is consistent with their partial incorporation into pulp via radical-coupling reactions. After Soxhlet-extraction with acetone aiming at removing the fraction of phenolic compound simply adsorbed onto pulp, samples from the L stage still showed kappa number higher than that for control pulp, in particular, the FRC- and CLD-treated samples had a kappa number greater than SNC- and SLD-treated samples. The less marked grafting observed in the dimethoxylated compounds may have reflected increased steric hindrance and stability in their phenoxy radicals precluding condensation of C_5 in the aromatic ring with residual lignin in the pulp fibres. All natural mediators diminished brightness in the L stage, especially SLD and SNC, which were found to cause the formation of a substantial amount of chromophoric groups during the enzyme treatment. After the P stage, pulp treated with HBT showed substantial delignification and brightness increase with respect to control pulp, whereas those treated with natural mediators exhibited a comparatively small decrease of kappa number and lower brightness than control pulp, with exception of SLD-treated pulp that showed (slightly) increased brightness. Viscosity after the L stage was retained in the pulp samples treated with natural mediators, while it was significantly decreased in the presence of HBT. The P stage caused an important loss of viscosity; furthermore, pulps treated with HBT, SNC and FRC showed a decreased viscosity relative to control. Probably, during the L stage, phenolic acids and HBT caused an alteration of functional groups in cellulose leading to easier degradation in the strong

alkaline medium used in the subsequent stage. The analysis of surface anionic charge in pulps samples from the L stage revealed the presence of higher amounts of anionic charge in samples treated with phenolic acids, and the increases with respect to control pulp echoed those in kappa number, confirming that FRC tends to graft onto fibre more markedly that does SNC.

Concerning the effluent properties, none of the L treatments increased COD significantly with respect to the control; among the natural mediators, SNC and SLD resulted in greater COD than FRC and CLD. The effluents from the P stage had much lower COD values than those from the L stage due to the absence of sodium tartrate buffer and lower concentration of chemical species dissolved in the former. However, the effluents resulting from the bleaching of FRC-and CLD-treated pulp samples had increased COD values relative to SNC- and SLD-treated samples. These differences may be related to the higher tendency of FRC and CLD to couple to fibres, which makes them to be less concentrated in the effluents from the L stage and contribute more markedly to COD in those from the subsequent bleaching stage. The enzyme treatments involving HBT, SNC and SLD produced effluents with markedly strong colour relative to FRC and CLD due to the presence of an increased amount of chromophoric species resulting from the oxidation and/or degradation of lignin and the mediators. The effluents from the bleaching of HBT-, SNC- and SLD-treated samples exhibited dramatically reduced colour, which, however, was similar to that of the FRC- and CLD-treated samples after the L stage. Application of LMS treatment increased the effluent toxicity relative to the value observed with laccase alone, to a different extent depending on the type of mediator used. Thus, FRC and CLD resulted in the smallest increase, followed by HBT with a twice greater effect, and, at an even greater extent, by SNC and SLD. The solutions containing the mediators in their reduced forms exhibited lower toxicity than that obtained in the L treatments, by exception of CLD solution that unexpectedly resulted in very high toxicity relative to the other studied compounds. The increase toxicity observed in the enzyme treatment can be ascribed to various factors including the formation of oxidized /radical species and degradation products from both lignin and the mediators.

Further bleaching assays, consisting of an L and a P stage, were performed with laccase from *Trametes villosa* (*Tv*L) and the synthetic mediator violuric acid (VA), and laccase from *Myceliophthora thermophila* (*Mt*L) and the natural mediator methyl syringate (MS). The results were compared with those obtained with *Tv*L and 1-

hydroxybenzotriazole (HBT) or sinapyl aldehyde (SLD). The latter was assayed at different doses and in the presence of two different enzyme doses, to better understand its potential for delignification. *Tv*L+HBT and *Tv*L+VA systems resulted in similar delignification effect, but the latter showed higher bleaching efficiency. *Mt*L+MS system resulted in higher kappa number and lower brightness than those provided by *Tv*L+HBT and *Tv*L+VA systems. Compared to *Tv*L+SLD at the same conditions of laccase and mediator doses, *Mt*L+MS system provided better results of brightness (especially after L stage), but slightly less delignification after the P stage. The combination of SLD with 40U/g odp of laccase yielded a final kappa number similar to those obtained with synthetic mediators, although brightness was somewhat lower.

