Novel enzymatic processes for the functionalisation of lignocellulose materials, and mechanistic insights

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CONTENTS

Abstract

Biotechnological modification of wood surface using enzyme processes offers an eco-friendly alternative to current physico-chemical methods. To this end, novel enzymatic processes have been developed for functionalising lignocellulose materials based on direct grafting and use of anchor groups. Using the 62 kDa *Trametes hirsuta* laccase, three fluorophenols, 4-[4-(trifluoromethyl)phenoxy]phenol, 4-(trifluoromethoxy)phenol and 4-fluoro-2-methylphenol were successfully grafted onto beech (*Fagus sylvatica*) veneers leading to an increase in hydrophobicity of up to 65 % when compared to treatment without laccase. The increase in hydrophobicity was related to the amount of fluorophenol incorporated and the fluorine content in the treated beech veneers, according to XPS-analysis. 4-[4-(trifluoromethyl)phenoxy]phenol was particularly effective with a 77.1 % increase in binding (compared to simple adsorption), resulting in fluorine content of 6.39 % and a 65.5 % increase in hydrophobicity. Mechanistic studies using complex lignin models showed covalent coupling of the fluorophenols to guaiacylglycerol β-guaiacyl ether via 5-5 coupling and to syringylglycerol β-guaiacyl ether through 4-*O*-5 linkages, to give the corresponding 1:1 adducts, leaving the fluorine molecule free (as proved by LC-MS and NMR) – a condition necessary for the hydrophobic properties. In a related study, laccase-mediated reactivity of long chain alkylamines to lignin moieties was demonstrated using putative lignin monomers, lignin models and lignocellulose materials. Multiple molecules of dodecylamine (DA) and dihexylamine (DHA) were readily coupled onto simple lignin molecules while coupling onto complex lignin models only yielded 1:1 coupling products. Surface analysis of beech veneers enzymatically grafted with DA showed a nitrogen content of 3.18 % compared to 0.71 % in laccase only treated controls while the O/C ratio decreased from 0.52 to 0.46. Concomitantly the coupling of DHA or DA onto beech veneers resulted in a 53.8 % and 84.2 % increase in hydrophobicity, respectively when compared to simple adsorption.

Direct grafting of hydrolysable tannins, particularly tannic acid, and the condensed cationic mimosa tannin, significantly inhibited the growth of *S. aureus* on wood veneer and pulp and also showed modest antibacterial effect on *E. coli*, while condensed (mimosa) tannin imparted less resistance against these bacteria. Mechanistic studies showed covalent coupling of tannins to lignin model molecules in the presence of laccase. The structures of the coupling products proposed showed 5-5, 4-*O*-5 and *β*-*O*-4 as the predominant linkages. The efficacy of the different tannins as antibacterial agents was in line with their minimum inhibitory concentrations (MIC) for *S. aureus* and *E. coli* and their reactivity toward laccase.

Since some of the radicals or coupling sites are quenched as the radical tries to stabilize itself, a new method was developed in which reactive amines were grafted to lignocellulose material as anchor groups, creating a stable reactive surface for further functionalisation. This approach increased coupling of antifungal agents by up to 58 %, when compared to direct laccase-mediated coupling. Further mechanistic studies provided evidence for the covalent coupling of tyramine (anchor group) onto syringylglycerol β–guaiacylether via a 4-*O*-5 bond, leaving the -NH_2 group free for further attachment of functional molecules. Laccase-mediated coupling of functional molecules (phenolic amines, fluorophenols, and selected wood preservatives) was also demonstrated for the first time using the recently discovered lignin model, dibenzodioxocin.

A novel non-selective chemoenzymatic process targeting all structural components of lignocellulose material, based on lipase-generated oxiranes as anchor groups, was developed. Using a lipase and hydrogen peroxide, soybean oil and linoleic acid were epoxidised resulting in a 52.7 % and 92.4 % relative conversion to oxirane, respectively. A commercial oxirane, 1,2 epoxyoctane and oxiranes generated from epoxidation of oils (using lipase and hydrogen peroxide) were successfully coupled onto long chain alkylamines while the oxirane was also successfully coupled to various lignin monomers and models. Treatment of beech veneers with dodecylamine using this process resulted in an increase in hydrophobicity which is superior to targeted laccase-mediated hydrophobicizing with the same molecule.

Zusammenfassung

Biotechnologische Modifikationen von Holzoberflächen unter Verwendung von Enzymen stellen eine umweltfreundliche Alternative zu chemisch physikalischen Methoden dar. Zur Funktionalisierung von Lignocellulose wurde ein enzymatischer Prozess entwickelt, der auf Grafting oder der Einführung von Ankergruppen beruht. Mit Hilfe der 62kDA großen Laccase von *Trametes hirsuta* wurden drei Fluorophenole, nämlich 4-[4-(Trifluoromethyl)phenoxy]phenol, 4-(Trifluoromethoxy)phenol und 4-Fluoro-2-methylphenol erfolgreich an Buchenholzoberflächen gekoppelt. Im Vergleich zu Laccase-freier Behandlung ist eine um 65% erhöhte Hydrophobizität zu beobachten. Der Anstieg der Hydrophobizität steht laut Ergebnissen aus XPS-Untersuchungen im Bezug zur im behandelten Holz eingelagerten Fluorophenol-Menge und zum Fluorgehalt. Besonders wirkungsvoll erwies sich 4-[4-(Trifluoromethyl)phenoxy]phenol mit einer um 77.1% erhöhten Bindung im Vergleich zur normalen Adsorption, einem Fluorgehalt von 6.39% und einer Steigerung der Hydrophobizität um 65.5%. In Versuchen an Lignin Modellsubstraten wurde eine kovalente Bindung der Fluorophenole an Guajacylglycerol β-guaiacyl ether und Syringylglycerol β-guaiacyl ether im Verhältnis 1:1, sowie Fluor, was für die Hydrophobizität notwendig ist, mittels LC-MS und NMR Analysen nachgewiesen. In ähnlichen Versuchen wurde die durch Laccasen vermittelte Reaktivität langkettiger Alkylamine gegenüber Ligninresten unter Verwendung von Ligninmonomeren, Lignin-Modellsubstraten und Lignocellulosen demonstriert. Einzelne Ligninmoleküle konnten mit mehreren Molekülen Dodecylamin (DA) und Dihexylamin (DHA) gekoppelt werden, während eine Kopplung an komplexe Lignin Modelle nur im Verhältnis 1:1 möglich war. Oberflächenanalysen von Buchenfurnieren, welche enzymatisch mit DA gekoppelt wurden zeigten einen Stickstoffgehalt von 3.18% im Vergleich zu 0.71% bei Kontrollproben, welche nur mit Laccase behandelt wurden. Das O/C Verhältnis sank von 0.52 auf 0.46. Weiters zeigte die Kopplung von DHA oder DA eine um 53.8% bzw. 84.2% gesteigerte Hydrophobizität im Vergleich zu normaler Adsorption.
Werden Tannine, insbesondere Gerbsäure und das kondensierte kationische Mimosa Tannin direkt an die Buchenholzfurniere bzw. den Zellstoff gekoppelt, ist das Wachstum von *S. aureus* erheblich gehemmt und eine mäßig antibakterielle Wirkung auf *E.coli* ist beobachtbar, wohingegen das kondensierte Mimosa Tannin eine geringere Resistenz gegen diese Bakterien vermitteln. In mechanistischen Studien wurde die kovalente Bindung von Tanninen an ein Lignin Modellmolekül in Anwesenheit von Laccase gezeigt. In der Struktur der angenommenen Kopplungsprodukte erwiesen sich die 5-5, 4-*O*-5 und β-*O*-4 Bindungen als dominant. Die antibakterielle Wirksamkeit der unterschiedlichen Tannine stimmt mit ihrer minimalen Hemmkonzentration (MHK, MIC Minimal Inhibitory Concentration) für *E. coli* und ihrer Reaktivität gegenüber der Laccase überein.
Da einige Radikale oder Bindestellen aus Stabilitätsgründen gequencht werden, wurde einen neue Methode entwickelt, in der reaktive Amine als Ankergruppen an Lignocellulose angebracht wurden um eine stabile, reaktive Oberfläche für eine weitere Funktionalisierung zu erhalten. Dieser Ansatz erhöht die Kopplungswahrscheinlichkeit von antifungalen Wirkstoffen um bis zu 58% im Vergleich zu Laccase-indizierter Kopplung. Durch weitere mechanistische Studien konnte eine kovalente Bindung von Tyramin (Ankergruppe)an den Syringylglycerol β-guaiacylether über eine 4-*O*-5 Bindung nachgewiesen werden, wobei eine $–NH_2$ Gruppe als Bindestelle für weitere funktionelle Moleküle frei bleibt. Die durch Laccase vermittelte Bindung von funktionellen Molekülen (phenolische Amine, Fluorophenole und ausgesuchte Holzschutzmittel) wurde unter Verwendung des kürzlich entdeckten Lignin Modells Dibenzodioxocin erstmalig nachgewiesen.
Es wurde außerdem ein neuer, nicht-selektiver chemoenzymatischer Prozess, dem als Substrat alle Lignocellulose Strukturen dienen und durch Lipase erzeugte Oxirane als Anker-Gruppen zugrunde liegen, entwickelt. Unter Verwendung einer Lipase und Wasserstoffperoxid wurden

das Sojaö und Linolsäure epoxidiert wodurch 52.7% bzw. 92.4% relative Umwandlung zu Oxiran gegeben ist. Ein kommerzielles Oxiran, 1,2 Epoxyoctan und durch Epoxidation von Ölen entstandene Oxirane (unter Verwendung von Lipase und Wasserstoffperoxid) wurden erfolgreich an langkettige Alkylamine gekoppelt während die Oxirane außerdem an unterschiedliche Lignin Monomere und Modelle gekoppelt wurden. Eine Behandlung der Buchenholzfurniere mit Dodecylamin mit Hilfe dieses Prozesses führte zu einer gesteigerten Hydrophobizität, welche selbst die durch gezielte Behandlung mit Laccase erreichten Hydrophobizität übertrifft.

Preamble

This thesis is organised into eight chapters. Chapter 1 introduces important subjects of this work, highlighting the motivation behind the current work and providing a background which reviews literature on the key elements of this study. Particular attention is given to the current problems with regards use of physico-chemical methods for wood modification, the advances made in enzymatic modification of lignocellulose materials culminating in new perspectives in laccase-mediated wood modification which set the general direction for the current work. Chapters 2, 3 and 4 describe the novel applications developed based on direct grafting of fluorophenols, alkylamines and tannins, respectively. The feasibility of using anchor groups to create stable reactive surfaces is investigated in Chapters 5 while mechanistic evidence of coupling using lignin models is provided in Chapter 6 using a *Bacillus* spore laccase together with some unique properties of the *Bacillus* spore laccase. Further mechanistic studies with a recently discovered lignin model dibenzodioxocin are provided in Chapter 7. The final chapter explores the possibility of using lipase-generated oxiranes as anchor groups in a non-targeted approach to lignocellulose material functionalisation. The thesis ends with summarised conclusions of the current work also incorporating future perspectives.

1

General Introduction

Humankind has always recognised the value of lignocellulose materials in particular wood. The use of wood and wood-based products is an integral part of human life with the history of mankind closely intertwined with wood utilization. It has been used as a fuel since time immemorial and as a construction material since humans began building shelters. Today the major wood product categories used for different purposes have broadened thanks to many new processing technologies which have given rise to new products which include sawn timber, wood-based panels, woodchips, paper and paper products, poles and railway sleepers. Over the years wood has been used so extensively because of desirable properties such as aesthetic appearance, low density, low thermal expansion, good mechanical strength and low energy requirement to produce a usable end product. However, wood like all lignocellulose fibres, is naturally hydrophilic and being a natural organic material, is prone to biodeterioration. For example the exterior application of wood composites increases exposure to moisture, and consequently, to fungal decay and attack by insects. Although some tree species are naturally resistant to decay, they are either in short unsustainable supply, expensive, less versatile or are not grown close to markets hence the need to resort to fast-growing susceptible (softwood) species which require processing for enhanced protection.

It has been proposed that wood preservation goes back to the time when wood was first used (Richardson, 1978). There are records of wood preservation dating back to the time of Alexander the Great where olive oil was used as preservative while biblical quotations (from Genesis, 6:13-14) suggest use of wood preservatives as far back as the time of Noah. Over the years a number of technologies have been developed to preserve wood materials starting with simple practice to keep wood dry by placing on stones. The first successful processes were recorded in the nineteenth century and they included soaking wood in dilute water solutions of mercuric chloride by Kyan (1832); Margary process (1837) of soaking in

copper sulphate, and the Burnett process (1838) of impregnation under pressure with zinc chloride (Graham, 1973). These early processes have influenced a lot of research in development of wood treating antimicrobial and hydrophobicity enhancing chemicals. Subsequently a number of reviews and books have been written summarising the advances that have been made in this regard (Schultz et al., 2007; Evans, 2003; Freeman et al., 2003; Ibach 1999; Rowell and Banks, 1985). However, preservation effectiveness is not only determined by the protective value of the preservative material, but also by the method of application of the preservative and the extent of penetration and retention of the preservative in the treated wood. Unfortunately, in most cases, the chemicals are not covalently bonded to the wood and therefore are easily leached into the environment. For example water repellent substances seem to be bonded to the cell wall only by relatively weak Van der Wall forces (Razzaque, 1982) and over a long time and due to continuous exposure to water, the repellents are displaced by water and washed off as the weak Van der Waals forces between the wood and repellant are replaced by stronger wood-water hydrogen bonds (Banks, 1973, Razzaque, 1982).

Surface coating (which involves formation of a surface film of polymeric materials, such as varnish, lacquer, or paint) has the disadvantage that it is only effective if there are no cracks, openings or other imperfections that can allow water to enter the wood. As such most treatments using high pressure in which the wood is impregnated in closed vessels under considerable pressure. Unfortunately such methods are not only energy consuming but are associated with an increase in temperature which can negatively affect the wood or the preservative. Wood may shrink if it loses moisture during treatment while some preservatives may precipitate at elevated temperatures (Ibach, 1999). In addition, both high pressure and low pressure treatments methods are generally not eco-friendly when used for volatile preservatives or those that leach out of the products which then easily contaminate the environment and may affect plant and animal life. There is growing evidence that some preservatives like the fire retardant polybrominated diphenyl ether persist in the environment and accumulate in living organisms, as well as toxicological testing that indicates these chemicals may cause liver toxicity, thyroid toxicity, and neurodevelopmental toxicity (Betts, 2001; Alae et al., 2003).

In more recent years there has been strong interest in non-biocidal, chemical and thermal, wood modification systems which reduce wood affinity for water thereby reducing biodeterioration while also improving dimensional stability. This can be achieved chemically by substituting hydroxyl groups on wood's structural polymers with other functional groups,

and bulking the cell wall with chemicals. For example acetylation results in esterification of hydroxyl groups by acetyl groups thereby reducing the number of accessible OH-groups while introducing bulk groups which reduce interaction with water (Rowell, 2006; Obataya et al., 2002; Vick and Rowell, 1990). Heat treatment can also improve hydrophobicity through thermal degradation of the carbohydrate components of the wood cell wall (Viitaniemi and Jämsä, 1996), catalysed by acetic acid formed by deacetylation of hemicellulose. Although these processes have been commercialized, the chemical and thermal modification reactions are carried out under harsh and high energy consuming conditions which alters the structural integrity of wood leading to reduction in mechanical properties such as tensile strength (Ramsden et al., 1997; Obataya et al., 2002).

The shortcomings of chemical and physical methods, growing environmental concerns, legal restrictions and increasing scientific knowledge have prompted a shift to biotechnological approaches in wood processing. Enzymes now offer an alternative to current practices used to produce a variety of value added products. The pioneering works of Dordick and coworkers during the early 1990's (Popp et al., 1991; Blinkovsky and Dordick, 1993) which demonstrated the ability of heme peroxidases to oxidatively graft phenols onto lignin (*in vitro*) resulting in the formation of phenolic resins, heralded the beginning of a new era in polymer functionalization. Subsequently, several studies have demonstrated the possibility of laccase-catalysed bonding of low molecular weight compounds onto lignin/synthetic molecules as summarised in the detailed reviews by Witayakran and Ragauskas (2009), Mikolasch and Schauer (2009), Widsten and Kandelbauer (2008), Kunamneni et al. (2008a,b) and Nyanhongo et al. (2007). The further demonstration by Yamaguchi et al. (1994), Felby et al. (1997, 2002) of the ability of laccases to mediate the production of fibre boards increased the interest in applying oxidoreductases for surface functionalization of lignocellulose materials. Thus, oxidoreductases have emerged as important potential catalysts in wood functionalisation as they are both eco-friendly and work under mild conditions. This enzymatic approach takes advantage of the ability of the enzymes to oxidize molecules on the surface of wood, thereby creating radicals to which molecules of interest can be covalently bound. However, much research is now focusing on laccase since peroxidases require hydrogen peroxide as mediator which adds an extra cost. Laccases work with air (oxygen) releasing water as the only by product. Consequently a number of studies as reviewed by Widsten and Kandelbauer (2008) have been carried out to improve wood properties using laccase. However, most studies rather focused on macroscopic effects and material properties while the elucidation of the mechanisms behind has often been neglected.

This work tries to explain mechanistically the effect of laccases in lignocellulose functionalisation while complex model substrates were one of the important tools used. Also, the current study seeks to expand the current biotechnological processes of wood functionalisation by developing novel applications and new enzymatic processes for lignocellulose material functionalisation.

Aim:

to develop novel enzymatic processes for lignocellulose material functionalisation based on coupling/grafting of functional molecules and elucidation of the mechanisms behind using model reactions

Objectives

1. to develop laccase –mediated methods for direct coupling of functional molecules onto lignocellulose materials
2. to prepare reactive surfaces through laccase-mediated introduction of reactive stable anchor groups onto the surfaces of lignocellulose material.
3. to develop a novel enzymatic non-targeted process for lignocellulose material functionalisation.
4. to elucidate mechanisms of enzyme coupling based on *in vitro* modeling of the functionalisation systems using lignin monomers and lignin synthetic molecules

1.1 Background

1.1.1 Structure of wood as lignocellulose material

Lignocellulose materials have lignin, cellulose and hemicellulose as the main building components. Briefly cellulose is a polymer of glucose; hemicellulose is a copolymer of different C5 and C6 sugars including e.g. xylose, mannose and glucose while lignin is a branched polymer of aromatic compounds. The primary lignocellulose material is wood which is a natural, abundant and one of the few renewable natural resources. On a chemical level wood is composed primarily of carbon, hydrogen and oxygen, carbon being the predominant element (Table 1). The chemical elements are organised into the polymeric

components cellulose, hemicellulose and lignin (Fig 1). The composition of the polymers varies depending on wood species (Table 2). However, a general classification into hardwood and softwood reveals the composition of the polymers as shown in Table 3.

Table 1: Chemical composition of wood

Element	Dry weight (%)
Carbon	49
Hydrogen	6
Oxygen	44
Nitrogen	>0.1
Ash	0.2 – 0.5

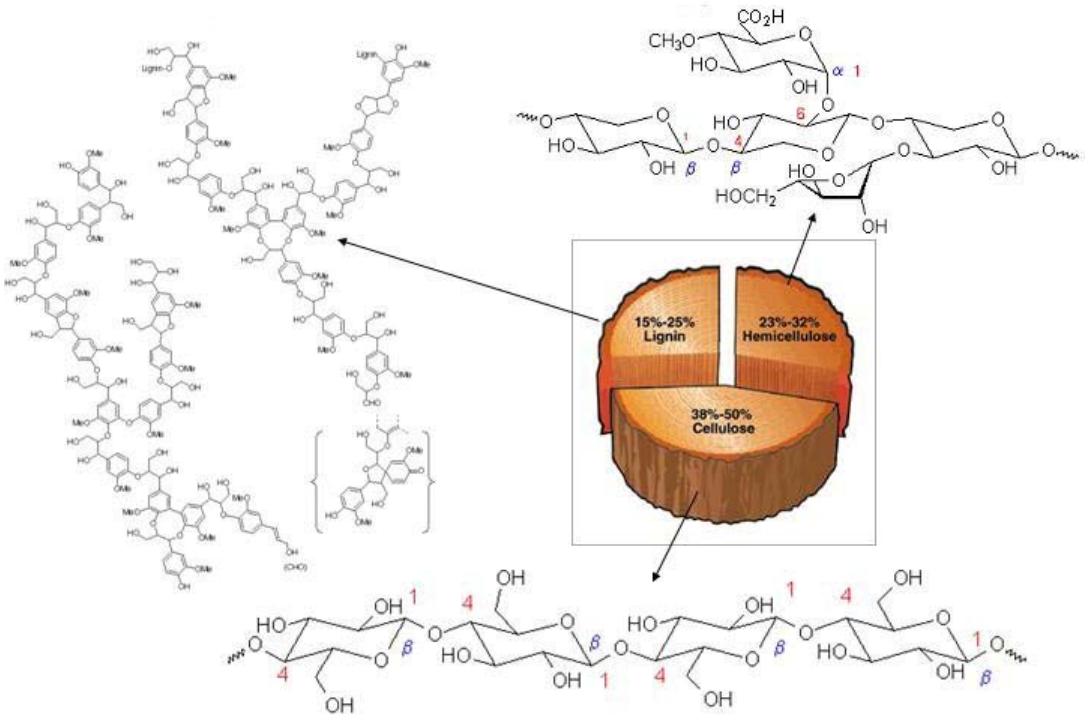


Figure 1: Main components of wood. Source: Department of Chemistry, University of Maine, website: chemistry.umeche.maine.edu/Fort/Cole-Fort.html

Table 2: Chemical composition of some wood species (Sjöström, 1993)

Constituent	Scots Pine *(Pinus sylvestris)*	Spruce *(Picea glauca)*	Eucalyptus *(Eucalyptus camaldulensis*	Silver Birch (*Betula verrucosa)*
Cellulose (%)	40	39.5	45.0	41.0
Hemicellulose				
-Glucomannan (%)	16.0	17.2	3.1	2.3
-Glucuronoxylan (%)	8.9	10.4	14.1	27.5
-Other polysaccharides (%)	3.6	3.0	2.0	2.6
Lignin (%)	27.7	27.5	31.3	22.0
Total extractives (%)	3.5	2.1	2.8	3.0

Table 3: Polymeric components of wood (Betts et al., 1991)

Wood type	Components (% of dry weight)		
	Cellulose	Hemicellulose	lignin
Softwood	45-50	25-35	25-35
Hardwood	45-55	24-40	18-25

Structurally wood is basically a series of tubular fibers or cells cemented together. The fibre walls are composed of cellulose, hemicellulose and lignin organised as proposed in Fig 2 (Mosier et al., 2005). The cellulose fibrils are embedded in a network of hemicellulose and lignin. Cellulose is considered the structural component responsible for strength in the wood fiber because of its high degree of polymerization and linear orientation (Winandy and Rowell, 2005). The hemicelluloses act as a matrix for the cellulose, and increase the packing density of the cell wall. It is also suspected that hemicelluloses act as a highly specific coupling agent capable of associating both with the more random (non-crystalline) areas of hydrophilic fibrous cellulose and the more amorphous hydrophobic lignin (Winandy and Rowell, 2005). Lignin acts as 'glue' which holds the wood fibers together and helps bind carbohydrate molecules together within the cell wall of the wood fiber. Winandy and Rowell (1984) proposed that the chemical components of wood responsible for mechanical properties can be viewed from three levels which are macroscopic (cellular), microscopic (cell wall), and

molecular (polymeric) level. Ultimately, the physical and chemical properties exhibited by the wood are determined by the tubular structure and the polymeric construction.

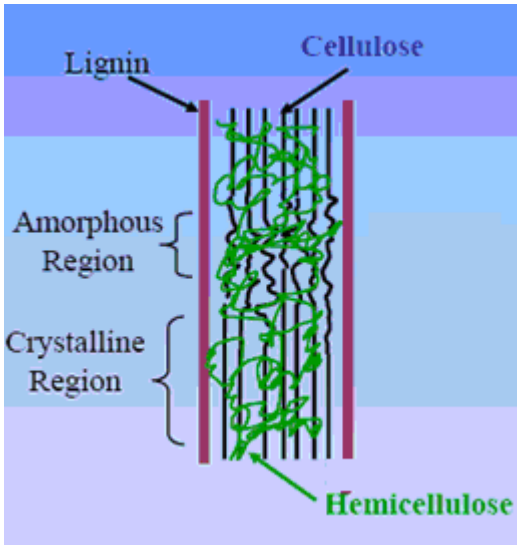


Figure 2: Proposed lignocellulose model (Mosier et al., 2005)

The next sections review in brief the polysaccharide components of lignocellulose material while a more detailed review of lignin is provided as it is the target molecule of the key enzyme (laccase) used in this study.

Cellulose

Cellulose is a straight chain homopolymer of D-anhydroglucose units which condense through β(1→4)-glycosidic bonds. The degree of polymerisation (average number of sugar units in one molecular chain) ranges from 5000 to 15000 where a DP of 10,000 would mean a linear chain length of approximately 5 µm in wood. Generally, the degree of polymerisation is 5000 to 7500 in primary plant cell walls, 10,000 in cellulose from wood and 15,000 in cellulose from cotton (O'Sullivan, 1997). Since each glucose residue is tilted by 180° towards its neighbours, the structural subunit of cellulose is considered to be cellobiose. This is supported by the fact that all the allormorphs of cellulose have a fiber repeat of about 10.3 Å in common, which indicates a two residue repeat as confirmed by the virtual bond length of a cellobiose unit in single structure investigation (Zugenmaier, 2001). At least 5 polymorphic forms of cellulose have been identified using X-ray crystallography (Fig 3) (O'Sullivan, 1997).

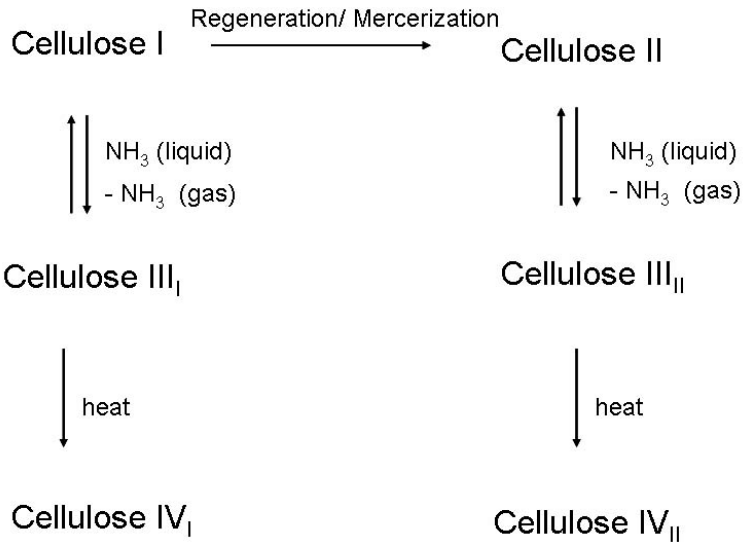


Figure 3: Interconversion of the polymorphic forms of cellulose

Cellulose I (native cellulose) consists of an array of parallel chains linked by intermolecular hydrogen bonds which hold the chains together and intramolecular hydrogen bonds between the atoms of adjacent glucose residues. The molecular chains pack in layers that are held together by van der Waals' forces (Pettersen, 1984). In nature cellulose I exists as low crystalline sub-allormorphs cellulose I_α and I_β which have been confirmed by NMR (Atalla and VanderHart, 1984; VanderHart and Atalla, 1984). The celluloses produced by primitive organisms (bacteria, algae etc.) are enriched in the I_α form which is a triclinic P1 crystal with one cellobiose residue per unit cell (Heiner and Teleman, 1996) whereas the cellulose of higher plants (woody tissues, cotton, ramie etc.) consists mainly of the I_β phase which is monoclinic in nature with two cellobiose moieties per unit cell (Finkenstadt and Millane, 1998).

Celluose II is the most thermodynamically stable allomorph and is formed from cellulose I by either regeneration (extruding a solution of cellulose in sodium hydroxide into water - solubilization and recrystallization) or mercerization (swelling native cellulose fibres in concentrated sodium hydroxide – alkali treatment). Regeneration has been found to give higher yields than mecerization (Kolpak and Blackwell, 1976). Although it can be produced from cellulose I by chemical processes, natural forms of cellulose II have been reported (Nyburg, 1961; Kuga et al., 1993). Unlike cellulose I, the chains in cellulose II lie antiparallel to one another (Sarko and Muggli, 1974; Kolpak and Blackwell, 1976; Stipanovic and Sarko, 1976).

Cellulose III is obtained when either cellulose I or II is treated with liquid ammonia, monomethylamine or monoethylamine. Cellulose IV is obtained by heating cellulose I or II in glycerol at 260 ^{o}C for 20 minutes (Hayashi et al., 1975) or by boiling the cellulose ethylene diamine complex in dimethylformamide. Both cellulose III and IV seldom occur in nature. Table 4 summarises the dimensions of the unit cells of the various allormorphs of cellulose.

Table 4: Unit cells of allomorphs of cellulose

Type	Spacer group	Number of chains	Unit cell (Å, ^{o})						References
			a	b	c	α	β	γ	
Iα	P1	1	6.74	5.93	10.36	117	113	81	Sugiyama et al. (1991)
Iβ	$P2_1$	2	7.85	8.27	10.38	90	90	96.3	Finkenstadt and Millane (1998)
I-ethylenediamine	$P2_1$	2	12.88	9.52	10.35	90	90	118.8	Lee et al. (1984)
Na-C I	$P2_1$	4	8.83	25.28	10.29	90	90	90	Nishimura and Sarko (1991)
II (macerated)	$P2_1$	2	8.10	9.05	10.31	90	90	117.1	Langan et al. (2001)
II hydrazine	$P2_1$	4	9.37	19.88	10.39	90	90	120	Lee et al. (1983)
II hydrate	$P2_1$	2	9.02	9.63	10.34	90	90	116	Lee and Blackwell (1981)
III_I	$P2_1$	2	10.25	7.78	10.34	90	90	122.4	Sarko et al. (1976)
IV_I	P1	2	8.03	8.13	10.34	90	90	90	Gardiner and Sarko (1985)
IV_{II}	P1	2	7.99	8.10	10.34	90	90	90	Gardiner and Sarko (1985)
Na-C IV	$P2_1$	2	9.57	8.72	10.35	90	90	122	Zugenmaier (2001)

Hemicellulose

Hemicelluloses are heteropolysaccharides synthesized in wood consiting of backbones containing glucose and mannose (galactoglucomannans) or xylose (arabinoglucuronoxylans). Additionally, these polysaccharide contain 1-6 linked side groups such as galactose or arabinose and 4-*O* methylglucuronic acid and may be acetylated (Pettersen, 1984). Hardwood hemicelluloses are generally termed xylans and are formed by polymerization of the anhydro forms of xylose, arabinose, and 4-methylglucuronic acid whereas softwood hemicelluloses are generally termed galactoglucomannans and are formed by polymerization of residues of galactose, glucose and mannose. Generally hardwood xylans contain an average of two xylan branching chains per macromolecule and may contain small amounts of L-sugars and trace amounts of rhamnose (Pettersen, 1984). Table 5 shows the major hemicellulose components found in wood. Hemicelluloses consist of shorter chains of 500-3000 sugar units per polymer and are branched. The sugars in the hemicellulose structure exhibit hydrogen bonding both within the hemicellulose chain as well as between other hemicellulose and amorphous cellulose regions. Unlike cellulose which is crystalline and ordered, hemicellulose has a

random, amorphous structure which is easily hydrolyzed by dilute acid or base as well as myriad hemicellulase enzymes. Together with pectin, hemicelluloses are intimately associated with cellulose, acting as a matrix for the cellulose and also increasing the packing density of the cell wall. As pointed out earlier, it is also suspected that they act as a highly specific coupling agent capable of associating both with the non-crystalline portions of cellulose and the more amorphous hydrophobic lignin (Winandy and Rowell, 1984). As such they appear to contribute as a structural component in the plant. This is supported by the fact that some hemicelluloses are present in abnormally large amounts when the plant is under stress; e.g., compression wood has a higher than normal galactose content as well as a higher lignin content (Pettersen, 1984).

Table 5: The major hemicellulose components (Pettersen, 1984)

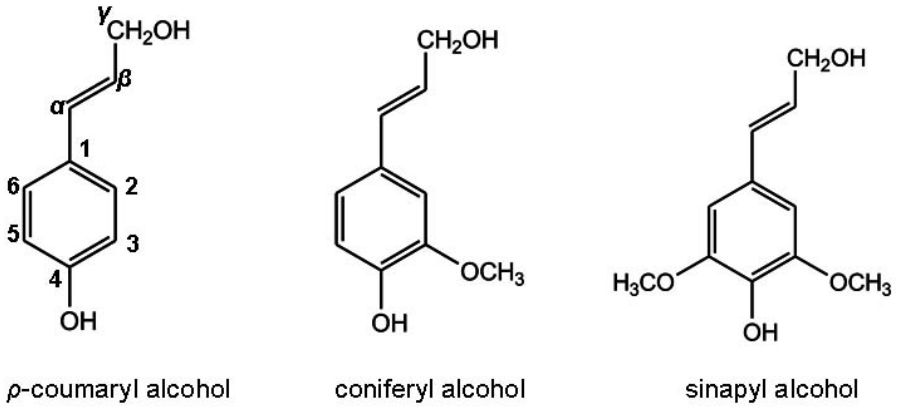
Hemicellulose Type	Occurrence	Amount (% of wood type)	Units	Molar ratio	Linkage	Solubility	$\overline{DP}_n$
Galactoglucomannan	Softwood	5-8	β-D-Man*p* β-D-Glu*p* α-D-Gal*p* Acetyl	3 1 1	1→ 4 1→ 4 1→ 6	Alkali, water*	100
(Galacto)Glucomannan	Softwood	10-15	β-D-Man*p* β-D-Glu*p* α-D-Gal*p* Acetyl	4 1 0.1 1	1→ 4 1→ 4 1→ 6	Alkaline borate	100
Arabinoglucuronoxylan	Softwood	7-10	β-D-Xyl*p* 4-O-Me-α-DGlupA α-L-Ara*f*	10 2 1.3	1→ 4 1→ 2 1→ 3	Alkali, dimethyl sulfoxide*, water*	100
Arabinogalactan	Larchwood	5-35	β-D-Galp α-L-Ara*f* β-L-Ara*p* β-D-Glu*p*A	6 2/3 1/3 Little	1→ 3 1→ 6 1→ 6 1→ 3 1→ 6	Water	200
Glucuronoxylan	Hardwood	15-30	β-D-Xyl*p* 4-O-Me-α-DGlupA Acetyl	10 1 7	1→ 4 1→ 2	Alkali, dimethyl sulfoxide*	200
Glucomannan	Hardwood	2-5	β-D-Man*p* β-D-Glu*p*	1-2	1→ 4 1→ 4	Alkaline borate	200

* - partially soluble

$\overline{DP}_n$ average degree of polymerisation, usually obtained by osmometry

Lignin

Lignin is a heterogeneous optically inactive three dimensional polymer of hydroxylated and methoxylated phenylpropane units, linked in an irregular manner through oxidative coupling to form ether and C-C bonds. It is considered the most chemically complex and least understood of the three wood structural components. Unlike carbohydrates, it is phenolic in nature. It is the binding agent which holds cells together, giving rigidity to the cell. Its hydrophobic nature and its ability to act as an encrusting agent on and around the carbohydrate fraction, limits the influence of water on the carbohydrate fraction which helps to reduce dimensional change with moisture content - enabling wood to retain its strength and stiffness as moisture is introduced to the system (Winandy and Rowell, 2005). This is supported by the observation that dry delignified wood has nearly the same strength as normal dry wood, but wet delignified wood has only approximately 10 % of the strength of wet normal wood (Lagergren et al., 1957). Lignin also adds to wood's toxicity, reducing decay and insect attack. However, in actively growing plants the ability of lignin to decrease the permeation of water through the cell walls of the xylem, plays an intricate role in the transport of water and nutrients. Lignin also plays an important function in a plant's natural defense against degradation by impeding penetration of destructive enzymes through the cell wall (Sjöström, 1993). There are three monolignol monomers; *ρ*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (Fig 4) that make up all lignin found in nature. *ρ*-Coumaryl alcohol is a minor component of grass and forage type lignins and is a also a minor precursor of softwood and hardwood lignins. Coniferyl alcohol is the predominant lignin monomer found in softwoods. Both coniferyl and sinapyl alcohols are the building blocks of hardwood lignin. However, acylated (e.g. acetylated, *p*-coumaroylated, *p*-hydroxybenzoylated) lignins (at the γ-carbon) have also been observed (Martínez et al., 2008). Due to plasticity in lignin structure, its composition is generally characterized by the relative abundance of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units (derived from each of the 3 primary monolignols, respectively) and by the distribution of interunit linkages in the polymer (Ralph et al., 2004).



p-coumaryl alcohol coniferyl alcohol sinapyl alcohol

Figure 4: lignin precursor molecules

The pathway for lignin biosynthesis is relatively well characterized (Baucher et al., 1998; Li et al., 2000; Dixon et al., 2001; Li et al., 2001; Humphreys and Chapple, 2002; Baucher et al., 2003; Boerjan et al., 2003). Generally, the process of lignin synthesis is initiated by the abstraction of a proton from the lignin monomer by peroxidases or laccases, forming a radical. A series of radicals which are in equilibrium with one another are then formed through reasonance stabilization (Fig 5). The radicals then couple to form polymers generating a series of inter-unit linkages such as *β*-*O*-4, *β*-5, 5-5, *β*-*β*, 5-*O*-4 and the more recently discovered dibenzodioxocin 5-5-*O*-4 (Karhunen et al., 1995a;b) (Fig 6). The percentage abundance of the linkages has been determined and it has been shown that the *β*-*O*-4 linkage is the predominant linkage estimated to be as high as 50 % in softwood and 60 % in hardwood (Argyropoulos et al., 2002; Kukkola et al., 2003; Adler, 1977; Sakakibara, 1980), (Table 6).

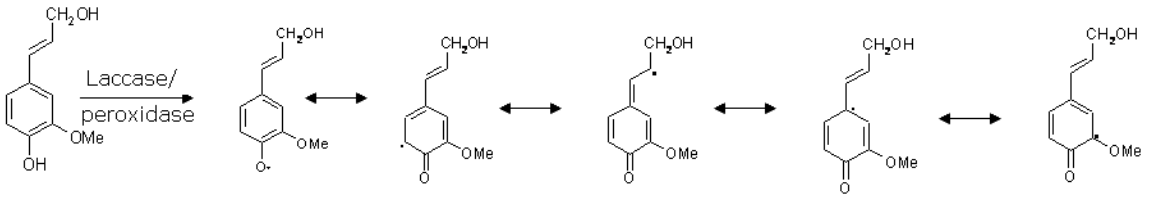


Figure 5: The major resonance stabilized free radicals of coniferyl alcohol

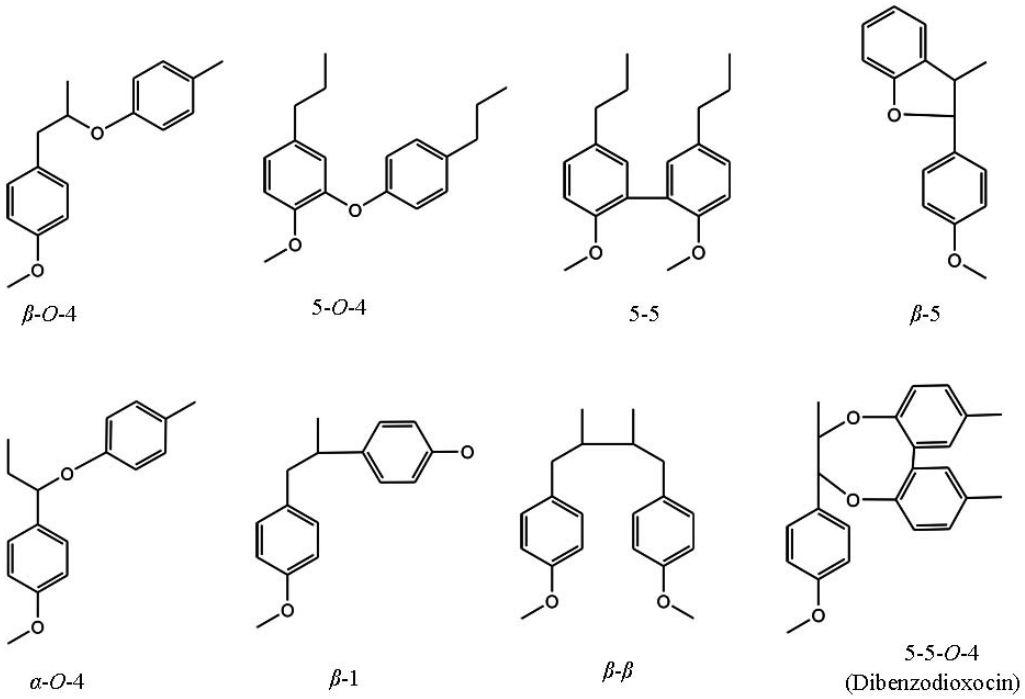


Figure 6: Common linkages in lignin

Table 6: The frequency of the major linkages in softwood and hardwood linkages

Type of linkage	**% of total linkages**	
	Softwood lignin	Hardwood lignin
β-*O*-4 (β-Aryl ether)	45 – 48	60
5-5 (Biphenyl) and dibenzodioxocin	18 – 25	4.5*
β-5 (Phenylcoumaran)	9 – 12	6
β-1	7 – 10	8
α-*O*-4 (α-Aryl ether)	6 – 8	6 – 8
4-*O*-5 (5-*O*-4) (Diphenyl ether)	3.5 – 8	6.5
β–*β* (Pinoresinol)	3	-
α-*O*-*γ* (α-Alkyl ether)	Small	small

* Value refers to percentage of 5-5 linkages only

The reactivity of the lignin polymer is determined to a significant extent by the functional groups on the polymer. The common functional groups are shown in Table 7. The proportion of phenolic hydroxyl groups is low as most are occupied in linkages to neighbouring phenylpropane linkages. Similarly carbonyl and alcoholic hydroxyl groups are incorporated into the lignin structure during enzymatic dehydrogenation and coupling reactions.

Table 7: Frequency of functional groups in softwood lignin per 100 phenyl propane units

Functional Group	**Abundance per 100 C9 units**
Methoxyl	92-96
Phenolic hydroxyl (free)	15-30
Benzyl alcohol	15-20
Carbonyl	10-15

The first comprehensive model of softwood lignin structure (Fig 7) was developed by Sakakibara (1980). A similar structure was proposed for hardwood lignin (*Fagus silvatica* L.) except that there are three times as many syringylpropane units as guaiacylpropane units (Pettersen, 1984). The Sakakibara model was based upon the degradation products formed from lignin samples as a result of hydrolysis in aqueous dioxane and catalytic hydrogenolysis. The most prevalent interunit linkages were thought to be the *β–O–*4 alkyl aryl ether, 5–5 biphenyl, *β* –5 and *β* –1 alkyl arene, and *α–O–*4 benzyl aryl ether linkages.

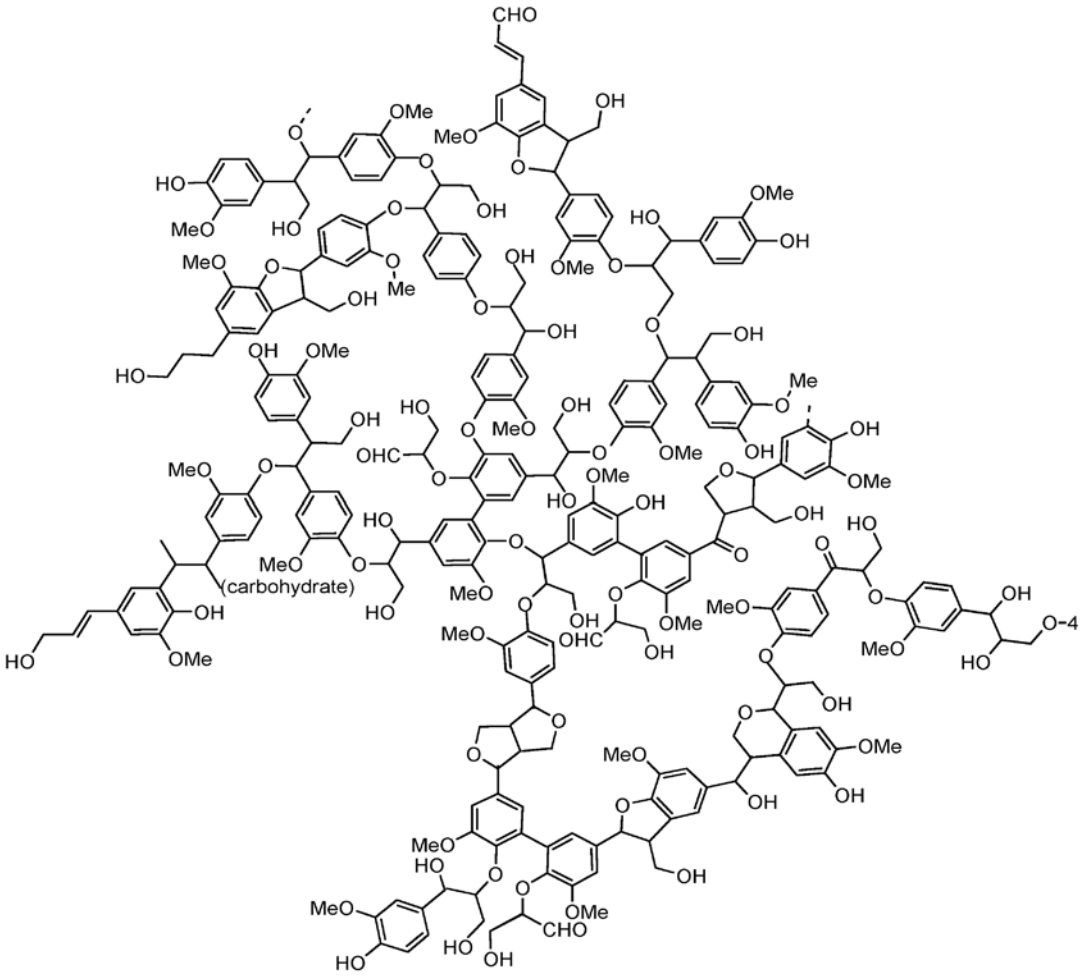


Figure 7: Structural model of gymnosperm lignins according to Sakakibara (1980).