- **Comparing TCF biobleaching sequences involving laccase and xylanase stages**

The evolution of lignin and hexenuronic acid (HexA) content in sisal pulp was followed during two different TCF bleaching sequences performed with and without a xylanase pre-treatment (an X stage), and including a LMS treatment (an L stage) where either the natural compound sinapyl aldehyde (SLD) or the synthetic compound violuric acid (VA) were employed as laccase mediators. Two control sequences were performed where the L stage was carried out with 20 and 40 U/g odp laccase, in the absence of VA and SLD, respectively.

Xylanase pre-treatment resulted in an important reduction of kappa number relative to initial pulp, ascribed to a delignifying effect resulting from the removal of lignin trapped between xylan chains, as well as to the removal of HexA bonded as side groups to xylans. $L20_{VA}QP_O$ sequence showed high efficiency in decreasing kappa number in all stages, particularly when an X stage was included, which confirmed the bleach boosting effect of the xylanase pre-treatment. The application of laccase-SLD system resulted in a significant increase of kappa number in L and Q stages compared to control sequence, which may have resulted from adsorption or partial condensation of the phenol on the pulp via radical-coupling reactions. However, the final bleaching stage yielded a kappa number smaller than control value and similar to that for $XL20_{VA}QP_O$ when an X stage was applied, suggesting that the natural mediator can be simultaneously involved in coupling and oxidative degradation reactions during the L stage, the effect of the latter

only being observed at the end of the bleaching stage. Laccase-VA system exhibited a high efficiency in raising pulp brightness in all stages, particularly when an X stage was applied. Laccase-SLD adversely affected pulp brightness in the L and Q stages, especially in the absence of an X stage. After the P_O stage, however, SLD-treated pulp samples exhibited increased brightness with respect to control pulp, but lower than that achieved with laccase-VA system. Using the LMS afforded important reductions in hydrogen peroxide dose.

The L and Q stages in all bleaching sequences led to similar pulp viscosity values, with no appreciable loss from the initial pulp. After the P_O stage, L-treated pulps showed a more marked viscosity reduction than control pulps; probably, the mediators altered cellulose functional groups, thus making the pulp vulnerable to degradation in the strong alkaline medium used in the bleaching stage. The increased viscosities obtained at the end of the sequences including an X stage may have resulted from partial removal of xylans from pulp.

The HPLC analysis of xylan content of enzymatically treated pulps as calculated after acid hydrolysis showed the xylanase treatment to considerably reduce xylan content of initial pulp and the xylan removal to be boosted by the subsequent L stage with both mediators.

The xylanase treatment was found to remove a substantial amount of HexA in the initial pulp. Control treatment with laccase alone reduced the HexA content, more markedly at higher enzyme dose. Contrary to laccase-SLD system, laccase-VA proved efficient in reducing HexA content, probably due to its ability to destroy HexA by oxidizing their double bonds similarly to electrophilic bleaching agents.

Measurements of KN_{lig} showed that the xylanase pre-treatment had a substantial delignification effect on the initial pulp. Interestingly, the final KN_{lig} value for the $XL40_{SLD}QP_O$ sequence was slightly smaller than that for the $XL20_{VA}QP_O$ sequence in spite of the significant increase obtained in the L stage.

Xylanase treatment produced a high COD accounting for more than 40% of the total value for the sequences including an X stage. COD after the L stage was markedly higher than it was after Q and P_O; this was mainly a result of the presence of sodium tartrate buffer and the use of commercial laccase. In all cases, the colour of effluents decreased in

the sequence L>Q>P_O. The effluents from the L stage performed in the presence of a laccase-mediator system exhibited a marked increase in colour relative to the control effluents, especially in the case of laccase-SLD system. This was ascribed to the increased amount of chromophoric species resulting from oxidation and/or degradation of lignin and the mediators. Effluents resulting from treatments performed with xylanase and laccase in the absence of mediator led to low toxicity values, which were increased by the application of LMS. The increase was especially marked with the laccase-SLD treatment, the resulting effluent being roughly 6 times more toxic than that from the laccase-VA treatment. VA was found to introduce no toxicity in the effluents, whether in its reduced or oxidized form; by contrast, SLD led to a dramatically increased toxicity level after laccase oxidation, probably through the formation of stable radicals and by-products from the mediators. Very low toxicity levels were found in effluents obtained from Q and P_O stages involving LMS-treated pulps.

The pulp samples from the X, L and P_O stages of the different sequences were analysed for the lipophilic extractives by GC/MS. It was found that the application of laccase alone at higher dose reduced the content of free sterols in the L stage and led to a decreased amount of sterol glycosides in the subsequent bleaching stage. Laccase-SLD system proved ineffective for further reducing the amount of lipophilic compounds compared to the corresponding control. In contrast, laccase-VA system enabled a significant reduction of alkanes, sterol glycosides and free sterols, being the latter the main responsible for pitch deposits. The xylanase stage did not reduce the content of lipophilic extractives, but did enhance their removal in the subsequent bleaching stage.

- **Biografting of phenolic compounds**

Sisal pulp treated with laccase and the *p*-hydroxycinnamic compounds sinapic acid (SNC), ferulic acid (FRC), coniferyl aldehyde (CLD) and sinapyl aldehyde (SLD) was analysed in terms of kappa number and optical properties prior and subsequently to a Soxhlet extraction with acetone. A straightforward method was developed to obtain an estimation of the minimum amount of grafted phenol on the base of a calibration line originated from kappa number measurements. Analytical pyrolysis coupled to GC/MS was used in an attempt to evidence the covalent binding of the phenols to fibres after the enzymatic treatments.

After acetone extraction, a slight decrease of kappa number was observed in all pulps; however all the laccase-treated pulps still presented a kappa number higher than the control pulp, thus indicating that a binding of these simple phenols onto fibres affectively occurred. The kappa numbers of the acetone-extracted pulps indicated a higher degree of grafting for the guaiacyl-type phenolic compounds (FRC and CLD), than for the syringyl-type phenolic compounds (SNC and SLD), the difference being ascribed to the steric effects of the structure of the phenols. The minimum amount of grafted phenols provided by the calibration line were 6.6, 9.6, 17.3 and 16.9 μmol/g of pulp for SLD, SNC, CLD and FRC, respectively. No improvement of brightness was obtained in the treated pulps after acetone extraction, by exception of that treated with SLD, in which the removal of the adsorbed products of oxidation resulted in considerable increase of brightness. Similarly to brightness, Chroma (C*) and Lightness (L*) properties of FRC- and CLD-treated pulps did not result in any differences with respect to control pulp, while a marked increase of colour saturation was induced by SNC and SLD. The removal of adsorbed phenols by acetone extraction resulted in enhanced L* in all enzymatically treated pulps, especially SLD-treated pulp, whereas no C* decrease was observed in any case.

The analysis of the laccase-treated samples by Py-GC/MS and Py/TMAH demonstrated the incorporation of some of these phenolic compounds into fibres. The analysis revealed that FRC was covalently bound to fibres as such, while CLD was found incorporated into fibres in a different form, probably as vanillin or and/or ferulic acid. In the case of SNC, it was probably incorporated into fibres in the form of syringic acid, whereas no compound indicative of the incorporation of SLD into fibres was detected.

- **Investigating biografting of ferulic acid and its potential for improving paper strength**

Since FRC was found to incorporate into fibres as such and in the greatest extent among the assayed phenolic compounds, laccase-catalysed grafting of FRC was further investigated on unbleached sisal fibres and the extent of phenol coupling to fibres under different reaction conditions was evaluated via selected pulp properties.