Although the model showed fairly frequent long-chain branching, experimental estimates for cross-linking density in (gymnosperm) lignins have actually been quite low, ~1/19 for example (Yan et al., 1984). In addition, two-dimensional NMR spectroscopy of isolated milled wood lignins, has shown very low levels, if any of β –1 and noncyclic α –*O*–4 linkages (Kilpeläinen et al., 1994; Ede and Kilpeläinen, 1995). Moreover, it has been observed that many of the 5–5 biphenyl interunit linkages are etherified with phenylpropanoid units to form 5–5–*O*–4 dibenzodioxocin structures (Karhunen et al., 1995). These dibenzodioxocin structures also embody cyclic β–*O*–4 alkyl aryl and α–*O*–4 benzyl aryl ethers, and thus account altogether for about 12 % of the interunit linkages in gymnosperm lignins (Argyropoulos et al., 2002; Kukkola et al., 2003). Consequently, a new improved model which took into account the new knowledge was developed (Chen and Sarkanen, 2003) (Fig 8). However, a newer model is inevitable since more aspects of lignin structure have

since been resolved e.g. the identity of the putative precursor of *β*–1 interunit linkage (presented as an insert in figure 8).

Lignin is covalently linked to hemicellulose to form lignin carbohydrate complexes (Björkman, 1957; Erikkson and Lindgren, 1977). The xylan residues appear to be a major interface between lignin and the carbohydrate components (Jeffries, 1990; Cornu et al., 1994).

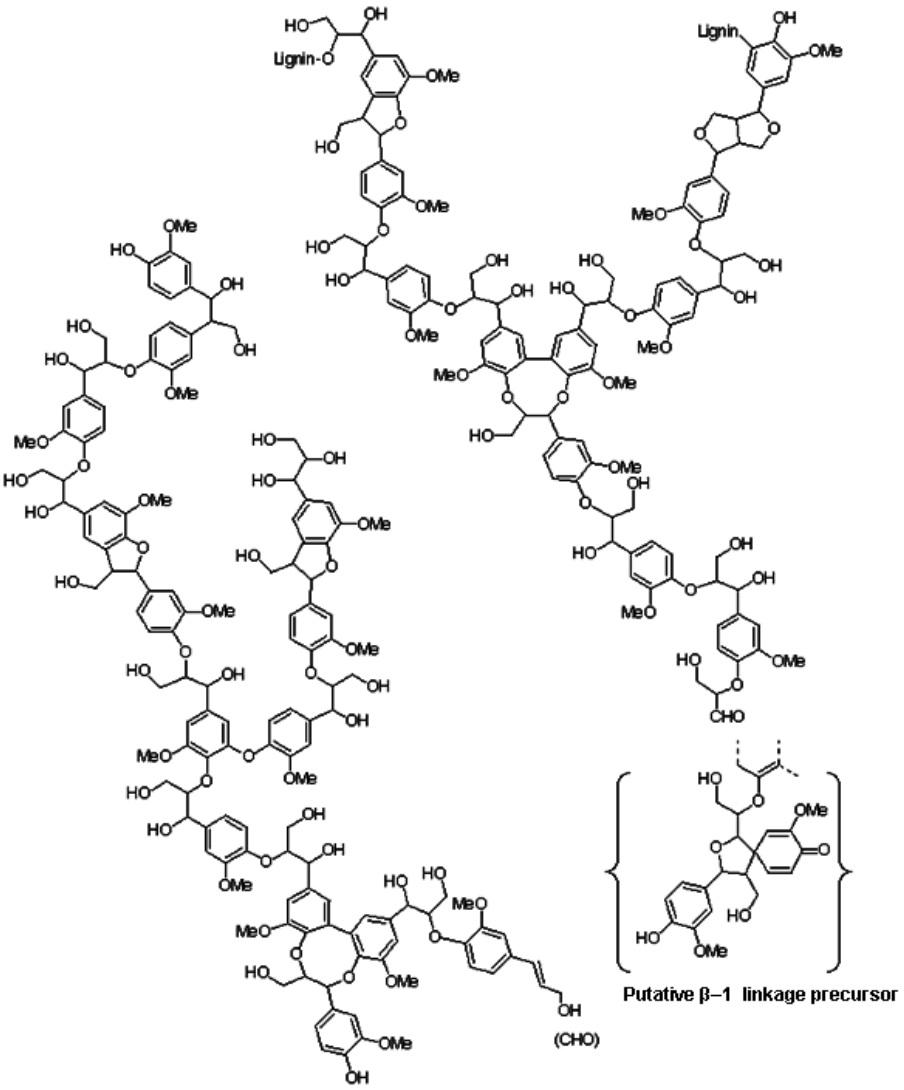


Figure 8: A model of the **s**tructural features of gymnosperm lignins according to Brunow et al., 1998 (Adapted from Chen and Sarkanen, 2003).

1.1.2 Wood Processing Technologies

A number of technologies have been developed in the wood industry mainly in the area of wood preservation. The main objectives include increasing resistance to biodeterioration and fire, and preservation/improving strength and aesthetic properties of the wood. Wood preservation has spanned several generations and technology has improved from crude technology of simply laying wood on stones to keep it dry to the various physico-chemical processes which still dominate the wood industry today. However, as society is fast becoming eco- and energy sensitive, a lot of attention is now being paid to biotechnological processes which are mild and eco-friendly. These concerns are likely to shape the future of the wood processing industry.

Traditional methods of wood treatment

The major technologies are centred around a) use of preservatives which make wood toxic to organisms that use it for food or shelter b) flame retardants which reduce flame spread and prevent wood from supporting its own combustion c) water repellents which slow moisture changes in wood and also reduce biodeterioration, and d) chemical modification to reduce/prevent biological deterioration or make it resistant to acids and alkalis.

As pointed out earlier, the conservation of wooden objects is almost as old as its usage. However, the first records of patents were filed in the early eighteenth century and they dealt with poisonous substances and water repellent coatings (Graham, 1973). The first successful processes were recorded in the nineteenth century and they included soaking wood in dilute water solutions of mercuric chloride by Kyan (1832); Margary process (1837) of soaking in copper sulphate; the Burnett process (1838) of impregnation under pressure with zinc chloride; use of water repellent creosote by Moll (1836) and elaborated by Boulton (1884) (Graham, 1973). The work of these early inventors has shaped the wood preservation industry as we know it today. A lot of reviews have thus been written summarising the various methods of wood conservation (Schultz et al., 2007; Evans, 2003; Ibach, 1999; Rowell and Banks, 1985). Generally wood preservatives can be broadly grouped into oilborne preservatives and waterborne preservatives that are applied as water solutions (Ibach, 1999). Oil based preservatives include coal-tar Creosote, pentachlorophenol solutions, copper naphthenate, chlorothalonil, chlorothalonil/chlorpyrifos, oxine copper (copper-8-quinolinolate), zinc naphthenate, bis(tri-n-butyltin) oxide, 3-iodo-2-propynyl butyl carbamate, alkyl ammonium compound, propiconazole, 4,5-dichloro-2-N-octyl-4-isothiazolin-3-one, tebuconazole, and chlorpyrifos (Ibach, 1999). Each of these preservatives has its advantages

and disadvantages. However, in general, on the positive side, wood does not swell from treatment with preservative oils, volatile oils or solvents with oilborne preservatives; if removed after treatment, oil preservatives leave the wood cleaner, and wood treated with some preservative oils can be glued satisfactorily. However, wood may shrink if it loses moisture during the treating process, and heavy, less volatile petroleum oils may adversely influence wood cleanliness, odor, color, paintability, and fire performance (Ibach, 1999). Oil based preservatives also include water-repellents which are treated using non-pressure methods e.g. impregnation with hydrophobic oil (Ulvcrona et al., 2006) and using wood binders containing hydrophobic diluents **(**US Patent 6352661). Water repellants are usually applied to wood by immersion or vacuum impregnation, in solutions of organic solvents (Rowell and Banks, 1985). Although water repellents increase dimensional stability, they are non-chemically bonded materials (Borgin and Corbett, 1970, Feist and Mraz, 1978, Razzaque, 1982). They seem to be bonded to the cell wall only by relatively weak Van der Wall forces and are displaced by water and washed off as the weak Van der Waals between the wood and repellent can easily be replaced by stronger wood-water hydrogen bonds (Banks 1973, Razzaque, 1982). As such, many of the developed water repellents, like the classical wood preservatives, have the drawback of being detrimental to the environment. Surface coating (which involves formation of a surface film of polymeric materials, such as varnish, lacquer, or paint) has the disadvantage that it is only effective if there are no cracks, openings or other imperfections that can allow water to enter the wood.

Waterborne preservatives include acid copper chromate, ammoniacal copper zinc arsenate, chromated copper arsenate, ammoniacal copper quat, copper bis(dimethyldithiocarbamate), ammoniacal copper citrate, copper azole–Type A, inorganic boron (Borax/Boric Acid) (Ibach, 1999). Waterborne preservatives are preferred when cleanliness and paintability of the treated wood are required. However, as already pointed out, preservation effectiveness is not only determined by the protective value of the preservative material, but also by the method of application of the preservative and the extent of penetration and retention of the preservative in the treated wood. Although several formulations involving combinations of copper, chromium, and arsenic have shown high resistance to leaching and very good performance in service, the majority of waterborne preservatives easily leach into the environment. As such most treatments use high pressure in which the wood is impregnated in closed vessels under pressures considerably above atmospheric. Unfortunately such methods are not only energy consuming but are associated with an increase in temperature which can negatively affect the wood or the preservative.

Wood may shrink if it loses moisture during treatment while some preservatives may precipitate at elevated temperatures (Ibach, 1999). The other disadvantage with waterborne preservatives is that water, which is added to the wood in the treatment process, must be removed by drying to the moisture content required for the end use intended, after treatment.

Emerging technologies

The main emerging technologies in wood preservation have been reviewed by Evans (2003). These include systems based on the metals copper, zinc, iron, quaternary ammonia salts, aluminum azole complexes and aluminates as well as metal-free systems. He also reviewed advances in plant design, treatment processes and as well as chemical modification of wood to increase dimensional stability. Copper-based preservatives have been receiving increased attention particularly due to the efficacy of copper as a fungicide, its low mammalian toxicity and cost, and its long history of use as a wood preservative and as a biocide in other fields such as horticulture and viticulture (Evans, 2003). For example, Battershell et al. (1998) filed a patent application, which describes a process for depositing copper dimethydithiocarbamate in wood by infiltrating wood with gaseous carbon disulphide and then impregnating it with an aqueous solution of copper ions. On the other hand zinc-based preservatives are attractive because of low cost, long history of use as a wood preservative, and ability to be formulated in a colorless form (Evans, 2003). Chemical modification of wood cellular structure reduces the natural hydrophilic nature of wood while imparting hydrophobicity. This can be accomplished through heat or chemical treatment. For example acetylation results in esterification of hydroxyl groups by acetyl groups thereby reducing the number of accessible OH-groups while introducing bulk groups which reduce interaction with water and improves dimensional stability (Obataya et al., 2002; Vick and Rowell, 1990). Heat treatment can also improve hydrophobicity through thermal degradation of the hemicellulose components of the wood cell wall (Viitaniemi and Jämsä, 1996). However these chemical and thermal modification reactions are carried out under harsh and high energy consuming conditions which alters the structural integrity of wood leading to reduction in mechanical properties such as tensile strength (Ramsden et al., 1997; Obataya et al., 2002).

In general chemical methods of preservation including those used as fire retardants are becoming increasingly unpopular due to health, environment and energy concerns. For example, although chromated copper arsenate has an excellent performance and environmental record, public perceptions regarding potential arsenic exposure have led to a

voluntary withdrawal of CCA-treated wood from the residential market (with the notable exception of wood treated for permanent wood foundations) by 2004 in the United States and in Canada. Many chemicals such as the fire retardant polybrominated diphenyl ether persist in the environment and accumulate in living organisms, while toxicological testing indicates these chemicals may cause liver toxicity, thyroid toxicity, and neurodevelopmental toxicity (Betts, 2001; Alae et al., 2003). In fact, the future use of any metal-containing wood preservatives has been called into question in some places such as the European Union where there are lobbies to put limits on the number of active substances that can be used in wood preservation (Evans, 2003). While metal free systems which are based mainly on organic biocides offer hope, there are concerns over their efficacy and cost-effectiveness while further research is required to examine their effects on a host of wood properties, including corrosivity, paintability, gluability, electrical conductivity, strength, fire resistance, and ease of disposal and recycling (Evans, 2003). Natural biocides derived from plants and microorganisms such as plant alkaloids extracted from the Neem tree (*Azadirachta indica*), salicylic acid, and wood vinegar, as well as bacterial metabolites and cysts are too expensive and unsustainable. Although environmentally friendly water repellents like extractives from trees and natural resins have been successfully tested in the laboratory (Passialis and Voulgaridis, 1999; Var and Öktem, 1999), they are not effective when wood is in contact with the ground or water (hazard class 4), where moisture levels in the wood are permanently over 20 % (Hyvönen et al., 2005*)*. Like natural biocides, their commercialisation is also likely to be limited by cost and supply considerations. It is against this background that new biotechnological processes in particular enzymes are now being seriously considered as the future in wood technology.

Enzymatic modification of lignocellulose materials

Over the years enzyme technology has evolved significantly thanks largely to increased understanding of enzyme reaction mechanisms, increased enzyme production facilitated by molecular techniques, and the new global thrust aimed at developing green technologies. Traditionally used for the synthesis of small molecules, enzymes are emerging as important catalysts in the biotransformation of complex natural/synthetic polymers. Research into the modification of lignocellulose materials has mainly been targeted onto the lignin component mainly because it can be altered without adversely affecting the structural integrity of wood.

The obvious candidates for use in modification of the lignin component are the enzymes involved in lignin degradation and lignification and these include laccase and the peroxidases, manganese peroxidase, lignin peroxidase, and versatile peroxidase (Eriksson et al., 1990; Reid, 1995; Highley and Dashek, 1998; Bajpai et al., 1999; Mester and Tien, 2000; Ryan et al., 2006). The ability of heme peroxidases to oxidatively graft phenols onto lignin resulting in the formation of phenolic resins (Popp et al., 1991; Blinkovsky and Dordick, 1993) motivated a lot of research in peroxidase-mediated polymer functionalization. Subsequently a similar enzyme laccase, was also investigated as a candidate for lignocellulose material modification. The demonstration by Yamaguchi et al. (1994) and Felby et al. (1997, 2002), of the ability of laccases to mediate the production of fibre boards increased the interest in applying this technology for surface functionalization of polymers. Consequently, to date, the major research in enzymatic modification of lignin has been carried out using laccase and peroxidases. The ability of these enzymes to oxidise a variety of phenolic molecules, generating reactive radicals which can cross couple, forms the basis of their importance in polymer chemistry. Coupling and grafting reactions form the basis of attaching new molecules to lignocellulose materials (introducing new functionalities) or formation of natural adhesives as a way of enhancing strength properties. The next sections review these key enzymes and the progress made so far in their use for lignocellulose material modification/functionalisation.

Peroxidases – brief review of sources and catalysis

Peroxidases (EC 1.11.1.7) are heme-containing enzymes that oxidise a variety of molecules (organic and inorganic) using hydrogen peroxide by electron abstraction generating radical cations. They typically have a ferriprotoporphyrin IX prosthetic group located at the active site (Ryan et al., 2006). Peroxidases have been isolated from a variety of organisms including animals, plants and fungi (Conesa et al., 2002). In fungi, peroxidases are limited to certain basidiomycetes. A review of the various sources of fungal peroxidase is available (Hofrichter, 2002). Horseradish peroxidase (HRP) and soybean peroxidases (SBP) are the main peroxidases found in plants while the mammalian family of peroxidases includes enzymes such as lactoperoxidase and myeloperoxidase.

The reaction mechanism of peroxidase generally involves a sequence of three steps (Fig 9) (Conesa et al., 2002). The first step in the catalytic cycle is the reaction between H_2O_2 and the resting state of the enzyme [(Fe3+)Pox] to generate compound I, a two electron deficient highly oxidising intermediate comprising an Fe(IV) oxoferryl centre and a

porphyrin-based cation radical. Compound I oxidises substrates by one electron and forms compound II, an Fe(IV) oxoferryl species that is one oxidising equivalent above the resting state. Compound II can in turn oxidise a second substrate and return the enzyme to its resting state. Although both compound I and II have high redox potential above +1 V, in the presence of excess hydrogen peroxide and poor substrates, compound II can be converted into compound III which is an inactive form. It is suspected that veratryl alcohol, a favourable substrate for compound II functions to convert it back to its resting state. In vivo, H_2O_2 is generated from oxidation of low molecular weight aldehydes catalysed by glyoxal oxidase (Breen and Singleton, 1999).

$(Fe^{3+})Pox + H_2O_2 \longrightarrow (Fe(IV)=O)Pox^{*} + H_2O$

Compound I

$(Fe(IV)=O)Pox^{*} + A \longrightarrow (Fe(IV)=O)Pox + A^{*}$

Compound I Compound II

$(Fe(IV)=O)Pox + A \longrightarrow (Fe^{3+})Pox + A^{*}$

Compound II

Figure 9: A simplified reaction mechanism of the typical hemoperoxidase reaction (Conesa et al., 2002).

Although fungal peroxidases like the lignin (LiP) and manganese (MnP) peroxidase (from *Phanerochaete chrysosporium* and other white-rot basidiomycetes), which have been implicated in lignin degradation, are the obvious candidates for lignin modification, HRP was the first peroxidase to be employed for oxidative polymerization studies during the 1980s (Schnitzer et al., 1984; Dordick et al., 1987). However, it was the work of Dordick and coworkers using HRP (Popp et al., 1991; Blinkovsky and Dordick, 1993) which heralded the beginning of special interests in the application of plant peroxidases for grafting. Today the bulk of what is known in application of peroxidases in coupling or grafting reactions is based on plant peroxidises, HRP and SBP.

Application of peroxidases in modification of lignocellulose materials

The main functions of peroxidases in lignocellulose materials technology are based on the ability of the enzyme to generate radicals on lignocellulose materials which can be used to graft molecules of interest to form functional polymers or cross couple to form an adhesive.

The work initiated by Dordick and coworkers (1991; 1993) was motivated by the need to replace chemically synthesised resins with phenolic resins synthesized using enzyme preparations. Phenolic resins are widely used in surface coatings, adhesives, laminates, molding, friction materials, abrasives, flame retardants, carbon membranes, glass fiber laminates, fiberboards, and protein-based wood adhesives etc (Eker et al., 2009).Traditionally thermosetting adhesives used in the manufacture of wood composites such as medium density fibreboards and particle boards, are urea-formaldehyde (UF), melamin-urea-formaldehyde (MUF) and phenol-formaldehyde (PF). However, these chemicals are expensive, toxic to humans and have limited recycle options. As such there has been an increase in research efforts to manufacture synthetic resin-free composite boards and lignin-based resins using enzymes, in particular peroxidases and laccases. Despite being the second most abundant polymer on Earth constituting 30% of non-fossil organic carbon (Boerjan et al 2003), lignin is underutilised. It is estimated that only 2 % (1 million tons/year lignosulphonates and less than 100,000 tons/year of kraft lignins) of approximately 50 million tonnes produced annually as a by-product of the pulp and paper industry, is being used commercially and its disposal has become a source of ecological problems (Mai et al., 1999; Gargulak and Lebo, 2000; Lora and Glasser, 2002; Gosselink et al., 2004). It is therefore appropriate that lignin should be considered for possible use as substrates for enzyme prepared adhesives. Some of the substrates which have been investigated during peroxidase-mediated production of phenolic resins are summarised in Table 8.

Table 8: Some of the substrates used for peroxidase mediated formation of phenolic resins

Substrates	**References**
Lignin + cresol	Liu et al 1999, Popp et al 1991, Liu et al 2001
Kraft lignin + phenols	Blinkovsky and Dordick, 1993
Milled wood lignin	Grönqvist et al 2005
2,6-Dimethylphenol	Ikeda et al 1996
Lignocatechol Lignocresol	Xia et al 2003, Yoshida et al 2005 Eker et al 2009
Brown rotted wood	Li and Geng 2005
Cardanol	Ikeda et al 2000, Kim et al 2003, Kim et al 2005, Kim et al 2007, Park et al 2009, Won et al 2004
Anarcardic	Chelikani et al 2009

The enzymatically prepared resins are not only less toxic and cheaper but they have also shown some superior performance qualities. For example enzymatically prepared poly(*p*-phenylphenol) and poly(*p*-cresol) had high melting points while the poly(*p*-phenylphenol) showed much higher electrical conductivity than phenol-formaldehyde resins (Dordick et al., 1987).

Ikeda et al. (1996) successfully produced polyphenol resin Poly(2,6-dimethyl-1,4-oxyphenylene) (poly(phenylene oxide from a putative lignin monomer 2,6 dimethoxyphenol using peroxidases HRP and SBP, and laccase. Such a process can be an alternative for production of conventional phenol resins like novolak and resol resins, which involves the use of toxic formaldehyde. Similarly, peroxidase mediated polymerization of lignocatechol and lignocresol was shown to produce phenolic resins that have potential application in adhesives, as bonding agents and engineering materials (Xia et al., 2003; Yoshida et al., 2005). Recently, a highly thermostable polymer with good thermosetting properties was produced using SRP in ionic liquids (Eker et al., 2009). Treatment of brown-rot-fungus-decayed wood with sodium borohydride followed by mixing with polyethylenimine resulted in a formaldehyde free, strong and water-resistant wood adhesive (Li and Geng, 2005). Brown-rot fungi preferentially degrade cellulose and hemicelluloses in wood and in the process oxidize and demethylate lignin resulting in oxidized ortho-quinone structures (Li and Geng, 2005).

There have also been attempts to use lignosulphonates oxidized by peroxidase or laccase as binders for the production of particle boards (Nimz et al., 1972; 1976). Particle boards that surpassed the requirements of transverse tensile strength (DIN 52365 test) of 0.35 MPa specified by European standard EN 312-4, were successfully produced. Unfortunately the particle boards swelled in water due to the sulphonate groups present in lignosulphonate, making them suitable for indoor use only. Therefore subsequent investigations focused on promoting auto adhesion of lignin in wood without any additional gluing material during production of wood laminates, particle boards (PB) and medium density fibre boards (MDF). HRP and laccase treatment of brown rotted wood produced laminates with relatively good shear strength although lower than those obtained with adhesives (Jin et al., 1991). Unfortunately the wood laminates swelled in water leading to delamination. However, forced-air-drying of MDF treated with laccase increased internal bond strength and reduced swelling in water (Felby et al., 1997; 1998; 2002; Unbehaun et al., 2000). The enzymatic process involved oxidizing the lignin rich middle lamella exposed on the fibre surface of wood which promoted cross-linking of fibers during hot pressing of the boards. This resulted in MDFs with comparable properties to traditional MDF. Both laccase and peroxidase bonded MDF

boards achieved the European standard CIN DIN 622-5. Although peroxidases gave the same results as boards treated by laccase, the requirement of hydrogen peroxide and its rapid degradation was its main drawback (Kharazipour et al., 1998). Nevertheless, these studies demonstrated the possibility of producing MDF using peroxidases as an alternative to the use of chemically synthesized resins.

Another interesting application of peroxidases is in the grafting of functional molecules to polymeric materials in order to obtain functional polymers with new/improved properties (Fig 10). For example the findings of (Fukuoka et al., 2004) indicate that *p*- and *m*-substituted phenols can be oxidatively coupled to polymeric materials yielding novel functional polymers. It was shown that the co-polymerization reaction did not occur with acetylated lignin or methylated lignin (phenolic groups blocked), indicating the necessity for the peroxidase to form phenoxy radicals with both the ρ-cresol and the lignin. Blinkovsky and Dordick (1993) demonstrated the ability of HRP to catalyze the copolymerization of phenols with kraft lignin in aqueous-organic solvent mixtures. Similarly, Grönqvist et al. (2005) also showed the ability of HRP to oxidatively polymerize milled wood lignin. The copolymerization products had markedly lower glass transition temperatures and higher (and more uniform) curing exotherms. Liu et al. (1999) also successfully copolymerized lignin with cresol (Fig 11) and controlled the molecular weight of the copolymer by adjusting the surfactant concentration. The hybrid copolymer also showed quite different properties compared to native lignin, including lower glass transition temperature and higher curing exotherm. In subsequent studies, Liu et al. (2001) produced a polymer with good thermoset properties and which could be easily recovered after precipitation by controlling Mw of the lignin/cresol polymer during peroxidase-catalyzed copolymerization of lignin with cresol in the reversed micellar system. They did this by adjusting the concentration of surfactant, enzyme, cresol, lignin and the ratio of alcohol to hydrocarbon in the organic phase. Although significant progress has been made using peroxidases, a lot of research is also focussing on laccase as a candidate for lignocellulose material modification.

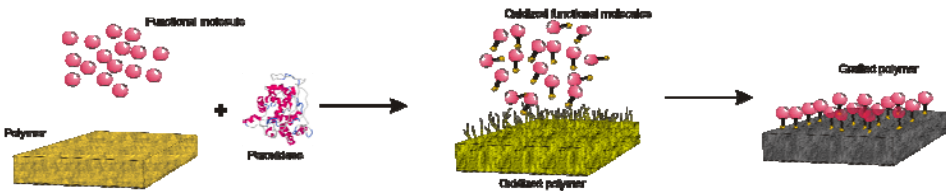


Figure 10: Grafting of functional molecules - a general scheme

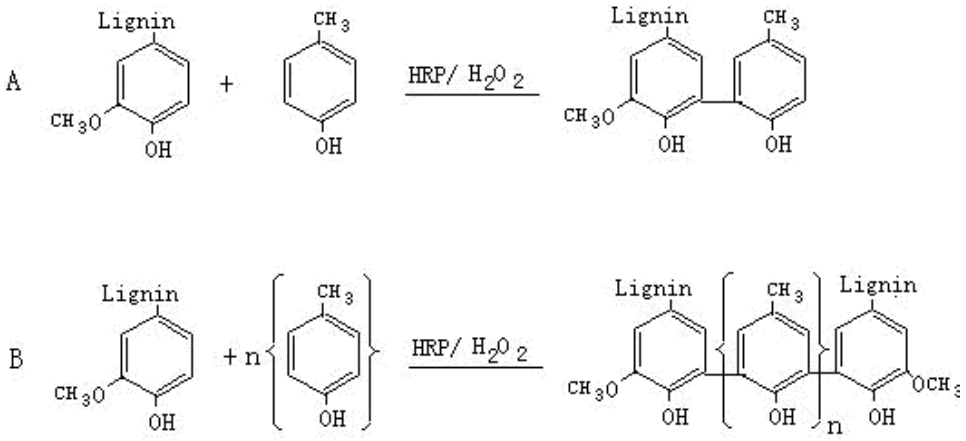


Figure 11: HRP catalyzed copolymerization of lignin with cresol (Liu et al., 1999)

Laccases: brief review of sources and catalysis

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing blue enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates such as diphenols, methoxy substituted monophenols and aromatic amines (Claus, 2004; Riva, 2006; Xu, 1996). Laccases have been isolated in higher plants (Yoshida, 1883; Ranocha et al., 2002; Caparrós-Ruiz et al., 2006), prokaryotes (Claus, 2003), insects (Kramer et al., 2001), and are widespread in fungi (Thurston, 1994; Baldrian, 2006). Recent reviews covering their distribution have acknowledged the ubiquitous nature of laccases (Mayer and Staples, 2002; Claus, 2004, Riva, 2006; Baldrian, 2006).

Laccase catalyses the four electron reduction of O_2 to H_2O, coupled with single-electron oxidation of four hydrogen-donating substrates (Baldrian, 2006). This is accomplished by a cluster of four copper atoms (type 1 copper; type 2 copper and two type 3 copper atoms) that form the enzyme active site (O' Malley et al., 1993; Riva, 2006). Type 1 copper, which confers the typical blue colour to the enzyme due to intense electronic absorption of covalent copper-cysteine bonds, is the site where monoelectronic oxidation of substrate takes place. Type 2 and type 3 copper form a trinuclear cluster, to which the electrons are transferred and reduce molecular oxygen to water. The net result is the oxidation of four molecules of suitable substrate to produce four radicals while reducing one molecule of oxygen to two molecules of water (Fig 12). The reactive radicals can then undergo a number of non-enzymatic reactions which include i) covalent coupling to form dimers, oligomers and polymers through C-C, C-O and C-N bonds (Claus, 2003; 2004) ii)

Degradation of complex polymers by cleavage of covalent bonds especially alkyl-aryl- (sometimes in the presence of mediators) releasing monomers (Dean and Eriksson, 1994; Thurston, 1994; Claus and Filip, 1998; Breen and Singleton, 1999) and iii) ring cleavage of aromatic compounds (Kawai et al., 1988; Duran and Esposito, 2000; Claus et al., 2002).

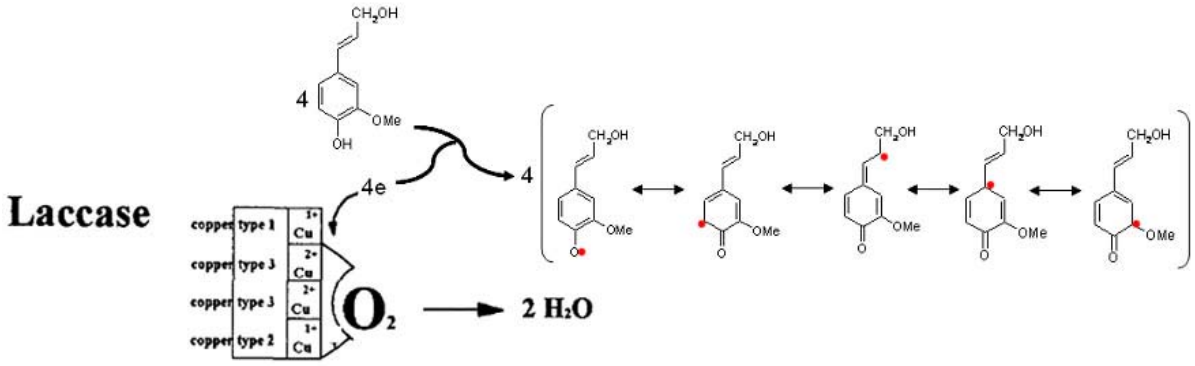


Figure 12: A simplified reaction mechanism of laccase oxidation of suitable substrate using coniferyl alcohol as an example

Application areas of laccases are increasing mainly because they have a wide substrate range. Basically laccase can oxidise any substrate with characteristics similar to a ρ-diphenol and some fungal laccases can also oxidise monophenols such as cresol while some are able to oxidise ascorbic acid (Mayer and Staples, 2002). Substrates of interest which cannot be oxidised solely because of steric hindrance or they have a particularly high redox potential can be oxidised by small laccase-radicalised mediators. As such the substrate range has widened to include non-phenolic molecules such as veratryl alcohol (Enriqueta Arias et al., 2003) and adlerol (Barecca et al., 2003). Thus research on laccase applications especially involving coupling reactions (both cross-coupling and homocoupling) has widened in recent years, opening up new opportunities for a number of industries.

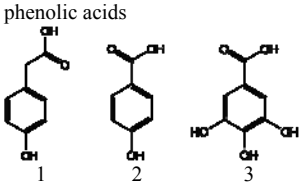
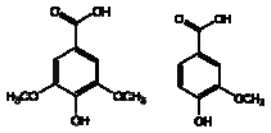
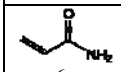
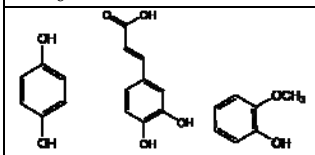
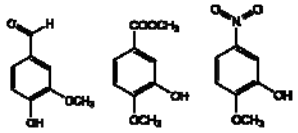
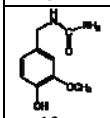
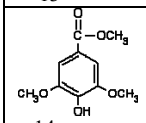
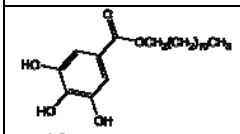
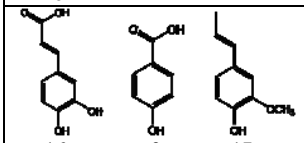
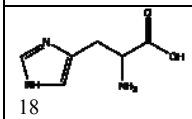
Laccase-mediated modification of lignocellulose materials

Laccase-mediated modification of lignocellulose materials is accomplished through two main ways: coupling of low-molecular weight compounds onto lignocellulosic materials and laccase mediated cross-linking of lignin molecules in-situ. Laccase-mediated coupling of low-molecular weight compounds provides a mild eco-friendly method for functionalizing wood. A number of authors have demonstrated the possibility of coupling low molecular weight compounds onto lignocellulose material. Lund and coworkers reported grafting of 4-hydroxyphenylacetic acid (PAA) and guaiacol sulfonate to kraft lignin (Lund and Ragauskas,

2001; Lund et al., 1998). Lund and Ragauskas (2001) further established that there seems to be a correlation between substitution pattern and the degree of incorporation of phenolic molecules, with only phenols having an electron-donating group (methoxy on guaiacol sulfonate and the methylene group on 4-hydroxyphenylacetic acid) being incorporated. Grönqvist et al. (2006) reported the grafting of 3-hydroxytyramine to TMP. They observed that bleached TMP was radicalised more efficiently than unbleached TMP and the bleached TMP accepted a higher amount of 3-hydroxytyramine. In the same study, it was also reported that created lignin radicals are unstable (up to 90 % were quenched within a few hours), and thus the optimal time for the activation and addition of the chemical to be bonded is an important parameter.

A number of other researchers have successfully grafted functional molecules onto lignocellulose material which can improve properties of the material (Table 9). Most of the molecules grafted are phenolic in nature. However, coupling of acrylamide (Milsten et al., 1994; Mai et al., 1999) indicates that it is also possible to graft aliphatic molecules.

Table 9: Examples of molecules grafted onto lignocellulose materials using laccase

Molecule grafted	Raw material	Application/ Potential Application	Source of laccase	References
phenolic acids  1 2 3  4 5	Kraft paper, Softwoods pulp	Improving strength properties (increase in burst, tear and tensile indexes)		Kenealy et al., 2004; Chandra, 2003; Chandra and Ragauskas, 2001 ; Chandra and Ragauskas, 2002 ; Chandra and Ragauskas, 2002a ; Chandra et al., 2004 ; Chandra et al., 2004a Milsten et al 1994
 6	Technical lignin	Introduction of 'plastic' side chains into lignin backbone	*Trametes versicolor*	Milstein et al. 1994; Mai et al., 1999
 7 8 9  10 11 12	Flax fibres	Improvement of colour and antimicrobial properties	*Trametes hirsuta*	Schroeder et al.2007
 13	wood (spruce) chips	Increase in internal bond	*Trametes villosa*	Fackler et al. 2008
 14	Kraft pulp	Improvement in wet strength (wet tensile index doubled)	*Aspergillus oryzae*	Liu et al. 2009
 15	Bleached and unbleached TMP Handsheets	Improvement of hydrophobicity	*Trametes hirsuta*	Suurnäkki et al., 2006;
 16 2 17	Unbleached kraft liner fibres	Improvement of antimicrobial properties of prepared handsheets; oligomeric forms of caffeic acid and isoeugenol were most effective against both Gram positive and Gram negative bacteria	*T. pubescens*	Elegir et al., 2008
 18	Pulp and paper	Improving strength properties	*Trametes villosa*	Witayakran and Ragauskas, 2009

4-Hydroxyphenylacetic acid **2**. 4-Hydroxybenzoic acid **3**. Gallic acid **4**. Syringic acid **5**. Vanillic acid **6**. Acrylamide **7**. Hydroquinone **8**. Ferulic acid **9**. Guaiacol **10**. Vanillin **11**. Methyl-3-hydroxy-4-methoxybenzoate **12**. 2-Methoxy-5-nitrophenol **13**. 4-Hydroxy-3-methoxybenzylurea **14**. Methyl syringate **15**. Lauryl gallate **16**. Caffeic acid **17**. Isoeugenol **18**. Histidine.

The effect of modifying the fiber chemistry of lignocellulosic materials using laccase is being actively pursued particularly in the pulp and paper industry, with a lot of research centred on improving mechanical properties of paper. A lot of success has been achieved in increasing strength properties of kraft pulp using phenolic acids (Chandra and Ragauskas, 2001; Chandra and Ragauskas, 2002a,b; Chandra et al., 2003, 2004a,b). This is ascribed to the increase in carboxyl groups which promote hydrogen bonding and also the crosslinking of phenoxy radicals in the paper sheet (Chandra, 2003). Although treatment of pulp with laccase alone leads to improvement in wet strength, treatment of pulp with laccase in the presence of a low-molecular weight laccase substrate like methyl syringate leads to a higher increase in wet strength (Liu et al., 2009). It is suggested that the methyl syringate accelerated the oxidation of lignin within fibers and enhanced the activation of fibers - essentially also worked as a mediator. Therefore enhanced wet strength improvement results from a combination of the deposition of the polymerized phenolic molecules, their grafting onto fibers and the accelerated oxidation of lignin within fibers which results in much more covalent bonds between fibers due to the crosslinking of radicals. In related studies, laccase-facilitated grafting of celestine blue to pulp fibers resulted in tensile strength increases of 10-15% (Chandra et al., 2003). Low-molecular weight ultra-filtered lignin can also improve mechanical properties of kraft liner pulp and chemi-thermo-mechanical pulp (Elegir et al., 2007). Laccase-mediated cross-linking of lignocellulose fibres using ultra-filtered lignin resulted in a two-fold increase in wet strength of kraft liner pulp handsheets without loosing other critical mechanical properties. Although a laccase/ABTS mediator system showed a greater increase in wet tensile strength, other mechanical properties such as dry tensile strength, compression strength and Scott Bond internal strength were negatively affected (Elegir et al., 2007). However, laccase-mediator systems have been found to be more effective in improving paper properties (Lund and Felby, 2000; Lund and Felby, 2001; Wong et al., 1999). Copolymerisation of lignin with vanillic acid (Milsten et al., 1994) could also have strength improvement properties in a similar manner as other phenolic acids. Yamaguchi et al. (1992, 1994) utilized laccase to polymerize various phenolic compounds forming dehydrogenative polymers which were subsequently coupled to TMP with peroxidase for the formation of paperboard with high tensile strength. By incorporating phenolics from renewable sources (vanillic acid, catechol, mimosa tannin and tannic acid) as crosslinking agent in the presence of peroxidase from shoots of bamboo or laccase from *T. versicolor,* it was possible to produce paper boards with good tensile and ply-bond strength comparable to hot-pressed TMP–phenol paper boards and significantly higher than those produced in the

absence of phenols (Yamaguchi et al., 1991, 1992, 1994). A patent describing a process for producing linerboard or corrugated medium using *Polyporous* laccase has been filed (Hansen et al., 1995). Similarly, addition of lignin-rich extractives to laccase-treated high-yield kraft pulp resulted in an improvement in paper wet strength (Lund and Felby, 2001). Wong et al. (2000) reported an improvement in handsheet properties (increased tensile index and burst indices) made from laccase-treated pressurized refiner mechanical pulp. It has also been reported that laccase pretreated kraft lignin grafted with spruce sulfite pulp resulted in handsheets with higher tear strength and wet strength than untreated controls (Huttermann et al., 1998). However, the authors did not show the results of their paper tests. In related studies pretreatment of wood chips with white-rot fungus *Trametes hirsuta* and the brown-rots *Gloeophyllum trabeum* (Unbehaun et al 1999, 2000), *Coniophora puteana* and *Fomitopsis pinicola* (Körner et al., 2001) reduced the required refining energy by 40 % in thermo-mechanical pulping (TMP) as compared with untreated wood chips. In addition, the pretreated TMPs had 3.5-times higher bending strength, 3-times higher modulus of elasticity and at least 60 % reduction in thickness swelling in water (24 h), when compared to boards pressed from untreated fibres (Unbehaun et al., 2000, Körner et al., 2001).

Although a lot of research has focused on structural properties of paper, recently there has been an increase in innovative research aimed at developing functional packaging material with antimicrobial or antioxidant properties. For example Elegir et al. (2008) reported laccase mediated grafting of phenols possessing antimicrobial activities onto unbleached kraft liner. The resulting paper sheets showed a greater efficacy against the Gram positive *Staphylococcus aureus* and the Gram negative *Escherichia coli* than paper sheets treated only with monomeric phenol derivatives. Similarly, Suurnäkki et al. (2006) reported an increase in hydrophobicity of thermomechanical pulp treated with lauryl gallate in the presence of laccase. In a related study but with protein-based material (wool), Hossain et al. (2008) reported laccase-mediated grafting of lauryl gallate onto wool resulting in a multifunctional textile material with antioxidant, antibacterial and water repellent properties. Laccase-mediated coupling of methoxyphenols and hydroquinone to modify colour of flax fibres and impart antimicrobial properties has been investigated (Schroeder et al., 2007). Ferulic acid and hydroquinone significantly inhibited growth of *Bacillus subtilis* and *Staphylococcus aureus* while fibre colouration depended on the molecule grafted although all tested molecules showed weak fastness properties. It was proposed that the antibacterial properties could be due to hydroxyl groups and carbonic acid groups (Park et al., 2001).

Coupling of in-situ laccase-created radicals in wood chips have been investigated as a way of producing binderless (artificial adhesive-free) wood such as medium density fibreboards (MDFs) and particle boards (PBs). Traditionally, urea-formaldehyde, melamin-urea-formaldehyde and phenol-formaldehyde are used as adhesives in the manufacture of these boards while an additional binder is required for high-density fiberboards (HDF boards, hardboards). Unfortunately as pointed out earlier, these adhesives are expensive (Maloney, 1996), hazardous (Sellers, 2001; Youngquist, 1999), limit reuse options, and increase mass of the boards. Consequently, there has been an increase in research dealing with the manufacture of binderless fiberboard (MDF and HDF) and PB using laccase (Widsten et al., 2003, 2004; Felby et al., 2004; Felby et al., 2002; Felby et al., 1997; Kharazipour et al., 1997; Kharazipour et al., 1998) and some of the work has been patented (Kharazipour et al., 1993; Qvintus-Leino et al., 2003; Hüttermann et al., 1998; Viikari et al., 1998a,b). Generally, the process exploits the ability of laccase to form phenoxy radicals which couple when the fibres are pressed into boards. Other factors which contribute to improved bonding include surface molecular entanglements, condensation of hemicellulose degradation products and hydrogen bonding, (Felby et al., 2004; Widsten et al., 2003, 2004). Although, laccase catalyzed bonding requires higher pressing temperatures and longer pressing times (Felby et al., 2002), fiberboards made from laccase-treated fibers have a high, up to industrial standard wet strength compared to boards made from untreated fibers (Felby et al., 2002, 2004; Wisten et al., 2003, 2004). However, more work is required to improve dimensional stability which is still below industrial standards (Felby et al., 2002, 2004; Widsten et al., 2003, 2004). A variant processes has been described in which a laccase oxidized lignin based adhesive with 1% methylene diphenyl diisocyanate resulted in particle boards with doubled tensile strength and reduced swelling in water (Hüttermann and Kharazipour, 1996, Hüttermann et al., 2001).

1.1.3 Conclusion and future perspectives

Generally significant advances have been made in lignocellulose material modification. The processes have evolved over the years from crude processes of drying wood through physico-chemical methods to the emerging technologies focussing on natural biodegradable preservatives and mild enzymatic processes. It is likely that enzymatic processes will shape the future of lignocellulose material modification. The foregoing discussion has highlighted some advances made with the lignin modifying enzymes peroxidases and laccases. Although peroxidases generally give the same results as laccases, the requirement of the expensive hydrogen peroxide and its rapid degradation makes their

exploitation expensive and cumbersome (Kharazipour et al., 1998). It is therefore likely that laccases will attractive more attention in future research efforts as they are cheaper (only require oxygen as electron acceptor), eco-friendly (water is the only by-product) and work under mild conditions. Despite the large number of investigations describing novel approaches of enzymatic material functionalisation, the mechanistic basis is only discussed and investigated in few cases. Therefore, an important aspect of this work was the elucidation of the effect of enzymes on a molecular scale.

1.1.4 List of References

Adler, E. (1977) Lignin chemistry: past, present and future. *Wood Sci Technol* **11**: 169-218.

Alae, M., Arias, P., Sjödin, A. and Bergan, A. (2003) An overview of commercially used brominated flame retardants, their applications, their use patterns in different countries/regions and possible modes of release. *Environment International* **29**: 683-689.

Argyropoulos, D.S., Jurasek, L., Krištofová, L., Xia, Z., Sun, Y. and Paluš, E. (2002). Abundance and Reactivity of Dibenzodioxocins in Softwood Lignin. *J. Agric. Food Chem* **50** (4): 658–666.

Atalla, R. H. and VanderHart, D. L. (1984) Native cellulose: a composite of two distinct crystalline forms. *Science* **223**: 283-285.

Bajpai, P., Bajpai, P.K. and Kondo, R. (1999) Biotechnology for environmental protection in the pulp and paper industry. Springer, Berlin. 266 p.

Baldrian, P. (2006) Fungal laccases-occurrence and properties. *FEBS Microbiol. Rev.* **30**: 215–242.