Four pulp treatments were performed where the enzyme dose, FRC dose or time was changed with respect to the conditions used in the previous study. Grafting was evaluated

in differently treated pulps via changes in kappa number, brightness, Klason lignin and surface anionic charge after Soxhlet extraction with acetone. In order to determine the contribution to kappa number of lignin and the lignin-derived compound FRC, HexA were removed from treated pulps and their content was determined. The HexA content was found to decrease after treatment with laccase alone, and in the presence of FRC probably as a consequence of a coupling reaction between HexA double bond and HexA in its phenoxy radical form. KN_{lig} was found to significantly increase with FRC dose from 1.5% to 3.5% (w/w) and laccase dose from 20 to 40 U/g odp; the results of brightness showed that this property was affected not only by coupling of the phenolic compound, but also by the formation of chromophores absorbing light at 457 nm via structural changes in FRC and/or lignin. Further evidence of FRC grafting was provided by the increased amount of Klason lignin and surface anionic charge, which echoed that in the KN_{lig} and clearly showing that the treatment using 40U/g odp laccase and 3.5% FRC for 4 h was that leading to the greatest extent of grafting.

Residual laccase activity and effluent toxicity were assessed after each treatment. Residual activity was found not to be adversely affected by the presence of FRC, and effluent toxicity to increase to twice greater values when the higher FRC dose was used.

The conditions ensuring the highest degree of FRC grafting were subsequently used to study the enzymatic functionalization on refined and unrefined pulps in order to confirm whether the increased surface area obtained by effect of fibrillation during the mechanical treatment resulted in enhanced grating. Pulps refined before and after laccase-FRC treatment were used to form handsheets which were analysed for dry tensile, wet tensile, burst and tear strength properties. Leftover handsheets from the physical tests were Soxhlet-extracted with acetone and, after disintegration, analysed in terms of anionic charge, kappa number and brightness. The changes in pulp properties caused by the laccase-FRC treatment relative to the control was significantly more marked in the samples refined before treatment (RT) than in those refined after treatment (TR). The results obtained clearly showed that grafting can be increased by refining the pulp prior to the enzyme treatment as this increases its fibre surface and the accessibility of the functionalizing reactant. Laccase-FRC treatment resulted in a slight increase of dry tensile index and a substantial gain in wet tensile index in both TR and RT handsheets, ascribed to the increase of surface acid groups in pulp fibres and the formation of water-resistant covalent bonds between fibres by coupling of phenoxy-radicals, respectively. In

all cases, the enzymatic treatment resulted in improved paper strength when applied after refining.

- **Wet strength development by laccase-TEMPO system**

The oxidation of sisal pulp by laccase-TEMPO system was investigated in order to evaluate the potential of this approach for improving the paper strength properties. The oxidative treatment was performed at varying dose of TEMPO, and the effect on pulp fibres was examined in terms of carboxyl groups content and viscosity of pulp fibres. The resulting papers were analysed for strength properties, water absorbency capabilities and fibre morphology.

In a preliminary study, conducted by using 1% (w/w) TEMPO, laccase-TEMPO treatment was found to cause a slight loss or no improvement of the dry-strength properties despite the increased yield in acid groups obtained; however the treatment had a significant beneficial effect on wet strength of the modified paper. This result suggested the formation of inter-fibre covalent bonds in the handsheets through hemiacetal linkages between aldehyde groups on fibres surface formed as intermediates groups and hydroxyl groups in adjacent fibre surfaces.

In the next step of the study, pulp was treated with laccase and a varying amount of TEMPO (from 1% to 8%). Pulp viscosity was measured in the absence and in the presence of a prior reductive treatment with sodium borohydride (borohydride viscosity), the latter being necessary to inactivate carbonyl groups present in pulp and exclude the effect of depolymerization reactions by β-elimination promoted by the alkaline measurement medium. An increasing loss of borohydride viscosity was observed in laccase-TEMPO – treated pulp as the mediator load was increase from 1% to 8%, which reflected the occurrence of degradation reactions in cellulose chains during the oxidative treatment. The slightly acidic medium used by the laccase–TEMPO system hinders β-elimination, so cellulose degradation was most probably a result of homolytic processes involving some active radical species formed *in situ* as by-products during the oxidation treatment. When no reductive treatment was applied, TEMPO-oxidized pulps exhibited a dramatic drop of viscosity compared to the reduced samples, the difference increasing with the increase in TEMPO dose. This suggested the formation of substantial amounts of carbonyl groups during the laccase-TEMPO treatment, increasing with the TEMPO dose.

The intrinsic strength of fibres was estimated from the wet zero span tensile strength of handsheets. It was found that the fibre strength was negatively affected by the oxidative treatment, more markedly at high TEMPO dose, and that a correlation between borohydride viscosity and wet zero span tensile index existed.

A modest increase of carboxyl groups was observed after treatments with 1% and 2% of TEMPO with respect to control pulp, and increasing amounts were obtained with increasing mediator doses. Measurements of vertical wicking were performed to confirm whether the increase in carboxyl groups enhanced the water absorbency capabilities of the handsheets. Samples treated with laccase and TEMPO were found to wick at a faster rate than did the control sample, indicating their increased hydrophilicity. Although the higher TEMPO doses increased the carboxyl content, no further increase was observed in the wicking rate. This result can be ascribed to the formation of increasing amounts of hemiacetal linkages implying the reduction of free hydroxyl groups in fibres.

Handsheets obtained from pulps treated with increasing amounts of TEMPO were analysed for wet tensile strength and wet burst strength. Both properties were dramatically improved as TEMPO dose was raised from 1% to 8%, suggesting the formation of increasingly large amounts of inter-fibre hemiacetal linkages during sheet-making.

- **Assessing the effect of process variables on fibres and handsheets properties in the laccase–TEMPO oxidation**

Further investigation was carried out concerning the laccase-TEMPO oxidation of sisal pulp, and the influence of process variables including the laccase and TEMPO doses, and reaction time, on various properties of the oxidized fibres and handsheets made from them was assessed by using a three variable statistical plan.

In a preliminary study, a series of treatments were performed in reactor under different operating conditions (reaction time, temperature, applied oxygen pressure and pulp consistency). Aldehyde and carboxyl contents were determined for pulps, and dry tensile, wet tensile, burst and tear indices were determined for handsheets. The variation of some parameters in the process, such as raising the temperature to 50 °C, removing the applied O_2 pressure and reducing the reaction time to 4 h resulted in significantly lower

aldehyde and carboxyl contents and wet tensile index. The results showed applying a longer reaction time, refining prior to the enzyme treatment, and, especially, using a higher consistency to increase the yields of carboxyl and aldehyde groups. Aldehyde groups were found to be correlated with the wet tensile index, while samples with increased carboxyl content exhibited an also increased dry tensile index. The tear index of the laccase-TEMPO – treated samples was very low in relation to the control samples, which was ascribed to the loss of fibre strength. The burst index was seemingly influenced by both the carboxyl content and fibre strength.

A total of 13 laccase-TEMPO treatments were performed according to the experimental design, where the independents variables were studied in the following ranges: 20-100 U/g odp (laccase dose), 2-8% odp (TEMPO dose) and 8-20 h (reaction time). The studied response variables were wet tensile index, dry tensile index, aldehyde content and borohydride viscosity. Available data allowed no accurate model for the carboxyl content to be constructed from the process variables. Based on the mathematical models derived, using a high TEMPO dose and a long reaction time favoured the formation of aldehyde groups and led to improved wet strength in the resulting handsheets; in addition, a clear-cut trend fitting a straight line was revealed by a graphical comparison of these properties. Based on the model for dry tensile index, the responses peaked at high TEMPO doses used in combination with either a low laccase dose and long reaction time or a high laccase dose and short reaction time. Although no accurate model for carboxyl content could be established, the improvement peaked under those conditions yielding the highest dry tensile index, which suggests that carboxyl groups promote inter-fibre hydrogen bonding in the resulting handsheets. The model for borohydride viscosity showed a weak influence of laccase dose and a strong influence of TEMPO dose. Borohydride viscosity dropped as the TEMPO dose was raised, especially from a low dose to medium one. The highest borohydride viscosity in the studied plan was obtained at a low reaction time and an also low TEMPO dose in combination with a medium laccase dose, which however provided low degree of aldehyde and carboxyl functionalization and almost no improvement of dry and wet tensile index.