Banks, W.B. (1973) Water uptake by Scots pine sapwood, and its restriction by the use of water repellents. *Wood Sci. Technol*.**7**: 271 – 284.

Barreca, A.M., Fabbrini, M., Galli, C., Gentili, P. and Ljunggren, S. (2003) Laccase/mediated oxidation of a lignin model for improved delignification procedures. *J. Mol. Cat. B: Enzymatic* **26** (1-2): 105-110.

Battershell, R.D., Jacobson, B.M., Abraham, A.C., Pereira, B.M. and Kempf. **J.V.** (1998) Method for preserving wood using single reactor and active dimethyldithiocarbamate agent produced in situ. Patent Appl. US 97-786823.

Baucher, M., Halpin, C., Petit-Conil, M. and Boerjan, W. (2003) Lignin: Genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology* **38**(4): 305–350.

Baucher, M., Monties, B., Van Montagu, M. and Boerjan, W. (1998) Biosynthesis and genetic engineering of lignin. *Crit. Rev. in Plant Sci.* **17**(2): 125–197.

Betts , K.S. (2001) Rapidly rising PBDE levels in North America. Science News - December 7.

Betts, W.B., Dart, R.K., Ball, A.S. and Pedlar, S.L. (1991) Biosynthesis and structure of lignocellulose. In: Biodegradation: Natural and Synthetic Materials W.B. Betts (Ed) (pp 139–155). Springer-Verlag, Berlin.

Björkman, A. (1957) Studies on Finley Divided Wood Part 3. Extraction of Lignin-Carbohydrate Complexes with Neutral Solvents. *Svensk Papperstidning* **60**(7): 243-251.

Blinkovsky, A.M.and Dordick, J.S. (1993) Peroxidase-catalyzed synthesis of lignin-phenol copolymers. *J. Polymer Sci. Part A: Polymer Chem*. (31)7: 1839 – 1846.

Boerjan, W., Ralph, J. and Baucher, M. (2003) Lignin biosynthesis. *Ann. Rev. Plant Biol*. **54**: 519-546.

Borgin, K. and Corbett, K. (1970). The stability and weathering properties of wood treated with various oils. *Plastics, Paint and Rubber* **14**(3): 69 – 72.

Breen, A. and Singleton, F.L. (1999) Fungi in lignocellulose breakdown and biopulping. *Curr. Opin. Biotechnol*.**10**(3): 252-258.

Brunow, G., Kilpeläinen, I., Sipilä, J., Syrjänen, K., Karhunen, P., Setälä, H. and Rummakko, P. (1998) Oxidative coupling of phenols and the biosynthesis of lignin. In Lignin and Lignan Biosynthesis (pp. 131–147). Lewis NG & Sarkanen S (eds). ACS Symp. Ser. 697. American Chemical Society, Washington DC, USA.

Caparrós-Ruiz, D., Fornalé, S., Civardi, L., Puigdomènech, P. and Rigau, J. (2006) Isolation and characterisation of a family of laccases in maize. *Plant Science* **171**: 217–225.

Chandra, R.P. (2003) Chemo-enzymatic modification of high-kappa kraft pulps with laccase. Ph.D. Thesis, Institute of Paper Science and Technology, Georgia Institute of Technology.

Chandra, R.P., Felby, C. and Ragauskas, A.J. (2004) Improving laccase-facilitated grafting of 4-hydroxybenzoic acid to high-kappa kraft pulps. *J. Wood Chem. Technol*. **24**: 69-81.

Chandra, R.P., Lehtonen, L.K. and Ragauskas, A.J. (2004) Modification of high lignin content kraft pulps with laccase to improve paper strength properties. 1. Laccase treatment in the presence of gallic acid. *Biotechnol. Progr*. **20**: 255-261.

Chandra, R.P. and Ragauskas, A.J. (2001) Laccase: the renegade of fiber modification. Tappi Pulping Conference, Seattle, WA, Nov. 4-7, 2001, pp. 1041-1051.

Chandra, R.P. and Ragauskas, A.J. (2002). Elucidating the effects of laccase on the physical properties of high-kappa kraft pulps. *Prog Biotech* **21**: 165-172.

Chandra, R.P. and Ragauskas, A.J. (2002) Evaluating laccase-facilitated coupling of phenolic acids to high-yield kraft pulps. *Enzyme Microb. Tech.* **30**: 855-861.

Chandra, R.P., Wolfaardt, F. and Ragauskas, A.J. (2003) Biografting of celestine blue onto a high kappa kraft pulp, *ACS Sym Ser* 855: 66–80.

Chelikani, R., Kim, Y., Yoon, D-Y., Kim, D-S. (2009) Enzymatic Polymerization of Natural Anacardic Acid and Antibiofouling Effects of Polyanacardic Acid Coatings. *Appl. Biochem. Biotechnol*. **157**: 263-277.

Chen, Y. and Sarkanen, S. (2003) Macromolecular lignin replication: A mechanistic working hypothesis. *Phytochemistry Reviews* **2**: 235–255.

Claus, H. (2004) Laccases: structure, reactions, distribution. *Micron* **35**: 93-96.

Claus, H. (2003) Laccases and their occurrence in prokaryotes. *Arch microbial* **179**: 145-150.

Claus, H., Faber, G. and König. H. (2002) Redox-mediated decolorization of synthetic dyes by fungal laccases. *Appl. Microbiol. Biotechnol*. **59** (6): 672-678.

Claus, H. and Filip, Z. (1998) Degradation and transformation of aquatic humic substances by laccase-producing fungi *Cladosporium cladosporioides* and *Polyporus versicolor*. *Acta hydrochimica et hydrobiologica* **26**(3): 180-185.

Conesa, A., Punt, P.J. and van den Hondel, C.A.M.J.J. (2002) Fungal peroxidases: molecular aspects and applications. *J. Biotechnol*. **93**: 143–158.

Cornu, A., Besle, J.M., Mosoni, P. and Grent, E. (1994) Lignincarbohydrate complexes in forages: Structure and consequences in the ruminal degradation of cell-wall carbohydrates. *Reprod. Nutr. Dev*. **24**: 385–398.

Dean, J.F.D. and Eriksson, K. (1994) Laccase and the deposition of lignin in vascular plants. *Holzforschung* **48**: 21-33

Dixon, R.A., Chen, F., Guo, D.J. and Parvathi, K. (2001) The biosynthesis of monolignols: a "metabolic grid", or independent pathways to guaiacyl and syringyl units? *Phytochemistry* **57**(7): 1069–1084.

Dordick, J. S. (1987) Production of phenolic polymers catalyzed by Horseradish Peroxidase in organic solvents. Proc. Materials Biotechnology Symp. pp 225-239.

Dordick, J.S., Marletta, M.A. and Klibanov, A.M. (1987) Polymerization of phenols catalyzed by peroxidase in nonaqueous media. *Biotechnol Bioeng*. **30**(1): 31-36

Durán, N and Esposito, E. (2000) Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis B: Environmental* **28**(2): 83-99.

Ede, R.M. and Kilpeläinen, I. (1995) Homo- and hetero-nuclear 2D NMR techniques–unambiguous structural probes for non-cyclic benzyl aryl ethers in soluble lignin samples. *Res. Chem. Intermediates* **21**: 313–328.

Eker, B., Zagorevski, D., Zhu, G., Linhardt, R. J. and Dordick, J.S. (2009) Enzymatic polymerization of phenols in room-temperature ionic liquids. *J. Mol. Catal B: Enzym*. **59**: 177-184.

Elegir, G., Bussini, D., Antonsson, S., Lindström, M. E. and Zoia, L. (2007) Laccase-initiated cross-linking of lignocellulose fibres using a ultra-filtered lignin isolated from kraft black liquor. *Appl. Microbiol. Biotechnol*. **77**: 809–817.

Elegir, G., Kindl, A., Sadocco, P. and Orlandi M. (2008) Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds. *Enzyme Microb. Technol*. **43**: 84-92.

Enriqueta Arias, M. Arenas, M., Rodríguez, J., Soliveri, J., Ball, A.S. and Hernández, M. (2003) Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335**.** *Appl. Environ. Microbiol*. **69**(4): 1953-1958.

Eriksson, K.E.L., Blanchette, R.A. and Ander, P. (1990) Microbial and enzymatic degradation of wood and wood components. Springer, Berlin. pp 225–334.

Erikkson, O. and Lindgren, B.O. (1977) About the Linkage Between Lignin and Hemicelluloses in Wood. *Svensk Papperstidning* **80**(2): 59-63.

Evans, P., (2003) Emerging technologies in wood protection. *Forest Prod. J.* **53**: 14–22.

Fackler, K., Kuncinger, T., Ters, T. and Srebotnik, E. (2008) Laccase-catalyzed functionalization with 4-hydroxy-3-methoxybenzylurea significantly improves internal bond of particle boards. *Holzforschung* **62**: 223–229.

Feist, W.C. and Mraz, E.A. (1978). Protecting millwork with water repellents. *Forest Prod. J.* **28** (5): 31–35.

Felby, C., Hassingboe, J. and Lund, M. (2002) Pilot-scale production of fiberboards made by laccase oxidized wood fibers: board properties and evidence for cross-linking of lignin. *Enzyme Microb. Technol*. **31**(6): 736-741.

Felby, C., Olesen, P.O. and Hansen, T.T. (1998) Laccase catalyzed bonding of wood fibers. Enzymes in fiber processing, ACS Symposium Series 687: pp. 88-98.

Felby, C., Pedersen, L. S. and Nielsen, B.R. (1997) Enhanced auto adhesion of wood fibers using phenol oxidases. *Holzforschung* **51**: 281-286.

Felby, C. Thygesen, L.G. Sanadi A. and Barsberg, S. (2004) Native lignin for bonding of fiber boards-evaluation of bonding mechanisms in boards made from laccase-treated fibers of beech (Fagus sylvatica). *Ind. Crop Prod.* **20**: 181–189.

Finekenstadt, V.L. and Millane, R.P. (1998) Crystal structure of Valonia cellulose Iβ. *Macromolecules* **31**: 7776-7783.

Finkenstadt, V.L. and Millane, R.P. (1998) Crystal structure of Valonia cellulose Iβ. *Macromolecules* **31**: 3776–3778.

Freeman, M.H., Shupe, T.F., Vlosky, R.P. and Barnes, H.M. (2003) Past, present, and future of the wood preservation industry. *Forest Prod. J.* **53**(10): 8-15.

Fukuoka, T., Uyama, H., and Kobayashi, S. (2004) Effect of phenolic monomer structure of precursor polymers in oxidative coupling of enzymatically synthesized polyphenols. *Macromolecules* **37**: 5911-5915.

Gardiner, E.S. and Sarko, A. (1985) Packing analysis of carbohydrates and polysaccharides. 16. The crystal structures of cellulose IV_1 and IV_2. *Can J. Chem* **63**: 173–180.

Gargulak, J. D. and Lebo, S. E. (2000) Commercial use of lignin-based materials. In: Commercial Use of Lignin-Based Materials. W.G. Glasser, R.A. Northey and T.P. Schultz, (Eds.) American Chemical Society pp. 304-320.

Gosselink, R. J. A., Abächerli, A., Semke, H., Malherbe, R., Käuper, P., Nadif, A. and van Dam, J. E. G. (2004) Analytical protocols for characterisation of sulphur-free lignin. *Ind Crops Prod* **19**: 271-281.

Graham, R.D. (1973) History of wood preservation. In Wood deterioration and its prevention by preservative treatments Volume 1 Degradation and Protection of Wood. D.D. Nicholas, (Ed.). Syracuse University Press, New York. 380 p.

Grönqvist S, Rantanen K, Alen R Mattinen, M. Buchert, J.and Viikari L. (2006) Laccase-catalysed functionalisation of TMP with tyramine. *Holzforschung* **60**: 503-508.

Grönqvist, S., Viikari, L., Orlandi, M., Canevali, C. and Buchert, J. (2005) Oxidation of milled wood lignin with laccase, tyrosinase and horseradish peroxidase. *Appl Microbiol Biotecnol* **67**:489-494.

Hansen, T.T., Holm, H.C. and Franks, N.E. (1995) A process for production of linerboard and corrugated medium. Int Pat Appl WO 9509946.

Hayashi, J., Sufoka, A., Ohkita J. and Watanabe, S. (1975) The confirmation of existence of cellulose III_1. III_2, IV_1 and IV_2 by the X-ray method. *Polym Lett* **13**: 23–27.

Heiner, A.P. and Teleman, O. (1996) Structural reporter parameters for the characterization of crystalline cellulose. *Pure Appl. Chem*. **68**(11): 2187-2192.

Highley, T.L. and Dashek, W.V. (1998) Biotechnology in the study of brown- and white-rot decay. In Forest products biotechnology. A. Bruce and J.W. Palfreyman (eds.). Taylor and Francis, London, pp 15–36.

Hofrichter, M. (2002) Review: lignin conversion by manganese peroxidase (MnP). *Enzyme Microb. Technol*. **30**: 454–466.

Hossain, Kh.M.G., González, M.D., Lozano, G.R. and Tzanov, T. (2009) Multifunctional modification of wool using an enzymatic process in aqueous-organic media. *J. Biotechnol*. **141**: 58-63.

Humphreys, J.M. and Chapple, C. (2002) Rewriting the lignin roadmap. *Curr. Opin. Plant Biol*. **5**(3): 224–229.

Hüttermann, A. and Kharazipour, A. (1996) Enzymes as polymerization catalysts. In: New catalysts for a clean environment. A. Maijanen and A. Hase (eds.). VTT Symposium 163, Technical Research Centre of Finland (VTT), Espoo, Finland, pp 143–148.

Hüttermann, A., Kharazipour, A., Schindel, K., Fastenrath, M., Noetzold, S., and Schroeter, M. (1998) Enzymatic Reactions of Activated Lignin with Nucleophiles as a Basis for the Design of Compound Materials with Lignin. in 7th International Conference on Biotechnology in the Pulp and Paper Industry. Vancouver, Canada. A207-A209.

Hüttermann, A., Mai, C. and Kharazipour, A. (2001) Modification of lignin for the production of new compounded materials. *Appl. Microbiol. Biotechnol*. **55**: 387-394.

Hüttermann, A., Nonninger, K. and Kharazipour, A. (1998) Intermediate for the production of lignin polymerizates and their use in the production of derived timber products. Int Pat Appl WO9831729.

Hyvönen, A., Piltonen, P. and Niinimäki, J. (2005). Biodegradable substances in wood protection. In Sustainable use of renewable natural resources - from principles to practices. Anneli Jalkanen and Pekka Nygren (eds.). University of Helsinki Department of Forest Ecology Publications 34.

Ibach, R.E. (1999) Wood preservation. In Forest Products Laboratory. Wood handbook—Wood as an engineering material. Gen. Tech. Rep. FPL–GTR–113. Madison, WI: U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 463 p.

Ikeda, R., Sugihara, J., Uyama, H. and Kobayashi, S. (1996) Enzymatic Oxidative Polymerization of 2,6-Dimethylphenol. *Macromolecules* **29**: 8702-8705.

Ikeda R, Tanaka H, Uyama H and Kobayashi, S. (2000) A new crosslinkable polyphenol from a renewable resource. *Macromol. Rapid Commun*. **21**:496-499.

Jeffries, T.W. (1990) Biodegradation of lignin-carbohydrate complexes. *Biodegradation* **1**: 163–176.

Jin, L., Nicholas, D.D. and Schultz, T.P. (1991) Wood laminates glued by enzymatic oxidation of brown-rotted lignin. *Holzforschung* **45**: 467-468.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G. and Kilpeläinen, I. (1995a). Dibenzodioxocins; a novel type of linkage in softwood lignins. *Tetrahedron Lett*. **36**: 169-170.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G. and Kilpeläinen, I. (1995b). The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. *Tetrahedron Lett*., **36**: 4501-4504.

Kawai, S., Umezawa, T., Shimada, M. and Higuchi, T. (1988) Aromatic ring cleavage of 4,6-di(tert-butyl)guaiacol, a phenolic lignin model compound, by laccase of *Coriolus versicolor*. *FEBS Lett*. **236**(2): 309-311.

Kenealy, W., Klungness, J., Tshabalala, M., Horn, E., Akhtar, M., Gleisner, R., Buschle-Diller, G. (2004) Laccase modification of the physical properties of bark and pulp of loblolly pine and spruce pulp. ACS Sym Ser 2004; 889: 126-138.

Kharazipour, A., Hüttermann, A., Kühne, G. and Rong, M. (1993) Process for glueing wood chips and articles produced by this process. Eur Pat Appl EP0565109.

Kharazipour, A. Hüttermann, A. and Lüdemann, H.D. (1997) Enzymic activation of wood fibers as a means for the production of wood composites. *J. Adhes. Sci. Technol*. 11: 419–427.

Kharazipour, A., Mai, C. and Hüttermann, A. (1998) Polyphenoles for compounded materials. *Polym. Degrad. Stabil.* **59**: 237-243.

Kilpeläinen, I., Sipilä, J., Brunow, G., Lundquist, K. and Ede, R.M. (1994) Application of two-dimensional NMR spectroscopy to wood lignin structure determination and identification of some minor structural units of hard- and softwood lignins. *J. Agric. Food Chem*. **42**: 2790–2794.

Kim, Y. H., An, E. S., Park, S. Y. and Song, B.K. (2007) Enzymatic epoxidation and polymerization of cardanol obtained from a renewable resource and curing of epoxide-containing polycardanol. *J Mol Catal B: Enzym.* **45**: 39-44.

Kim, Y. H., An, E.S., Song, B.K. Kim, D.S. and Chelikani, R. (2003) Polymerization of cardanol using soybean peroxidase and its potential application as anti-biofilm coating material. *Biotechnol. Lett*. **25**: 1521-1524.

Kim, Y. H., Won, K., Kwon, J. M., Jeong, H.S., Park, S.Y., An, E.S. and Song, B.K. (2005) Synthesis of polycardanol from a renewable resource using a fungal peroxidase from Coprinus cinereus. *J Mol Catal B: Enzym.* **34**: 33-38.

Kolpak, F. J. and Blackwell, J. (1976) Determination of the structure of cellulose II. *Macromolecules* **9**: 273-278.

Körner, I., Kühne, G. and Pecina, H. (2001) Unsterile Fermentation von Hackschnitzeln - eine Holzvorbehandlungsmethode für die Faserplattenherstellung. Zeitschrfit: *Holz als Roh- und Werkstoff* **59**: 334-341.

Kramer K.J., Kanost M.R., Hopkins T.L., Jiang H., Zhu Y.C., Xu R., Kerwin J.L. and Turecek F. (2001) Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* **57**: 385–392.

Kuga, S., Takagi, S. and Brown, R. M. Jr. (1993) Native folded-chain cellulose II. *Polymer* **34**: 3293-3297.

Kukkola, E.M., Koutaniemi, S., Gustafsson, M., Karhunen, P., Ruel, K., Lundell, T.K., Saranpää, P., Brunow, G., Teeri, T.H. and Fagerstedt, K.V. (2003) Localization of dibenzodioxocin substructures in lignifying Norway spruce xylem by transmission electron microscopy-immunogold labeling. *Planta.* **217**(2): 229-237.

Kunamneni, A., Camarero, S., García-Burgos, C., Plou, F.J., Ballesteros, A. and Alcalde, M. (2008a). Engineering and Applications of fungal laccases for organic synthesis. *Microbial Cell Factories* **7**: 32.

Kunamneni, A., Plou, F.J., Ballesteros, A. and Alcalde, M. (2008b) Laccases and their applications: a patent review. 1: *Recent Pat Biotechnol.* **2**(1): 10-24.

Lagergren, S., Rydholm, S. and Stockman, L. (1957) Studies on the interfibre bonds of wood. Part 1. Tensile strength of wood after heating, swelling and delignification. *Sven. Papperstidn*. **60**: 632–644.

Langan, P., Nishiyama, Y. and Chanzy, H. (2001) X-ray structure of mercerized cellulose II at 1 Å resolution. *Biomacromolecules* **2**(2):410–416.

Lee, D.M. and Blackwell, J. (1981) Structure of cellulose II hydrate. *Biopolymers* **20**: 2165–2179.

Lee, D.M., Blackwell J. and Litt, M.H. (1983) Structure of a cellulose II-hydrazine complex. *Biopolymers* **22**: 1383–1399.

Lee, D.M., Burnfield K.E. and Blackwell, J. (1984) Structure of a cellulose I-ethylenediamine complex. *Biopolymers* **23**: 111–126.

Li, L., Cheng, X.F., Leshkevich, J., Umezawa, T., Harding, S.A. and Chiang, V.L. (2001) The last step of syringyl monolignol biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *Plant Cell* **13**(7): 1567–1585.

Li, K. and Geng, X. (2005) Formaldehyde-Free Wood Adhesives from Decayed Wood. *Macromol. Rapid Commun*. **26**: 529-532.

Li, L., Popko, J.L., Umezawa, T. and Chiang, V.L. (2000) 5-Hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms. *J. Biol. Chem*. **275**(9): 6537–6545.

Liu, J., Yang, F., Xian, M. Qiu, L. and Ye, L. (2001) Molecular Weight and Distribution of the Copolymer of Lignin/Phenol in the Copolymerization Catalyzed by Peroxidase. *Macromol. Chem. Phys*. **202**: 840-848.

Liu, J., Yuan, W. and Lo, T. (1999) Copolymerization of lignin with cresol catalyzed by peroxidase in reversed micellar systems. *EJB Elect J of Biotechnol.* **2**: 82-87.

Liu, N., Shi, S., Gao, Y. and Qin, M. (2009) Fiber modification of kraft pulp with laccase in presence of methyl syringate. *Enzyme Microb. Technol*. **44**(2): 89-95.

Lora, J. H. and Glasser, W. G. (2002) Recent industrial applications of lignin: A sustainable alternative to nonrenewable materials. *J. Polym. Environ*. **10**: 39-48

Lund, M. and Felby, C. (2001) Wet-strength improvement of unbleached kraft pulp through laccase-catalyzed oxidation. *Enzyme Microb. Technol.* **28**: 760–765.

Lund, M. and Felby, C. (2000) Production of paper materials with improved wet strength and use of a phenol-oxidizing enzyme and a mediator therein. Int Pat Appl WO0068500.

Lund, M. Felby, C. and Bjerrum, M. (1998) Modification of kraft pulp and lignin by copolymerization of phenolic compounds initiated by laccase, International conference on

biotechnology in the pulp and paper industry 7th, Vancouver, B.C., June 16–19, pp. C139–C142.

Lund, M. and Ragauskas, A.J. (2001) Enzymatic modification of kraft lignin through oxidative coupling with water-soluble phenols. *Appl. Microbiol. Biotechnol.* **55**: 699–703

Mai, C., Milstein O. and Hüttermann, A. (1999) Fungal laccase grafts acrylamide onto lignin in presence of peroxides. *Appl. Microbiol. Biotechnol*. **51**: 527–531.

Maloney, T.M. (1996) The family of wood composite materials. *Forest Prod. J*. **46**: 19–26.

Martínez, A.T., Rencoret, J., Marques, G., Gutiérrez, A., Ibarra, D., Jiménez-Barbero, J. and del Río, J.C. (2008) Monolignol acylation and lignin structure in some nonwoody plants: A 2D NMR study. *Phytochemistr*y 69(16): 2831-2843.

Mayer, A.M. and Staples, R.C. (2002) Laccases: new functions for an old enzyme. *Phytochemistry* **60**: 551–565.

Mester, T. and Tien, M. (2000) Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. *Int Biodeter Biodegrad* **46**: SI51–SI59.

Mikolasch, A. and Schauer, Frieder (2009). Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. *Appl. Microbiol. Biotechnol*. **82**: 605–624.

Milstein, O., Hüttermann, A., Fründ, R. and Lüdemann, H.D. (1994) Enzymatic co-polymerization of lignin with low-molecular mass compounds. *Appl. Microbiol. Biotechnol.* **40**: 760–767.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M. and Ladisch, M. (2005) Features of promising technologies for pre-treatment of lignocellulosic biomass. *Bioresour. Technol*. **96** (6): 673-86.

Nimz, H., Gurang, I. and Mogharab, I. (1976) Untersuchungen zur Vemetzung technischer Sulfitablauge. Liebigs Annalen der Chemie:1421-1434.

Nimz, H., Razvy, A., Maqharab, I. and Clad, W. (1972) Bindemittel bzw. Klebemittel zur Herstellung von Holzwerkstoffen sowie zur Verklebung von Werkstoffen verschiedener Art. DOS 2 221 353 German.

Nishimura, H. and Sarko, A. (1991) Mercerization of cellulose. 6. Crystal and molecular structure of Na–cellulose IV. *Macromolecules* **24**: 771–778.

Nyanhongo, G.S., Gübitz, G., Sukyai, P., Leitner, C., Haltrich, D. and Ludwig, R. (2007) Oxidoreductases from Trametes spp. in Biotechnology: A Wealth of Catalytic Activity. *Food Technol. Biotechnol*. **45** (3): 248–266.

Nyburg, S. C. (1961) Fibrous macromolecular substances. In X-ray Analysis of Organic Structures. L. F. Fieser and M. Fieser (eds.). Academic Press, New York, pp. 302-314.

Obataya, E., Sugiyama, M. and Tomita, B. (2002). Dimensional stability of wood acetylated with acetic anhydride solution of glucose pentaacetate. *J. Wood Sci.* **48**: 315-319.

O'Malley, D.M., Whetten, R., Bao, W., Chen, C.L. and Sederoff, R. (1993) The role of laccase in lignification. *Plant J*. **4**: 751–757.

O'Sullivan, A.C. (1997) Cellulose: The structure slowly unravels. *Cellulose* **4**: 173-207.

Park, E. S., Moon, W. S., Song, M. J., Kim, M. N., Chung, K.H. and Yoon, J.S. (2001) Antimicrobial activity of phenol and benzoic acid derivatives. *Int. Biodeterior. Biodeg*. **47**: 209–214.

Park, S. Y., Kim, Y. H., Won, K. and Song, B.K. (2009) Enzymatic synthesis and curing of polycardol from renewable resources. *J. Mol. Catal. B: Enzym*. **57**: 312-316.

Passialis, C.N. and Voulgaridis, E.V. (1999) Water repellent efficiency of organic solvent extractives from Aleppo pine leaves and bark applied to wood. *Holzforschung* **53** (2): 151–155.

Pettersen, R. C. (1984) The chemical composition of wood. In The chemistry of solid wood. Advances in chemistry series 207. Roger M. Rowell (Ed.). American Chemical Society, Washington DC 1984.

Popp, J. L., Kirk, T. K. and Dordick, J. S. (1991) Incorporation of p-cresol into lignins via peroxidase-catalysed copolymerization in nonaqueous media. *Enzyme Microb. Technol*. **13**: 964-968.

Popp, J. L., Kirk, T. K. and Dordick, J. S. (1991) Enzymic Modification of Lignin for Use as a Phenolic Resin, *Enzyme Microb. Technol.* **13**: 964-968.

Qvintus-Leino, P., Widsten, P., Tuominen, S., Laine, J., Kunnas, J. (2003) Method of producing compressed layered structures such as fiberboard or similar wood-based product. Int Pat Appl WO03047826.

Ralph, j., Lundquist, K., Brunow, G., Lu, F., Kim, H., Schatz, P.F., Marita, J.M., Hatfield, R.D., Ralph, S.A., Christensen, J.H. and Boerjan, W. (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids. *Phytochemistry Reviews* **3**: 29–60.

Ramsden, M.J., Blake, F.S.R. and Fey, N.J. (1997) The effect of acetylation on the mechanical properties, hydrophobicity, and dimensional stability of *Pinus sylvestris*. *Wood Sci. Technol*. **31**: 97-104.

Ranocha, P., Chabannes, M., Chamayou, S., Danoun, S., Jauneau, A., Boudet, A.M. and Goffner, D. (2002) Laccase down-regulation causes alterations in phenolic metabolism and cell wall structure in poplar. *Plant Physiol.* **129**: 145–155.

Razzaque, M.A. (1982) The effect of concentration and distribution on the performance of water repellents applied to wood. University of Wales, Ph.D. Thesis.

Reid, I.D. (1995) Biodegradation of lignin. *Can J Bot* **73**: S1011–S1018.

Richardson, B.A. (1978) Wood preservation. Construction Press, Lancaster. 238 p.

Riva, S. (2006) Laccases: blue enzymes for green chemistry. *Trends Biotechnol*. **24** (5): 219-226.

Rowell, R., (2006) Acetylation of wood: journey from analytical technique to commercial reality. *Forest Prod. J*. **56**(9): 4-12.

Rowell, R.M. and Banks, W.B. (1985) Water repellency and dimensional stability of wood. Gen. Tech. Rep. FPL-50. Madison, WI, U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 24 p.

Ryan, B. J., Carolan, N. and Ó'Fágáin, C. (2006). Horseradish and soybean peroxidases: comparable tools for alternative niches? *Trends Biotechnol*. **24** (8): 355-363.

Sakakibara, A. (1980) A structural model of softwood lignin. *Wood Sci. Technol*. **14**: 89–100.

Sarko, A. and Muggli, R. (1974) Packing analysis of carbohydrates and polysaccharides. III. Valonia cellulose and cellulose II. *Macromolecules* **7**: 486-494.

Sarko, A., Southwick J. and J. Hayashi (1976) Packing analysis of carbohydrates and polysaccharides. 7. Crystal structure of cellulose III1 and its relationship to other cellulose polymorphs. *Macromolecules* **9**: 857–867.

Schnitzer, M., Barr, M. and Hartenstein, R. (1984) Kinetics and characteristics of humic acids produced from simple phenols. *Soil Biol. Biochem*. **16**: 371-376.

Schröder, M., Aichernig, N., Gübitz, G.M. and Kokol, V. (2007) Enzymatic coating of lignocellulosic surfaces with polyphenols. *Biotechnol. J*. **2**(3): 334-341.

Schultz,T.P., Nicholas, D.D. and Preston, A.F. (2007) Perspective – A brief review of the past, present and future of wood preservation. *Pest Manag. Sci.* **63**: 784–788.

Sellers, T. (2001) Wood adhesive innovations and applications in North America, *Forest Prod. J*. **51**: 12–22.

Sjöström, E. (1993) Wood Chemistry. Fundamentals and Applications. Second edition ed. Academic press, San Diego. 293 p.

Stipanovic, A. J. and Sarko, A. (1976) Packing analysis of carbohydrates and polysaccharides. 6. Molecular and crystal structure of regenerated cellulose II. *Macromolecules* **9**: 851-857.

Sugiyama, J., Vuong R. and Chanzy, H. (1991) Electron diffraction study on the two crystalline phases occurring in native cellulose from an algal cell wall. *Macromolecules* **24**: 4168–4175.

Suurnäkki, A., Buchert, J., Grönqvist, S., Mikkonen, H., Peltonen, S. and Viikari, L. (2006) Bringing new properties to lignin rich fiber materials, VTT Symposium 244, 61-70.

Thompson, J. A. Sarpeshkar, A. M. Rosthauser, J. W. Markusch, P. H. (2002) PMDI wood binders containing hydrophobic diluents US Patent 6352661

Thurston, C.F., 1994. The structure and function of fungal laccases. *Microbiology* **140**: 19–26.

Ulvcrona, T., Lindberg, H. and Bergsten, U. (2006) Impregnation of Norway spruce (*Picea abies* L. Karst.) wood by hydrophobic oil and dispersion patterns in different tissues. *Forestry* **79**(1): 123-134.

Unbehaun, H., Dittler, B., Kühne, G. and Wagenführ A. (2000) Investigation into the biotechnological modification of wood and its application in the wood-based material industry. *Acta Biotechnol*. **20**: 305-312.

Unbehaun H, Wolff M, Kühne G Schindel, K., Hüttermann, A., Cohen, R. and Chet, I. (1999) Mechanismen der mykologischen Transformation von Holz für die Holzwerkstoffherstellung. *Holz als Roh- und Werkstoff* **57**: 92.

Var, A.A. and Öktem, E. (1999) Reduction by natural resin of water uptake in various wood species. *Tr. J. of Agriculture and Forestry* **23**: 413-418.

Viikari, L., Hase, A., Qvintus-Leino, P., Kataja, K., Tuominen, S. and Gädda, L. (1998a) Lignin-based adhesive for particleboard manufacture. Int Pat Appl WO9831764.

Viikari, L., Hase, A., Qvintus-Leino, P., Kataja, K., Tuominen, S. and Gädda, L. (1998b) Lignin-based adhesives and a process for the preparation thereof. Int Pat Appl WO 9831763.

VanderHart, D. L. and Atalla, R. H. (1984) Studies of microstructure in native celluloses using solid state 13C NMR. *Macromolecules* **17**(8): 1465-1472.

Vick, C.B. and Rowell, R.M. (1990) Adhesive Bonding of Acetylated Wood. *Inter. J. Adh. Adhes*. **10**(4): 263-272.

Viitaniemi, P. and Jämsä, S. (1996) Modification of wood with heat treatment (in Finnish). Espoo 1996, Technical Research Centre of Finland. VTT Julkaisuja – Publikationer 814, 57.

Widsten, P. and Kandelbauer, A. (2008) Laccase applications in the forest products industry: A review. *Enzyme Microb. Technol*. **42**(4): 293-307.

Widsten, P. Laine, J.E. Tuominen S. and Qvintus-Leino, P. (2003) Effect of high defibration temperature on the properties of medium-density fiberboard (MDF) made from laccase-treated hardwood fibers. *J. Adhes. Sci. Technol*. **17**: 67–78.

Widsten, P. Tuominen, S. Qvintus-Leino P. and Laine, J.E. (2004) The influence of high defibration temperature on the properties of medium-density fiberboard (MDF) made from laccase-treated softwood fibers. *Wood Sci. Technol*. **38**: 521–528.

Winandy, J.E. and Rowell, R.M. (2005). The chemistry of wood strength.wood chemistry and wood composites. Roger M. Rowell. (Ed.). CRC Press. Boca Raton, Florida, pp 303-347.

Winandy, J.E., and Rowell, R.M. (1984) The chemistry of wood strength. p211–255. In The chemistry of solid-wood. Roger M. Rowell (Ed.). ACS Sym Series 208, Washington DC.

Witayakran, S. and Ragauskas, A.J. (2009) Synthetic applications of laccase in green chemistry, *Adv. Synth. Catal.*, **351**(9): 1187-1209.

Witayakran, S. and Ragauskas, A.J. (2009) Modification of high-lignin softwood kraft pulp with laccase and amino acids, *Enzyme Microb. Technol*. **44**(3): 176–181.

Wong, K.K.Y., Anderson, K.B. and Kibblewhite, R.P. (1999) Effects of the laccase-mediator system on the handsheet properties of two high kappa kraft pulps. *Enzyme Microbial. Technol.* **25**: 125–131.

Won, K., Kim, Y. H., An, E. S. Lee, Y.S. and Song, B.K. (2004) Horseradish Peroxidase-Catalyzed Polymerization of Cardanol in the Presence of Redox Mediators. *Biomacromolecules* **5**: 1-4.

Wong, K.K.Y., Richardson, J.D. and Mansfield, S.D. (2000) Enzymic treatment of mechanical pulp fibers for improving papermaking properties, *Biotechnol Prog.* **16**: 1025–1029.

Xia, Z., Yoshida, T. and Funaoka, M. (2003) Enzymatic synthesis of polyphenols from highly phenolic lignin-based polymers (lignophenols). *Biotechnol. Lett*. **25**: 9-12.

Xu, F. (1996) Oxidation of phenols, anilines, and benzenethiols by fungal laccases: correlation between activity and redox potentials as well as halide inhibition. *Biochemistry* **35**: 7608–7614.

Yamaguchi, H., Maeda, Y. and Sakata, I. (1992) Applications of phenol dehydrogenative polymerization by laccase to bonding among woody-fibers. *Mokuzai Gakkaishi* **38**: 931-937.

Yamaguchi, H., Maeda, Y. and Sakata, I. (1994) Bonding among woody fibers by use of enzymic phenol dehydrogenative polymerization. Mechanism of generation of bonding strength. *Mokuzai Gakkaishi* **40**: 185-190.

Yamaguchi, H., Nagamori, N. and Sakata, I. (1991) Application of the dehydrogenative polymerization of vanillic acid to bonding of woody fibers. *Mokuzai Gakkaishi* **37**: 220-226.

Yan, J.F., Pla, F., Kondo, R., Dolk, M. and McCarthy, J.L. (1984) Lignin 21: Depolymerization by bond cleavage reactions and degelation. *Macromolecules* **17**: 2137–2142.

Yoshida, H. (1883) Chemistry of lacquer (urushi). *J. Chem. Soc*. **43**, 472–486.

Yoshida, T., Xia, Z., Takeda, K., Katsuta, T., Sugimoto, K. and Funaoka M. (2005) Peroxidase-catalyzed polymerization and copolymerization of lignin-based macromonomer (lignocresol) having high content of p-cresol and thermal properties of the resulting polymers. *Polym. Adv. Technol.* **16**: 783-788.

Youngquist, J.A. (1999) Wood-based composites and panel products, Wood handbook—wood as an engineering material, USDA Forest Products Laboratory p. 10-1–10-31.

Zugenmaier, P. (2001) Conformation and packing of various crystalline cellulose fibers. *Prog. Polym. Sci*. **26**(9): 1341-1417.

2

Laccase catalyzed covalent coupling of fluorophenols increases lignocellulose surface hydrophobicity

This work presents for the first time the mechanistic evidence of a laccase-catalysed method of covalently grafting hydrophobicity enhancing fluorophenols onto *Fagus sylvatica* veneers. Coupling of fluorophenols onto complex lignin model compounds guaiacylglycerol β-guaiacyl ether and syringylglycerol β-guaiacyl ether was demonstrated by LC-MS and NMR. Laccase-mediated coupling increased binding of 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP) and 4-(trifluoromethoxy)phenol (4-F3MP) to veneers by 77.1 and 39.2 %, respectively. XPS studies showed that laccase-catalyzed grafting of fluorophenols resulted in a fluorine content of 6.39 % for 4,4-F3MPP, 3.01 % for 4-F3MP and 0.26 % for 4-fluoro-2-methylphenol (4,2-FMP). Grafting of the flurophenols 4,2-FMP, 4-F3MP and 4,4-F3MPP led to a 9.6, 28.6 and 65.5 % increase in hydrophobicity, respectively, when compared to treatments with the respective fluorophenols in the absence of laccase, in good agreement with XPS data.

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2.1. Introduction

Among the construction materials which are used by industry, wood holds a special place because of its impressive range of attractive properties, including low thermal extension, low density and high mechanical strength. Despite these attractive properties, wood is hygroscopic and susceptible to biodegradation, factors which are partly responsible for the continued search for better wood processing technologies. Traditionally two approaches, chemical and thermal wood processing technologies, have been developed to improve wood hydrophobicity. Chemical methods include use of silicon compounds (Mai and Militz, 2004), impregnation with hydrophobic oil (Ulvcrona et al., 2006), application of water repellants like waxes, oils, natural or synthetic resins (Borgin and Corbett,1970; Feist and Mraz, 1978; Razzaque, 1982; Hyvönen et al., 2006) and using wood binders containing hydrophobic diluents. However, these physico-chemical methods are becoming increasingly unpopular as society becomes eco-and energy-sensitive. Water repellent substances seem to be bonded to the cell wall only by relatively weak Van der Waal forces which, over a long time and due to continuous exposure to water, are displaced and washed off (Razzaque, 1982). Although chemical modification of wood through for example acetylation (Rowell, 2006; Obataya et al., 2002) and thermal degradation of hemicellulose components (Viitaniemi and Jämsä, 1996) increases dimensional stability and hydrophobicity, it is also known that these treatments reduce mechanical properties such as tensile strength (Vick and Rowell, 1990; Ramsden et al., 1997). Modifications with silicon compounds usually involves complicated and expensive multi-step processes while, due to high chemical and weathering stability, such treatments are usually recommended for wood exposed to conditions of hazard class III (EN 335, outside exposure without soil contact) (Mai and Militz, 2004).

In response to shortcomings of chemical and physical methods, enzymes such as laccases have emerged as important biotechnological catalysts as they are both eco-friendly and work under mild conditions. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are multi-copper glycoproteins that catalyze the monoeletronic oxidation of phenols and aromatic or aliphatic amines to reactive radicals in a redox reaction in which molecular oxygen is simultaneously reduced to water (Claus, 2004; Riva, 2006). The reaction usually involves oxidation of lignin moieties on the wood surface to create a radical-rich reactive surface to which oxidized (radical-containing) or non-oxidized molecules of interest can be grafted (Kudanga et al., 2008). Investigations into the possibility of using laccases to covalently attach molecules of interest to wood material are increasing (Grönqvist et al., 2006;

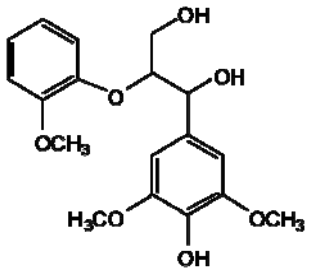
Widsten and Kandelbauer, 2008). However, a review of the literature shows that only one attempt by Suurnäkki et al., 2006 has been made to increase wood hydophobicity by enzymatic grafting although no mechanistic proof was provided for covalent attachment and the type of bonding.

In an effort to use green chemistry technology to increase wood surface hydrophobicity, this work presents for the first time a laccase-mediated method of covalently grafting fluorophenols onto beech (*Fagus sylvatica*) veneers. Further, evidence for covalent binding of fluorophenols to complex lignin model substrates is provided by LC-MS and NMR in order to gain mechanistic information on the coupling reaction.

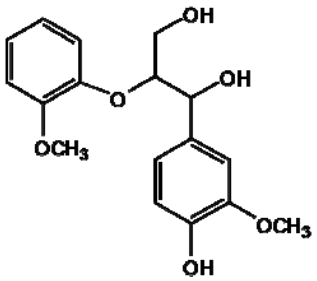
2.2. Materials and Methods

2.2.1 Chemicals

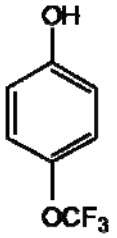
The lignin model compounds guaiacylglycerol β-guaiacyl ether **E** (erol) and syringylglycerol β-guaiacyl ether **G** (Figure 1) were synthesized following the procedure described by Sipilä and Syrjänen, 1995. All other chemicals including the fluorophenol molecules 4-fluoro-2-methylphenol (4,2-FMP), 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP) and 4-(trifluoromethoxy)phenol (4-F3MP) were purchased from Sigma-Aldrich.



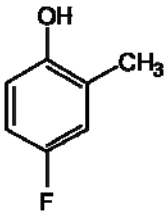
Syringylglycerol β-guaiacyl ether (G)



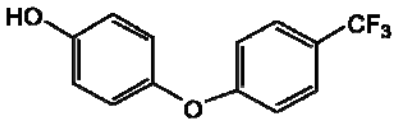
Guaiacylglycerol β-guaiacyl ether (E)



4-(Trifluoromethoxy)phenol (4-F3MP)



4-Fluoro-2-methylphenol (4,2-FMP)



4-[4-(Trifluoromethyl)phenoxy]phenol (4,4-F3MPP)

Figure 1. Chemical structures of the lignin model compounds and fluorophenols used in the coupling reactions.

2.2.2 Veneers

The wood veneers used in the present investigation were prepared from European beech (*Fagus sylvatica*) and measured 10 mm x 10 mm x 1 mm. Prior to their use in grafting experiments, they were Soxhlet-extracted with acetone overnight to remove lipophilic extractives which could interfere with oxidized molecules and also affect analysis of modified surface (Gutiérrez et al., 1998; Nzokou and Kamdem, 2004).

2.2.3 Laccases

The 62 kDa laccase from *Trametes hirsuta* was produced and purified as previously reported by Almansa et al., 2004. The activity of the laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε_{436} = 29,300 $M^{-1}cm^{-1}$) as substrate at 436 nm in 50 mM succinate buffer at pH 4.5 and 37 °C as described by Niku-Paavola et al. 1988, with some modifications. Briefly, the reaction mixture

contained 30 µl laccase, 350 µl ABTS (1 mM) and 50 mM succinate buffer pH 4.5 to make a final volume of 1.5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100 °C for 10 minutes. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 minutes.

2.2.4 Oxidation of fluorophenols

The ability of the *T. hirsuta* laccase to oxidize the three fluorophenols (4,2-FMP, 4-F3MP and 4,4-F3MPP) was monitored by UV/Vis- spectrophotometry and confirmed by HPLC analysis. To start the reaction, laccase with a final activity of 13.4 nkat ml^{-1} was added to a reaction mixture containing 0.1 mM fluorophenol in 50 mM succinate buffer pH 4.5. The reactions were monitored by UV/Vis-spectrophotometry in the wavelength scan mode from 900 nm – 200 nm at 3 min per cycle for a total of 18 min to identify oxidation products.

2.2.5 Coupling of fluorophenols to lignin model compounds

The reaction mixture contained a fluorophenol (2.0 mM) and one of the lignin model compounds **E** and **G** (4.0 mM) in the molar ratio of 1:2 (in order to increase the probability of coupling) and 13.4 nkat ml^{-1} final laccase activity in 50 mM succinate buffer (pH 4.5). Reactions were carried out at 37 ºC while shaking at 700 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg Germany). The lignin model compound was initially incubated with the laccase for 0.5 h; after which the fluorophenol was added to the reaction mixture which was further incubated for 3 h. The coupling products were analyzed by HPLC and LC-MS.

2.2.6 HPLC analysis of reaction products

To the incubation mixtures an equal volume of ice cold methanol was added to precipitate the protein. The mixture was centrifuged at 0 ºC for 15 minutes at 14000 g and 600 µl aliquots were transferred into vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 x 4.6 mm with precolumn, Knauer GmbH, Berlin, Germany) using a linear gradient of acetonitrile (solvent B) and 0.1 % H_3PO_4 (solvent A) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 25 °C. The gradient was set as shown in Table 1.

Table 1. Gradient set up for HPLC analysis

Time (min.)	Initial	5	20	21	35	35.1	45(end)
Solvent A%	80	80	25	10	10	80	80
Solvent B %	20	20	75	90	90	20	20

2.2.7 LC-MS analysis of reaction products

The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionisation coupled to the Dionex HPLC-UVD-system as described above and initial separation of coupling products was carried out using the same protocol except that 0.1 % formic acid was used instead of 0.1 % H_3PO_4. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350 °C and the nebulizer pressure set to 70 psi. Maximum accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30000.

2.2.8 NMR analysis of coupling products

Coupling products were separated by using preparative thin layer chromatograpy (TLC). The most prominent coupling product according to HPLC-MS was further analysed by NMR. NMR spectra were measured on a Varian INOVA 500MHz NMR-Spectrometer (^{1}H: 499.82 MHz, ^{13}C: 125.12 MHz, ^{19}F: 475.21 MHz). Proton-1D-spectra was measured for a solution of the sample in a mixture of 200 µl D_2O and 300 µl CD_3OD. Water suppression using transmitter-presaturation was applied for the proton spectra. However, due to lack of material no 1D ^{13}C-NMR spectrum could be obtained. In addition 2D-spectra such as DQCOSY, HSQC and HMBC (both giving information about the ^{13}C-NMR shifts) were acquired.

2.2.9 Coupling of fluorophenols onto veneer wood pieces

Soxhlet-extracted beech veneer (10 mm x 10 mm x 1.0 mm) were pre-oxidized by laccases (26.8 nkat ml^{-1}) for 1 h and then further incubated for 4 h in the presence (5mM) of each of the fluorophenols (4,2-FMP, 4-F3MP and 4,4-F3MPP). The reaction was carried out in 50 mM succinate buffer at pH 4.5 and 37 °C while shaking at 150 rpm. The incubation solution was collected and the wood pieces were resuspended in 10 ml 75 % ethanol and washed for 16 hours while shaking at 150 rpm. The washing solution was collected and the pieces were washed again for a further 10 minutes with 10 ml 75 % ethanol before transferring them into

10 ml 75 % acetone for another 10 min wash. After that the incubation and washing solutions containing unbound flurophenols were pooled and analyzed using a UV/Vis-spectrophotometer at the respective maximum absorbances of the fluoromolecules (4,4-F3MPP at 230nm and 4-F3MP at 275nm). However, 4,2-FMP was not determined as it was oxidized by laccase.

2.2.10 Contact angle measurements

After washing-off unbound phenols from the veneers and conditioning to equilibrium moisture content, the sessile contact angle of water was measured in the grain direction five seconds after drop deposition according to the Young-Laplace method using a DataPhysics OCA 35 goniometer and SCA20 software. The drop volume was 1.5 µl. For each veneer, the contact angle was measured at five spots and the results averaged. For each treatment triplicate samples were measured.

2.2.11 XPS measurements

Binding of the fluorophenols to veneers was determined using X-ray photoelectron spectroscopy (XPS). XPS measurements were performed at Joanneum Research, Graz, Austria, with a multiprobe UHV surface analysis system from Omicron Nanotechnology. Equipment and parameters: x-ray source: DAR400 aluminium-anode; quartz-crystal monochromator XM 500; x-ray excitation energy: 1486.70eV (Al Kα1-line); monochromated x-ray line width (FWHM): 0.3 eV; energy analyzer: hemispherical analyzer, type EA 125; signal-detection with pulse-counting channeltron (five channels for count-rate enhancement); total energy resolution (x-ray source + hemispherical analyzer): 0.45 eV for detailed scans at 10 eV pass-energy (+ lifetime broadening of the electronic state); charge neutralization for non-conducting samples: electron emission gun for low-energy electrons (0.1–500 eV): FG 15/40 (SPECS). The analyzed surface area was ~ 1 mm^2; five spots were analyzed per sample and the results averaged.

2.3. Results and Discussion

To establish the mechanistic basis for surface modification of lignocellulose with fluorophenols, the fluorophenols were first coupled to complex lignin model substrates *in vitro* using laccase. Practical application was then assessed by functionalizing beech veneers with the fluorophenols to increase hydrophobicity.

2.3.1 Oxidation of fluorophenols and lignin model compounds

Of the three fluorophenols investigated, only 4,2-FMP (t_R=14.9 min) was oxidized by the *Trametes hirsuta* laccase. Upon laccase oxidation, a distinct product peak emerged with a retention time of t_R=8.8 min; smaller product peaks emerged at retention times t_R=17.4 min and t_R=19.9 min. The other fluorophenols (4,4-F3MPP and 4-F3MP) were not readily oxidized most likely since they lack an electron donating substituent *ortho* to a hydroxyl group which is usually a pre-requisite for non-mediated laccase oxidation (Paice, 1995). However, some fungal laccases can oxidize simple monophenolic molecules (Takahama, 1995). Both of the lignin model compounds, guaiacylglycerol β-guaiacyl ether (**E**) and syringylglycerol β-guaiacyl ether (**G**) are oxidized by laccase (Kudanga et al., 2008; Rittstieg et al., 2002; 2003).

2.3.2 HPLC analysis of coupling of fluorophenols to lignin model compounds

All the fluorophenols were readily coupled onto the lignin model compounds **G** and **E**. As previously demonstrated for other substrates (Kudanga et al 2008), coupling reactions to **E** and **G** occurred when at least one of the molecules involved is accepted by the laccase as a substrate. It has also been previously highlighted for less complex substrates that coupling reactions proceeded following creation of radicals, and once radicals were formed, further reaction could proceed without additional enzymatic action (Grönqvist et al., 2003; Schroeder et al., 2007). The coupling reactions were followed on HPLC by monitoring the emergence of new peaks. An example is shown in Figure 2 for coupling of the flurorophenols 4,4-F3MPP and 4,2-FMP onto **G**. Under the chromatographic conditions used, the parent compound **G** and its oxidized product had retention times of t_R=10.5 min and 13.9 min, respectively. The concentration of the latter obviously decreased in the coupling reaction unless an excess of **G** was used. The fluoromolecules had retention times of t_R=20.8 min (4,4-F3MPP), t_R=8.6 min (4,2-FMP) and t_R=17.3 min (4-F3MP). New coupling product peaks were observed for 4,4-F3MPP at retention times t_R=22.0 min and t_R=13.7; for 4,2-FMP at retention time t_R=18.5 min (Figure 2) while for 4-F3MP, two products were observed at retention times t_R=4.5 and t_R=20.0 min.

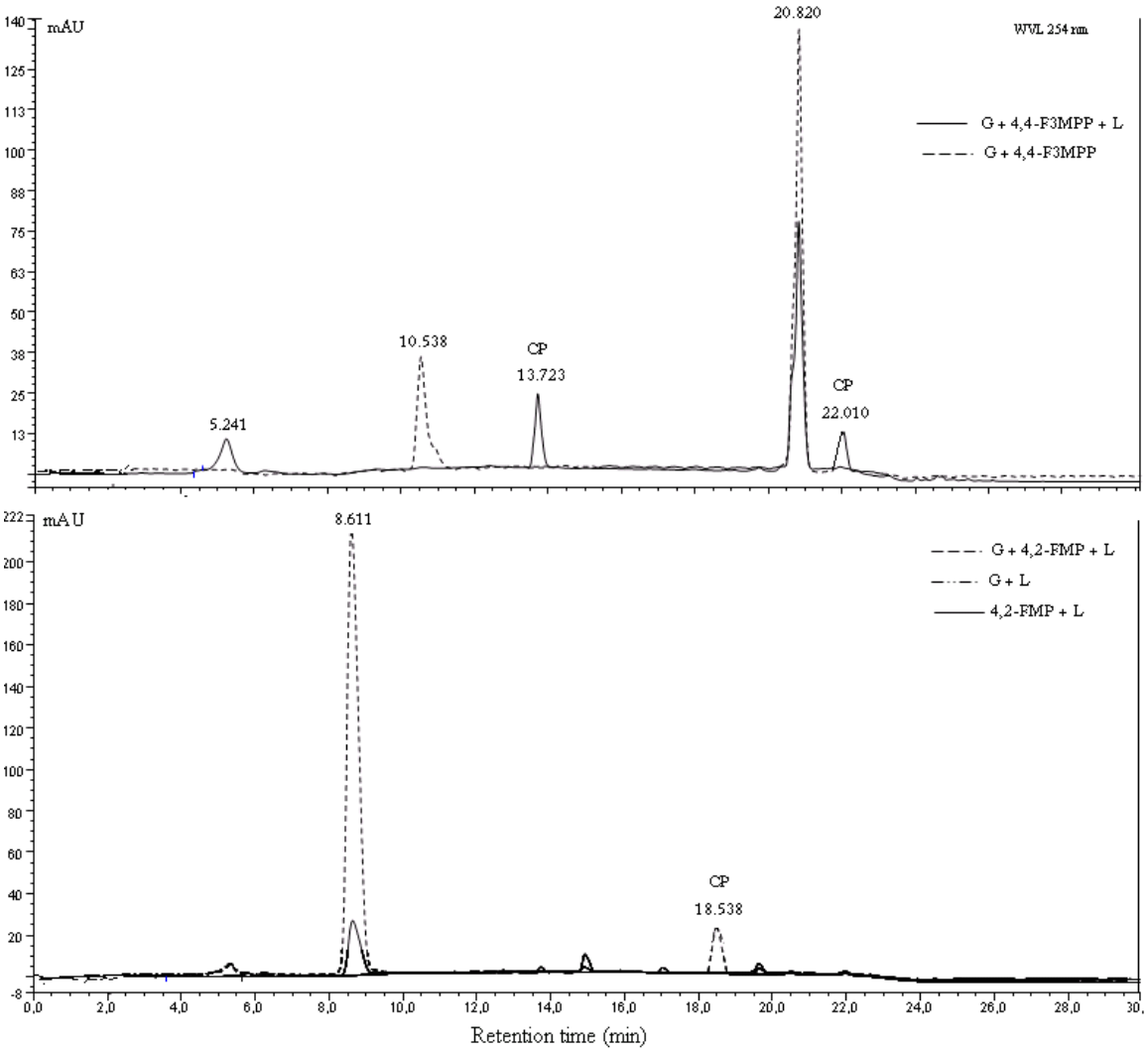


Figure 2. HPLC chromatograms of coupling products (CP) of the fluorophenols 4,4-F3MPP and 4,2-FMP onto syringylglycerol-β-guaiacyl ether (**G)** using *T. hirsuta* laccase (L).

2.3.3 LC-MS and NMR analysis

The molecular masses for **G** and **E** are 350.363 and 320.337, respectively, while the molecular mass for the fluoromolecules are 126.13 (4,2-FMP), 178.11 (4-F3MP) and 254.21 (4,4-F3MPP). LC-MS of the laccase-mediated coupling products observed in the positive ion mode showed dominant signals at *m/z* 601.3 and *m/z* 625.3 (G+4,4-F3MPP), *m/z* 525.3 (G+4-F3MP), *m/z* 473.3 (G+4,2-FMP) and *m/z* 571.3 (E+4,4-F3MPP) (Figure 3). These measured molecular weights suggest 1:1 coupling products. The molecular ion *m/z* 625.3 is a Na adduct and its molecular weight was confirmed to be 602.3 when the reaction was run in alternating (positive and negative) mode. Ions smaller by two units possibly result from oxidation of the benzyl alcohol group to an α-ketone (**A** to **B** in Figure 4). Additional $[M+H]^+$ ion signals were observed at *m/z* 586.5 (G+4,4-F3MPP) and at *m/z* 556.3 (E+4,4-F3MPP) suggesting cleavage

of the benzyl-hydroxyl group from the coupling adduct to give a benzyl cation (**GP3** and **EP1**, Figure 4), which is highly likely in the acidic conditions used in the experiment.

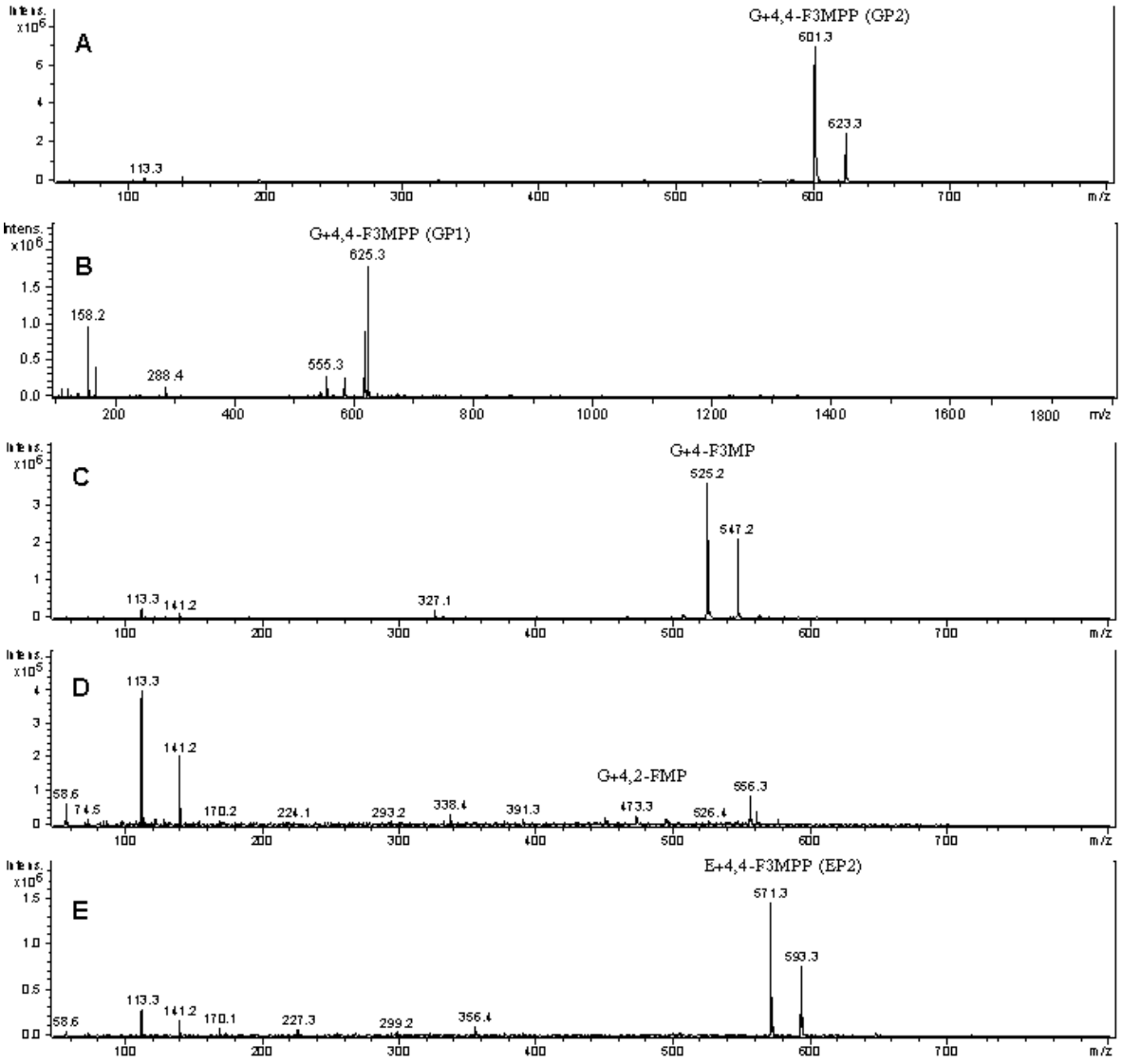


Figure 3. Mass spectra of of coupling products of the fluorophenols 4,4-F3MPP, 4-F3MP and 4,2-FMP onto syringylglycerol-β-guaiacyl ether (**G)** and guaiacylglycerol β-guaiacyl ether (**E**) using *T. hirsuta* laccase (L).

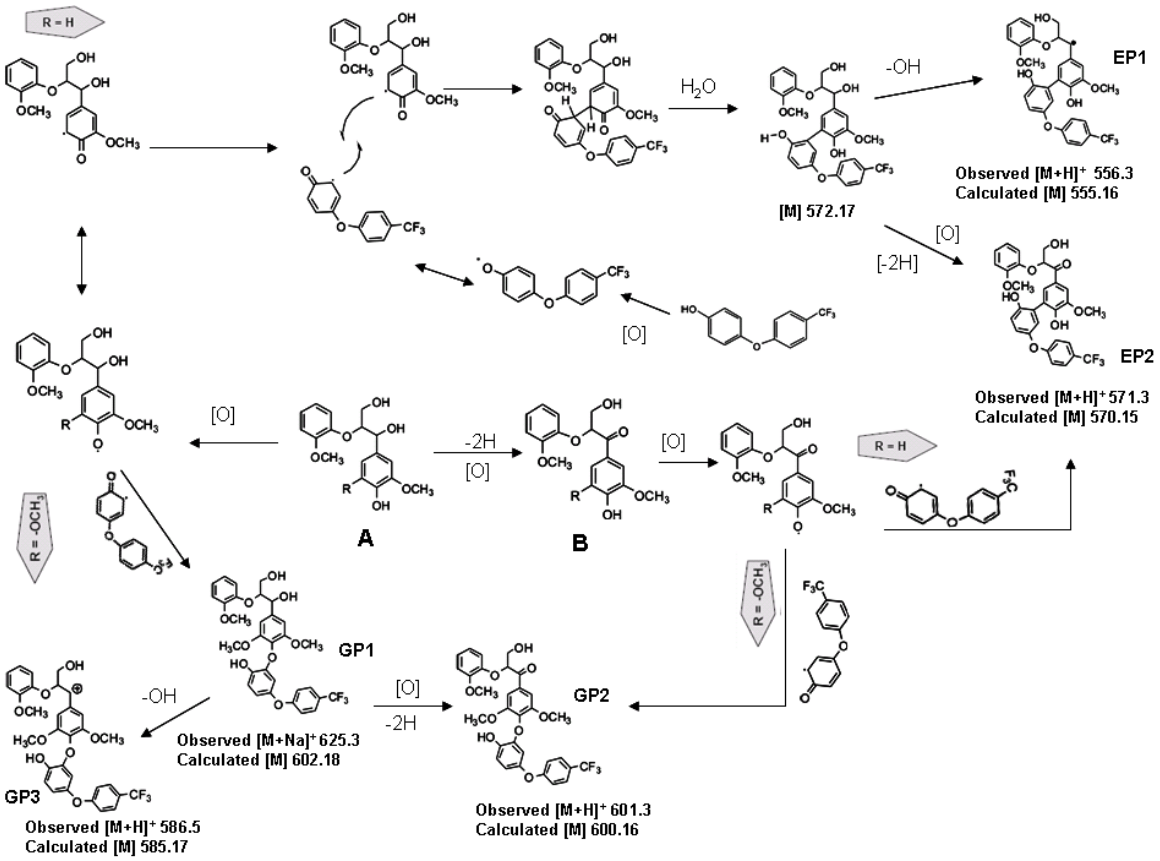


Figure 4. Proposed reaction pathway for laccase-catalysed coupling of 4,4-F3MPP onto the lignin model compounds **G** and **E.**

NMR spectra of the major coupling product between **G** and 4,4-F3MPP (**GP1**) support the molecular structure shown in Figure 5. NMR data revealed that the three methoxyl-groups (integral ratio 2:1, marked 1 and 2 in Figure 5) were still present in the molecule, two of them (in ring B) showing the same chemical shift.

The two aromatic protons in ring B (marked H_b) gave a singlet also suggesting symmetric substitution for ring B while ^{19}F-NMR analysis revealed one signal consistent with a CF_3-group (-61.5 ppm). Ring D analysis confirmed *para*-substitution in the DQCOSY spectrum revealing two doublets for the four protons (Hd_1 and Hd_2). Further, ring C analysis showed two protons appearing as doublets (Hc_1 and Hc_2) while Hc_3 appeared as a singlet, suggesting a BC-coupling pattern. The four protons in ring A were shown to be strongly coupled. Analysis of the C1, C2 and C3 showed that ^{13}C-NMR chemical shifts (C1: 62.1 ppm, C2: 83.8 ppm, C3: 72.9 ppm) were still very similar compared with the ^{13}C-NMR chemical shifts of the starting material (C1: 62.3 ppm, C2: 84.1 ppm, C3: 74.3 ppm), suggesting no change in this part of the molecule.

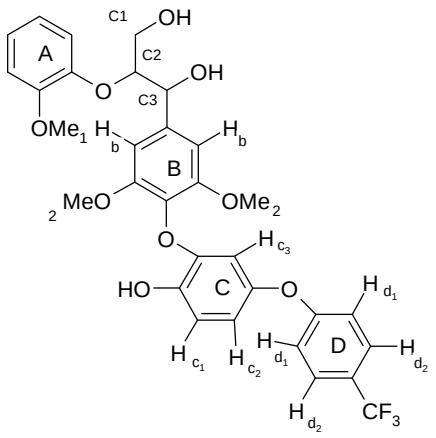


Figure 5. Structure of the laccase-mediated coupling product between **G** and 4,4-F3MPP (**GP1**) in D2O/CD3OD. Measurement time was 69 hours on a Varian INOVA 500 MHz spectrometer.

Based on the results from LC-MS, the scheme shown in Figure 4 is proposed as the possible reaction pathway for the coupling of the fluorophenol 4,4-F3MPP to lignin models **G** and **E** in either their **A** or **B** form. As depicted in Figure 4, laccase initiates the reaction by attacking the *para* hydroxyl group of compound **A** and **B** resulting in a radical which forms a resonance structure. The generated radicals of **A** and **B** probably act as mediators in the oxidation of 4,4-F3MPP into a radical which spontaneously forms a quinone through resonance stabilization. Further, the oxidized 4,4-F3MPP attacks oxidized **E** on position 5 thereby establishing a 5-5 covalent bond . However, in the case of **G**, 4,4-F3MPP can only covalently bind through a 4-O-5 linkage since the other positions are either sterically hindered or occupied.

2.3.4 Effect of fluorophenols on wood hydrophobicity

Effect of incorporation of fluorophenols onto wood materials was investigated using beech veneers. Interestingly, with all three fluorophenols, the contact angle is higher when laccase is included in the treatment than when only the corresponding phenol is used (Figure 6). Laccase-mediated treatment with 4,4-F3MPP showed the highest contact angle of 89.6°, representing a 65.5 % increase when compared to the contact angle (54.1^{o}) of beech wood treated with the same fluorophenol in the absence of laccase. Laccase-catalyzed grafting of 4,2-FMP and 4-F3MP resulted in 9.6 and 28.6 % increase in hydrophobicity, respectively. In a similar enzyme mediated coupling of functional molecules onto paper materials, dodecyl gallate-containing paper coatings resulted in increased hydrophobicity owing to the alkyl side group (Suurnäkki et al., 2006). However, the fact that these authors did not wash the sheets

with organic solvents to remove the unbound dodecyl gallate makes it difficult to attribute the hydrophobicity to laccase-mediated coupling. Although Zhang and Huang, 2000 and Tsukada et al. 2006 have chemically applied fluorophenols to improve hydrophobicity of polyesters and ionic liquids, respectively, this study has demonstrated for the first time the ability of an environmentally friendly method to increase wood hydrophobicity using fluorophenols.

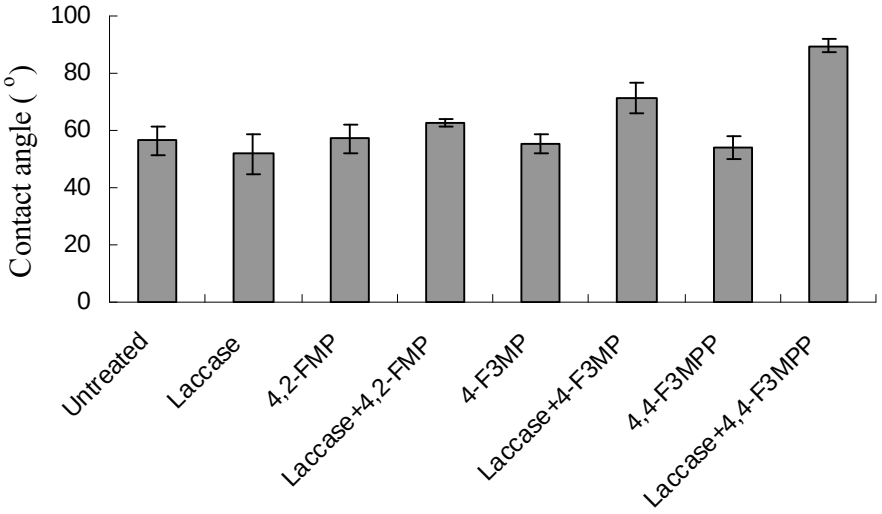


Figure 6. Effect of laccase-mediated grafting of fluorophenols on hydrophobicity of beech (*Fagus sylvatica*) wood veneers.
*All values are means of three replicates ± standard deviation

2.3.5 XPS analysis of fluorophenols grafted onto veneers

The estimated percentage of incorporated fluorine was 6.39% for 4,4-F3MPP; 3.01 % for 4-F3MP and 0.26 % for 4.2-FMP (Table 2). XPS data on the amount of fluoro compounds bound onto wood veneers positively correlates with the increase in contact angle. This finding is consistent with the previous observation that hydrophobicity increases with an increase in the content ratio of the fluorine molecule (Tsukada et al 2006. Further evidence of incorporation is given by the corresponding increase in the F/C ratio (Table 2). Analysis of the O/C ratio showed a decrease from 0.52 (laccase only) to 0.34 (laccase-4,4-F3MPP), 0.37 (laccase-4-F3MP) and 0.48 (4,2-FMP) which can be attributed to the low O/C ratios (0.15, 0.29 and 0.14 respectively) of surface-grafted 4,4-F3MPP; 4-F3MP and 4,2-FMP molecules. The low fluorine content obtained when 4,2-FMP was used is possibly because it is a laccase substrate and therefore the radicals formed can undergo homomolecular polymerization in addition to coupling onto the wood pieces. Laccase generated radicals are already known to undergo many reactions which include oligomerization, quinone formation and polymerization (Lundquist and Kristersson, 1985; Mai et al., 2002). This is also supported by

LC-MS data indicating, in contrast to 4,4-F3MPP (non laccase substrate), the formation of several products apart from a coupling product with lignin model (Figure 3D). Spectrophotometric studies also showed much higher increase in binding of fluorophenols during laccase treatment of veneers when 4,4-F3MPP was used (77.1 %) as compared to 4-F3MP (39.2 %) which is consistent with XPS data.

Table 2. XPS analyses of beech veneer surfaces after treatment with fluorophenols

Treatment	Wood surface						Phenol used with laccase[1]	
	O/C ratio	SD	F/C ratio	SD	% F	SD	O/C ratio	F/C ratio
Untreated	0.54	0.02	--	--	--	--	--	--
Laccase only	0.52	0.02	--	--	--	--	--	--
Laccase + 4,2-FMP	0.48	0.01	0.004	0.00	0.26	0.02	0.14	0.14
Laccase + 4-F3MP	0.37	0.01	0.045	0.051	3.01	3.33	0.29	0.43
Laccase + 4,4F3MPP	0.34	0.01	0.09	0.00	6.39	0.18	0.15	0.23

[1]Given as % as calculated from the molecular formulae and the O/C and F/C ratios

2.4. Conclusion

In summary, we have successfully demonstrated covalent attachment of fluorophenolics onto wood surfaces by using laccase resulting in an increase in hydrophobicity. Furthermore, *in vitro* studies with complex lignin models demonstrated the possibility of covalent coupling with fluorophenols and more importantly leaving the fluorine molecule free, a condition necessary for the hydrophobic properties. The advantage of laccase-mediated covalent binding of molecules onto wood surface is that the grafted molecules are not readily displaced and released into the environment. This eco-friendly process, which is conducted under mild conditions, provides an attractive alternative in improving wood surface hydrophobicity.

Acknowledgements

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2.5. References

Almansa, E., Kandelbauer, A., Pereira, L., Cavaco-Paulo, A., Guebitz, G.M., 2004 Influence of structure on dye degradation with laccase mediator systems. Biocat. Biotrans. 22 (5/6), 315-324.

Borgin, K., Corbett, K., 1970. The stability and weathering properties of wood treated with various oils. Plastics, Paint and Rubber 14(3) 69 – 72.

Claus, H., 2004. Laccases: structure, reaction, distribution. Micron 35, 93 – 96.

Feist, W.C., Mraz, E.A., 1978. Protecting millwork with water repellents. Forest Products Journal 28 (5), 31–35.

Grönqvist, S., Rantanen, K., Raimo, A., Mattinen, M., Buchert, J., Viikari, L., 2006. Laccase catalysed functionalization of TMP with tyramine. Holzforschung 60, 503–508.

Gronqvist, S., Buchert, J., Rantanen, K., Viikari, L., Suurnakki, A., 2003. Activity of laccase on unbleached and bleached thermomechanical pulp. Enzyme Microb. Technol. 32, 439- 445.

Hyvönen, A., Piltonen P., Niinimäki, J., 2006. Tall oil/water – emulsions as water repellents for Scots pine sapwood. Holz als Roh- und Werkstoff 64, 68–73.

Lundquist, K., Kristersson, P. 1985. Exhaustive laccase-catalysed oxidation of a lignin model compound (vanillyl glycol) produces methanol and polymeric quinoid products. Biochem J. 229(1), 277–279.

Gutiérrez, A., del Río, J.C., González-Vila F.J., Romero, J., 1998. Variation in the composition of wood extractives from *Eucalyptus globulus* wood during seasoning. J. Wood Chem. Technol. 18 (4), 439-446.

Kudanga, T., Prasetyo, E.N, Sipilä, J., Nousiainen, P., Widsten, P., Kandelbauer, A., Nyanhongo, G.S., Guebitz, GM., 2008. Laccase-mediated wood surface functionalization. Eng. Life Sci. 8(3), 297-302.

Mai, C., Militz, H., 2004. Modification of wood with silicon compounds. Treatment systems based on organic silicon compounds - a review. Wood Sci. Technol. 37 (6)453 - 461.

Mai, C., Schormann, W., Hüttermann, A., Kappl, R., Hüttermann, J., 2002. The influence of laccase on the chemo-enzymatic synthesis of lignin graft-copolymers. Enzyme Microb. Technol. 30 (1), 66-72.

Niku-Paavola, M. L., E.Karhunen, Salola, P., Raunio, V., 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. 254, 877–884.

Nzokou, P, Kamdem, D.P., 2004. Influence of wood extractives on moisture sorption and wettability of red oak (*Quercus rubra*), Black Cherry (*Prunus serotina*), and Red Pine (*Pinus resinosa*). Wood Fiber Sci. 36(4), 483-492.

Obataya, E., Sugiyama, M., Tomita, B., 2002. Dimensional stability of wood acetylated with acetic anhydride solution of glucose pentaacetate. J. Wood Sci. 48, 315-319.

Paice, M.G., Bourbonnais, R., Reid, I.D., 1995. Bleaching kraft pulps with oxidative enzymes and alkaline hydrogen peroxide. Tappi J. 78(9), 161–169.

Ramsden, M.J., Blake, F.S.R., Fey, N.J., 1997. The effect of acetylation on the mechanical properties, hydrophobicity, and dimensional stability of *Pinus sylvestris*. Wood Sci. Technol. 31, 97-104.

Razzaque, M.A., 1982. The effect of concentration and distribution on the performance of water repellents applied to wood. University of Wales, Ph.D. Thesis.

Rittstieg, K., Suurnakki, A., Suortti, T., Kruus, K., Gübitz, G., Buchert, J., 2002. Investigations on the laccase polymerization of lignin model compounds using size-exclusion HPLC, Enzyme Microb. Technol. 31, 403–410.

Rittstieg, K., Suurnakki, A., Suortti, T., Kruus, K., Gübitz, G., Buchert, J., 2003. Polymerization of guaiacol and a phenolic β-O-4-substructure by *Trametes hirsuta* laccase in presence of ABTS, Biotechnol. Prog. 19, 1505–1509.

Riva, S., 2006. Laccases: blue enzymes for green chemistry. Trends Biotechnol. 24, 219-226.

Rowell, R., 2006. Acetylation of wood: journey from analytical technique to commercial reality, Forest Prod. J., 56(9), 4-12.

Schröder, M., Aichernig, N., Gübitz, G.M., Kokol V., 2007. Enzymatic coating of lignocellulosic surfaces with polyphenols, Biotechnol J. 2, 334–341.

Sipila, J., Syrjanen, K., 1995. Synthesis and ^{13}C NMR spectroscopic characterization of six dimeric arylglycerol-β-aryl ether model compounds representative of syringyl and *p*-hydroxyphenyl structures in lignins. On the aldol reaction in β-ether preparation. Holzforschung 49, 325-331.

Suurnakki, A., Buchert, J., Gronqvist, S., Mikkonen, H., Peltonen, S., Viikari, L., 2006. Bringing new properties to lignin rich fiber materials. VTT Symposium 244, 61-70.

Takahama, U., 1995. Oxidation of hydroxycinnamic acid and hydroxycinnamyl alcohol derivatives by laccase and peroxidase-interactions among p-hydroxyphenyl, guaiacyl and syringyl groups during the oxidation reactions. Physiologia Plantarum 93, 61- 68.

Tsukada, Y., Iwamoto, K., Furutani, H., Matsushita, Y., Abe, Y., Matsumoto, K., Monda, K., Hayase, S., Kawatsura, M., Itoh, T., 2006. Preparation of novel hydrophobic fluorine-substituted-alkyl sulfate ionic liquids and application as an efficient reaction medium for lipase-catalyzed reaction. Tetrahedron Lett. 47, 1801–1804.

Ulvcrona, T., Lindberg, H., Bergsten, U., 2006. Impregnation of Norway spruce (*Picea abies* L. Karst.) wood by hydrophobic oil and dispersion patterns in different tissues. Forestry 79(1), 123-134.

Vick, C.B., Rowell, R.M., 1990. Adhesive Bonding of Acetylated Wood. Inter. J. Adh. Adhes. 10(4), 263-272.

Viitaniemi, P., Jämsä, S., 1996. Modification of wood with heat treatment (in Finnish). Espoo 1996, Technical Research Centre of Finland. VTT Julkaisuja – Publikationer 814, 57.

Widsten, P., Kandelbauer, A., 2008. Laccase applications in the forest products industry: A review. Enzyme Microb. Technol. 42(4), 293-307.

Zhang, L., Huang, W.Y., 2000. Synthesis of polyfluorinated aromatic polyesters. J. Fluorine Chem. 102, 55-59.

3

Reactivity of long chain alkylamines to lignin moieties: implications on hydrophobicity of lignocellulose materials

Enzymatic processes provide new perspectives for modification of lignocellulose materials. In the current study, laccase catalysed coupling of long chain alkylamines to lignin model molecules and lignocellulose was investigated. Multiple molecules of dodecylamine (DA) and dihexylamine (DHA) were readily coupled onto simple phenolic molecules like guaiacol, catechol and ferulic acid while coupling onto complex lignin models syringylglycerol β-guaiacyl ether, guaiacylglycerol β-guaiacyl ether and dibenzodioxocin only yielded 1:1 coupling products. Surface analysis of beech veneers enzymatically grafted with DA showed a nitrogen content of 3.18 % compared to 0.71 % in laccase only treated controls while the O/C ratio decreased from 0.52 to 0.46. Concomitantly the coupling of DHA or DA onto beech veneers resulted in a 53.8 % and 84.2 % increase in hydrophobicity, respectively when compared to simple adsorption. Therefore laccase-mediated treatment of lignocellulose with long chain alkylamines is a credible alternative for improving wood hydrophobicity.

Submitted as Kudanga et al. to *Journal of Biotechnology*

Introduction

Enzyme technology has evolved from the synthesis of small molecules to biotransformation of complex natural/synthetic polymers, thanks to increased understanding of enzyme reaction mechanisms and the new global thrust aimed at developing green technologies. Among the enzymes being actively pursued for biotransformation of polymers are laccases. Laccases (benzenediol: oxygen oxidoreductases, EC.1.10.3.2) are multicopper containing enzymes that catalyze the oxidation of various aromatic compounds, especially phenolic compounds, concomitantly reducing molecular oxygen to water (Nyanhongo et al., 2005). The generated reactive species (phenoxy radicals) provides ideal sites for cross coupling of lignin and functional molecules.

The pioneering works of Dordick and coworkers during the early 1990s (Popp et al., 1991; Blinkovsky and Dordick, 1993) which demonstrated the ability of heme peroxidases to oxidatively graft phenols onto lignin (*in vitro*) resulting in the formation of phenolic resins heralded the beginning of a new era in polymer functionalization. Subsequently, several studies have demonstrated the possibility of laccase-catalysed bonding of low molecular weight compounds onto lignin/synthetic molecules as summarised in the detailed reviews by Witayakran and Ragauskas, 2009, Mikolasch and Schauer, 2009; Widsten and Kandelbauer, 2008, Kunamneni et al., 2008 and Nyanhongo et al., 2007. Based on the knowledge acquired on laccase mediated modification of lignin, the application of laccases has now been extended to surface modification of lignocellulose material. The further demonstration by Yamaguchi et al., 1994, Felby et al., 1997, 2002 of the ability of laccases to mediate the production of fibre boards increased the interest in applying this technology for surface functionalization of polymers. This is evidenced by the recent development of lignocellulosic materials and textile fibers with antimicrobial (Kudanga et al., 2008) and antioxidant properties (Gaffar Hossain et al., 2009), new aesthetic look (Calafell et al., 2007, Hadzhiyska et al 2006), and reactive surfaces (Kudanga et al., 2008, Kudanga et al., 2009). Despite the attractive properties of lignocellulosic materials (low thermal extension, low density and high mechanical strength), they are highly hygroscopic. Although Suurnäkki et al. (2006) and Hossain et al. (2008) reported an increase in hydrophobicity in enzymatically treated polymers, hardly any evidence of covalent binding of the functional molecules used was given.

In this study the possibility of using laccase to mediate the coupling of alkylamines (docecylamine and dihexylamine) as functional molecules to enhance hydrophobicity of lignocellulose material is investigated in a hierarchical manner. The amines were first coupled

onto different simple phenolic molecules, then onto complex lignin model compounds and finally onto wood material. Further, the ability to transform a hydrophilic surface (wood material) into a hydrophobic material is investigated.

Materials and methods

Chemicals and wood veneers

The lignin model compounds (Fig. 1) guaiacylglycerol β-guaiacyl ether (erol), syringylglycerol β-guaiacylether and dehydrodivanillyl alcohol type dibenzodioxocin were synthesized as previously reported (Sipilä and Syrjänen, 1995; Karhunen et al. 1995). All other chemicals including phenolic molecules, dodecylamine (DA) and dihexylamine (DHA) (Fig. 1d and 1j) were purchased from Sigma-Aldrich. Wood veneers were prepared from European beech (*Fagus sylvatica*) and measured 10 mm x 10 mm x 1 mm. Prior to their use in grafting experiments, they were Soxhlet-extracted with acetone overnight to remove lipophilic extractives which could interfere with the designed reactions and also affect analysis of modified surface (Nzokou and Kamdem, 2004).

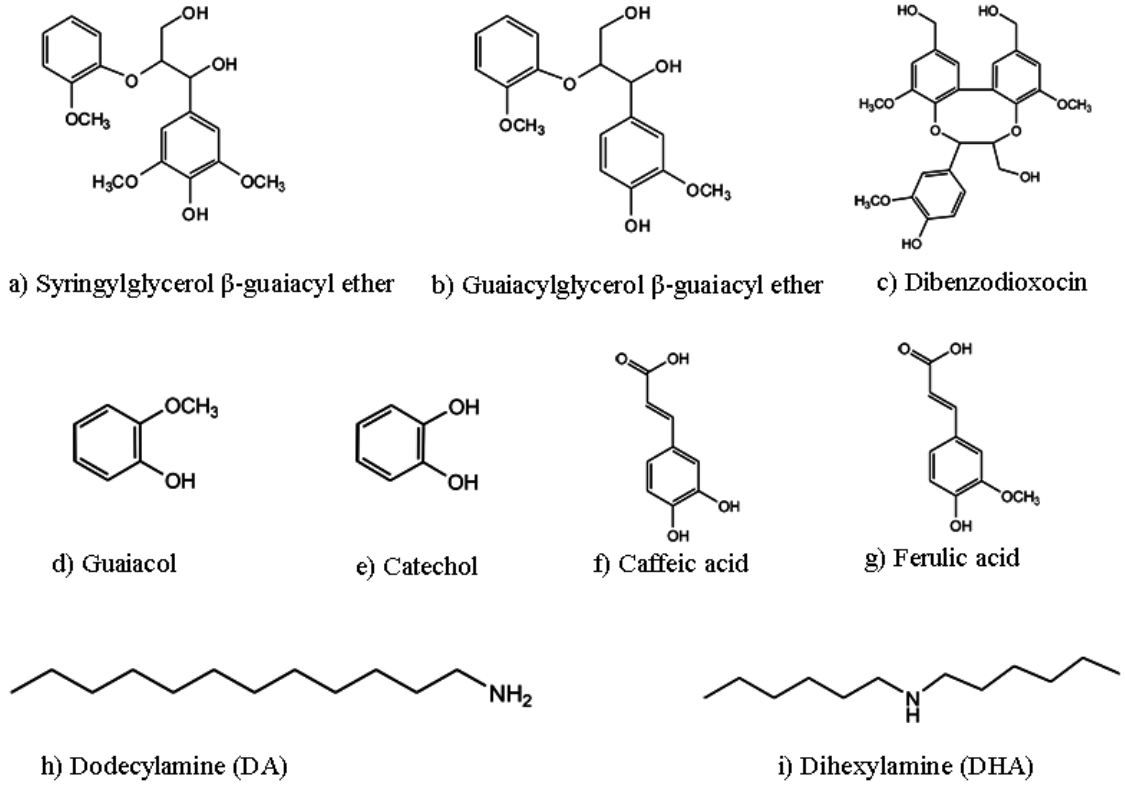


Fig. 1. Chemical structures of alkylamines, lignin models and phenolic molecules used in coupling reactions

Laccases

The 62 kDa laccase from *Trametes hirsuta* was produced and purified as previously reported by Almansa et al. (2004). The activity of the laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε_{436} = 29,300 $M^{-1}cm^{-1}$) as substrate at 436 nm in 50 mM succinate buffer at pH 4.5 and 37 °C as described by Niku-Paavola et al. (1988) with some modifications. Briefly, the reaction mixture contained 30 µl laccase, 350 µl ABTS (1 mM) and 50 mM succinate buffer pH 4.5 to make a final volume of 1.5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100 °C for 10 minutes. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 minutes.

Coupling of dodecylamine and dihexylamine onto lignin monomers and models

The reaction mixture contained one of the alkylamines and one of the lignin monomers or model compounds in the molar ratio of 1:1 and 13.4 nkat ml^{-1} laccase in 50 mM succinate buffer (pH 4.5). Reactions were carried out at 37 ºC while shaking at 650 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg Germany). The reactions were incubated for 1 h when phenolic molecules were used as models, 2 h when lignin model erol was used and 3 h for the other model compounds. The coupling products were analyzed by HPLC -MS.

HPLC-MS analysis of reaction products

To the incubation mixtures an equal volume of ice cold methanol was added to precipitate protein. The mixture was centrifuged at 0 ºC for 15 minutes at 14000 g and 650 µl aliquots were transferred into clean vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 x 4.6 mm with precolumn, Knauer GmbH, Berlin, Germany) using a linear gradient of acetonitrile (solvent B) and 0.1 % H_3PO_4 (solvent A) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30 °C. The gradient was set as shown in Table 1.

Table 1

Gradient set up for HPLC analysis

Time (min.)	Initial	5	20	21	35	35.1	45(end)
Solvent A%	80	80	25	10	10	80	80
Solvent B %	20	20	75	90	90	20	20

The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with electrospray ionisation coupled to the Dionex HPLC-UVD-system described above and using the same protocol except that 0.1 % formic acid was used instead of 0.1 % H_3PO_4. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350 °C, nebulizer to 70 psi. Maximal accumulation time was fixed to 300 msec and the loading of the trap was controlled by the instrument with an ICC of 30000.

Coupling of alkylamines onto wood veneers

Soxhlet-extracted beech veneer (10 mm x 10 mm x 1.0 mm) were pre-oxidized by laccases (26.8 nkat ml^{-1}) for 1 h and then further incubated for 4 h in the presence (5mM) of each of the alkylamines (dodecylamine and dihexylamine). The reaction was carried out in 50 mM succinate buffer at pH 4.5 and 37 °C while shaking at 150 rpm. The incubation solution was decanted and the veneers were resuspended in 10 ml 75 % ethanol and washed for 16 hours while shaking at 150 rpm to remove unbound alkyamines. The wood pieces were further rinsed with 10 ml 75 % ethanol for 10 minutes before transferring them into 10 ml 75 % acetone for another 10 min wash.

Contact angle measurements

After washing-off unbound alkylamines from the veneers and conditioning to equilibrium moisture content, the sessile contact angle of water was measured in the grain direction five seconds after drop deposition according to the Young-Laplace method using a DataPhysics OCA 35 goniometer and SCA20 software. The drop volume was 1.5 µl. For each veneer, the contact angle was measured on five spots and the results averaged. For each treatment triplicate samples were measured.

XPS measurements

Binding of the alkylamines to veneers was determined using X-ray photoelectron spectroscopy (XPS). XPS measurements were performed at Joanneum Research, Graz, Austria, with a multiprobe UHV surface analysis system from Omicron Nanotechnology. Equipment and parameters: x-ray source: DAR400 aluminium-anode; quartz-crystal monochromator XM 500; x-ray excitation energy: 1486.70eV (Al Kα1-line); monochromated x-ray line width (FWHM): 0.3 eV; energy analyzer: hemispherical analyzer, type EA 125; signal-detection with pulse-counting channeltron (five channels for count-rate enhancement); total energy resolution (x-ray source + hemispherical analyzer): 0.45 eV for detailed scans at 10 eV pass-energy (+ lifetime broadening of the electronic state); charge neutralization for non-conducting samples: electron emission gun for low-energy electrons (0.1–500 eV): FG 15/40 (SPECS). The analyzed surface area was ~ 1 mm^2; five spots were analyzed per sample and the results averaged.