The number of scissions due to the oxidative treatment itself and to β-elimination promoted by carbonyl groups was calculated from the intrinsic viscosity values obtained in the presence and absence of a reductive treatment. A linear relationship was found

between the aldehyde content and the number of chain scissions due to carbonyl groups as determined in the experimental plan.

- **Obtaining a biorefining effect of sisal pulp fibres by applying a laccase-TEMPO treatment of enhanced effectiveness**

The most effective laccase-TEMPO treatment of sisal pulp for improving paper strength found in the statistical plan was applied using an increased pulp consistency (5% instead of 1%) and after refining at different intensities with a view to determining whether the improved interaction between fibres and oxidant, and the increased fibre surface area would lead to enhanced functionalization.

Laccase-TEMPO treatment introduced aldehyde and carboxyl groups in pulp to an increasing extent as the refining intensity was increased, which indicated that refining intensity rendered fibre walls more accessible to the oxidative system. Pulp consistency was found to exert an important effect on the extent of functionalization: the higher consistency resulted in considerably higher aldehyde and carboxyl groups contents with respect to pulp treated under the same conditions but at 1% consistency. This testifies to the boosting effect of improved oxidant-fibre interactions by using an increased consistency in laccase-TEMPO treatments. As a result of the increased carboxyl group content, oxidized pulps showed considerably increased WRV values with respect to the initial pulp, particularly after refining. Increasing pulp consistency additionally reduced pulp viscosity more markedly during the enzyme treatment performed in this study, especially at high refining intensity, which had a detrimental effect on fibre strength, and consequently on tear strength that was significantly reduced in refined pulps. The analysis of morphological characteristics by kajaani showed that pulp treatments had no adverse effect on fibre length and induced a slight increase in fibre width and a decrease in fibre curl.

Dry tensile index and burst index were increased by the oxidative treatment at all refining levels by effect of the increased inter-fibre hydrogen bonding provided by carboxyl groups introduced in enzymatically treated pulps. Handsheets obtained from pulp refined for 3000 rev and then treated exhibited higher dry tensile strength than paper from the initial pulp refined for 4500 rev; a similar effect was observed in burst strength. Based on these results, treating sisal fibres with laccase-TEMPO system facilitates

development of their properties and allows a target level of tensile and burst strengths to be obtained by using less energy at the refining stage. These results, together with the increase in WRV and the changes of morphological properties, shows the potential of laccase-TEMPO oxidation for biorefining sisal pulp. Moreover, the use of higher pulp consistency providing considerably higher aldehyde group contents resulted in better wet tensile strength. Finally, one interesting feature of the handsheets obtained from oxidized pulp was their increased porosity relative to the initial pulp, despite their slightly higher density, an uncommon result of mechanical refining or biorefining with cellulase, where the increase in sheet density is usually accompanied by a decrease in porosity and an increase of drainage resistance.

- **Thermogravimetric study of laccase-modified sisal pulp fibres**

The different laccase-based systems used in this thesis for performing biobleaching, biografting and cellulose oxidation in sisal pulp were investigated in terms of effects induced on cellulose microfibril surface by using thermogravimetry analysis. The analyses were performed on pulps obtained from the biobleaching sequences involving laccase-SLD system (with and without X pre-treatment), the biografting treatments performed with high and low doses of enzyme and FRC, the laccase-TEMPO oxidation treatments under different conditions of enzyme and mediator doses, reaction time and pulp consistency. The addition of SLD resulted in virtually no change of the thermal degradation path of the initial pulp, indicating that laccase-SLD system basically exerted its action on the lignin component of fibres. The xylanase effect of removing xylans deposited on fibres surface and providing a cleaner microfibril surface was visible in the thermal degradation profile of the X-treated pulp showing a sharper peak than that observed in the absence of an X stage. In contrast to SLD, FRC was found to significantly increase the amount of the paracrystalline fraction of cellulose, probably as a consequence of its incorporation into fibres. The presence of TEMPO, especially at those conditions boosting the oxidative functionalization, was found to cause an intense degradation of cellulose and the formation of a substantial amount of amorphous cellulose degrading at low temperature.