Results and Discussion

Coupling of alkylamines onto simple phenolic molecules

Laccase catalysed coupling of alkylamines was first studied using simple phenolic molecules as models for the phenolic moieties in lignin. LC-MS spectra showed that both dodecylamine (DA) and dihexylamine (DHA) were readily coupled onto the phenolic molecules used, all of which are good laccase substrates (Kudanga et al., 2009). Coupling of DA to lignin monomers yielded multiple coupling products with two molecules being coupled onto at least one phenolic molecule (Fig. 2). For example strong signals for the $[M + H]^+$ ions at *m/z* = 491.4 and *m/z* = 489.4 correspond to coupling of two molecules of DA to a guaiacol molecule and its quinone form, respectively (exact masses are 490.45 and 488.43, respectively). The monomers with a free C5 position easily formed dimers or trimers and molecular ions corresponding to coupling of the amines to these oligomers were observed (Fig. 2c-f). Thus molecular ions at *m/z* 753.2, *m/z* 584.7 and *m/z* 611.5 correspond to coupling products of two dodecylamine molecules to dimers of ferulic acid, catechol and guaiacol, respectively. A coupling product of two amines to a trimer of catechol was also observed (*m/z* 693.7) (Fig. 2f). Catechol easily oligomerised to form trimers due to the absence of an alkyl side chain usually found on phenyl propane units. Such side groups prevent addition of a third unit once a molecule has been bound through the most feasibly formed 5-5 linkage (Valencia and Marinez, 2005).

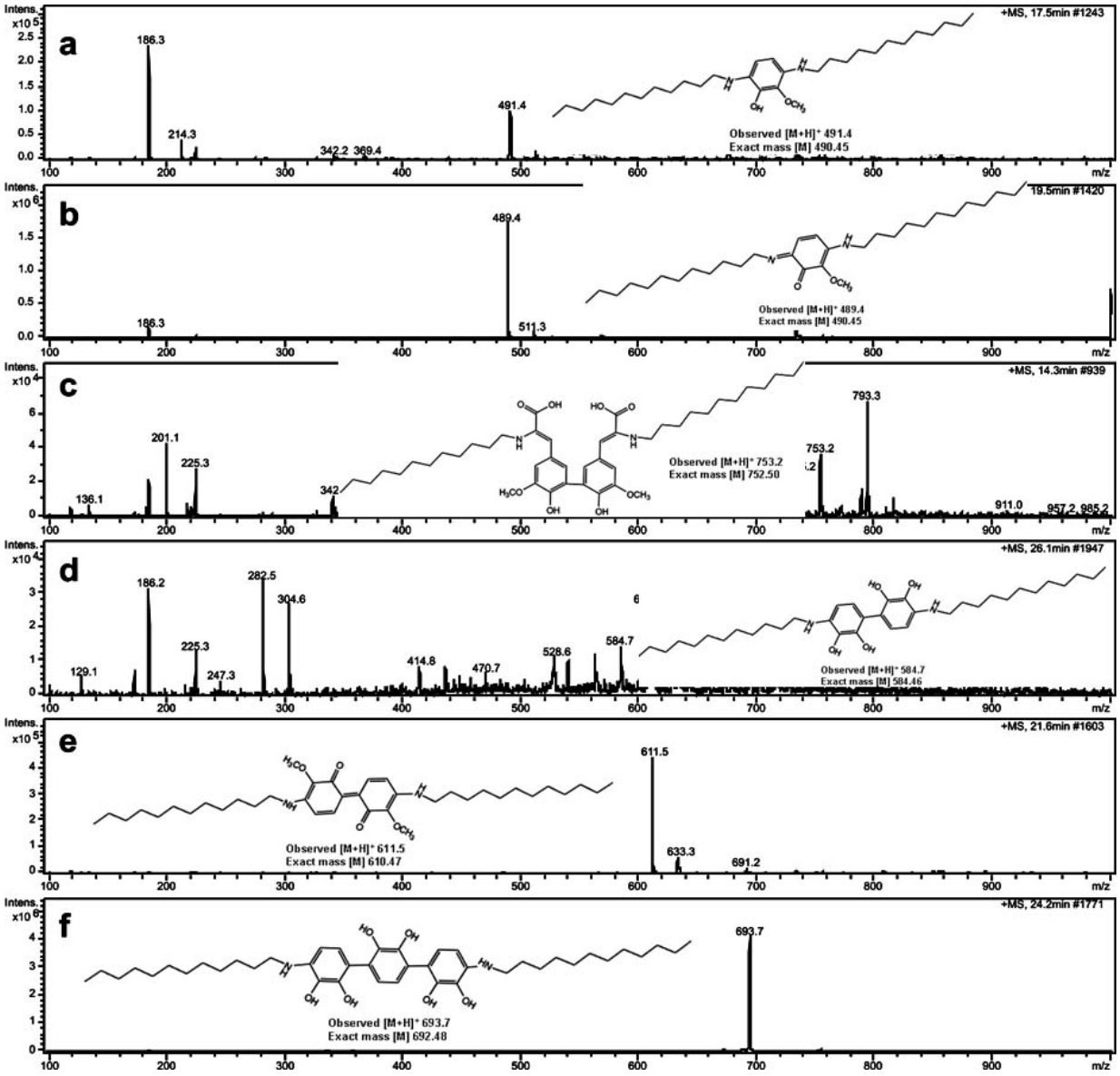


Fig. 2. Mass spectra of laccase-mediated coupling products of DA to phenolic molecules and suggested structures of coupling products: a) DA to guaiacol, b) DA to quinone form of guaiacol, c) DA to ferulic acid dimer, d) DA to catechol dimer, e) DA to guaiacol dimer, and f) 2DA to catechol trimer.

On the contrary, it was possible to couple one molecule of the amine when the less reactive DHA was used (Fig. 3). For example the $[M + H]^+$ molecular ions at *m/z* 294.3 and 542.5 (Fig. 3a,b) correspond to coupling of one molecule of DHA to catechol and to a dimer of caffeic acid, respectively (exact masses 293.24 and 541.27). However, like DA, multiple units of DHA were also successfully coupled to monomeric and oligomeric forms of guaiacol and catechol (Fig. 3c-e).

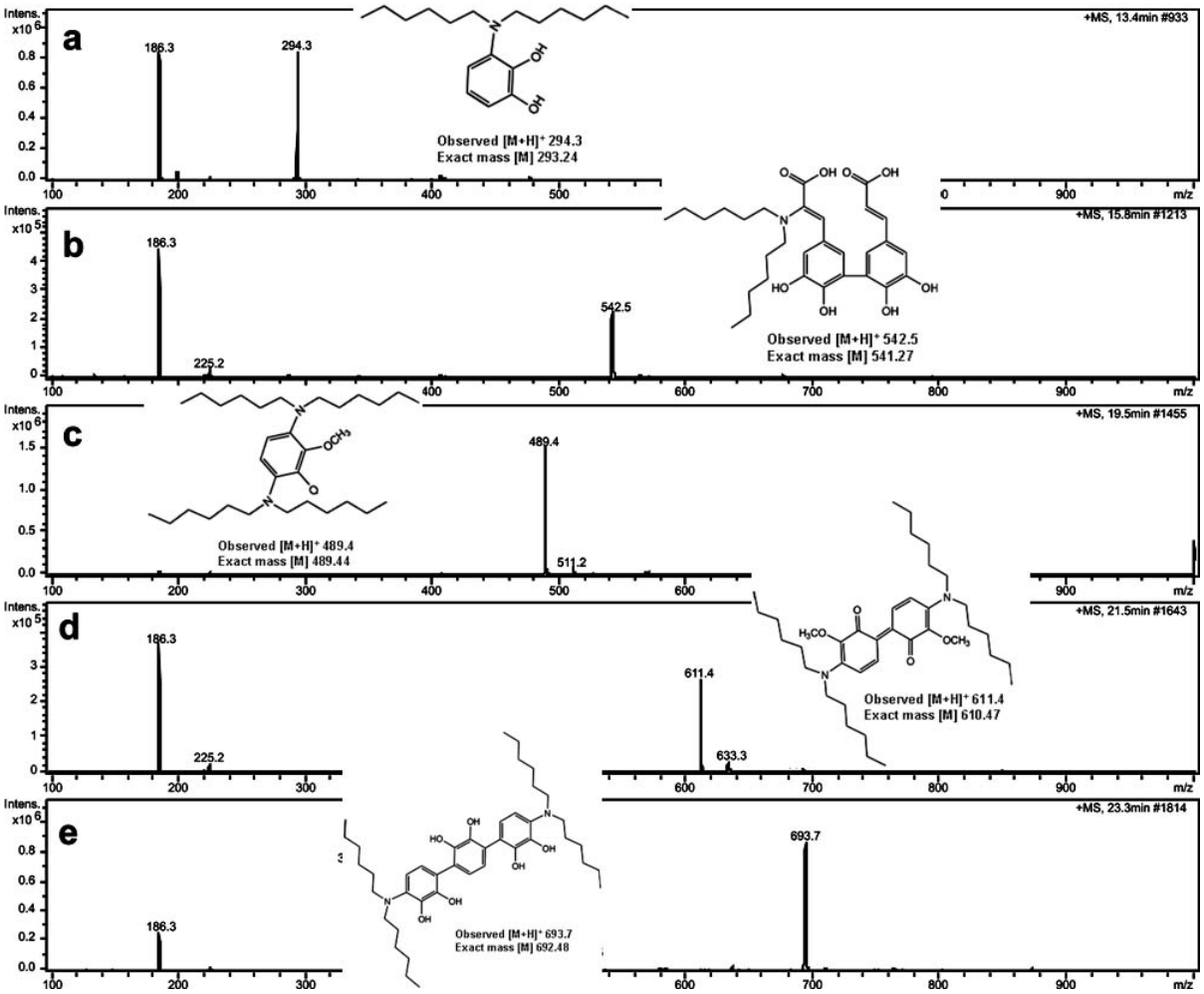


Fig. 3. Mass spectra of laccase-mediated coupling products of DHA to phenolic molecules and suggested structures of coupling products: a) DHA to catechol, b) DHA to caffeic acid dimer, c) DHA to guaiacol, d) DHA to guaiacol dimer, and e) DHA to catechol trimer.

Coupling alkylamines onto lignin models

The molecular weights of the lignin models are 350.363 (syringylglycerol β-guaiacyl ether), 320.337 (guaiacylglycerol β-guaiacyl ether) and 484.50 (dibenzodioxocin). Mass spectrometry in positive ion mode showed signals for the $[M + H]^+$ ion at *m/z* 533.7, *m/z* 504.6 and *m/z* 668.4 which suggests 1:1 coupling products between the amines and syringylglycerol β-guaiacyl ether, guaiacylglycerol β-guaiacyl ether and dibenzodioxocin, respectively (Fig. 4). Unlike the phenolic molecules, oxidative coupling onto lignin models yielded exclusively 1:1 coupling products possibly due to steric effects. Non-covalent hydrophobic interactions between the aromatic rings in these oligomeric models may hinder interactions between the molecules and reactants (Mattinen et al., 2008). On the contrary small phenolic molecules allowed coupling of multiple amine molecules as they provide better accessibility to reactants. These results suggest the need to understand the structural

components of the lignocellulose material in order to maximally exploit its structural properties for optimal functionalization.

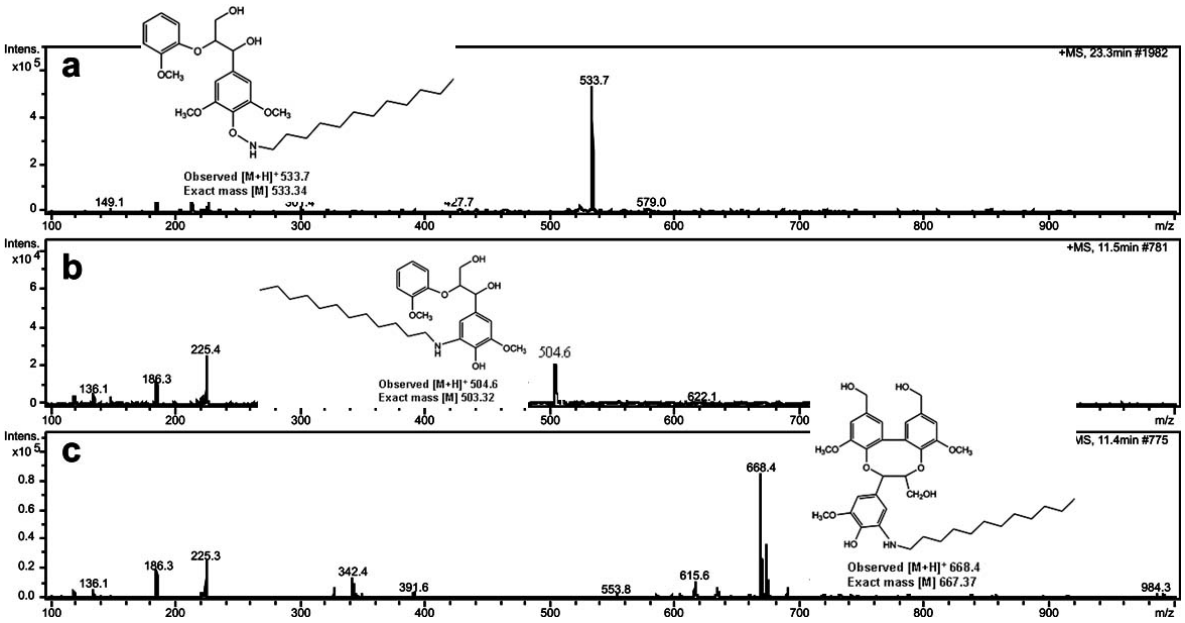
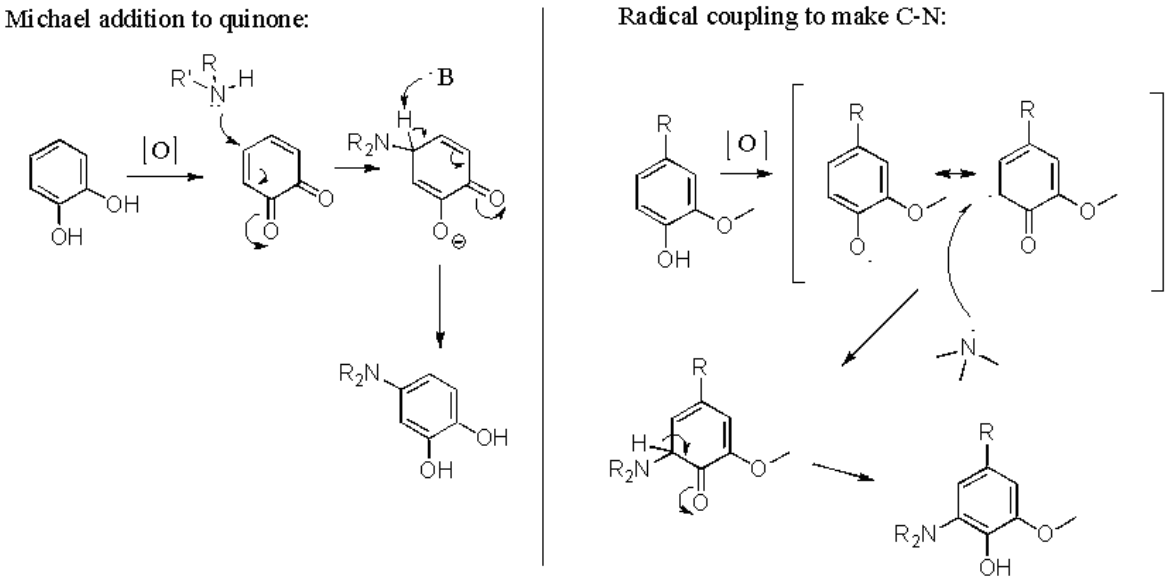


Fig. 4. Mass spectra of laccase-mediated coupling products of alkylamines to lignin models syringylglycerol β-guaiacyl ether (a), guaiacylglycerol β-guaiacyl ether (b) and dibenzodioxocin (c) and suggested structures.

It is well known that lignin molecules oligomerise through radical coupling with preformed lignin oligomers usually bonding through 5-5 and 4-O-5 linkages (Boerjan et al., 2003). This is supported by the observations that the 4-O-5 and 5-5 bonds have a lower heat of formation than the other possible linkages (Valencia and Marinez, 2005). The hydroxyl group on the benzene ring is *ortho* or *para* directing (Suparno et al., 2005; Schultz et al., 2001; Jonas et al., 2000) and molecules with free C-5 position usually cross-couple through 5-5 linkages due to stability of C-C bonds (del Río and Gutiérrez, 2008). As pointed out above, this allows molecules like catechol to easily oligomerise via 5-5 bonds. However, lignin molecules with syringyl or sinapyl units usually couple through 4-O-5 linkages to molecules with a free C5 position while the unsaturated side chains can be utilized for oxidative coupling if the phenolic carbons are sterically hindered. Laccase-mediated amine coupling can proceed via Michael addition (Witayakran and Ragauskas, 2009) or via radical coupling to make C-N bonds (Niedermeyer et al., 2005) as shown in scheme 1. Considering these facts together with the results from LC/MS analysis, structures embedded in the MS spectra figures were proposed (Fig. 2-4). Although these structures are based on radical coupling, similar products can also be obtained through Michael addition.



Scheme 1. Alternative coupling mechanisms of amines onto phenolic compounds using laccase

Grafting of alkylamines onto beech wood

XPS measurements

XPS results (Table 2) showed an increase in the percentage of nitrogen and a decrease in O/C ratio which indicates coupling of DA onto beech veneer surfaces. The increase in the percentage of nitrogen can obviously be attributed to the incorporation of the dodecylamine which also explains the decrease in the O/C ratio from 0.52 (laccase only) to 0.46 (laccase+DA) as the DA has a high carbon content and zero oxygen content. A small increase of the nitrogen content in the laccase only treated sample could results from adsorbed enzyme protein.

Table 2

XPS analysis of beech veneers treated with DA in the presence of laccase

Treatment	Wood surface			
	O/C ratio	SD	%N	SD
Untreated	0.54	0.02	0.45	0.07
Laccase	0.52	0.02	0.71	0.08
Laccase+DA	0.46	0.01	3.18	0.20

Contact angle measurements

Contact angle values of water (shown in Fig. 5) indicate that laccase-mediated grafting of alkylamines resulted in an increase in wood hydrophobicity. Laccase-assisted treatment of beech veneers with DA resulted in an increase in contact angle from 58.3° to 107.4°, effectively an 84.2 % increase in contact angle when compared with simple adsorption. However, laccase-assisted treatment with DHA was less effective and increased hydrophobicity by 53.8 %. When these results are compared to our recent findings with hydrophobicity enhancing fluorophenols (Kudanga et al., 2010), it is worth noting that DA performed better than all the fluorophenols used previously while DHA was only inferior to 4-[4-(trifluoromethyl)phenoxy]phenol. Thus the alkylamines present a credible alternative for enhancing wood hydrophobicity. These laccase assisted treatments covalently bind the molecules to the lignocellulose material hence the chemical agents are not readily washed off and released into the environment. In addition, this application presents an opportunity for multifunctional modification of wood since dodecylamine has already been successfully used in wood preservative formulations having activity against termites and fungi (Goettsche and Borck, 1994).

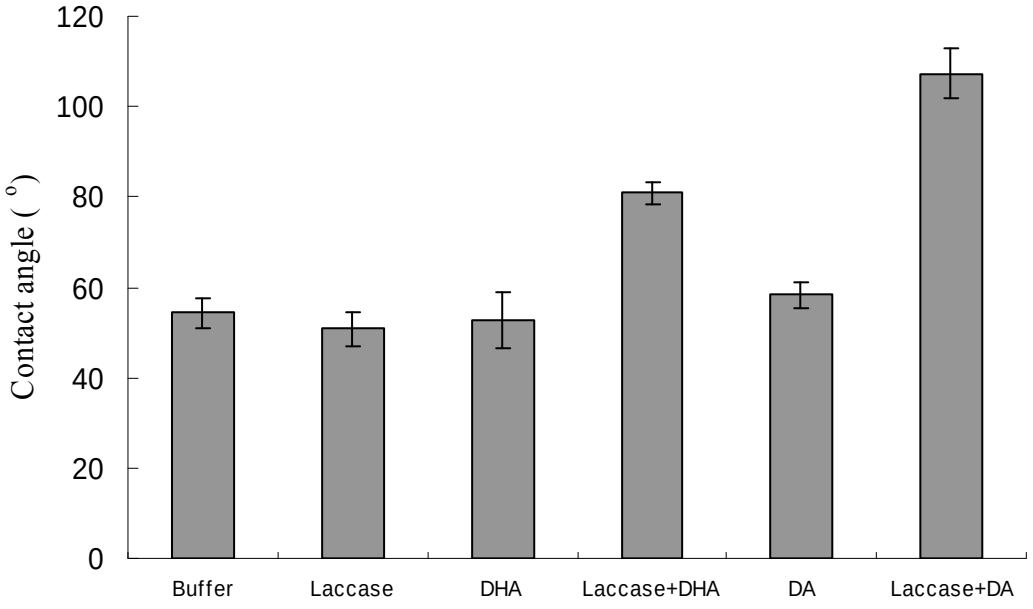


Fig. 5. Effect of laccase-mediated grafting of alkylamines, dodecylamine (DA) and dihexylamine (DHA) on hydrophobicity of beech (*Fagus sylvatica*) veneers.

*All values are means of three replicates ± standard deviation

Conclusion

In this work, we progressively demonstrated laccase-mediated coupling of long chain alkylamines to simple phenolic monomers, complex lignin models and to lignocellulose materials. The potential application of these reactions has been demonstrated by the increase in hydrophobicity of beech wood enzymatically treated with these amines. Since the amines are covalently bound and the reaction can be performed under mild conditions, this work presents a cost-effective and eco-friendly alternative to the current methods of improving wood hydrophobicity.

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References

Almansa, E., Kandelbauer, A., Pereira, L., Cavaco-Paulo, A., Guebitz, G.M., 2004. Influence of structure on dye degradation with laccase mediator systems. Biocat. Biotrans. 22, 315-324.

Blinkovsky, A.M., Dordick, J.S., 1993. Peroxidase-catalyzed synthesis of lignin-phenol copolymers. Journal of Polymer Science Part A: Poly. Chem. 31(7**)**, 1839 – 1846.

Boerjan, W., Ralph, J., Baucher, M., 2003. Lignin biosynthesis. Annu. Rev. Plant Biol. 54, 519-546.

Calafell, M., Díaz, C., Hadzhiyska, H., Gibert, J.M., Dagà, J.M., Tzanov, T., 2007. Bio-catalyzed coloration of cellulose fibers. Biocatal. Biotransform. 25, 336–340.

Felby, C., Pedersen, L.S., Nielsen, B.R., 1997. Enhanced auto-adhesion of wood fibers using phenol oxidases. Holzforschung 51, 281-286.

Felby C., Hassingboe J., Lund, M., 2002. Pilot-scale production of fiberboards made by laccase oxidized wood fibers: board properties and evidence for cross-linking of lignin, Enzyme Microb. Technol. 31(6), 736–741.

Goettsche, R., Borck, H.,1994. Wood preservative compositions having activity against termites and fungi. United States Patent 5276029.

Hadzhiyska, H., Calafell, M., Gibert, J.M., Dagà, J.M., Tzanov, T., 2006. Laccase-assisted dyeing of cotton. Biotechnol. Lett. 28, 755–759.

Hossain, Kh.M.G., González, M.D., Lozano, G.R., Tzanov, T., 2009. Multifunctional modification of wool using an enzymatic process in aqueous-organic media. J. Biotechnol. 141, 58-63.

Jonas, U., Hammer, E., Haupt, E.T.K., Schauer, F., 2000. Characterisation of coupling products formed by biotransformation of biphenyl and diphenyl ether by the white rot fungus *Pycnoporus cinnabarinus*. Arch. Microbiol. 174, 393-398.

Karhunen, P., Rummakko, P., Sipilä, J., Brunow, G., 1995. The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. Tetrahedron Lett. 36, 4501-4504.

Kudanga, T., Prasetyo, E.N., Sipilä, J., Nousiainen, P., Widsten, P., Kandelbauer, A., Nyanhongo, G.S., Guebitz, G., 2008. Laccase-mediated wood surface functionalization. Eng. Life Sci. 8(3), 297 -302.

Kudanga, T., Nugroho Prasetyo, E., Sipilä, J., Eberl, A., Nyanhongo, G.S., Guebitz, G.M., 2009. Coupling of aromatic amines onto syringylglycerol β–guaiacylether using *Bacillus* SF spore laccase. J. Mol. Catal. B: Enzym. 61, 143–149.

Kudanga, T., Nugroho Prasetyo, E., Widsten, P., Kandelbauer, A., Jury, S., Heathcote, C., Sipilä, J., Weber, H., Nyanhongo, G.S., Guebitz, G.M., 2010. Laccase catalyzed coupling of fluorophenols to lignin increases wood surface hydrophobicity. Biores. Technol. 101, 2793–2799.

Kunamneni, A., Camarero, S., García-Burgos, C., Plou, F.J., Ballesteros, A., Alcalde, M., 2008. Engineering and Applications of fungal laccases for organic synthesis. Microb. Cell Fact. **7:**32.

Mattinen, M-L., Suortti, T., Gosselink, R., Argyropoulos, D.S., Evtuguin, D., Suurnäkki, A., de Jong, E., Tamminen, T., 2008. Polymerization of different lignins by laccase. BioResources 3(2), 549-565.

Mikolasch, A., Schauer, F., 2009. Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl. Microbiol. Biotechnol. 82, 605–624.

Niedermeyer, T.H.J., Mikolasch, A., Lalk, M., 2005. Nuclear amination catalyzed by fungal laccases: Reaction products of *p*-Hydroquinones and primary aromatic amines. J. Org. Chem. 70, 2002-2008.

Niku-Paavola, M.L., Karhunen, E., Salola, P., Raunio, V., 1988. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem. J. 254, 877-884.

Nyanhongo, G. S., Schroeder, M., Steiner, W., Gübitz, G.M., 2005. Biodegradation of 2,4,6-trinitrotoluene (TNT): An enzymatic perspective. Biocatal. Biotransform. 23(2), 53-69.

Nyanhongo, G.S., Gübitz, G., Sukyai, P., Leitner, C., Haltrich, D., Ludwig, R., 2007. Oxidoreductases from *Trametes* spp. in Biotechnology: A wealth of catalytic activity. Food Technol. Biotechnol. 45(3), 248–266.

Nzokou, P., Kamdem, D.P., 2004. Influence of wood extractives on moisture sorption and wettability of red oak (*Quercus rubra*), black cherry (*Prunus serotina*), and red pine (*Pinus resinosa*). Wood Fiber Sci. 36(4), 483-492.

Popp, J.L., Kirk, T.K., Dordick, J.S., 1991. Enzymic modification of lignin for use as a phenolic resi. Enzyme Microb. Technol. 13, 964-968.

del Río, J.C., Gutiérrez, A., 2008. Rapid assessment of the lignin composition of lignocellulosic materials commonly used for paper pulp manufacturing by analytical pyrolysis. 18th Int Symp Anal Appl Pyr p. 99.

Schultz, A., Jonas, U., Hammer, E., Schauer, F., 2001. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. Appl. Environ. Microbiol. 67, 4377-4381.

Sipilä, J., Syrjänen, K., 1995. Synthesis and ^{13}C NMR spectroscopic characterization of six dimeric arylglycerol-β-aryl ether model compounds representative of syringyl and *p*-hydroxyphenyl structures in lignins. On the aldol reaction in β-ether preparation. Holzforschung 49, 325-331.

Suparno, O., Covington, A.D., Phillips, P.S., Evans, C.S., 2005. An innovative new application for waste phenolic compounds: Use of kraft lignin and naphthols in leather tanning. Res. Conserv. Recy. 45, 114-127.

Suurnakki, A.B., Buchert, J., Gronqvist, S., Mikkonen, H., Peltonen, S., Viikari, L., 2006. Bringing new properties to lignin rich fiber materials. In: Symp. on Appl. Mater. Proc. 244, 61–70.

Valencia, P., Marinez, L., 2005. Lignin in the restoration of the cultural heritage. Revista Colombiana de Física 37, 106-109.

Widsten P., Kandelbauer, A., 2008. Laccase applications in the forest products industry: A review. Enzyme Microb. Technol. 42(4), 293-307.

Witayakran, S., Ragauskas, A.J., 2009. Synthetic applications of laccase in green chemistry. Adv. Synth. Catal. 351(9), 1187-1209.

Witayakran, S. and Ragauskas, A.J., 2009. Modification of high-lignin softwood kraft pulp with laccase and amino acids. Enzyme Microb. Technol. 44(3), 176–181.

Yamaguchi, H., Maeda, Y., Sakata, I., 1994. Bonding among woody fibers by use of enzymatic phenol dehydrogenative polymerization: Mechanism of generation of bonding strength. Mokkuzai Gakkaishi 40, 185-190.

4

Enzymatic surface functionalisation of lignocellulosic materials with tannins for enhancing antibacterial properties

Grafting natural antibacterial phenols onto lignocellulosic materials is an environmentally-friendly way of imparting antibacterial properties to the substrates. In the present investigation, wood veneer and pulp were treated with tannins in the presence or absence of laccase. Treatments with hydrolysable tannins significantly improved the antibacterial resistance of veneers and paper made from tannin-treated pulp against a Gram-positive bacterium (*Staphylococcus aureus*) while a more modest protective effect was observed against a Gram-negative bacterium (*Escherichia coli*). Condensed tannin improved the antibacterial resistance against *S. aureus*, albeit less than hydrolysable tannin, but had little effect on *E. coli*. A cationic condensed tannin derivative bearing a quaternary amino group provided far better resistance to pulp against *S. aureus* and *E. coli* than the corresponding unmodified condensed tannin. These findings agree with the minimal inhibitory concentrations (MIC) of the tannins and their reactivities toward laccase as determined by O_2 consumption measurements. Due to a better retention of tannins via covalent bonding, treatments with laccase usually resulted in greater antibacterial effects than those without laccase. LC-MS investigations with monomeric tannin and lignin model compounds showed that covalent bonding of tannin to lignin via radical coupling occurred in the presence of laccase.

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4.1 Introduction

Tannins are natural polyphenols widely distributed in woody and herbaceous plants which can be divided into two distinct groups: condensed tannins based on polymeric flavonoids (proanthocyanidins) such as catechin and gallocatechin, and hydrolysable tannins which comprise mainly gallotannins and ellagitannins [1]. Gallotannins and ellagitannins are esters of gallic acid or oxidised gallic acid (hexahydroxydiphenic acid) and a polyol (usually glucose), respectively. Tannins can be extracted with water from different plant parts including wood, bark, galls, leaves, and roots. The main source of commercial condensed tannins is wattle (mimosa-acacia) and quebracho bark. Tannic acid (TA) is a commercial hydrolysable gallotannin product extracted from the galls and leaves of different tree species such as sumac and Aleppo oak, while chestnut tannin is predominantly of ellagitannin type and is extracted from wood. Hydrolysable and especially condensed tannins are extracted in large amounts for the leather tanning industry. Cationic tannin, which is produced from mimosa tannin, is used as a flocculant in wastewater treatment.

Tannins are known for their antimicrobial properties. The toxicity of tannins to various bacteria has been demonstrated in several studies [1-3]. The antibacterial effects of tannins may be largely due to their *o*-diphenol groups allowing them to act as iron chelators, thus depriving microorganisms of this essential element [1]. The hydroxyl groups may also play an important role in the molecule's ability to permeate the cell walls of bacteria [4]. According to Chung et al. [5], the ester linkage between gallic acid and glucose is important for the antibacterial properties of gallotannins; they found TA to strongly inhibit Gram-positive and Gram-negative bacteria while neither gallic acid nor ellagic acid showed this effect. In fact, tannins are often far more toxic to microorganisms than monomeric compounds such as gallic acid or catechin related to them but this cannot be generalised for all microorganisms [1].

Antimicrobial phenols can be potentially applied to lignocellulosic materials to prevent biodeterioration. However, they should preferably be immobilised to prevent their loss by leaching out when the substrates are exposed to water. Naturally, the mechanism of action should in this case not be dependent on phenol mobility. One method of phenol immobilisation is to graft them to polymer surfaces by using a phenol-oxidising enzyme such as laccase. In the case of lignocellulosic substrates, grafting is achieved by coupling of phenoxy radicals formed in lignin and the phenolic compounds to be grafted by laccase-assisted oxidation. Recently, we [6] and other groups [7,8] have reported that lignocellulosic substrates treated with simple phenols or their oligomeric coupling products in the presence of

laccase show improved antibacterial resistance toward Gram-positive and Gram-negative bacteria. Schröder et al. [6] showed that laccase-assisted modification of flax fibres with ferulic acid or hydroquinone monomers improved their antibacterial activity toward the Gram-positive *Bacillus subtilis* and Gram-negative *Escherichia coli* (*E. coli*) bacteria. In an investigation by Elegir et al. [7], the antibacterial properties of unbleached kraft paper toward the Gram-positive *Staphylococcus aureus* (*S. aureus*) and *E. coli* were boosted by their laccase-assisted modification with monomeric natural phenols such as caffeic acid or isoeugenol or their oligomeric coupling products. Interestingly, the antibacterial effects of the oligomeric phenols produced by laccase pretreatment of the monomeric phenols were far superior to those of the monomers, possibly because of higher radical stability arising from greater electron delocalisation and the branched structure of the oligomers. Further, Hossain et al. [8] reported that antibacterial properties could be imparted to wool fibres by laccase-mediated grafting of lauryl gallate.

A major problem regarding the commercialisation of the above-discussed methods is the lack of availability of suitable monomeric and antibacterial phenols in large amounts and at a reasonable cost while the possible need for their oligomerisation prior to application represents a further complication. Tannins have none of these drawbacks as they are oligomeric/polymeric, readily available in industrially relevant amounts and at a moderate cost, and possess a large number of antibacterial *o*-diphenol moieties. The goal of the present investigation was to study the laccase-assisted grafting of different tannins to solid wood and kraft pulp and their effectiveness for imparting antibacterial properties to these lignocellulosic materials.

4.2 Materials and methods

4.2.1Tannins and other chemicals

The tannin powders used in the present investigation are listed in Table 1 together with their actual tannin content according to the suppliers. TA was purchased from Carl Roth, Karlsruhe, Germany and the other tannins were provided by Christian D. Markmann GmbH, Hamburg, Germany. The tannin doses in this work are based on the total dry matter contents unless otherwise indicated. Microbiology reagents were purchased from Carl Roth Chemicals; only nutrient agar and nutrient broth were purchased from Merck, Germany. The rest of the chemicals were obtained from Sigma-Aldrich, Germany.

Table 1. Tannins used in the present investigation

Tannin	Classification	Tannin content, % of total solids[1]
Tannic acid (TA)	Hydrolysable gallotannin	100
Chestnut tannin (CH)	Hydrolysable ellagitannin	80 ± 1
Mimosa tannin (MT)	Condensed tannin	76.8 ± 2.0
Quebracho tannin (QT)	Condensed tannin	100
Cationic tannin (CT)	Mimosa tannin derivative	n/a

[1]According to suppliers' information

4.2.2 Laccase

The liquid Novozym 51003 laccase (*Myceliophtora thermophila*) preparation was supplied by Novozymes, Nieder-Olm, Germany. The activity is expressed in nanokatals (nkat) and was determined spectrophotometrically against ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at 30°C according to Niku-Paavola et al. [9].

4.2.3 Stock cultures

The microbial strains (*S. aureus* and *E. coli*) were obtained from the stock culture collection at Graz University of Technology. The cultures, maintained frozen at -80°C, were subcultured onto standard nutrient agar of the following composition (g l^{-1}): peptone, 15.0; yeast extract, 3.0; NaCl, 6.0; and D (+) glucose, 1.0. Cultures on nutrient agar plates were stored at 4°C and subcultured monthly.

4.2.4 Treatment of wood veneers with tannins and laccase

Norway spruce (*Picea abies*) veneers (20 mm x 10 mm x 1 mm) were immersed in an aqueous succinate buffer (pH 3.9) or phosphate (pH 5.8 or 6.6) buffer solution (25 mM) containing dissolved tannin (20 %, or 0.6 mmol g^{-1} based on the molecular weight of catechin, on dry wood basis) at 40 °C in a 100 ml glass beaker at a consistency of 10 %. After that, laccase (300 nkat g^{-1} on dry wood) was added and the mixture shaken at 200 rpm for 6h; the bottles were opened and thoroughly shaken by hand every hour. After reaction, the veneers were thoroughly washed by shaking in 100 ml of water (80 °C) for 15 min, and this procedure was repeated ten times with fresh water. After the last washing, the veneers were allowed to air-dry at 20 °C and 65 % RH. The colour change resulting from the treatment was recorded. Only distilled water was used in the work. The veneers for XPS studies were pre-extracted

with acetone in a Soxhlet apparatus overnight to remove interfering lipophilic wood extractives.

4.2.5 Treatment of kraft pulp with tannin and laccase and handsheet preparation

The temperature and pH of a 1.3% suspension of an industrial (Mondi Frantschach, Austria) never-dried and an unrefined softwood kraft pulp (kappa 41), dosed with various tannins and laccase mediator chemicals, were gradually altered over 30 minutes to give the required experimental conditions. The mixture was stirred at 440 rpm with a mechanical stirrer and the pH was adjusted with dilute sulphuric acid and sodium hydroxide solutions. After 30 minutes (when required), the mixture was dosed with laccase at 100 nkat g^{-1} fibre. The temperature of the mixture was controlled thermostatically at the required level and the mixture was stirred at 550 rpm. After a further 60 minutes, the contents of the beaker were diluted to 10 l and 75 g m^{-2} paper handsheets were produced by a standard method.

4.2.6 Colour measurements

The colour measurements of spruce veneers were carried out with a Gretagmacbeth ColourEye colorimeter using the CIELAB measurement system with a D65 light source and a 10^{o} angle. Tannin pickup on the veneer surfaces was determined semi-quantitatively by measuring the total difference in colour (ΔE^*) of the samples. Colour was measured once on either side of each veneer. The treatments and measurements were performed in triplicate using 20 veneers / experimental point, giving the 2 x 20 x 3 = 120 as the total number of measurements for each experimental point. The mean values were compared to the mean value obtained from a reference consisting of 20 untreated veneers.

4.2.7 Oxygen consumption measurements

The O_2 consumption measurements were performed with an YSI 5100 dissolved oxygen meter equipped with a self-stirring O_2 probe. Tannin, gallic acid, catechin, or kraft pulp was dissolved/dispersed in buffer solution (pH 6) in a 100 ml flask, the probe was introduced into the flask and the flask, full to the rim, sealed with parafilm. The mixture was allowed to equilibrate at 30°C to reach a stable O_2 reading after which laccase (500 nkat g^{-1}) was added to start the reaction. The oxygen uptake was recorded after 1 h.

4.2.8 XPS spectroscopic analyses of veneer surfaces

X-ray photoelectron spectroscopic (XPS) measurements on veneers were performed with a multiprobe UHV surface analysis system from Omicron Nanotechnology. Equipment and parameters: X-ray source: DAR400 aluminium-anode; quartz-crystal monochromator XM 500; X-ray excitation energy: 1486.70 eV (Al Kα1-line); monochromated X-ray line width (FWHM): 0.3 eV; energy analyser: hemispherical analyser, type EA 125; signal-detection with pulse-counting channeltron (five channels for count-rate enhancement); total energy resolution (x-ray source + hemispherical analyser): 0.45 eV for detailed scans at 10 eV pass-energy (+ lifetime broadening of the electronic state); charge neutralisation for non-conducting samples: electron emission gun for low-energy electrons (0.1-500 eV): FG 15/40 (SPECS). The analysed surface area was ~ 1 mm^2; five spots / sample were analysed and the results averaged.

4.2.9 Coupling of catechin and gallic acid to lignin model molecules

The reaction mixture contained catechin or gallic acid (4.0 mM), one of the other seven phenolic molecules shown in Fig. 1 (5.0 mM), and 13.4 nkat ml^{-1} laccase in 50 mM ammonium acetate buffer (pH 4.5). Reactions were carried out at 37ºC while shaking at 700 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg, Germany) for 1.5 h when guaiacol was used as lignin model monomer and for 4 h for the rest of the phenolic molecules. The coupling products were analysed by High-Performance Liquid Chromatography – Mass Spectroscopy (HPLC-MS).

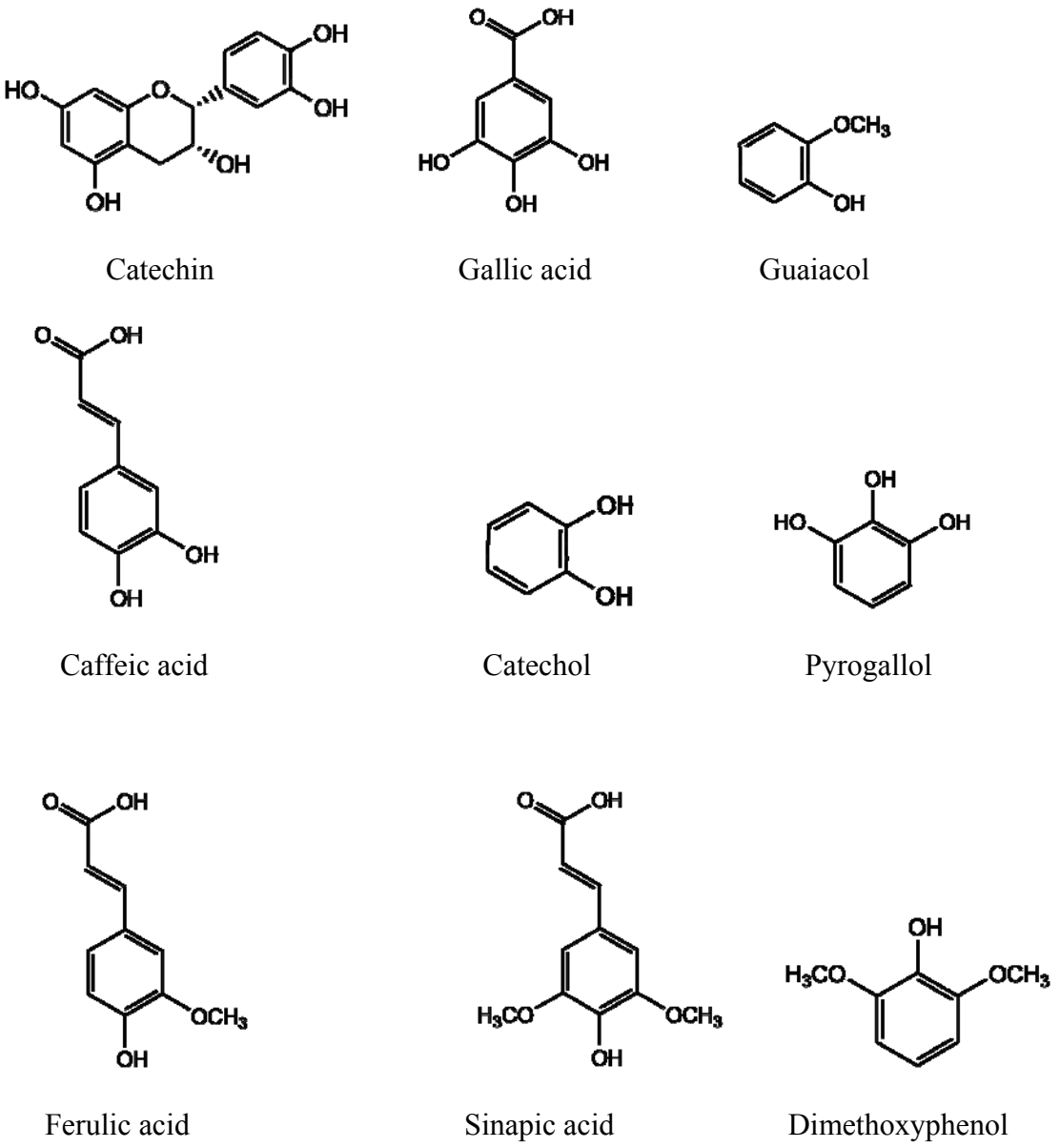


Figure 1. Catechin, gallic acid and lignin model molecules used in coupling reactions

4.2.10 HPLC-MS analysis of catechin/gallic acid-lignin model reaction products

To the incubation mixtures an equal volume of ice cold methanol was added to precipitate protein. The mixture was centrifuged at 0 ºC for 15 minutes at 14,000 g and 600 µl aliquots were transferred into vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler and a photodiode array detector (PDA-100) while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 x 4.6 mm with precolumn, Knauer GmbH, Berlin, Germany) using a linear gradient of acetonitrile (solvent B) and 0.1 %

formic acid (solvent A) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30°C. The gradient was set as shown in Table 2.

Table 2. Gradient set up for HPLC analysis

Time (min)	Initial	5	20	21	35	35.1	45 (end)
Solvent A, %	80	80	25	10	10	80	80
Solvent B, %	20	20	75	90	90	20	20

The mass spectra (MS) were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with electrospray ionisation (ESI) coupled to the Dionex HPLC-UVD-system as described above and initial separation of coupling products was carried out using the same protocol. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 10 l min^{-1} with a temperature of 350°C, and the nebuliser was set to 0.345 MPa. Maximum accumulation time was fixed to 300 ms and trap loading was controlled by the instrument with an ICC of 30,000.

4.2.11 Determination of antibacterial activity of tannin-treated veneers and paper handsheets

A modified procedure of AATCC Test Method 100-2004 was used to assess the antibacterial activity of veneer and paper handsheets. All bacterial pre-inoculum cultures were grown overnight in 100 ml buffered Erlenmeyer flasks containing 15 ml nutrient broth while shaking at 140 rpm at 30°C. The veneers and paper samples (2.3 × 2.3 cm) were aseptically placed in petri dishes containing sterile support media (NaCl 8.5 g; agar 15.0 g; distilled water 1000 ml). The pre-inocolum was diluted with nutrient broth and a 50 µl culture used to inoculate the veneers and paper sheets. The bacterial culture suspension was added dropwise so that several micro-droplets were approximately evenly distributed on the surface of the test material. The petri dishes containing the test samples were incubated overnight at 37 °C in a humidified chamber to prevent drying. To recover the bacteria on a veneer or paper sample, the sample was aseptically removed, placed in 10 ml sterile saline solution (0.85 % w/v), and shaken on an orbital shaker for 1 h at 140 rpm to resuspend the bacteria. The suspension was plated onto agar plates overnight at 37 °C and the viable counts determined. Reduction in viable counts with respect to the relevant controls and initial viable count was used to determine the efficacy of the tannin treatments. In the present work the percentage reduction in viable bacterial counts after 24 h as a result of a treatment with respect to the untreated control was calculated using the following formula [10]:

% reduction=$[(N_c-N_t)/N_c]\times 100$

where N_t is the number of bacteria cells recovered from the tannin-treated sample, and N_c is the number of bacteria recovered from the untreated or control sample.

4.3 Results and discussion

4.3.1 Oxygen consumption of laccase substrates

The reactivity of tannins, monomeric phenols related to tannins and kraft pulp toward laccase was assessed by measuring the O_2 consumption during laccase treatment which is an indicator of laccase-catalysed oxidative phenoxy radical formation in the substrate [11]. The likelihood of radical coupling reactions occurring between tannins and lignin in the substrate (wood or pulp) depends, however, also on the extent of self-coupling of the (tannin-based) phenoxy radicals and the number of available cross-linking sites in the aromatic units of tannins and the lignin. As shown by Fig. 2, the hydrolysable tannins (TA, and CH) displayed higher laccase reactivity than the condensed tannins or their cationic derivative (MT, QT, and CT), the gallotannin-type TA being the most reactive of them. The higher reactivity of MT as compared to QT may be due to the higher pyrogallol/catechol B-ring ratio of MT [12]; pyrogallol was also found to be more reactive than catechol (Fig. 2). The kraft pulp showed very low reactivity compared to the tannins, which is due to the low content of reactive lignin in the fibre surfaces accessible to laccase.

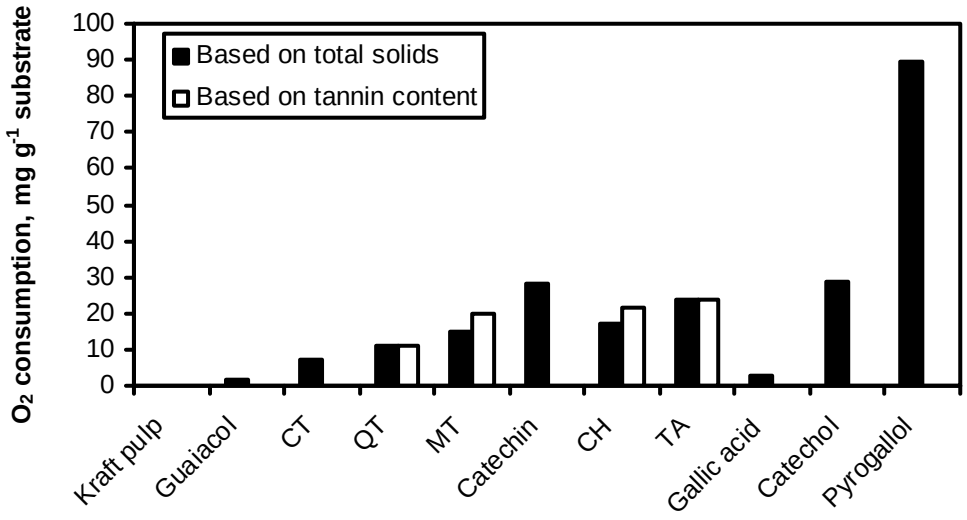


Figure 2. Oxygen uptake of kraft pulp, tannins and monomeric phenols during laccase treatment. For tannin contents, see Table 1

4.3.2 Retention of tannin in veneers

Tannin retention in veneers treated with TA or MT, as estimated by semi-quantitative colour measurements, is shown in Fig. 3. An increased retention, after leaching with water, of brown colour on treatment with tannin and laccase as compared to treatment with tannin alone, suggests covalent bonding of tannin to wood. In the case of TA, treatments at different pH levels produced a slight colour shift towards brown, indicating that not all non-covalently bonded TA had been washed off. Upon treatment with TA and laccase the brown colour intensified with an increase in pH. At the lowest pH level, however, the change was below that obtained with laccase alone. The colour change obtained with laccase alone was due to leach-resistant laccase retained on the wood surface and/or chromophoric groups formed upon laccase-catalysed oxidation of the wood surface. A comparison of the MT treatments performed with and without laccase shows that non-covalently bonded MT was more difficult to wash off than non-covalently bonded TA. The increase in brown colour was only slight at pH 3.9 but substantial at pH 6.6. The treatments with TA and MT cannot be compared directly because of their slightly dissimilar colours and because their laccase-mediated oxidation may have affected their colour to different extents. In both cases, however, the results indicate that the tannins were covalently bonded to wood in the presence of laccase.

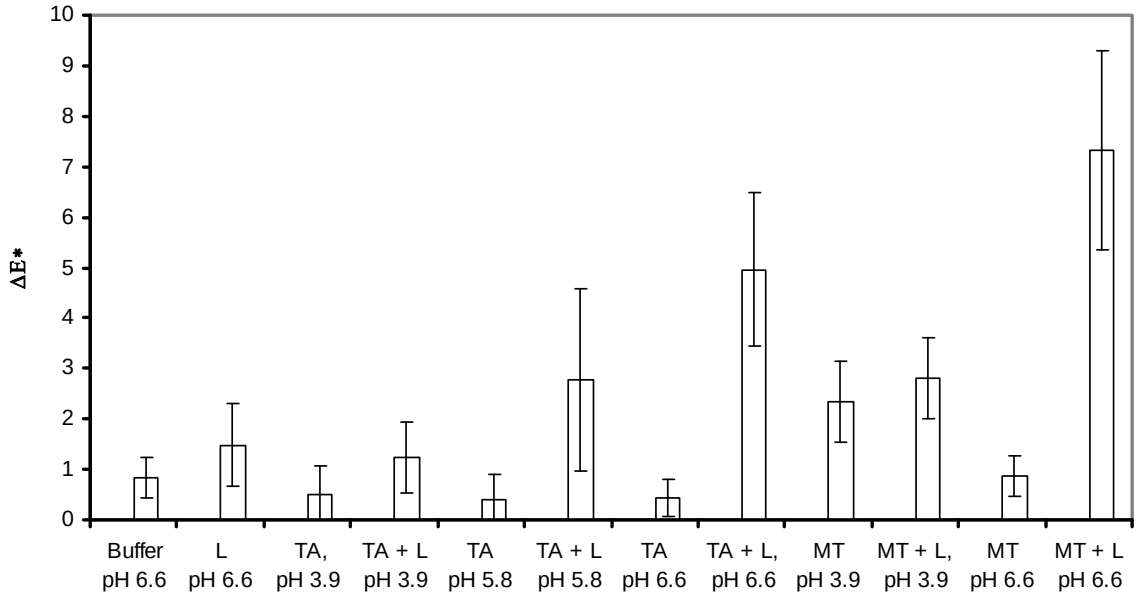


Figure 3. Colour change on treatment of veneers with TA or MT with or without laccase (L). Error bars indicate the standard deviation

4.3.3 XPS analysis of wood surfaces

As shown by XPS analysis (Fig. 4), the tannin treatment of veneers in the presence of laccase lowered the O/C ratio of the veneer surface compared to veneers that were untreated or treated with laccase only (lignocellulosic surfaces). The low theoretical O/C ratio of MT (~ 0.40-0.47 depending on the flavanoid unit types present) compared to that of the two lignocellulosic wood surfaces (> 0.5) and the observed O/C ratio (~ 0.38) of the veneer treated with MT and laccase suggest the presence of a significant amount of MT at the veneer surface. In the case of TA the results are less straightforward. Since the theoretical O/C ratio of pentagalloyl glucose (the main component of TA) is 0.63, the treatment with TA would be expected to give an O/C ratio which is not only higher than that of the MT-treated veneer but also higher than that of the lignocellulosic surfaces. The observed value (~ 0.48), however, was slightly below those of the lignocellulosic veneers, which at the moment defies explanation unless it was due to the presence of laccase at the surface. The percentage of nitrogen increased with all the treatments, indicating that some laccase was retained on the veneer surface, and was at its highest with the veneer treated with TA and laccase.

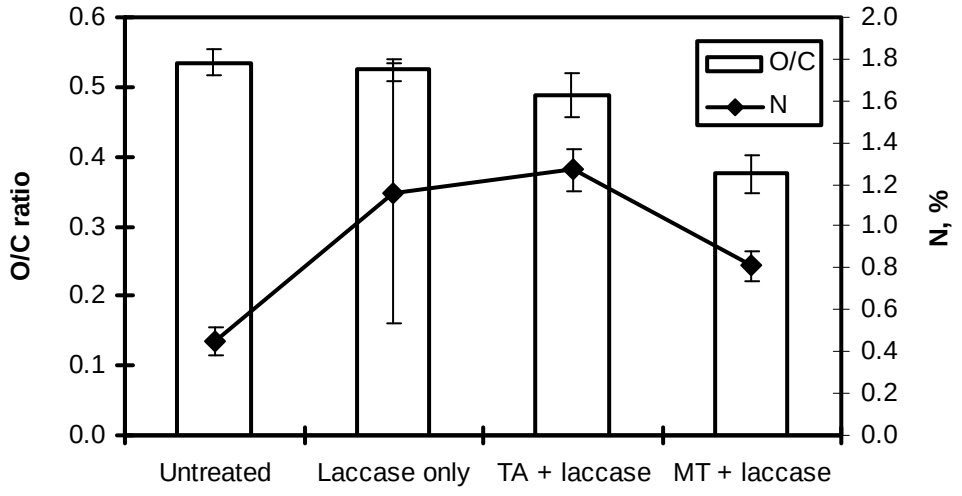


Figure 4. O/C ratio and nitrogen content of veneer surfaces

4.3.4 HPLC-MS of laccase-catalysed coupling products

In vitro coupling of catechin to phenolic molecules as model structural elements of lignin was used to mimic the grafting of flavonoid-based tannins (QT, CT, MT) onto lignin moieties of veneers while coupling of gallic acid provided the basis for the grafting of hydrolysable tannins (TA and CH) which are based on galloyl esters of glucose. LC-MS of laccase-mediated coupling products of lignin monomers to catechin showed dominant signals at *m/z*

413.2, t_R=10.0 min (guaiacol); *m/z* 469.2, t_R=5.3 min (caffeic acid); *m/z* 399.2, t_R=4.6 min (catechol); *m/z* 415.1, t_R=2.5 min (pyrogallol); *m/z* 483.1, t_R=12.0 min (ferulic acid); *m/z* 513.1, t_R=11.7 min (sinapic acid), and *m/z* 443.1, t_R=9.9 min (dimethoxyphenol) for the $[M+H]^+$ions (Fig 5). These measured molecular weights suggest 1:1 coupling products. Additional $[M+H]^+$ ion signals were observed at *m/z* 579.1, t_R=5.1 min and *m/z* 865.5, t_R=4.3 min, suggesting homomolecular coupling of catechin to form dimers and trimers. Similarly 1:1 coupling products were observed when gallic acid was coupled onto lignin molecules (Fig. 6). According to existing knowledge the hydroxyl group on the benzene ring is *ortho* or *para* directing [13-15] and molecules with a free C-5 position usually cross-couple through 5-5 linkages due to stability of C-C bonds [16]. However, lignin molecules with syringyl or sinapyl units usually couple through 4-O-5 linkages to molecules with a free C5 position. It is already known that coupling between preformed lignin oligomers results in units bonded through 5-5 and 4-O-5 linkages [17]. Considering these facts, structures for the coupling products were elaborated (Figs 7 and 8). Interestingly, additional β-O-4 coupling products (Fig. 6 H, I, J) corresponding to products P12, P14 and P16 (Fig. 8) were observed when gallic acid was coupled onto the unsaturated phenolic molecules caffeic acid, ferulic acid and sinapic acid respectively. Although these substructures are known to be the most abundant in lignin [18], they were not observed with catechin. This is probably because the position 5 is free in catechin molecules and 5-5 and 4-O-5 bonds are favoured. In addition, it has been observed that the 4-O-5 and 5-5 bonds have a lower heat of formation than the *β*-O-4 bonds [19]. Although *β*-O-4 linkages are more abundant in lignin, dimerisation and lignin synthesis are substantially different processes [20]. Preformed lignin oligomers usually couple through 5-5 and 4-O-5 linkages [17], while *β*-O-4 bonds are favoured in-vivo, which probably explains why in-vitro and in-vivo experiments give different lignin substructure distributions.

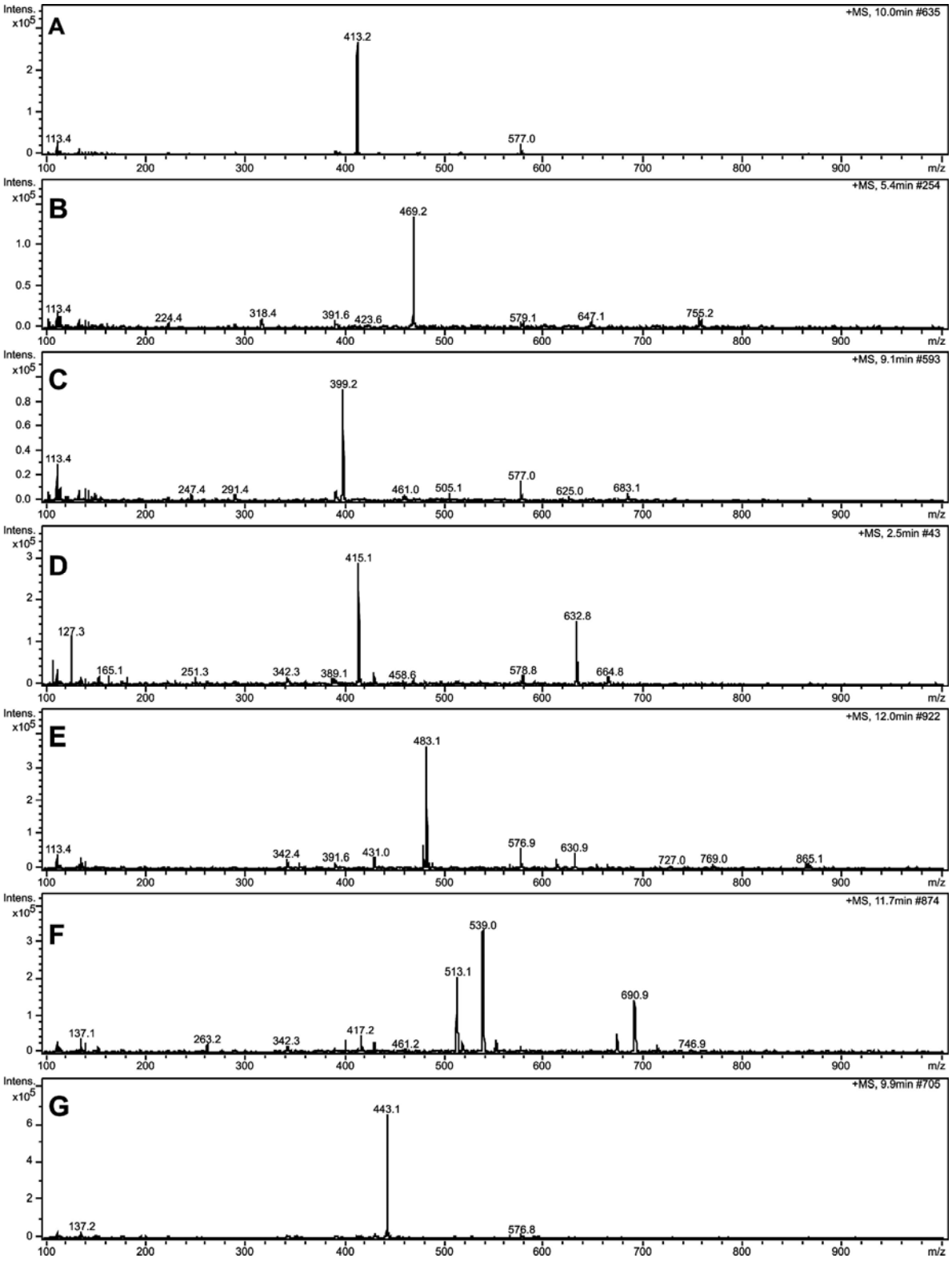


Figure 5. Mass spectra of laccase-mediated coupling products between catechin and lignin model molecules: (A) catechin+guaiacol; (B) catechin+caffeic acid; (C) catechin+catechol; (D) catechin+pyrogallol; (E) catechin+ferulic acid; (F) catechin+sinapic acid; (G) catechin+dimethoxyphenol

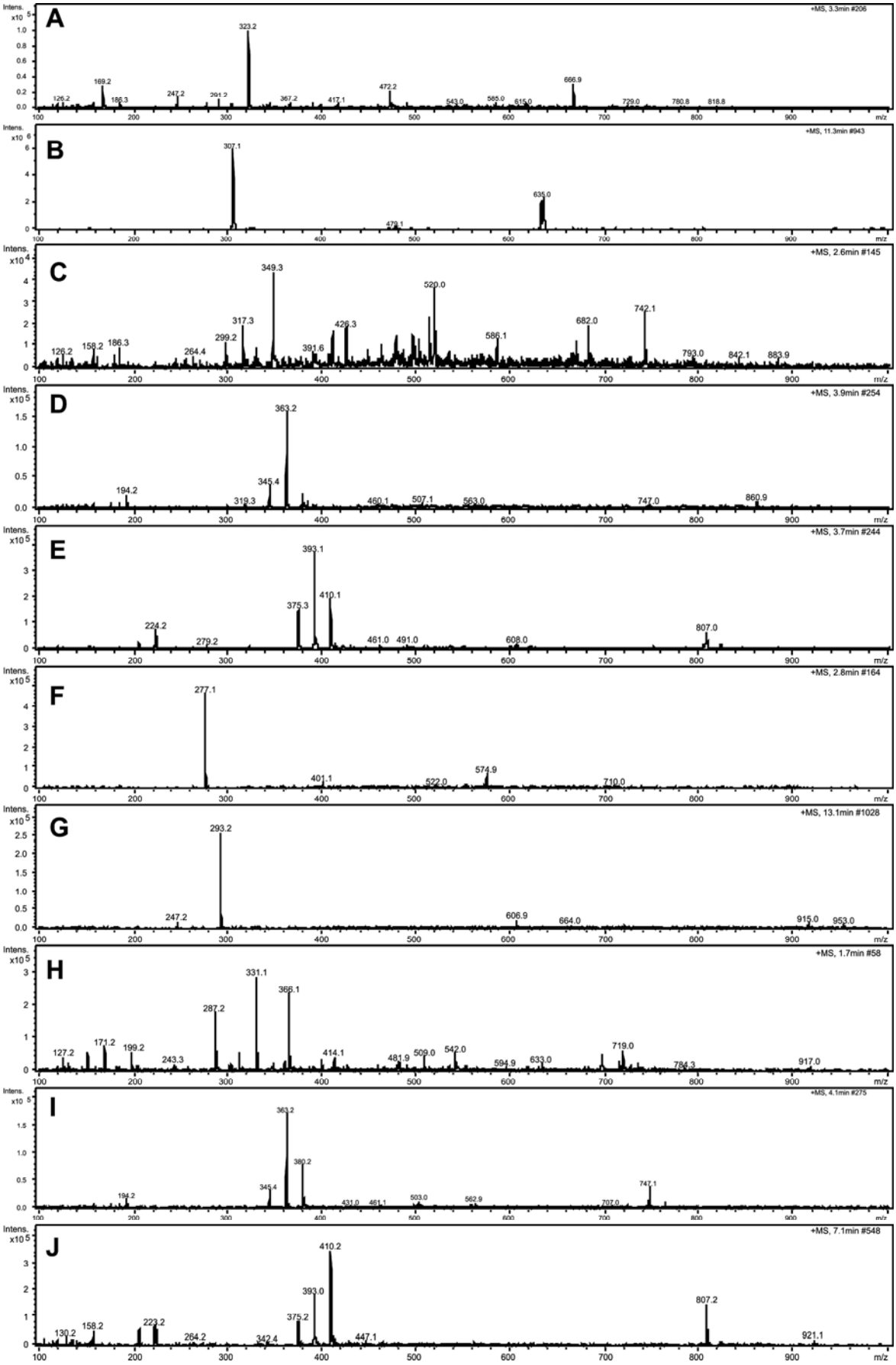
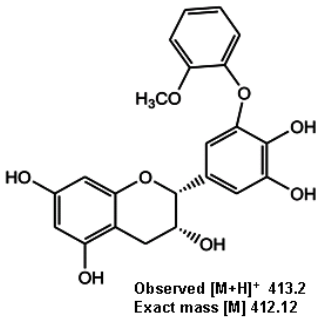
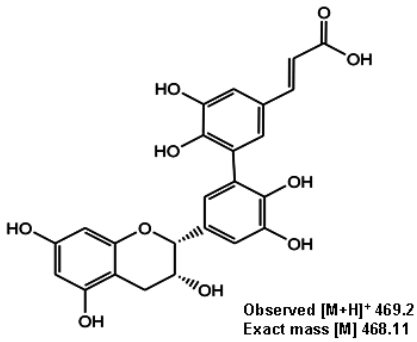


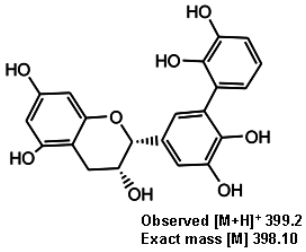
Figure 6. Mass spectra of laccase-mediated coupling of gallic acid to DMP (A and B), caffeic acid (C and H), ferulic acid (D and I), sinapic acid (E and J), catechol (F) and pyrogallol (G).



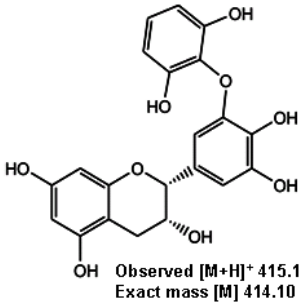
Catechin + guaiacol (P1)



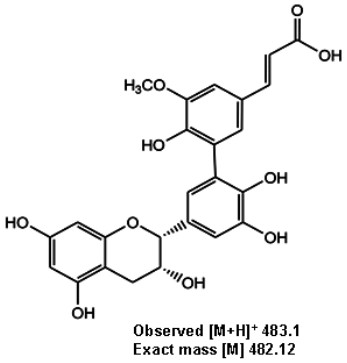
Catechin + caffeic acid (P2)



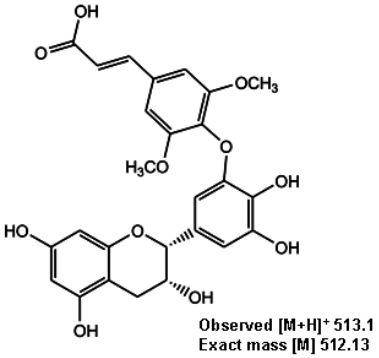
Catechin + catechol (P3)



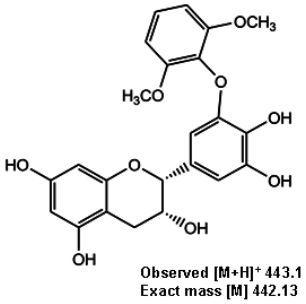
Catechin + pyrogallol (P4)



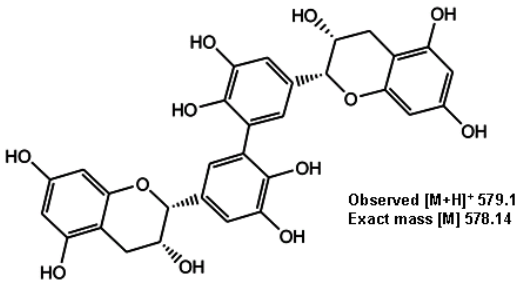
Catechin + ferulic acid (P5)



Catechin + sinapic acid (P6)



Catechin + dimethoxyphenol (P7)



Catechin + catechin (P8)

Figure 7. Proposed coupling products of catechin and lignin model phenols

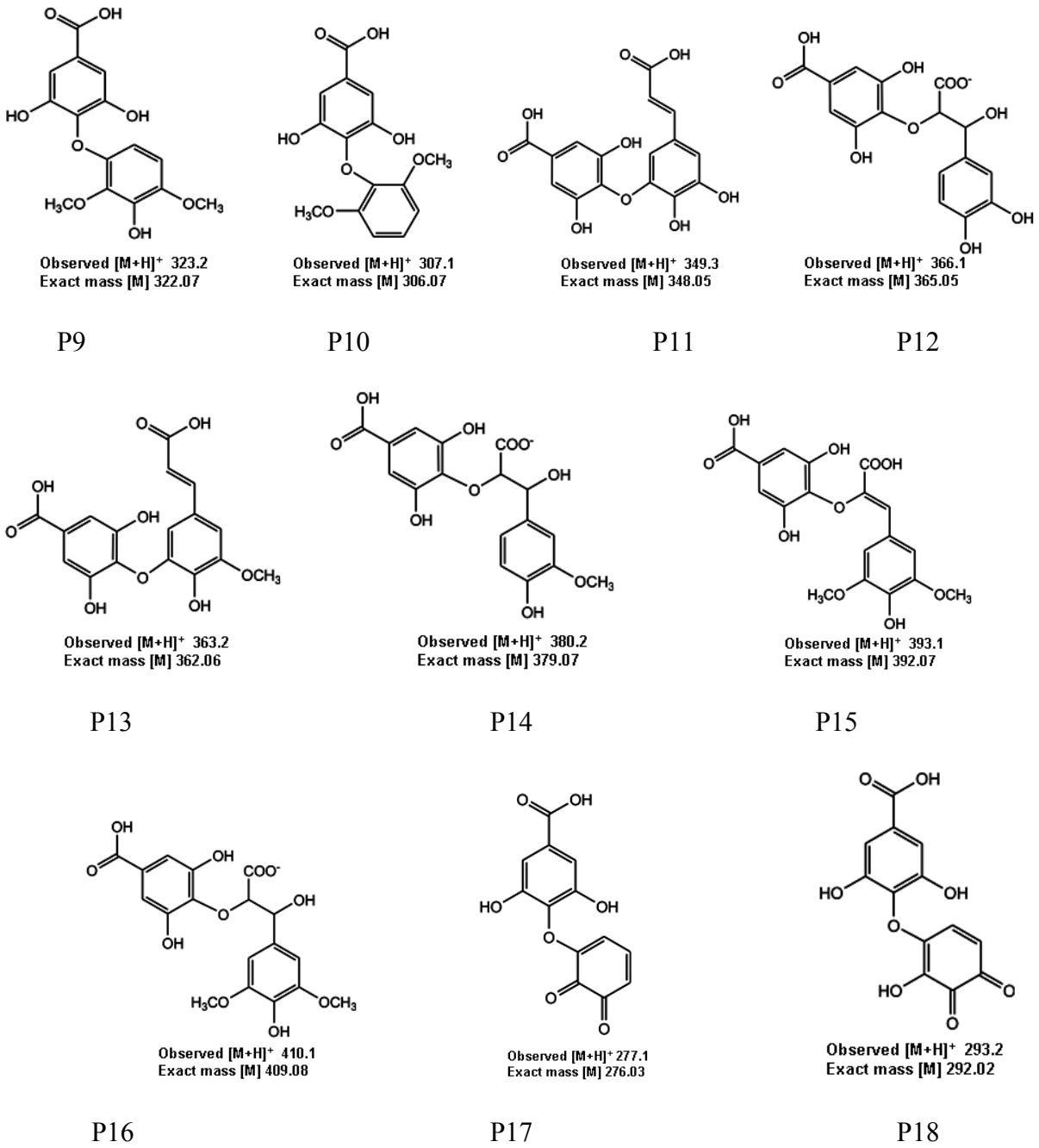


Figure 8. Proposed coupling structures of gallic acid to dimethoxyphenol (P9 and P10), caffeic acid (P11 and P12), ferulic acid (P13 and P14), sinapic acid (P15 and P16), catechol (P17) and pyrogallol (P18).

4.3.5 Antibacterial tests

The minimum inhibition concentration (MIC) of tannins for a Gram-positive bacterium (*S. aureus*) and a Gram-negative bacterium (*E. coli*) show that inhibition of *S. aureus* with the gallotannin TA was four times as high as with the ellagitannin CH and at least eight times as high as with the condensed tannins or the condensed tannin model compound catechin (Fig.

9). The higher tannin content of the TA preparation (Table 1) can only partly account for this difference unless the non-tannin components actually promoted bacterial growth. TA also had a much lower MIC than gallic acid which represents the phenolic moiety of major TA constituents (galloyl esters). CT was equally effective than TA and thus far more effective than the tannin from which it is derived (MT). This could be due to its positive charge, which may contribute to the antibacterial properties and/or improve the adsorption of the tannin onto an anionic substrate surface. At higher concentrations, most of the tannins also inhibited *E. coli*, CT being particularly effective. Mori et al. [4] found flavonoids structurally related to condensed tannins to show antibacterial activity against *S. aureus* and *Proteus vulgaris* (Gram-negative). The activity significantly depended on the number of phenolic hydroxyl groups, flavonoids possessing a 3-OH group and a 3,4,5-trihydroxy (pyrogallol) B-ring being more active than those with fewer phenolic hydroxyls while flavonoids with masked hydroxyl groups were inactive. The authors speculated that the hydrophilicity conferred to the molecules by the hydroxyl groups is critical for their ability to pass through bacterial cell walls.

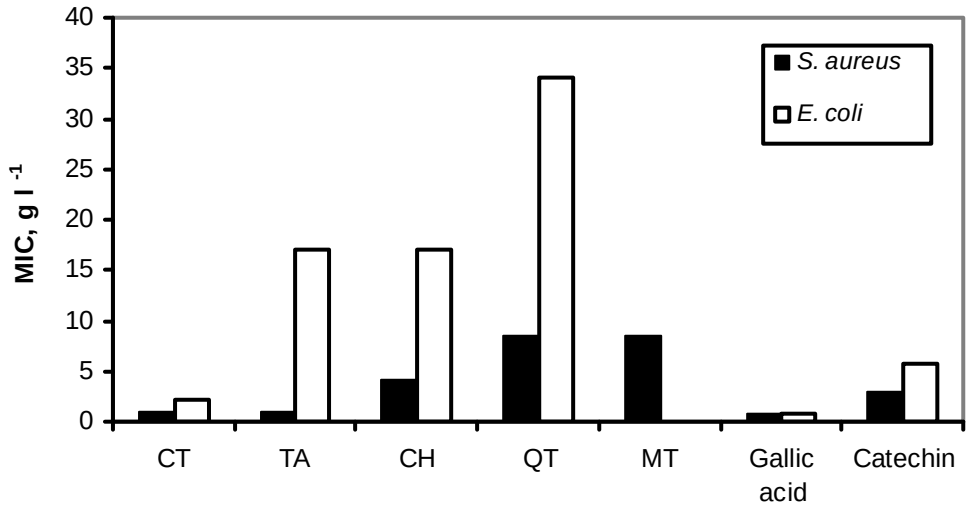


Figure 9. MIC of different tannins and monomeric phenols on *S. aureus* and *E. coli*. No inhibition of *E. coli* was observed with MT

To have a bacteriostatic or bacteriocidal effect, a treatment should give a 1 log (90%) or 2 log (99%) CFU reduction 24h after inoculation, respectively [JIS Z 2801:2000; 7].

The antibacterial effects of TA and MT on veneers are shown in Fig. 10. The treatments with TA and laccase had a bacteriocidal effect on *S. aureus* and reduced the growth of *E. coli* by ~ 50%. TA without laccase reduced the CFUs of *S. aureus* by up to 89%, showing the

effectiveness of even a slight amount of (non-covalently bound) TA. MT exhibited a bacteriostatic effect on *S. aureus* and was also somewhat less effective against *E. coli* than TA. This result is in agreement with those of Nishizawa et al. [21] regarding the toxicity of several gallo- and ellagitannins against *S. aureus* and *E. coli* as well as the MICs determined for TA and MT in the present study (Fig. 9).

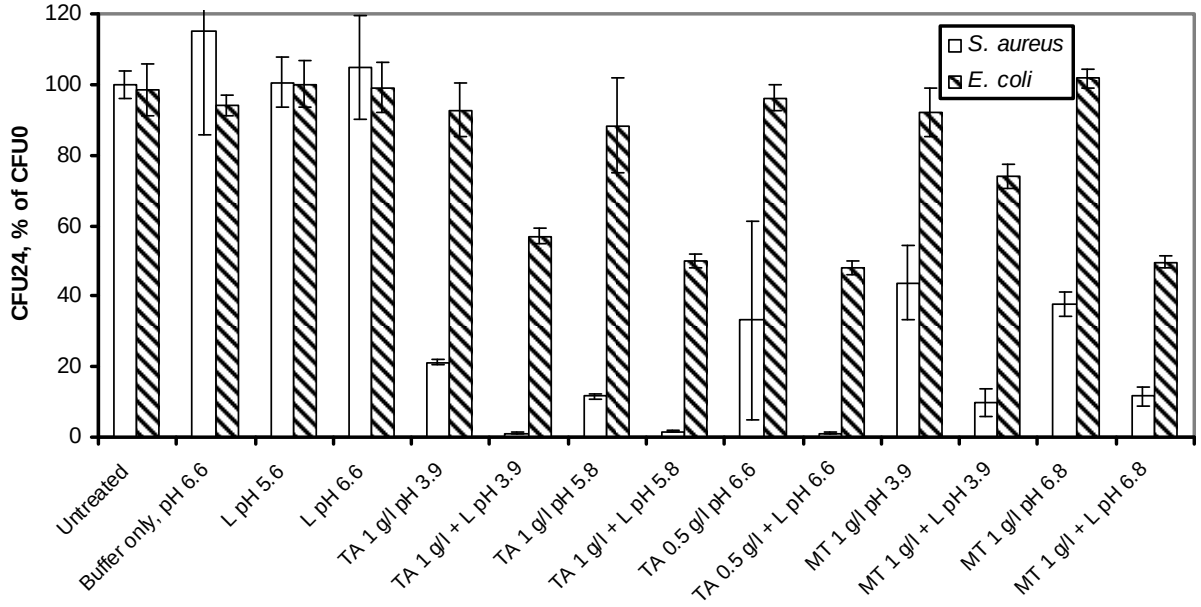


Figure 10. Colony-forming units (CFU24) as a percentage of the inoculated CFUs (CFU0) on wood veneers pre-treated with tannins with and without laccase (L). Error bars indicate the standard deviation

As for the paper samples, the hydrolysable tannins (TA and CH) improved the antibacterial resistance of the handsheets (Fig. 11), TA being more effective than CH against both *S. aureus* and *E. coli*. The condensed tannin, MT, were comparable to those of CH while CT, the cationic derivative of MT, mostly outperformed MT and produced similar reductions in CFU as TA when applied with laccase with or without the syringaldehyde (SA) laccase mediator. Compared to untreated pulp, the treatments with tannin and laccase without the mediator gave greater CFU reductions for *S. aureus* than the corresponding treatments with tannin alone. However, the opposite was true for *E. coli*, CT being an exception. The application of both laccase and mediator with tannin had a variable effect on CFU when compared to the corresponding non-mediated laccase/tannin treatments. The laccase mediator, SA, improved the effectiveness of some tannin-laccase treatments but in some cases detracted from the antibacterial properties. These results suggest that the effects of laccase, such as covalent bonding of tannins to pulp or polymerisation of the tannin molecules via radical

coupling on the antibacterial properties of tannins, are in many cases beneficial, depending on the type of tannin and bacterium. Overall, the largest CFU reductions, bacteriocidal or near-bacteriocidal, were seen for TA and CT applied together with laccase with or without the mediator SA. In most cases, the antibacterial effects on *S. aureus* were greater than those on *E. coli*.

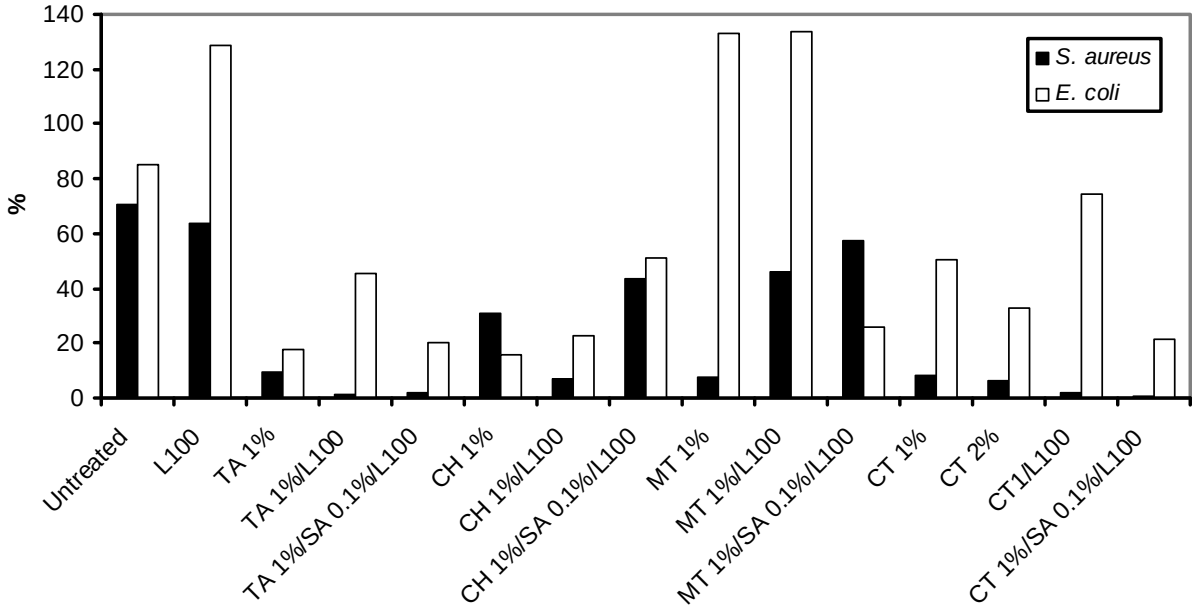


Figure 11. Colony-forming units (CFU24) as a percentage of the inoculated CFUs on paper handsheets pre-treated with different combinations of tannins, laccase, and laccase mediator. The bars show the mean values of duplicate experiments

4.4 Conclusions

- Hydrolysable tannins, particularly tannic acid, and cationic mimosa tannin significantly inhibit the growth of *S. aureus* on wood veneer and pulp and also show some antibacterial effect on *E. coli*, while condensed (mimosa) tannin imparts less resistance against these bacteria.
- Studies with tannin and lignin model compounds and the colour changes of wood samples on tannin treatment in the presence or absence of laccase suggest that grafting of tannin to wood lignin occurs when laccase is present.
- The growth inhibition of bacteria in veneers and most pulps is greater in the presence of laccase, improving tannin retention by its covalent bonding to the substrate.
- The effectiveness of the different tannins as antibacterial agents is in line with their minimum inhibitory concentrations (MIC) for *S. aureus* and *E. coli* and their reactivity

toward laccase. The MICs are lower for the tannins than for the tannin-related monomeric phenols.

- The results show that grafting of tannins onto lignocellulosic material is an environmentally-friendly way of boosting their antibacterial properties. Such materials could be used for packaging or during handling of foodstuffs and other sensitive goods.

Acknowledgements

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4.5 References

[1] Scalbert A. Antimicrobial properties of tannins. Phytochemistry 1991;30:3875-3883.

[2] Akiyama H, Fujii K, Yamasaki O, Oono T, Iwatsuki K. Antibacterial action of several tannins against *Staphylococcus aureus*. J Antimicrob Chemother 2001;48:487-491.

[3] Funatogawa K, Hayashi S, Shimomura H, Yoshida T, Hatano T, Ito H, Hirai Y. Antibacterial activity of hydrolyzable tannins derived from medicinal plants against *Helicobacter pylori*. Microbiol Immunol 2004;48:251-261.

[4] Mori A, Nishino C, Enoki N, Tawata S. Antibacterial activity and mode of action of plant flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*. Phytochemistry 1987;26:2231-2234.

[5] Chung K-T, Stevens SE, Lin W-F, Wei CI. Growth inhibition of selected food-borne bacteria by tannic acid, propyl gallate and related compounds. Lett Appl Microbiol 1993;17:29-32.

[6] Schröder M, Aichernig N, Gübitz GM, Kokol V. Enzymatic coating of lignocellulosic surfaces with polyphenols. Biotechnol J 2007;2:334-341.

[7] Elegir G, Kindl A, Sadocco P, Orlandi M. Development of antimicrobial cellulose packaging through laccase-mediated grafting of phenolic compounds. Enzyme Microbial Technol 2008;43:84-92.

[8] Hossain, KhMG, González MD, Lozano GR, Tzanov T. Multifunctional modification of wool using an enzymatic process in aqueous-organic media. J Biotechnol 2009;141:58-63.

[9] Niku-Paavola M-L, Karhunen E, Salola P, Raunio V. Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. Biochem J 1988;254:877-884.

[10] Jeong J-H, Byoun Y-S, Lee Y-S. Poly(styrene-alt-maleic anhydride)-4-aminophenol conjugate: synthesis and antibacterial activity. React Funct Polym 2002;50:257-263.

[11] Yaropolov AI, Skorobogatko OV, Vartanov SS, Varfolomeyev SD. Laccase: properties, catalytic mechanism and applicability. Appl Biochem Biotechnol 1994;49:257-280.

[12] Pizzi A. Advanced Wood Adhesives Technology. Marcel Dekker Inc., New York, 1994.

[13] Jonas U, Hammer E, Haupt ETK, Schauer F. Characterisation of coupling products formed by biotransformation of biphenyl and diphenyl ether by the white rot fungus *Pycnoporus cinnabarinus*. Arch Microbiol 2000;174:393-398.

[14] Schultz A, Jonas U, Hammer E, Schauer F. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. Appl Environ Microbiol 2001;67:4377-4381.

[15] Suparno O, Covington AD, Phillips PS, Evans CS. An innovative new application for waste phenolic compounds: Use of kraft lignin and naphthols in leather tanning. Res Conserv Recy 2005;45:114-127.

[16] del Río JC, Gutiérrez A. Rapid assessment of the lignin composition of lignocellulosic materials commonly used for paper pulp manufacturing by analytical pyrolysis. 18th Int Symp Anal Appl Pyr p. 237 (2008).

[17] Boerjan W, Ralph J, Baucher M. Lignin biosynthesis. Annu Rev Plant Biol 2003;54:519-546.

[18] Kandanarachchi PH, Autrey T, Franz JA. Model compound studies of the *β*-O-4 linkage in lignin: absolute rate expressions for *β*-scission of phenoxyl radical from 1-phenyl-2-phenoxyethanol-1-yl radical. J Org Chem 2002;67:7937-7945.

[19] Valencia P, Marinez L. Lignin in the restoration of the cultural heritage. Revista Colombiana de Física 2005;37:106-109.

[20] Adler E. Lignin chemistry - past present and future. Wood Sci Technol 1977;11:169-218.

[21] Nishizawa K, Nakata I, Kishida A, Ayer WA, Browne LM. Some biologically active tannins of *Nuphar variegatum*. Phytochemistry 1990;29:2491-2494.

5

Laccase-mediated wood surface functionalization (using phenolic amines as anchor groups)

Laccase-catalyzed grafting of functional molecules on wood surface presents an eco-friendly opportunity to improve wood properties. In a novel stepwise approach, functional molecules were bound to an enzymatically modified wood surface containing anchor groups. As anchor groups, phenolic amines were coupled to lignin moieties of wood using the 62 kDa *Trametes hirsuta* laccase. The coupling reaction to lignin was mimicked using the lignin model compounds 4-O-methyl guaiacylglycerol β-guaiacyl ether **A**, guaiacylglycerol β-guaiacyl ether **E**, dehydrodivanillyl alcohol type dibenzodioxocin **D** and syringylglycerol β - guaiacylether **G**. Using this approach it was possible to increase binding of the fungicides propiconazole and thiabendazole by 42 % and 58 %, respectively, when compared to coupling onto wood pieces which were oxidized by laccase only.

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5.1. Introduction

Enzymatic wood surface functionalization either for aesthetic purposes or for preservation of wood materials is a recent development which has drawn the interest of both researchers and relevant industries. Although there are established physical and chemical methods, enzymatic grafting represents an environmentally friendly alternative, reducing energy and chemical consumption and providing permanent functionalization due to covalent bonding.

Lignin, the natural adhesive in wood, is a three-dimensional hetero-polymer of coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, linked together in an irregular manner, which can be modified/degraded by oxidases such as lignin peroxidases, manganese peroxidases and laccases [1, 2, 3]. Among the enzymes able to oxidize lignin, laccases are preferred as they require only oxygen as co-substrate forming water as the only byproduct [4]. Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) are glycoproteins which catalyze the oxidation of phenols and aromatic or aliphatic amines to the corresponding reactive radical with concomitant reduction of molecular oxygen to water [5]. Upon laccase oxidation of phenolic lignin moieties on wood surfaces, reactive radical sites are introduced. It has been reported that enzymatically formed radicals on wood surfaces are unstable; up to 90 % were shown to be quenched within a few hours in laccase-treated thermo-mechanical pulp [2]. In the presence of suitable molecules further covalent binding can occur. Several authors have indicated the possibility of laccase-catalyzed bonding of low-molecular weight compounds such as syringic acid, vanillic acid, gallic acid, 4-hydroxybenzoic acid and nitroaromatic compounds to lignin or lignocellulosic materials [6-11].