11.2. Main conclusions

The aim of this thesis was to apply laccase-based systems for biobleaching and functionalization of sisal pulp fibres with a view to developing novel and/or improved fibre and paper properties in environmentally friendly processes. Natural, potentially cost-effective compounds were assayed as substitutes for expensive, potentially toxic laccase mediators. Efficient TCF biobleaching sequences involving xylanase and LMS showed the potential of the enzymatic system for providing high cellulose content sisal pulp and substantial savings in bleaching chemicals.

Two different approaches to fibre functionalization were assessed, aiming either at lignin modification (biografting) or cellulose modification (laccase-TEMPO oxidation). The application of laccase-based modifying system to an unconventional non-wood raw material such as sisal fibres was a novel aspect of this thesis. New methods for exposing covalent binding of phenolic compounds to fibres and evaluating the extent of grafting were developed. Enhanced grating efficiency was achieved, and improved strength properties were exhibited by the resulting papers. The use of laccase-TEMPO system to oxidatively modify cellulose and improve strength-related properties was evaluated as an environmentally friendly alternative to established halide-based systems. Laccase-TEMPO oxidation was found an efficient approach to considerably improve wet strength of sisal pulp and, additionally, allow energy saving in the refining stage by virtue of the biorefining effect it exerts during the enzyme treatment.

The analytical methods used -pyrolysis-GC/MS, polyelectrolyte titration, conductimetric titration, carbohydrate determination by HPLC, fibre morphology analysis by SEM, thermogravimetry- provided a better understanding of the reaction mechanisms behind the different laccase-based treatments.

The following are the novel and most salient aspects of this thesis:

- An unconventional raw material such as sisal fibres was used to obtain high added value pulps by means of biotechnology.

- The use of laccase-mediator system was an efficient approach for aiding sisal pulp delignification and bleaching

- Natural phenolic compounds were assayed for the first time as laccase mediators for aiding bleaching of sisal pulp

- Natural mediators seemed to be involved in both coupling and oxidative degradation reactions, the ultimate effect of laccase-natural mediator system being strongly dependent on the balance between these two types of reactions

- In contrast to HBT, natural mediators did not inactivate laccase during biotreatments where the presence of pulp exerted a protective role against inactivation

- TCF biobleaching sequences were applied for the first time to sisal pulp. A xylanase stage showed high efficiency for boosting the bleaching effect of LMS treatments. The bleaching sequence involving the natural mediator SLD showed similar delignifying effect than that involving the synthetic mediator VA, but with lower brightness and a stronger impact on effluents properties.

- The proposed TCF sequences enabled efficient bleaching of sisal pulp and saving of bleaching agents, as well as, when VA was used, removal of lipophilic extractives.

- HexA content was determined for the first time in sisal pulp. Xylanase, laccase alone and to a higher extent in combination with VA significantly reduced the content of HexA in sisal pulp.

- A reduction in the content of HexA was obtained in biografting and in laccase-TEMPO oxidation treatments, ascribed in the former case to coupling reactions between HexA double bond and HexA in its phenoxy radical form, and in the second case to the oxidation of their double bond similarly to electrophilic bleaching agents.

- Biografting of a phenolic compound was studied for the first time on a non-wood low-lignin-content raw material such as sisal pulp. The covalent

binding of FRC to sisal fibres during the enzyme treatment was exposed by pyrolysis-GC/MS.

- Biografting efficiency was enhanced by applying a refining stage prior to the enzyme treatment. Enhanced grafting provided improved strength properties in the resulting papers.

- A different approach to fibre functionalization based on the oxidation of cellulose by using the laccase-TEMPO system was assessed for the first time on sisal pulp.

- Laccase-TEMPO oxidation can be regarded as an efficient method to develop wet strength of paper, as well as a potential approach for biorefining pulp fibres.

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