In an effort to create stable anchor groups on wood surfaces, the laccase-mediated coupling of various aromatic amines to lignin moieties was investigated in the present study. This approach involves the enzymatic attachment of anchor groups (i.e., phenolic amines) to which functional molecules of interest can be bound either chemically, enzymatically or by enhanced adsorption. This involves a three-stage process in which laccase-oxidized wood forms radicals to which phenolic amines are grafted forming anchor groups for further functionalization.

5.2. Materials and methods

Chemicals

The lignin model compounds (Fig. 1) guaiacylglycerol β-guaiacyl ether **E** (erol), its 4-O-methyl derivative 1-(3,4-dimethoxyphenyl)-1-oxo-2-(2-methoxyphenoxy)-3-hydroxypropane

A (adlerol) and syringylglycerol β-guaiacylether **G** were synthesized following the procedure described by Sipilä and Syrjänen [12]. Dehydrodivanillyl alcohol type dibenzodioxocin **D** was synthesized as described in Karhunen *et al*. [13]. All other chemicals including tyramine, 3-hydroxytyramine, 4-O-methyldopamine, 3-(3,4-dihydroxyphenyl)-DL-alanine, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and 4-hydroxy-3-methoxybenzylamine were purchased from Sigma-Aldrich. Beech (*Fagus sylvatica*) wood samples were provided by Mitteramskogler GmbH, Austria.

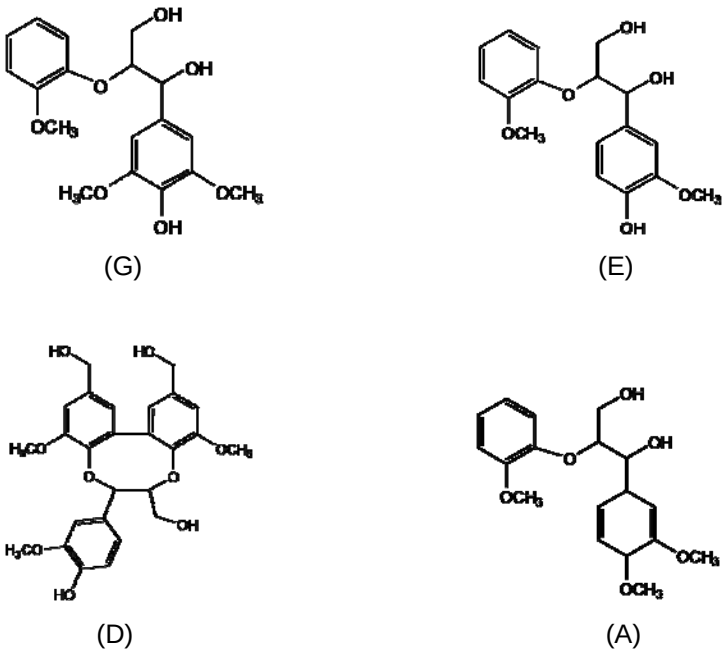


Fig. 1. Chemical structure of lignin model compounds. **G** syringylglycerol β-guaiacylether, **E** guaiacylglycerol β-guaiacyl ether (erol), **D** dehydrodivanillyl alcohol type dibenzodioxocin, **A** 1-(3,4-dimethoxyphenyl)-1-oxo-2-(2-methoxyphenoxy)-3-hydroxypropane (adlerol).

Laccases

The 62 kDa laccase from *Trametes hirsuta* was produced and purified as previously reported by Almansa et al. [14]. The activity of the laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε_{436} = 29,300 $M^{-1}cm^{-1}$) as substrate at 436 nm in 50 mM succinate buffer at pH 4.5 and 37 °C as described by Niku-Paavola et al. [15] with some modifications. Briefly, the reaction mixture contained 30 µl laccase, 350 µl ABTS (1 mM) and 50 mM succinate buffer pH 4.5 to make a final volume of 1.5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100 °C for 10 minutes. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 minutes.

Oxidation of lignin model substrates and phenolic amines

The ability of *T. hirsuta* laccase to oxidize four lignin model compounds (**A**, **E**, **D** and **G**) and six phenolic amines (Table 1, **1**-**6**) was investigated. To start the reaction, laccase with a total activity of 0.67 nkat ml^{-1} was added to a reaction mixture containing 0.1 mM lignin model compounds or phenolic amines in 50 mM succinate buffer pH 4.5. The reactions were monitored by UV/Vis-spectrophotometry in the wavelength scan mode from 900 nm – 200 nm at 3 min per cycle for a total of 18 min to identify the products. The kinetic studies were performed in triplicate and the data obtained fitted to a hyperbola by means of the Michaelis–Menten equation. The V_{max} values were then determined through non-linear regression analysis using the program OriginPro 7.0. The obtained V_{max} values were used to calculate the K_{cat} values.

Coupling of phenolic amines to lignin model compounds

The reaction mixture contained one of the phenolic amines and one of the lignin model compounds **A, D, E,** and **G** in the molar ratio of 1:2 (in order to increase the probability of coupling) and 0.67 nkat ml^{-1} laccase in 50 mM succinate buffer (pH 4.5). Reactions were carried out at 37 ºC while shaking at 700 rpm using a thermomixer. The lignin model compound was initially incubated with the laccase for 1.5 h; after addition of the phenolic amine, the reaction proceeded for further 1.5 h. The coupling products were analyzed by Thin Layer Chromatography (TLC) and HPLC.

Chromatographic analysis of reaction products

TLC separations were made on 0.2-mm-thick silica gel/TLC-cards with a 254 nm UV-fluorescent indicator (Sigma-Aldrich GmbH, Germany) using acetic acid and toluene (1:2, v/v) as solvent.

HPLC analysis of the coupling products was performed using an HPLC system from Dionex with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Identification and quantitative determination of amine substrates (after coupling) was done by reversed phase HPLC on a Discovery HS C18 column (5 µm; 15 cm x 4.6 mm, Supelco, Bellefonte, USA) using acetonitrile and 50 mM potassium phosphate buffer, pH 4.6 (1:49 v/v) as solvent at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C. For the detection of coupling products, a parallel analysis was also carried out using acetonitrile, 10 mM sulphuric

acid and deionized water (4: 3:13) as solvent at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C.

Coupling of antifungal molecules onto phenolic amine-functionalized wood surface

Beech wood pieces (30mm x 15 mm x 15 mm) were oxidized by laccases (0.67 nkat ml^{-1}) in the presence (1 mM) of one of the phenolic amines **1**-**6**. The reaction was carried out in 50 mM succinate buffer at pH 4.5 and 37 °C while shaking at 150 rpm for 2 h. The unbound phenolic amines were removed by successive washing with acetone, ethanol and distilled water. The phenolic amine-functionalized wood pieces were then incubated with 2 mM of either propiconazole or thiabendazole for 12 h. The unbound antifungal molecules were then quantified spectrophotometrically (propiconzole at 225 nm and thiabendazole at 299 nm) by reference to a calibration curve of the respective antifungal molecule after washing the wood pieces successively with acetone, ethanol and distilled water.

5.3. Results and Discussion

Oxidation of phenolic amines and lignin model compounds

Five of the six phenolic amines were oxidized by the 62 kDa *Trametes hirsuta* laccase (Table 1). However, the rate of oxidation varied with 4-hydroxy-3-methoxybenzylamine having the lowest (0.12 min^{-1}) and 4-O-methyldopamine the highest (4.46 min^{-1}) K_{cat} value. The phenolic amines studied here probably had an electron-withdrawing effect on the benzene ring which has been previously shown to decrease the reaction rate [16 -18]. When compared with other laccase substrates, the K_{cat} values of the phenolic amines investigated were at least 720 times lower than those of the common laccase substrates ABTS, syringaldazine and 2,6-dimethoxyphenol [14]. Tyramine was not readily oxidized as it lacks a substituent *ortho* to a hydroxyl group, as in the case of 3-hydroxytyramine used by Grönqvist et al. [2].

Table 1. Oxidation of phenolic amines by *T. hirsuta* laccase

	Substrate	K_{cat} (min^{-1})
1	Tyramine	No reaction
2	1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	0.48
3	3-Hydroxytyramine	4.11
4	3-(3,4-Dihydroxyphenyl)-DL-alanine	0.16
5	4-O-Methyldopamine	4.46
6	4-Hydroxy-3-methoxybenzylamine	0.12

Of the four lignin model substrates used, **D**, **E**, and **G** were laccase substrates while no oxidation of adlerol **A** was observed. Adlerol is a non-phenolic compound, which might explain this finding. This is consistent with the results of other authors who reported no oxidation of adlerol in the absence of mediators [19]. It is generally well-known that laccases only oxidize phenolic lignin moieties unless electron mediators are added [20]. Insignificant oxidation of adlerol has also been reported by Rittstieg et al. [21]. Enriqueta Arias et al. [22] only observed significant oxidation of a similar non-phenolic compound, veratryl alcohol, after 3 days in the presence of ABTS.

Coupling of phenolic amines to lignin model substrates

Coupling reactions were only observed when either the phenolic amine or the lignin model substrate was accepted by the laccase as a substrate. Tyramine, which is not a laccase substrate, was readily coupled to those lignin model molecules (**D**, **E**, and **G**) which were laccase substrates, as illustrated for tyramine and **E** in Figure 2. Consequently, adlerol was only coupled to those aromatic amine which were laccase substrates. Thus it was possible to couple adlerol to 3-hydroxytyramine, 3-(3,4-dihydroxyphenyl)-DL-alanine, 4-hydroxy-3-methoxybenzylamine, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and 4-O-methyldopamine, but there was no coupling when tyramine (not a laccase substrate) was used. This indicates that coupling reactions only proceeded following creation of radicals, and once radicals were formed, further reaction could proceed without additional enzymatic action as previously reported [23, 24].

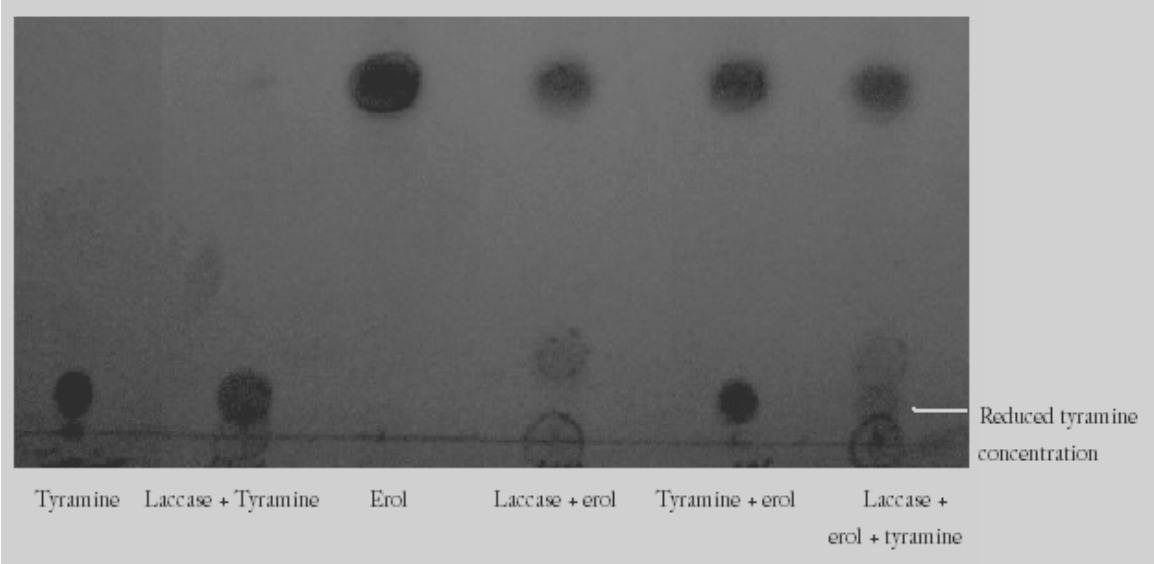


Fig. 2. Coupling of tyramine and erol in the presence of *T. hirsuta* laccase as shown by a reduction in concentration (qualitative analysis) of tyramine (last lane). The same concentration of tyramine (1.25 mM ml^{-1} in 50 mM succinate buffer pH 4.5) was added in each of the reactions.

Tyramine was chosen as a substrate for further studies since it is the only one out of **1**-**6** that is not a laccase substrate. This allowed quantification of the enzymatic coupling to lignin model compounds using HPLC based on the consumption of tyramine (Table 2). By optimizing the HPLC conditions for separation of the resulting coupling products, it was possible to observe novel peaks in coupling reactions with tyramine, 3-hydroxytyramine and syringylglycerol β-ether (Fig. 3). The parent syringylglycerol β-ether molecule had a retention time of 12.8 min and its oxidized radical had a retention time of 40.4 min (Fig. 3). In the presence of tyramine, a new coupling product peak with a retention time of 22.8 min was detected while in the presence of 3-hydroxytyramine a coupling product with retention time of 20.9 min was observed.

Table 2. Coupling of tyramine (initial concentration 1 mM) to laccase-oxidized lignin model substrates. Reactants were provided in the molar ratio of 1:2 (aromatic amine: lignin model)

Lignin model compound		% Transformation
Adlerol	**A**	0
Syringylglycerol β-ether	**G**	34.11 ± 3.2
Dibenzodioxocin	**D**	13.64 ± 3.3
Erol	**E**	31.53 ± 1.4
Wood	**W**	18.04 ± 2.1

***All values are means of three replicates ± standard deviation.

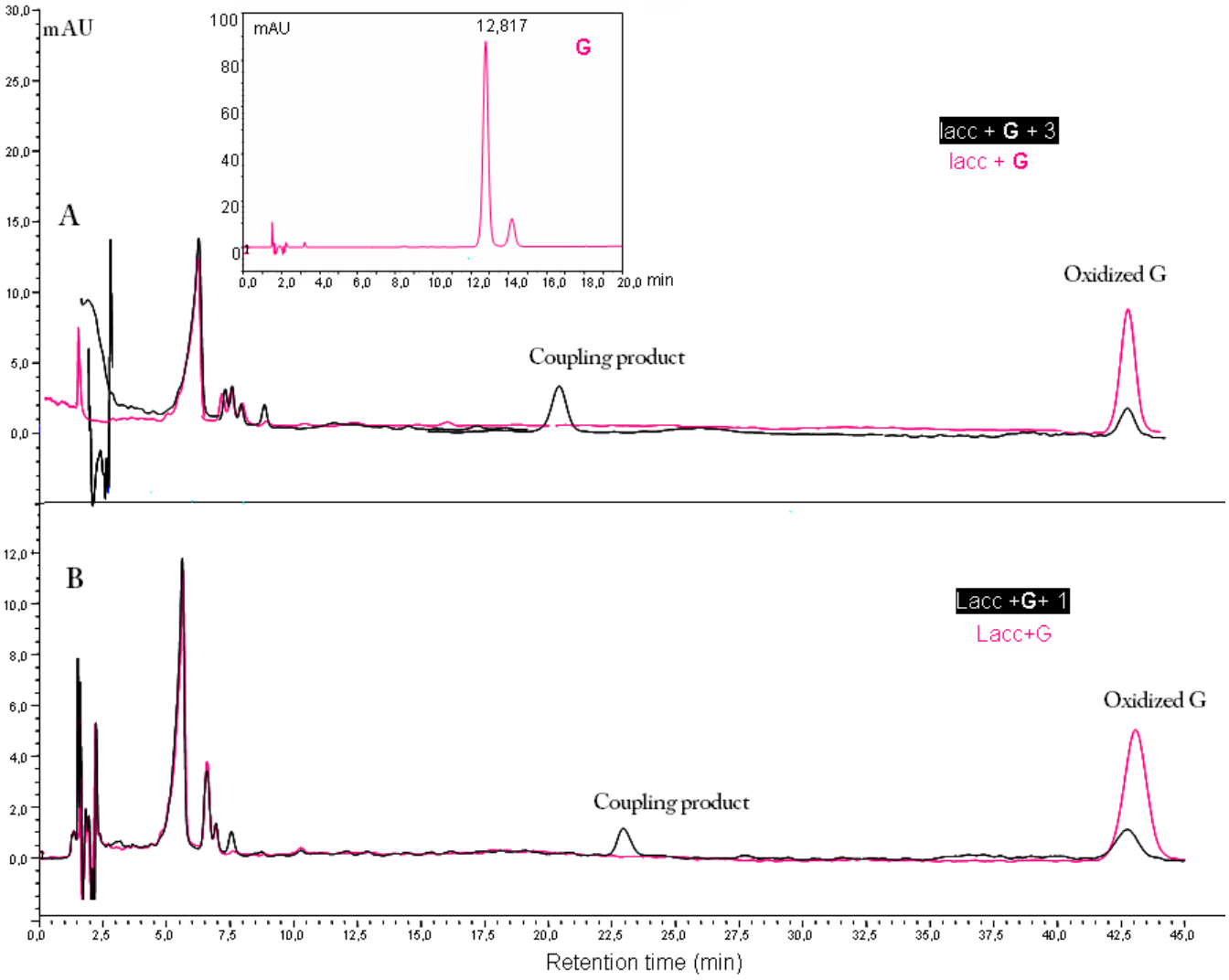


Fig. 3A Coupling of 3-hydroxytyramine (**3**) and syringylglycerol β-ether (**G)** and (**Fig. 3B**) of tyramine (**1**) and syringylglycerol β-ether (**G**) using *T. hirsuta* laccase. The insert shows the parent compound (**G**).

Coupling of antifungal molecules onto phenolic amine-functionalized wood surfaces

The functionalization of wood surface by either simple laccase oxidation or laccase oxidation followed by coupling of phenolic amines as anchor groups were compared as pretreatments for grafting of antifungal molecules. Increases of 33 % and 35 % were observed when laccase-oxidized wood pieces were incubated with thiabendazole and propiconazole, respectively (Figures 4 and 5). More interesting was the 77 % increase in coupling of propiconazole onto tyramine-functionalized wood (Fig. 4.) and the 91 % increase in coupling of thiabendazole onto 3-(3,4-dihydroxyphenyl)-DL-alanine-functionalized wood (Fig 5). This represents a 42 % and 58 % increase in coupling of propiconazole and thiabendazole, respectively when compared to coupling onto wood pieces which were simply "activated" by laccase only. However, all the phenolic amines used in this study enhanced coupling of both antifungals when compared to the effect of laccase treatment only.

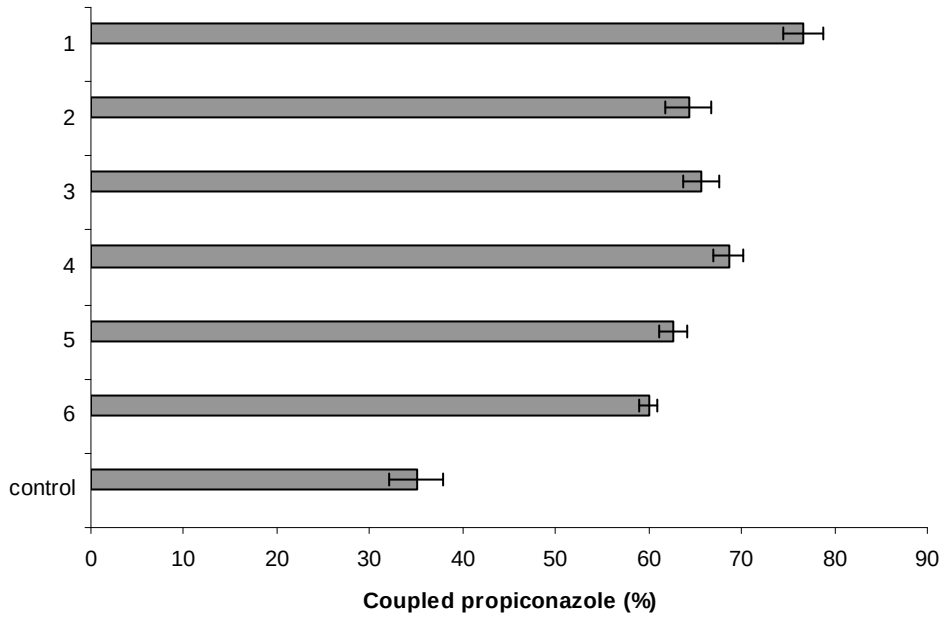


Fig. 4. Coupling of the fungicide propiconazole **P** (0.4 mM) onto phenolic amine functionalized wood samples. Before coupling of propiconazole, the wood samples were treated with laccase alone (control) or with laccase and different phenolic amines **1**-**6** (see table 1). The % values represent an increase in coupling when compared to wood samples treated with **P** alone. All values are means of three replicates ± standard deviation.

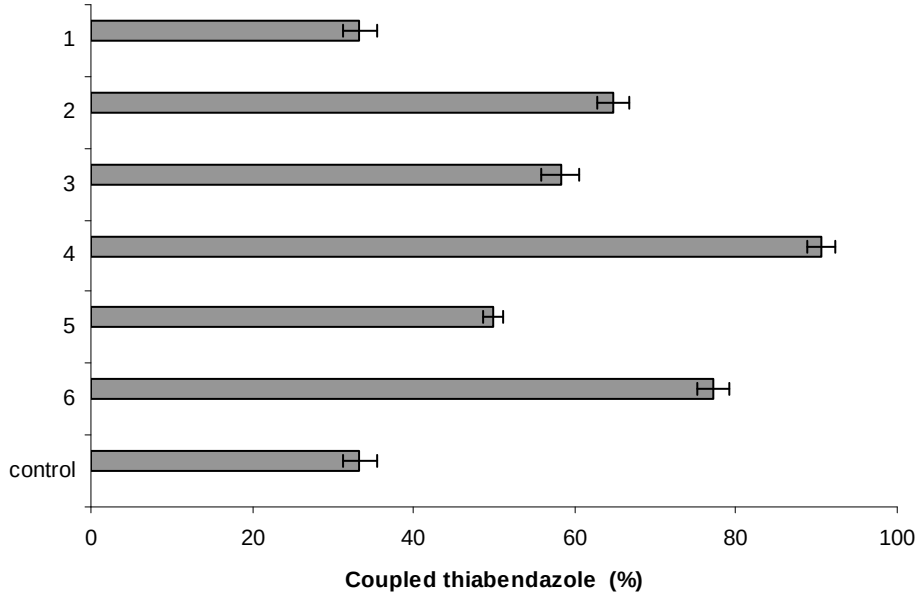
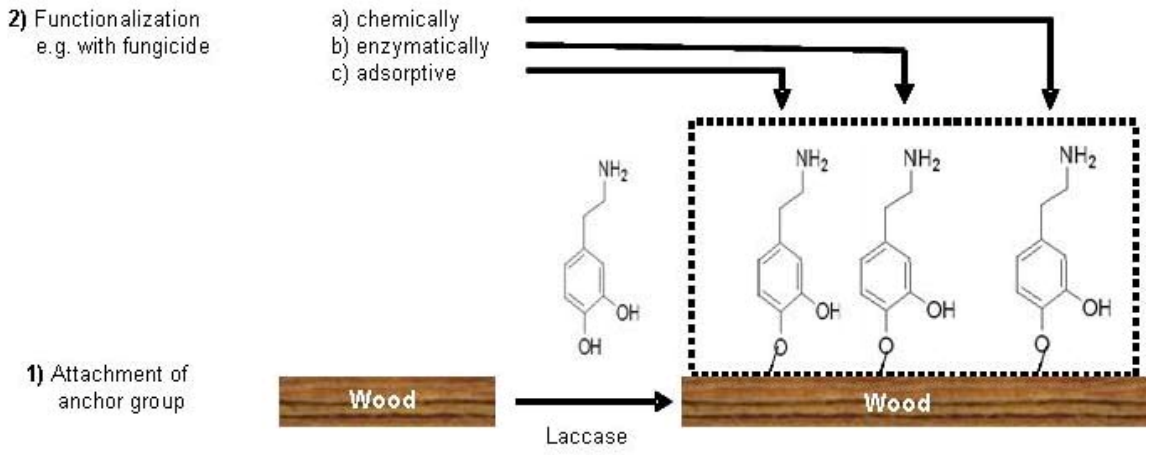


Fig. 5. Coupling of the fungicide thiabendazole **T** (0.4 mM) onto phenolic amine functionalized wood samples. Before coupling of propiconazole, the wood samples were treated with laccase alone (control) or with laccase and different phenolic amines **1**-**6** (see table 1). The % values represent an increase in coupling when compared to wood samples treated with **T** alone. All values are means of three replicates ± standard deviation

A previous study (using FTIR) on coupling of laccase-oxidized phenolic amines to lignin showed an increase in the formation of ether linkages [2] which indicates that it is the hydroxyl group that preferentially reacts with lignin radicals (Scheme 1). The amino group remains available for further reaction and has been shown to form C-NH bonds with phenolic monomers [25]. In general, amino anchor groups on wood surfaces could enhance binding of functional molecules via chemical or enzymatic reaction or simply by changing adsorption characteristics. The enzymatic coupling of phenolic molecules to amino groups on polymer surfaces was described only recently [26]

It can therefore be concluded that it is possible to insert phenolic amines as anchor groups to lignin moieties on wood surface. The amine-functionalized wood enhances binding of functional molecules as demonstrated for antifungal agents.



Scheme 1: Attachment of anchor groups (i.e. phenolic amines) to lignin moieties of wood for further functionalization.

Acknowledgements

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5.4. References

[1] E. Sjöström; Wood Chemistry: Fundamentals and application. Academic Press. Orlando, **1993**, 293pp

[2] S.Grönqvist, K.Rantanen, A. Raimo, M. Mattinen, J. Buchert, L.Viikari, Laccase-catalysed functionalization of TMP with tyramine. *Holzforschung,* **2006**, 60, 503–508

[3] A-M, Boudet, Lignins and lignification: Selected issues. Plant Physiol. Biochem., **2000**, 38 (1/2), 81-96.

[4] S. Riva, Laccases: blue enzymes for green chemistry. *Trends Biotechnol.,* **2006**, 24, 219-225.

[5] N. Durán, M. A. Rosa, A. D'Annibale, L. Gianfreda, Applications of laccases and tyrosinases (phenoloxidases) immobilized on different supports: a review. *Enzyme and Microbial Technol*., **2002**, 31(7), 907-931.

[6] R. Chandra, A.Ragauskas, Sculpting the molecular weight of lignin via laccase. In: Proceedings of the 11th International Symposium on Wood and Pulp Chemistry, Nice, France, **2001**, Part II. pp. 39–42.

[7] R. Chandra, A. Ragauskas, Fibre modification with laccase:you say you want a revolution. In: Abstracts of the 223rd ACS National Meeting, book 1. American Chemical Society, Washington, DC, **2002**a, p. 104.

[8] R. Chandra, A.Ragauskas, Evaluating laccase-facilitated coupling of phenolic acids to high-yield kraft pulps. *Enzyme Microbial Technol*., **2002**b, 30, 855–861.

[9] R. Chandra, L. Lehtonen, A. Ragauskas, Modification of high lignin content kraft pulps with laccase to improve paper strength properties. 1. Laccase treatment in the presence of gallic acid. *Biotechnol. Prog*., **2004**a, 20,255–261.

[10] R. Chandra, C. Felby, A. Ragauskas, Improving laccase-facilitated grafting of 4-hydroxybenzoic acid to high-kappa pulps. *J. Wood Chem. Technol*., **2004**b, 24, 69–81.

[11] G. S. Nyanhongo, S. Rodrıguez Couto, G. M. Guebitz, Coupling of 2,4,6-trinitrotoluene (TNT) metabolites onto humic monomers by a new laccase from *Trametes modesta*. *Chemosphere*, **2006**, 64, 359–370.

[12] J. Sipilä, K. Syrjänen. Synthesis and ^{13}C NMR spectroscopic characterization of six dimeric arylglycerol-β-aryl ether model compounds representative of syringyl and *p*-hydroxyphenyl structures in lignins. On the aldol reaction in β-ether preparation. *Holzforschung*, **1995**, 49, 325-331.

[13] P. Karhunen, P. Rummakko, J. Sipilä, G. Brunow. The Formation of Dibenzodioxocin Structures by Oxidative Coupling. A Model Reaction for Lignin Biosynthesis, *Tetrahedron Letters*, **1995**, 36, 4501-4504.

[14] E. Almansa, A. Kandelbauer, L. Pereira, A. Cavaco-Paulo, G.M. Guebitz, Influence of Structure on Dye Degradation with Laccase Mediator Systems. *Biocat. Biotrans*., **2004**, 22 (5/6), 315-324.

[15] M. L. E Niku-Paavola,. P.Karhunen, Salola, V. Raunio, Ligninolytic Enzymes of the white-rot fungus *Phlebia radiata*. *Biochem. J*., **1988,** 254, 877–884.

[16] S. Rodriguez Couto, J.L.Toca-Herrero. Industrial and biotechnological applications of laccases: A review. *Biotechnol. Advances*, **2006**, 24, 500–513.

[17] A. Kandelbauer, A. Erlacher, A. Cavaco-Paulo, G.M. Gübitz, Laccase catalyzed decolorization of the synthetic azodye Diamond Black PV and of some structurally related derivatives. *Biocat. Biotrans.,* **2004**, 22, 331-339.

[18] A, Kandelbauer, O, Maute, R. Kessler, A. Erlacher, G.M. Gübitz, Study on dye decolorization in an immobilized laccase enzyme-reactor using on-line spectroscopy. *Biotechnol. Bioeng,* **2004**, 87, 552-563.

[19] A. M. Barreca, M. Fabbrini, C. Galli, P. Gentili, S. Ljunggren, Laccase/mediated oxidation of a lignin model for improved delignification procedures. *J. Mol. Cat. B: Enzymatic,* **2003**, 26 (1-2), 105-110.

[20] Paice, M. G.; Bourbonnais, R.; Reid, I. D.; Archibald, F. S.; Jurasek, L. Oxidative Bleaching Enzymes - A Review. *J. Pulp. Pap. Sci*., **1995**, 21, 280-284.

[21] K. Rittstieg, A. Suurnakki, T. Suortti, K. Kruus, G. Guebitz, J. Buchert, Investigations on the laccase-catalyzed polymerization of lignin model compounds using size-exclusion HPLC. *Enzyme Microbial Technol*., **2002**, 31(4), 403-410.

[22] M. E. Arias, M. Arenas, J. Rodrıguez, J. Soliveri, A. S. Ball, M. Hernandez, Kraft pulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335. *Appl. Environ. Microbiol.*, **2003**, 69(4), 1953–1958.

[23] S. Grönqvist, J. Buchert, K. Rantanen, L.Viikari, A.Suurnäkii, Activity of laccase on unbleached and bleached thermomechanical pulp. *Enzyme Microbial Technol.,* **2003**, 32 (3-4), 439-445.

[24] M. Schroeder, N. Aichernig, G.M. Guebitz, V. Kokol, Enzymatic coating of lignocellulosic surfaces with polyphenols. *Biotechnol. J.*, **2007**, 2, 1- 7

[25] A. Mikolasch, E. Hammer, U. Jonas, K. Popowski, A. Stielow, F. Schauer, Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines using laccase. *Tetrahedron*, **2002**, 58, 7589–7593.

[26] S. Sampaio, P. Taddei, P. Monti, J. Buchet, G. Freddi. Enzymatic grafting of chitosan onto *Bombyx mori* silk fibroin: kinetic and IR vibrational studies. *J. Biotech*. **2006** 116, 21-33.

6

Coupling of aromatic amines onto syringylglycerol β–guaiacylether using *Bacillus* SF spore laccase: a model for functionalisation of lignin-based materials

The potential of *Bacillus* SF spore laccase for coupling aromatic amines to lignin model molecules as a way of creating a stable reactive surface was investigated. The Bacillus spore laccase was shown to be active within the neutral to alkaline conditions (pH 7- 8.5) and was more resistant to common laccase inhibitors than fungal laccases. Using this enzyme, tyramine was successfully covalently coupled onto syringylglycerol β–guaiacylether via a 4-O-5 bond, leaving the -NH_2 group free for further attachment of functional molecules. This study demonstrates the potential of *Bacillus* SF spore laccase for application in lignocellulose surface functionalisation and other coupling reactions which can be carried out at neutral to alkaline pH under extreme conditions which normally inhibit fungal laccases.

Published as Kudanga et al., *Journal of Molecular Catalysis B: Enzymatic* 61 (2009), pp. 143–149.

6.1. Introduction

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates such as diphenols, methoxy substituted monophenols and aromatic amines [1,2]. Although most of the known and used laccases are of fungal origin, reports of laccases in bacterial systems are increasing [3-7]. Bacterial laccases have properties different from the fungal laccases given the different physiological and environmental conditions which support them [7]. Among the bacterial systems, the discovery of laccases especially in spores of *Bacillus* has attracted a lot of scientific and industrial interest. Since *Bacillus* spores are naturally designed to resist a wide range of extreme physico-chemical conditions such as dry heat, desiccation, radiation, UV light and oxidising agents [8], it is speculated that this enzyme may also posses these properties. It has already been favourably established that *Bacillus subtilis* spore laccases are thermally stable at 80 °C with a half-life of about 2 h and have optimum temperature around 75 °C [8], as compared to fungal laccases which are optimally active below 60 °C. *Bacillus licheniformis* spore laccase was also reported to oxidize ABTS at 85 °C [9]. Bacillus spore laccase (CotA) is a component of the spore coat involved in formation of a brown pigment that protects spores against UV radiation and H_2O_2 [5,10]. The crystallographic structure of CotA has been elucidated by Enguita et al [11].

Apart from oxidation of the traditional laccase substrates [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate] (ABTS), syringaldazine (SGZ) and 2,6-dimethoxyphenol (DMP), recent reports show the ability of *Bacillus* spore laccases to oxidize dyes [6] and a few phenolic molecules [9,12]. However, their ability to oxidize a wide range of structurally diverse lignin molecules has not been investigated while existing knowledge on coupling of low molecular weight molecules onto lignin and lignocellulose materials is exclusively based on fungal laccases which are traditionally applied under acidic conditions [13-17]. In our earlier study we demonstrated that grafted phenolic amines can form a stable reactive wood surface, counteracting negative effects of quenching of unstable radicals [18]. This study demonstrates for the first time the ability of *Bacillus* SF spore laccase to mediate the coupling of these aromatic amines onto lignin model compound (syringylglycerol β–guaiacylether) under different pH conditions. The stability of the laccase against common laccase inhibitors was also studied. Further, the ability of *Bacillus* SF spore laccase to oxidize a wide variety of structurally different lignin molecules (simple phenolics, hydroxycinnamic acids,

hydroxybenzoic acids and phenolic amines) is investigated by monitoring both spectrophotometrical changes and oxygen consumption.

6.2. Materials and methods

6.2.1 Chemicals

The lignin model compound syringylglycerol β-guaiacylether was synthesized as previously described by Sipilä [19]. All other chemicals were purchased from Sigma-Aldrich.

6.2.2 Laccase activity assay

The *Bacillus* SF spore laccase was produced as previously reported [6]. The activity of the laccase was determined spectrophotometrically by monitoring the oxidation of 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonate (ABTS) (ε_{436} = 29,200 $M^{-1}cm^{-1}$) as the substrate at 436 nm in 50 mM citrate buffer at pH 7.0 and 37 °C as described by Niku-Paavola et al. [20] with some modifications. The effect of six different known fungal laccase inhibitors namely thymine, thiourea, NaF, H_2O_2, NaN_3 and KCN on the oxidation of ABTS was investigated. Inhibitors were preincubated with the laccase in 50 mM citrate buffer at pH 7.0 and 37 °C for 60 min and residual laccase activity measured using the assay above.

6.2.3 Oxidation of phenolic molecules and lignin model compounds

The reaction mixture contained 2.0 nkat ml^{-1} laccase as determined using ABTS as substrate and 0.1 mM phenolic molecules. Incubation was carried out at different pH levels ranging from 2.2 to 9.0. Citrate buffer (0.05 M) was used in the range 2.2 to 5.5, phosphate buffer (0.1 M) in the range 6.0 to 8.0 while 0.025 M borate buffer was used in the range 8.5 to 9.0. Oxidation was monitored by means of wavelength scans in the range 200 – 800 nm and by oxygen consumption using a Rank Brothers oxygen meter (Dual Digital Model 20, England). Actual oxygen consumption values were computed by reference to the oxygen saturation concentration of the control reaction mixture (enzyme replaced by buffer) as determined by the Winkler method [21].

6.2.4 Coupling reactions in solution

Tyramine (1.0 mM) or 3-hydroxytyramine (1.0 mM) was coupled onto the lignin model compound syringylglycerol β-guaiacylether (2.0 mM) in 0.1 M ammonium acetate buffer pH 4.5-7.0 and in 0.1 mM ammonium bicarbonate buffer pH 7.5-8.5. Reactions were

carried out at 37 ºC while shaking at 650 rpm. Syringylglycerol β-guaiacylether was initially incubated with the laccase for 30 min; after which 3-hydroxytyramine was added and the reaction continued for a further 75 min. In the case of tyramine (non-laccase substrate), the monomer and model were mixed simultaneously and the reaction was run for 1 hr 45 min.

6.2.5 Chromatographic analysis of oxidation and coupling products

Analysis of lignin molecules, their oxidation and coupling products was performed using HPLC. An equal volume of ice cold methanol was added to the incubation mixtures to stop the reaction and to precipitate proteins. The mixture was centrifuged at 0 ºC for 15 minutes at 14000 g and 700 µl transferred into vials. Analysis was performed by an HPLC-UV system from Dionex with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector. Identification and quantitative determination of products was done by reversed phase HPLC, on a Discovery HS C18 column (5 µm; 15 cm x 4.6 mm, Supelco, Bellefonte, USA) using acetonitrile, 10 mM sulphuric acid and deionized water (20:15:65) as solvent with isocratic elution at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C. Laccase oxidation of phenolic amines was monitored using acetonitrile and 50 mM potassium phosphate buffer pH 4.6 (2:98) as solvent (isocratic elution) at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C.

The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with electrospray ionisation coupled to the Dionex HPLC-UVD-system described above and using the same protocol except that 0.1 % formic acid was used instead of 10 mM sulphuric acid. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350 °C, nebulizer to 70 psi. Maximal accumulation time was fixed to 300 msec and the loading of the trap was controlled by the instrument with an ICC of 30000.

6.3. Results and Discussion

6.3.1 Oxidation of phenolic molecules and aromatic amines

The substrate specificity of fungal laccases on phenolic molecules and lignin model substrates is well established. However, there is only little known in this respect about bacterial laccases which show a lower redox potential [22]. The spore laccase from *Bacillus* SF oxidized a wide range of phenolic molecules according to UV-Vis spectrophotometry and oxygen consumption (Table 1). Approximately 1 mole oxygen was consumed for every 4

moles of substrates upon complete oxidation which is typical for laccase catalyzed reactions [23,24]. However, oxidation of 3,4,5-trihydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid and 3-hydroxy-4-methoxycinnamic acid (predominantly trans) was not complete within the given reaction time. The influence of substituents on position 3 and 5 on the benzene ring with respect to a hydroxyl group on position 4 was quite evident. The presence of methoxy and –hydroxyl substituents on position 3 and 5 makes the phenolic molecules laccase substrates (Table 1). However, the presence of only a hydroxyl group on position 4 is not sufficient to make the phenolic molecule a substrate of the *Bacillus* SF spore laccase as evidenced by the respective four molecules that were not oxidized (Table 1). A similar observation has been reported in previous studies using *Bacillus licheniformis* spore laccase [9], although Takahama reported oxidation of coumaric acid with *Pyricularia oryzae* laccase [25]. This is not surprising since bacterial laccases have been reported to exhibit a lower redox potential of about 0.5 V [22] compared to 0.5 - 0.8 V for fungal laccases [26,27]. Another interesting observation is that carboxyl groups which are electron withdrawing tend to make the molecules poor substrates when attached directly to the benzene ring as exemplified by the lower turnover (i.e. oxygen consumption) for 4-hydroxy-3-methoxybenzoic acid (Table 1). For example, this is quite evident when comparing 3,4,5-trihydroxybenzoic acid and 1,2,3-trihydroxybenzene, where the latter was oxidized to higher extent for the given time of the reaction. Indeed Koschorreck et al. reported that 4-hydroxy-3-methoxybenzoic acid (which was one of the poor substrates for our *Bacillus* SF spore laccase) was not oxidized by *Bacillus licheniformis* spore laccase [9]. Nevertheless, electron-donating methoxy groups seem to counteract the effect of electron with-drawing groups (compare for example 3,5-Dimethoxy-4-hydroxybenzoic acid and 4-Hydroxy-3-methoxybenzoic acid). Despite the narrower substrate range, generally the observed influence of the nature and position of functional groups on the benzene ring of various molecules on *Bacillus* SF spore laccase oxidation is consistent with that reported for fungal laccases [17, 28-34].

Table 1: Oxidation of phenolic molecules and syringylglycerol β –guaiacylether (lignin model compound) by *Bacillus* SF spore laccase after 30 min incubation time

Monomer	Absorption maxima of substrate (nm)	Absorption maxima of laccase oxidation products (nm)	Oxygen consumed by 1.0 mM substrate (mM)[a]
trans-4-Hydroxycinnamic acid	289; 310	-	0.00
4-Hydroxyphenoxyacetic acid	286; 331	-	0.00
4-Hydroxy-3-methoxybenzylamin hydrochloride	285	330	0.23 ± 0.021
4-Hydroxy-3,5-dimethoxy-cinnamic acid	314	510	0.26 ± 0.024
3,4-Dihydroxycinnamic acid	314	398	0.22 ± 0.019
Trans-4-Hydroxy-3-methoxycinnamic acid	319	+	0.28 ± 0.030
4-Hydroxybenzoic acid	286	-	0.00
3-Hydroxy-4-methoxycinnamic acid (pred. trans)	312 - 315	+	0.17 ± 0.024
Tyramine 2-(4-Hydroxyphenyl)ethylamine	285	-	0.00
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline hydrobromide	284	331	0.25 ± 0.033
3- Hydroxytyramine hydrochloride	280	475	0.24 ± 0.029
1,2,3-Trihydroxybenzene	225; 267	320, 380, 236 (broad peaks)	0.26 ± 0.024
3-(3,4-Dihydroxyphenyl)-DL-alanine	226; 281	312	0.28 ± 0.034
3,4,5-Trihydroxybenzoic acid	264	242,5 ; 396	0.17 ± 0.012
3,5-Dimethoxy-4 hydroxybenzoic acid	266	250; 289	0.23 ± 0.026
4-O-Methyldopamine hydrochloride	228; 281	475	0.20 ± 0.020
2-Methoxyphenol	287; 290	497	0.29 ± 0.028
1,2-Benzenediol	289; 295	386	0.28 ± 0.024
2,6 Dimethoxyphenol	300	469	0.24 ± 0.020
4-Hydroxy-3,5-dimethoxybenzaldehyde azine	357	530	0.23 ± 0.021
4-Hydroxy-3-methoxybenzaldehyde	279; 309	+	0.22 ± 0.020
4-Hydroxy-3-methoxybenzoic acid	256; 289	+	0.13 ± 0.014
Syringylglycerol β –guaiacylether	274	300	0.26 ± 0.014

(+) oxidized by *Bacillus* SF spore laccase (no product peak detected, substrate consumption was used as an indicator of a positive laccase reaction)
(-) not oxidized by *Bacillus* SF spore laccase
[a]All values are means of three replicates ± standard deviation

6.3.2 Effect of pH on oxidation of selected common laccase substrates

There was a wide variability among different substrates in terms of the pH optimum for oxidation with ABTS having a very low optimum pH of 2.5 while guaiacol requires pH 8.5 (Fig. 1 and 2). The optimal pH required to oxidize ABTS is similar to that of most fungal laccases [35] and our results for ABTS and syringaldazine are consistent with the findings for a *Bacillus subtilis* spore laccase [9]. In addition to standard substrates, in this study we have determined the pH optima for oxidation of typical lignin model substrates guaiacol, sinapic

acid, catechol and caffeic acid by the spore laccase (Fig. 2). It was observed that the common lignin model substrates generally require neutral to alkaline pH for effective oxidation with *Bacillus* SF spore laccase.

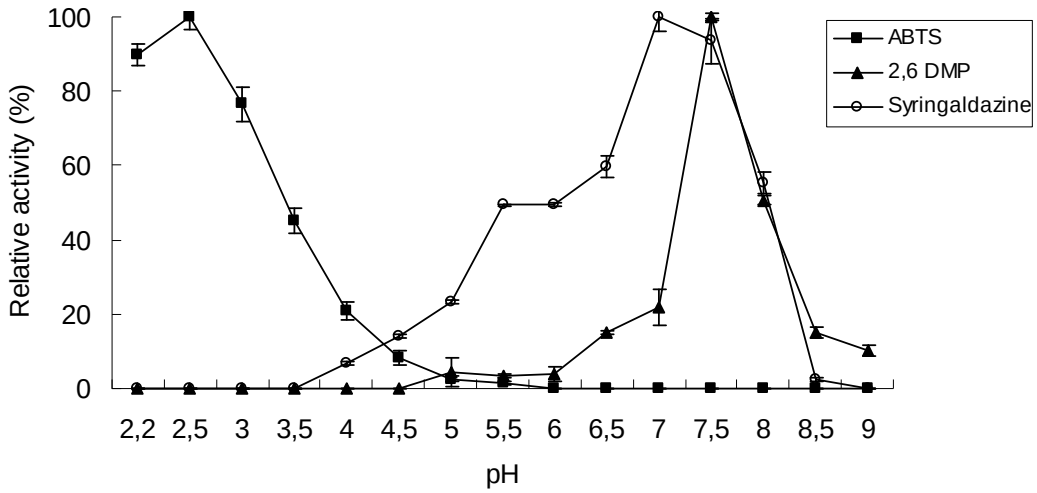

Fig. 1. Effect of pH on oxidation of common laccase substrates by *Bacillus* SF spore laccase
*All values are means of three replicates ± standard deviation and were obtained by monitoring product formation (ABTS at 415 nm; 2,6-DMP at 469 and syringaldazine at 530 nm)

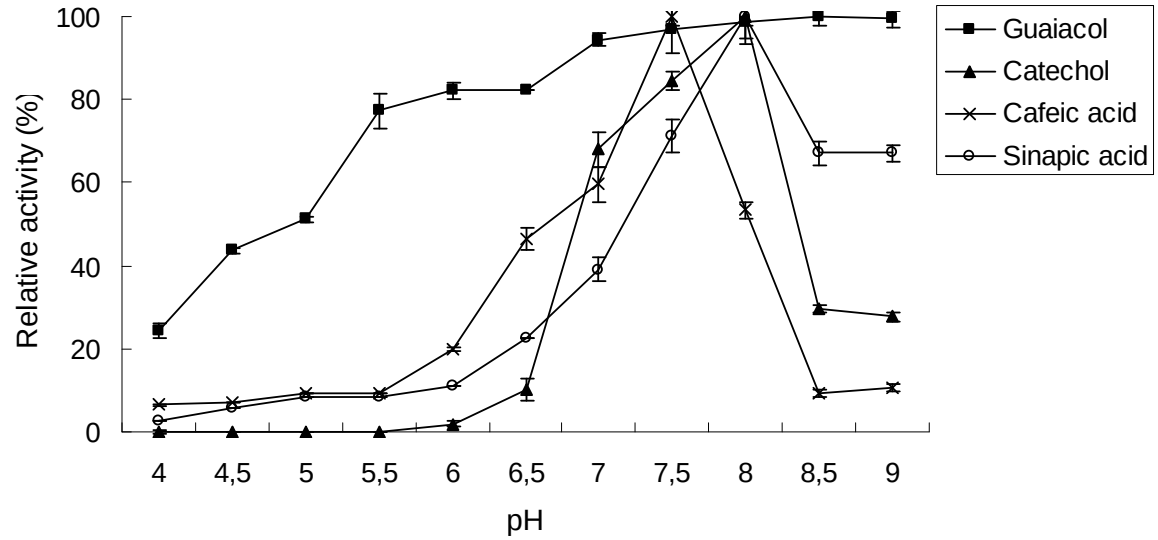

Fig. 2. Effect of pH on oxidation of selected lignin model compounds by *Bacillus* SF spore laccase. *All values are means of three replicates ± standard deviation obtained by monitoring product formation (guaiacol at 497 nm; catechol at 386 nm; caffeic acid at 398 nm and sinapic acid at 510 nm)

6.3.3. Inhibition of Bacillus SF *spore laccase by putative laccase inhibitors*

Like all known laccases, the *Bacillus* SF spore laccase was inhibited by the common laccase inhibitors thymine, thiourea, NaF, H_2O_2, NaN_3 and KCN (Table 2). Of particular interest is the inhibition by cyanide and azide which confirms the presence of a metal (i.e. copper) in the catalytic center while inhibition by fluoride has been reported to be typical for laccases [36]. However, it is worth noting that while the spore laccase has some IC_{50} values within the range of fungal laccases (Table 2), it is three times more resistant to thymine and seven times more resistant to KCN which suggests that it may tolerate harsher conditions than free fungal and bacterial laccases.

Table 2: Inhibition of *Bacillus* SF spores laccase by putative laccase inhibitors

Inhibitor	[a]I_{50} *Bacillus* SF spore laccase	I_{50} Fungal laccases	References
Thymine	2.8 mM	0.01 - 0.95 mM	[37, 38]
Thiourea	23.4 µM	1.5 mM	[17]
NaF	458.9 µM	12 µM - 10 mM	[17, 36, 38]
H_2O_2	24.7 mM	54 mM	[39]
NaN_3	38.8 µM	9 µM – 10 mM	[17,35,36, 38]
KCN	1.4 mM	130 µM -0.2 mM	[17, 36]

[a]I_{50} is the concentration of inhibitor required to achieve a 50 % drop in laccase activity

6.3.4 Coupling Reactions

In our earlier study, an increase in coupling of fungicides onto amine functionalized wood using *Trametes hirsuta* laccase was reported [18]. Here, due to its higher pH-optimum and stability, we used the *Bacillus* SF spore laccase for a mechanistic study to elucidate the possibility of coupling and the nature of the coupling products between phenolic amines and syringylglycerol β-guaiacylether (mimicking lignin). It was observed that the yield of coupling product is much higher when syringylglycerol β-guaiacylether was pre-oxidized by

the laccase before addition of the 3-hydroxytyramine. This reduced chances of formation of homo-oligomeric products. Consequently, in the case of tyramine (non-laccase substrate), there was no need for this preoxidation step. According to HPLC analyses, new peaks indicated coupling products between syringylglycerol β-guaiacylether and the phenolic amines, tyramine and 3-hydroxytyramine (Fig. 3). New peaks were observed in tyramine coupling reactions with retention times t_R=7.3 min t_R=21.8 min and t_R=28.1 min (there was a slight shift in retention times to t_R=9.5 min, t_R=20.3 and t_R=28.8 respectively, in LC/MS when 0.1 % formic acid was used instead of 10 mM H_2SO_4, Fig. 4). New peaks with retention times t_R=12.6 min, t_R=8.4 and t_R=7.7 were observed when 3-hydroxytyramine was used.

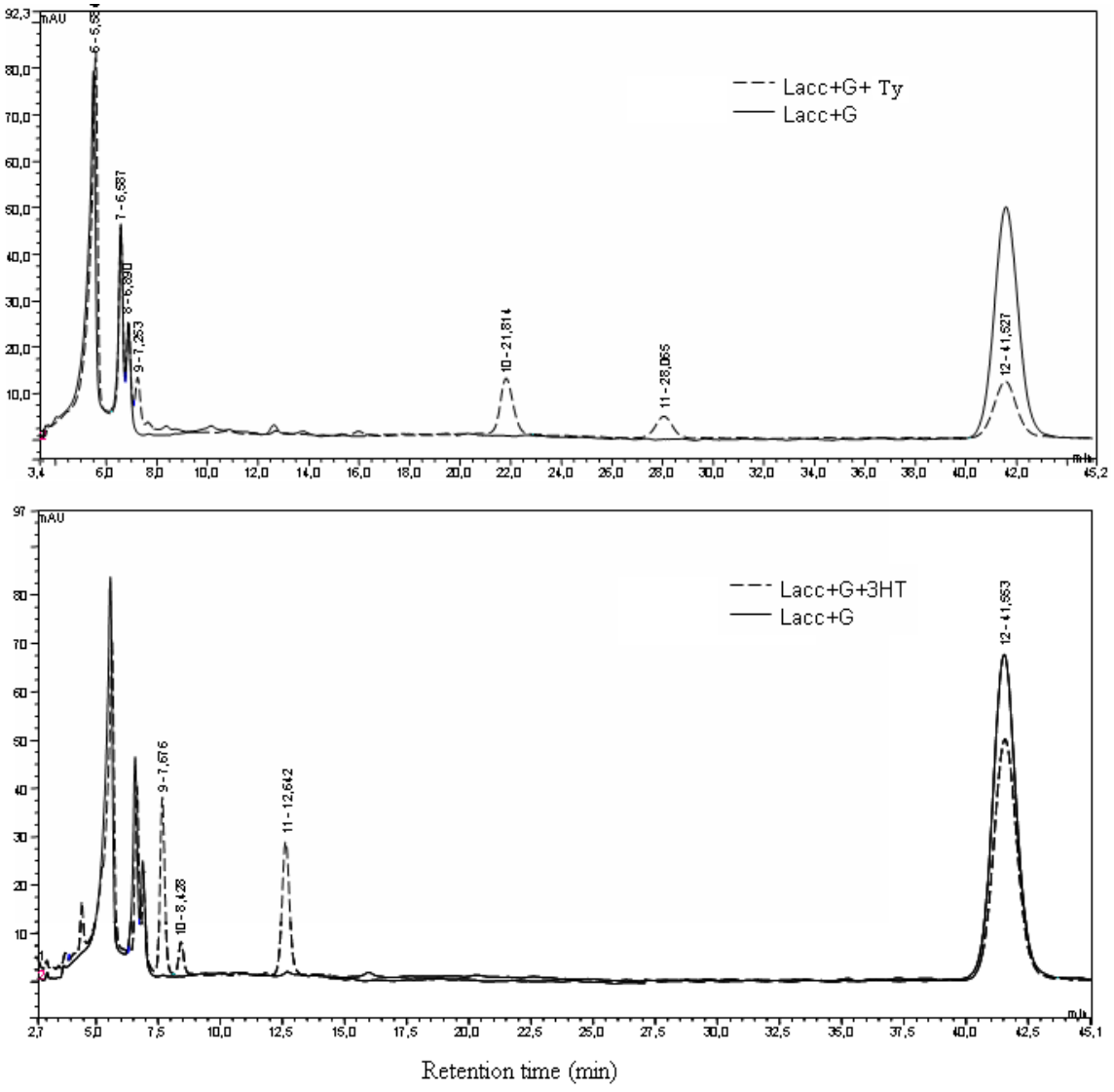

Fig. 3. Coupling of tyramine (Ty) and of 3-hydroxytyramine (3HT) onto syringylglycerol β-ether (**G**) using *Bacillus* SF laccase. **CP** - coupling product

LC-MS studies were carried out to confirm coupling between tyramine and syringylglycerol β-guaiacylether and to elucidate the structure of the coupling products. The molecular weight of syringylglycerol β-guaiacylether is 350.36 while that of tyramine is 137.18. LC-MS showed three main $[M+H]^+$ ions at *m/z* 486.2 *(*t_R 11.1 min), *m/z* 484.2 (t_R=29.0 min) and *m/z* 470.2 (t_R 9.5 min) (Fig. 4). The hydroxyl group on the benzene ring is known to be *ortho* or *para* directing [40-42] and in the absence of an unsaturated side chain, syringyl lignin moieties usually undergo 4-O-5 coupling with other molecules [43]. We therefore hypothesize that the electrophilic oxidized syringylglycerol β-guaiacylether cation radical preferentially attacks the sterically unhindered electron-rich *ortho* position on the tyramine molecule forming a 4-O-5 linkage as shown in Fig. 5. For this reaction to occur tyramine is probably oxidized in the presence of oxidized syringylglycerol β-guaiacylether radical acting as mediator. This results in the coupling product (**P1**) with the observed $[M+H]^+$ molecular ion *m/z* 486.2 (exact mass 485.2). This product was observed in all coupling reactions from pH 4.5 - 8.5. However, the acidic conditions used, seemed to facilitate cleavage of the benzyl α-hydroxyl group resulting in the coupling product (**P2**) with $[M+H]^+$ molecular ion *m/z* 470.2 (exact mass 469.21). This is further supported by the fact that this adduct was only observed in acidic conditions and was absent in neutral to alkaline conditions (pH 7-8.5). The product (**P3**) with molecular ion *m/z* 484.2 (exact mass 483.19) can be explained by the α-oxidation of the benzyl hydroxyl group to a keto group which may be quite a facile reaction.

The other coupling products; **P4**, *m/z* 500.2, t_R=20.3 min; and **P5**, *m/z* 514.2, t_R=36.9 min (observed on total ion chromatogram) are probably a result of nucleophilic attack by OH groups of water (calculated 499.2) and methanol (calculated 513.2) respectively. The laccase created radicals or their quinone form have been shown to undergo nucleophilic attack by substances having nucleophic groups like water or methanol present in the reaction mixture [44]. Similar reactions have also been previously reported [45-47]. It has been observed that hydroxylation is a transition step in laccase-mediated demethylation of lignin molecules [45]. Fig. 5 summarizes the possible reaction pathway for the laccase-mediated coupling of tyramine onto syringylglycerol β-guaiacylether.

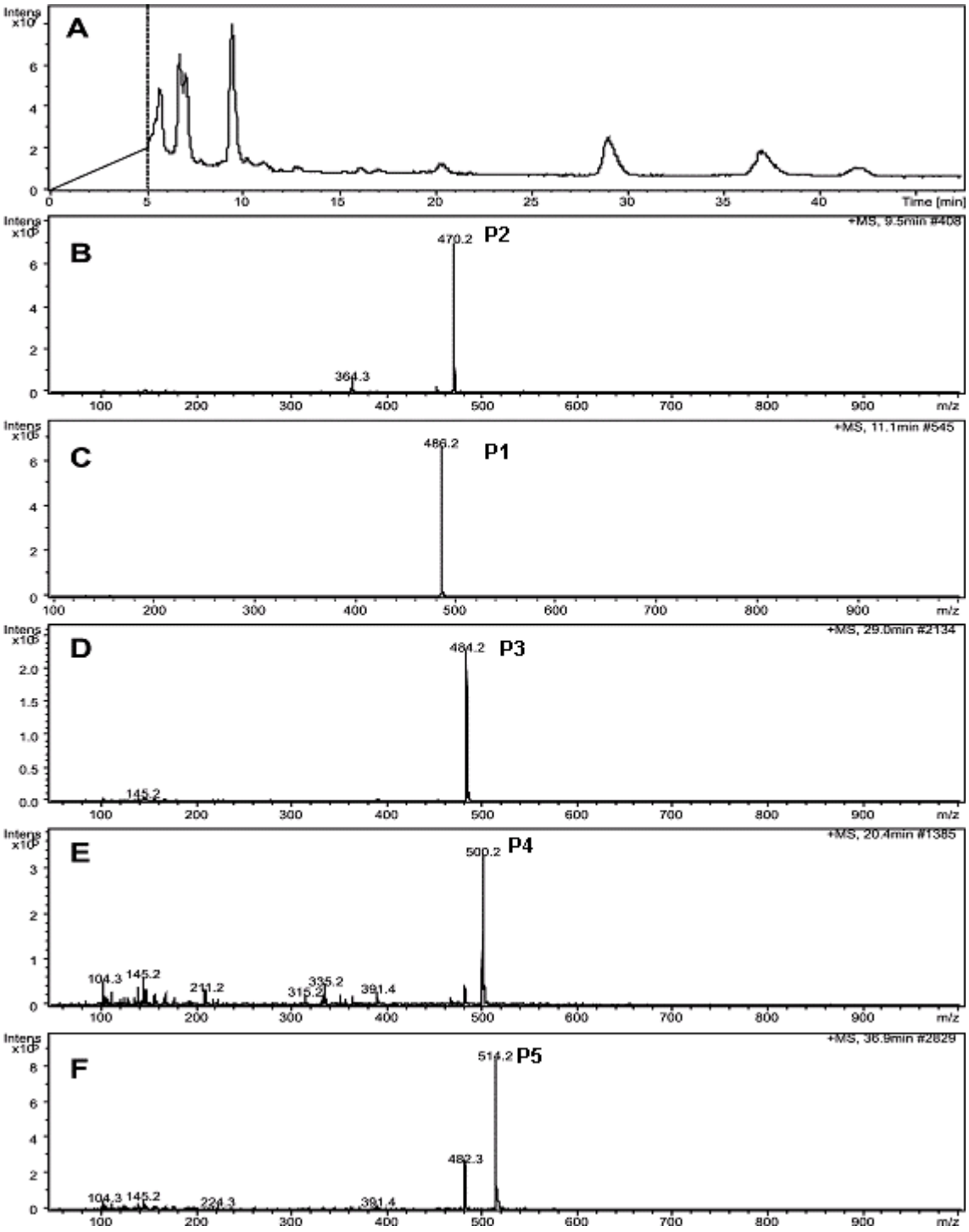

Fig. 4. A Total ion chromatogram for coupling products between tyramine and syringylglycerol β-guaiacylether. **B-F** Mass spectra of coupling products between tyramine and syringylglycerol β-guaiacylether (**G).**

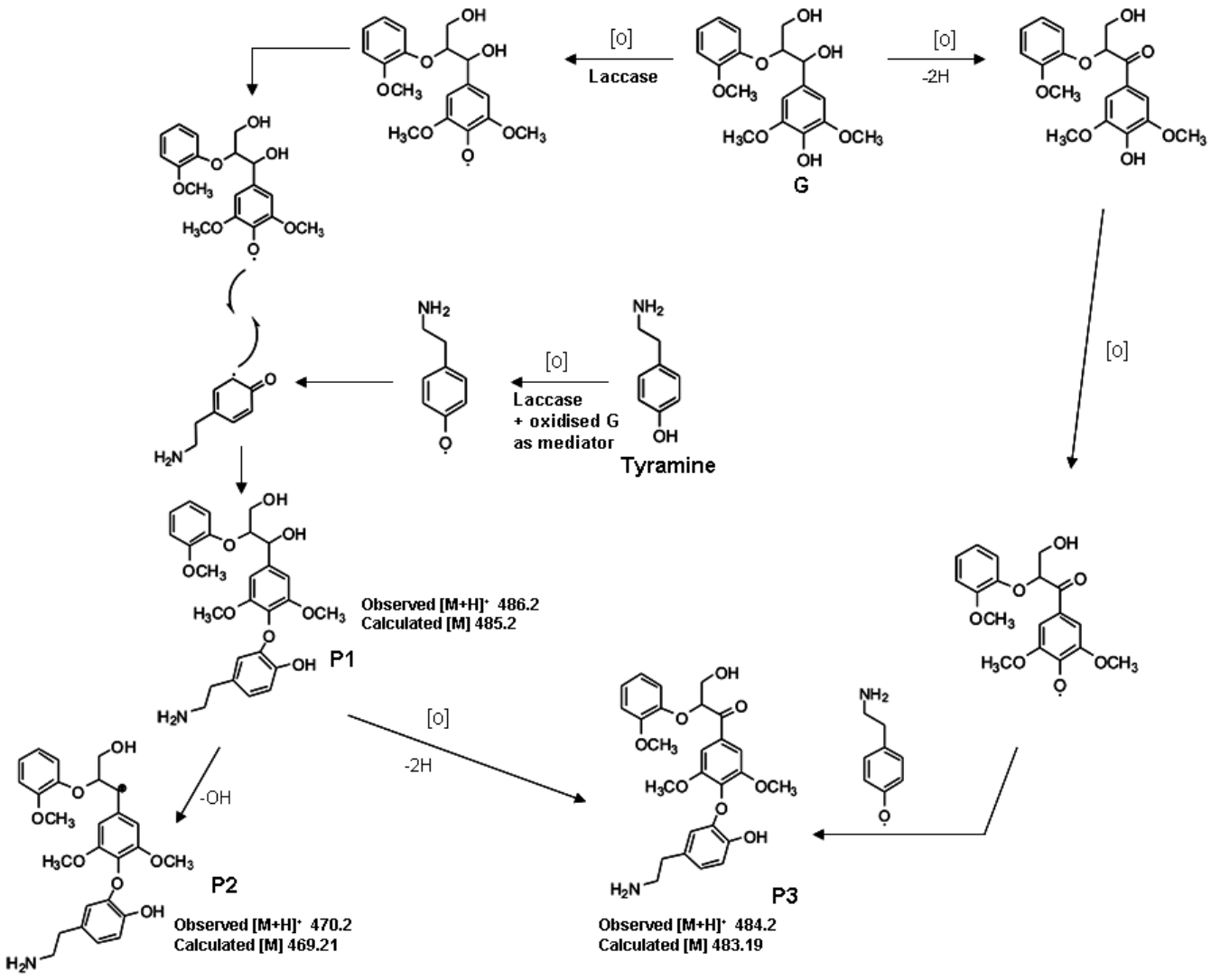

Fig. 5. Proposed reaction pathway scheme for the laccase-mediated coupling of tyramine onto syringylglycerol β-guaiacylether (**G).**

6.4. Conclusions

Previously we have shown that laccase-catalyzed amino-functionalization of lignocellulosic material creates a reactive surface for coupling of functional molecules such as antifungal agents [18] and possibly dyes, flame retardants etc. Here we present the mechanistic evidence that demonstrates the ability of laccases to mediate the covalent bonding of aromatic amines to a lignin model substrate via 4-O-5 coupling leaving the $–NH_2$ group free for further functionalisation. In addition, the ability of *Bacillus* SF spore laccase to oxidize a wide range of lignin molecules, work at relatively high pH and tolerate high laccase

inhibitor concentrations as shown in this study, coupled with the already known ability to tolerate high temperatures, makes it an attractive alternative to fungal laccases for application in lignocellulosic material functionalization or modification processes.

Acknowledgements

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6.5. Literature Cited

[1] F. Xu, Biochemistry 35 (1996) 7608-7614.

[2] A.M. Mayer, R.C. Staples, Phytochemistry 60 (2002) 551-565.

[3] H. Claus, Micron. 35 (2004) 93–96.

[4] H. Claus, Arch Microbiol. 179 (2003) 145-150.

[5] P.Sharma, R. Goel, N. Capalash, World J. Microbiol. Biotechnol. 23 (2007) 823-832.

[6] H. Held, A. Kandelbauer, M. Schroeder, A. Cavaco-Paulo, G.M. Guebitz, *Environ. Chem. Lett*. 3 (2005)74-77.

[7] C. Wang, X. Wei, M. Zhao, J. Biotechnol. 2008;- in press

[8] L.O. Martins, C.M. Soares, M.M. Pereira, M. Teixeira, T. Costa, G.H. Jones, A.O. Henriques, J. Biol. Chem. 277 (2002) 18849-18859.

[9] K. Koschorreck, S. Richter, A. Ene, E. Roduner, R. Schmid, V. Urlacher, App. Microbiol. Biotechnol. 79 (2008) 217-224.

[10] M.F. Hullo, I. Moszer, A. Danchin, I. Martin-Verstraete, J. Bacteriol. 183 (2001) 5426-5430.

[11] F.J. Enguita, O.L. Martins, A.O. Henriques, M.A. Carrondo, J. Biol. Chem. 278 (2003)19416-19425.

[12] H. Hirose, M. Nasu, H. Yokoi, Biotechnol. Lett. 25 (2003)1609-1612.

[13] R. Chandra, A.J. Ragauskas, In: 11th International Symposium on Wood and Pulping Chemistry, Nice, France, II; Tappi Press, Atlanta. 2 (2001) 39–43.

[14] R.P. Chandra, A.J. Ragauskas, Enz. Microb. Technol. 30 (2002) 855-861.

[15] C. Felby, R. Chandra , A.J. Ragauskas, J. Wood chem. technol. 24 (2004) 69-81.

[16] R.P. Chandra, L.R. Lehtonen, A.J. Ragauskas, Biotechnol. Prog. 20 (2004) 255-261.

[17] G.S. Nyanhongo, S. Rodríguez Couto, G.M. Guebitz, Chemosphere. 64 (2006) 359-370.

[18] T. Kudanga, E. Nugroho Prasetyo, J. Sipilä, P. Nousiainen, P.Widsten, A Kandelbauer, G.S. Nyanhongo, G.M. Guebitz, Eng. Life Sci. 8 (2008) 297-302.

[19] J. Sipilä, K. Syrjänen, Holzforschung 49 (1995) 325-331.

[20] M.L. Niku-Paavola, E. Karhunen, P. Salola, V. Raunio, *Biochem. J*. 254 (1988) 877-883.

[21] L. Winkler, Berichte der Deutschen Chemischen Gesellschaft, 21(1888) 2843–2855.

[22] E.P. Melo, A.T. Fernandes, P.Dura, L.O. Martins, Biochem. Soc. Trans. 35 (2007) 1579-1582.

[23] E. Nugroho Prasetyo, T. Kudanga, W. Steiner, M. Murkovic, G.S. Nyanhongo, G.M. Guebitz, Anal. Bioanal. Chem. 393 (2009) 679-687.

[24] S. Riva, Trends Biotechnol. 24 (2006) 219-226.

[25] U. Takahama, Physiol. Plantarum.1995;93(1):61-68.

[26] S.V.S. Kumar, P.S. Phale, S. Durani, P.P. Wangikar, Biotechnol. Bioeng. 83 (2003) 386-394.

[27] K.J. Kersten, B. Kalyanaraman, K.E. Hammel, B. Reinhammar, T.K. Kirk, Biochem. J. 268 (1990) 475-480.

[28] D. Litthauer , M.J. van Vuuren, A. van Tonder, W. F. Wolfaardt, Enz Microb Technol. 40 (2007) 563-568.

[29] S.D. Varfolomeev, A. Naki, A.L. Yaropolov, I.V. Berezin, Biokhimiya 50 (1985) 1411-1420.

[30] F. Xu, J.J. Kulys, K. Duke, K. Li, K. Krikstopaitis, H.J.W. Deussen, E. Abbate, V. Galinyte , P. Schneider, Appl. Envrion. Microbiol. 66 (2000) 2052–2056.

[31] K. Li, F. Xu, K.E.L. Eriksson, App. Environ. Microbiol. 65 (1999) 2654-2660.

[32] A. Robbles, R. Lucas, M. Martinez-Canamero, N.B. Omar, P. Perez, A. Galvez, Enz. Microb. Technol. 31 (2002) 516-522.

[33] C. Liers, R. Ullrich, M. Pecyn, D. Schlosser, M. Hofrichter, Enz. Microbial. Technol. 41 (2007) 785-793.

[34] T. Saito, P. Hong, K. Kato, M. Okazaki, H. Inagaki, M. Maeda, Y. Yokogawa, Enz. Microb. Technol. 33 (2003) 520–526.

[35] P. Baldrian, FEMS Microbiol. Rev. 30 (2006) 215-242.

[36] M. Scherer, R. Fischer, Arch. Microbiol. 70 (1998) 78-84.

[37] G. Iyer, B. B. Chattoo, FEMS Microbiol. Lett. 227 (2003) 121-126.

[38] S. Ryan, W. Schnitzhofer, T. Tzanov, A. Cavaco-Paulo, G.M. Gübitz, Enz. Microb. Technol. 33 (2003) 766-774.

[39] H. Palonen, M. Saloheimo, L. Viikari, K. Kruus, Enz. Microb. Technol. 33 (2003) 854-862.

[40] O. Suparno, A.D. Covington, P.S. Phillips, C.S. Evans, Resources Conservation and Recyling. 45 (2005) 114-127.

[41] A. Schultz, U. Jonas, E. Hammer, F. Schauer, Appl. Environ. Microbiol. 67 (2001) 4377–4381.

[42] U. Jonas, E. Hammer, E.T.K. Haupt, F. Schauer, Arch. Microbiol. 174 (2000) 393-398.

[43] J. Ralph, In: Proceedings of the 13th ISWFPC, Auckland, New Zealand, 1(2005) 3-13.

[44] K. Manda, D. Gördes, M. Mikolasch, E. Hammer, E. Schmidt, K. Thurow, F. Schauer, Appl. Microbiol. Biotechnol. 76 (2007) 407-416.

[45] A. Leonowicz, N.S. Cho, J. Luterek, A. Wilkolazka, M. Wojtas–Wasilewska, A. Matuszewska, M. Hofrichter, D. Wesenberg, J. Rogalski, J. Basic Microbiol. 41 (2001)185-227.

[46] S. Witayakran, A.J. Ragauskas, Eur. J. Org. Chem. 3(2009) 358-363.

[47] S. Witayakran, A.J. Ragauskas, Enz. Microb. Technol. 44 (2009) 176-181.

7

Enzymatic grafting of functional molecules to the lignin model dibenzodioxocin and lignocellulose material

Laccase-mediated grafting of functional molecules presents an eco-friendly approach to functionalize lignocellulose materials. In this study functional molecules in the form of reactive phenolic amines, hydrophobicity enhancing fluorophenols and selected wood preservatives, were for the first time successfully coupled onto the lignin model compound dibenzodioxocin (Db) as demonstrated by HPLC-MS analysis. A 1:1 - coupling was demonstrated for various combinations including Db and tyramine (*m/z* 620.5), Db and 3-O-methyldopamine (*m/z* 650.5), Db and 4-hydroxy-3-methoxybenzylamine (*m/z* 636.5), Db and 4-fluoro-2-methylphenol (*m/z* 609.5), and Db and 2 phenylphenol (*m/z* 653.5). Fungal laccases from *Trametes hirsuta* and *T. villosa* were more efficient in mediating the coupling of tyramine to dibenzodioxocin and beech (*Fagus sylvatica*) wood than a *Bacillus* sp. laccase with lower redox potential. This work presents for the first time a model for functionalizing of lignocellulose using the lignin model dibenzodioxocin.

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7.1. Introduction

Lignin constitutes 18-35 % of wood and is one of three major components which include cellulose and hemicellulose [1]. It is a three dimensional polymer of coumaryl alcohol, coniferyl alcohol and sinapyl alcohol joined together in an irregular manner through linkages which include *β*-O-4, *β*-5, 5-5, *β*-*β*, 5-O-4 and the more recently discovered dibenzodioxocin 5-5-O-4 [2-6]. In recent years, there has been an increase in research activities targeted at improving physico-chemical properties of lignocellulosic polymers based on the extensive knowledge acquired in enzymatic grafting of small molecules onto technical lignins and enzyme mediated synthesis of hybrid polymers. Laccases are among the enzymes being investigated. Laccase-mediated grafting of functional molecules onto lignin is thought to provide novel opportunities to impart desired functionalities. Laccases (EC 1.10.3.2, p-diphenol:dioxygen oxidoreductase) are glycoproteins which catalyse the monoelectronic oxidation of a suitable substrate molecule such as phenols and aromatic or aliphatic amines to the corresponding reactive radical coupled with reduction of molecular oxygen to water [7,8]. During wood functionalization, laccases oxidize lignin moieties on the wood surface to create a radical-rich reactive surface to which molecules of interest can be grafted [9]. A recent review summarised several studies that demonstrated the possibility of laccase-catalysed bonding of low molecular weight compounds such as syringic acid, vanillic acid, gallic acid, hydroxytyramine and 4-hydroxybenzoic acid onto lignin [10]. Lignin offers the best target for wood modification as it can be altered without excessively affecting the structural integrity of the wood material.

In previous studies we successfully demonstrated laccase mediated coupling of phenolic amines and imparting antifungal properties to beech veneers [9,11]. We also provided the mechanistic evidence for the coupling *in vitro* using lignin models syringylglycerol β-guaiacyl ether (G-S-β-ether) and guaiacylglycerol β-guaiacyl ether (erol). Nevertheless, such evidence does not exist for coupling functional molecules onto dibenzodioxocin despite the fact that it is an important substructure constituting up to 11 % in softwood lignin [12,13]. Further, evidence showed that the majority of the *5-5*-dihydroxybiphenyl structures are etherified with phenylpropanoid units to form dibenzodioxocin structures [14,15] and together with 5-5 linkages, dibenzodioxocin constitute 18-25 % of linkages in softwood lignin [13,16]. Therefore *in vitro* experiments using this molecule have significant implications on laccase mediated lignocellulose surface functionlization. This study is therefore aimed, for the first time, at investigating the

possibility of coupling different functional molecules (amines, fluorophenols, wood preservatives) onto dibenzodioxocin.

7.2. Materials and methods

Chemicals

The lignin model compound dehydrodivanillyl alcohol type dibenzodioxocin (Db) (Fig. 1a) was synthesized as described by Karhunen *et al*. [3]. The functional molecules tyramine, 3 hydroxytyramine (3HT), 3-O-methyldopamine (3-OMD), 4-hydroxy-3-methoxybenzylamine (4,3-HMB), 4-fluoro-2-methylphenol (4,2-FMP), 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP), triphenylphosphate (TPP) and 2 phenylphenol (2PP) (Fig. 1b-j) were purchased from Sigma-Aldrich. Beech (*Fagus sylvatica*) wood samples were provided by Mitteramskogler GmbH, Austria.

Source of laccases and activity determination

Three laccase enzymes were used namely; *Bacillus* SF spore laccase produced as reported by Held et al. [17], *Trametes hirsuta* laccase produced and purified as previously reported by Almansa et al. [18], and *Trametes villosa* laccase, a kind donation from Novozyme A/S, Denmark. The activity of laccases was measured by monitoring the rate of oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) at 436 nm according to Nugroho Prasetyo et al [19] with some modifications. Briefly, the reaction mixture contained 30 µl laccase, 350 µl ABTS (1 mM) and 50 mM succinate buffer pH 4.5 to make a final volume of 1.5 ml. A blank was set in the same way as the sample experiment except that the laccase was initially heat denatured at 100 °C for 10 minutes. The spectrometric measurements were done by recording the absorbance in the time scan mode for 2 minutes.

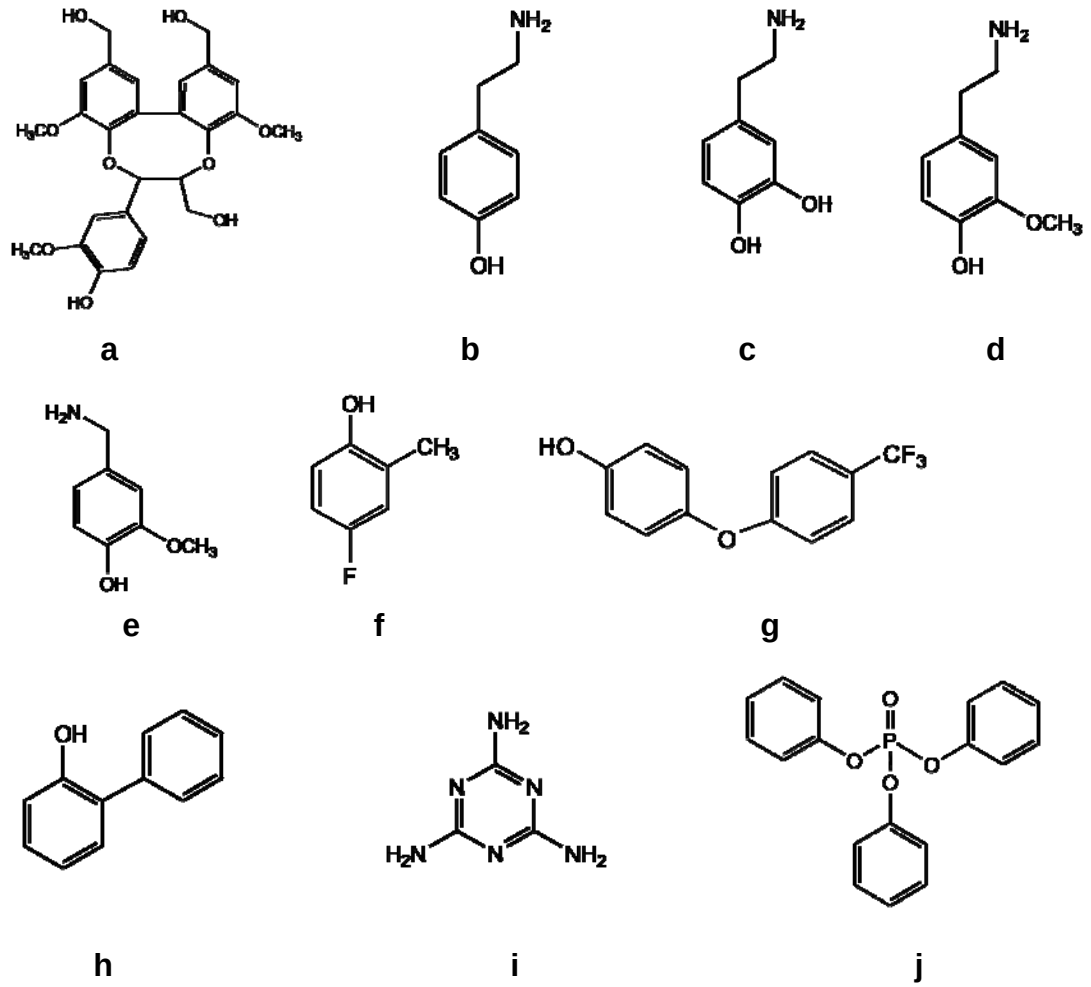


Figure 1. Chemical structures of the lignin model molecule dibenzodioxocin and functional molecules used in the coupling reactions: (**a**) dibenzodioxocin (**b**) tyramine (**c**) 3-hydroxytyramine (**d**) 3-O-methyldopamine (e) 4-hydroxy-3-methoxybenzylamine, (**f**) 4-fluoro-2-methylphenol (**g**) 4-[4-(trifluoromethyl)phenoxy]phenol (**h**) 2-phenylphenol (**i**) melamine, (j) triphenylphosphate.

Oxidation of functional molecules and dibenzodioxocin

The ability of the three laccases to oxidize the functional molecules was monitored by UV/Vis- spectrophotometry and confirmed by HPLC analysis. To start the reaction, laccase with a final activity of 2.0 nkat ml^{-1} was added to a reaction mixture containing 0.1 mM functional molecule in 50 mM succinate buffer pH 4.5 (0.1 M phosphate buffer pH 7.0 for *Bacillus* SF laccase). The reactions were monitored by UV/Vis-spectrophotometry in the wavelength scan mode from 900 nm – 200 nm at 3 min per cycle for a total of 18 min to identify oxidation products.

Coupling of functional molecules onto dibenzodioxocin and beech wood

The reaction mixture contained 1.0 mM functional molecule, 2.3 mM dibenzodioxocin and 2.0 nkat ml^{-1} laccase in 50 mM ammonium acetate buffer pH 4.5 (pH 7.0 for *Bacillus* SF laccase). Reactions were carried out at 37 ºC while shaking at 650 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg Germany). The reactions were incubated for 1 hour when 2 phenylphenol was used as functional molecule, 2 hours when 3-hydroxytyramine was used and 3 h for the rest of the molecules. The coupling products were analyzed by HPLC and LC-MS. The non-laccase substrate tyramine was also coupled onto beech wood so as to determine the extent of coupling. The beech wood was oxidised using the three laccases (2.0 nkat ml^{-1}) in the presence of tyramine (1.0 mM) in 50 mM succinate buffer pH 4.5 (0.1 M phosphate buffer pH 7.0 for *Bacillus* SF laccase) for 3 hours. The unbound tyramine was washed with 75 % ethanol for 16 hours while shaking at 150 rpm. The washing solution was collected and the pieces were washed again for a further 10 minutes with 10 ml 75 % ethanol before transferring them into 10 ml 75 % acetone for another 10 min wash. The washing solutions containing unbound tyramine were pooled and the residual (uncoupled) tyramine determined using HPLC analysis.

HPLC analysis of reaction products

To the incubation mixtures an equal volume of ice cold methanol was added to precipitate proteins. The mixture was centrifuged at 0 ºC for 15 minutes at 14000 g and 650 µl aliquots were transferred into clean vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 x 4.6 mm with precolumn, Knauer GmbH, Berlin, Germany) using a linear gradient of acetonitrile (solvent B) and 0.1 % H_3PO_4 (solvent A) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30 °C. The steps of the gradient were as follows: linear gradient from 80 % solvent A to 25 % in 15 min, from 25 % to 10 % in 1 min, held at 10 % for 14 min before increasing back to 80% followed by washing and reconditioning of the column. Alternatively 0.1 % formic acid was used instead of 0.1 % H_3PO_4 although this led to a slight shift in retention time. The coupling product of the phenolic amine 3-hydroxytyramine was observed under isocratic conditions using acetonitrile, 10 mM sulphuric acid and deionized water (4: 3:13) as solvent at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C. Identification and quantitative determination of amine substrates (after

coupling) was done by reversed phase HPLC on a Discovery HS C18 column (5 µm; 15 cm x 4.6 mm, Supelco, Bellefonte, USA) using acetonitrile and 50 mM potassium phosphate buffer, pH 4.6 (1:49 v/v) as solvent at a flow rate of 1 ml min^{-1} and an oven temperature of 25 °C.

LC-MS analysis of coupling products

MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionisation coupled to the Dionex HPLC-UVD-system as described above and initial separation of coupling products was carried out using the linear gradient method described above except that 0.1 % formic acid was used instead of 0.1 % H_3PO_4. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 12 l min^{-1} with a temperature of 350 °C and the nebulizer pressure set to 70 psi. Maximum accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30000.

7.3. Results and Discussion

Oxidation of functional molecules and of the lignin model compound dibenzodioxocin

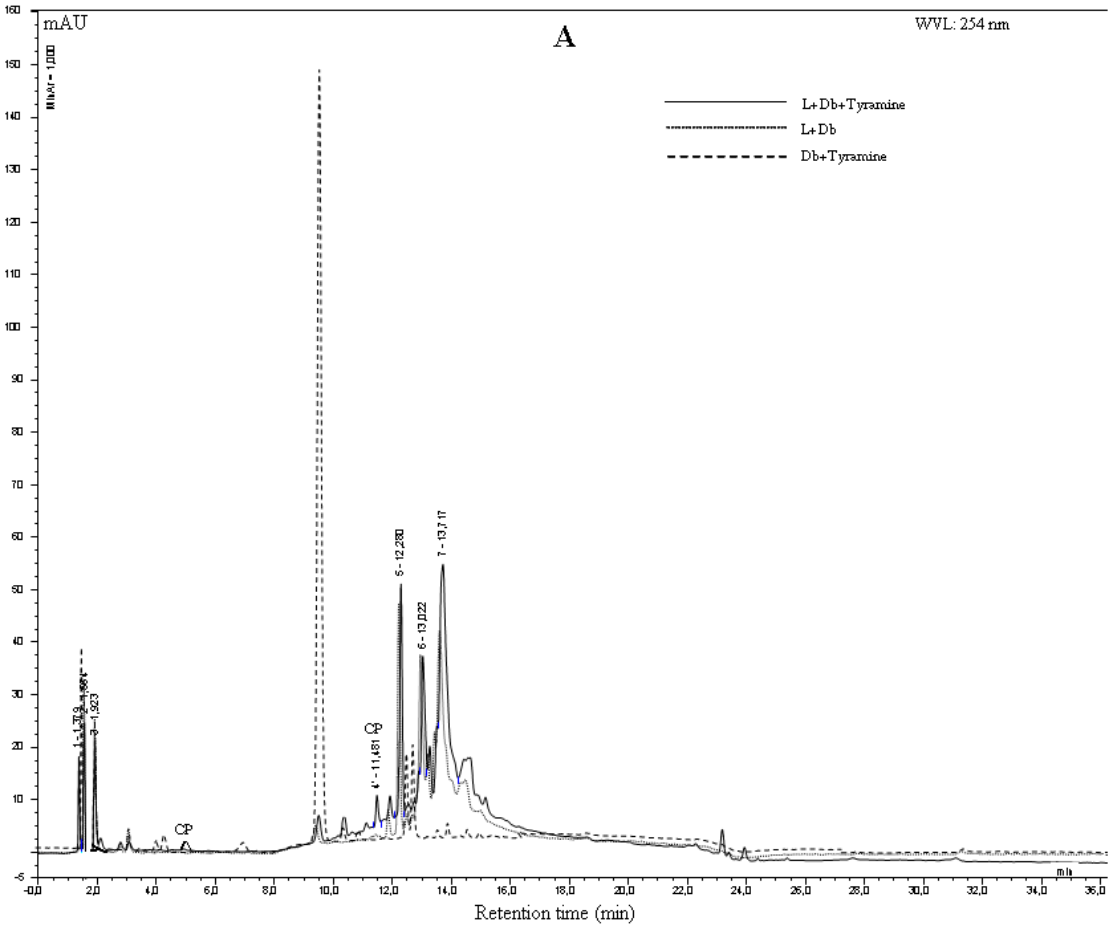
Initially oxidation of the various educts by laccase was investigated using HPLC analysis. The retention time of dibenzodioxocin was t_R=9.4 min. Upon laccase oxidation, 3 oxidation products (t_R=12.2 min t_R=13.0 min t_R=13.6 min) were observed. Similarly, laccase oxidation products were observed with the molecules 3-hydroxytyramine (t_R=37.8), 3-O-methyldopamine (t_R=37.9), 4-fluoro-2-methylphenol (t_R=5.4), 2 phenylphenol (t_R=23.9) and triphenylphosphate (t_R=5.4). More products were observed upon dibenzodioxocin oxidation probably due to enzymatic cleavage [20] or oligomerisation [21, 22]. However, tyramine was not readily oxidized by all laccases used probably because it lacks an electron donating substituent *ortho* to a hydroxyl group which is usually a pre-requisite for non-mediated laccase oxidation [23]. However, some fungal laccases can oxidize simple monophenolic molecules [24]. Triphenylphosphate is the only molecule which lacks a phenolic hydroxyl group and was oxidised probably because of high electron density as a result of the 3 benzene rings which makes electron abstraction easier. Generally it has been observed that the nature and position of functional groups attached to the benzene ring influence the reactivity of the molecule to laccase [11]. Electron donating groups e.g. methoxyl groups tend to make a

molecule more reactive while electron-withdrawing groups e.g. fluorine decreases the reactivity.

Coupling reactions

Coupling of phenolic amines onto dibenzodioxocin

Coupling or grafting reactions are negatively affected by quenching of radicals which usually occurs within few hours to a reported extent of 90% in laccase-treated thermo-mechanical pulp [25]. In our earlier study we reported an increase in coupling of fungicides onto amine functionalized wood using *Trametes hirsuta* laccase [9]. It was proposed that phenolic amines acted as anchor groups creating a stable reactive surface which counteract negative effects of radical quenching. In a follow up study, using *Bacillus* SF laccase, we provided the mechanistic evidence that tyramine was successfully covalently coupled onto syringylglycerol β–guaiacylether via a 4-O-5 bond, leaving the -NH_2 group free for further attachment of functional molecules [11]. In this study coupling of phenolic amines is demonstrated using the most recently discovered lignin subunit dibenzodioxocin. As dibenzodioxocin is a laccase substrate [9], it was possible to couple the laccase substrates 3-hydroxytyramine, 3-O-methyldopamine and 4 hydroxy-3methoxybenylamine and the non laccase substrate tyramine. According to HPLC analysis, peaks observed in coupling reactions and not in the control reactions indicate coupling products between the dibenzodioxocin and the amines (Fig. 2). Two new peaks were observed in tyramine coupling reaction with retention times t_R 5.1 min and t_R=11.4 min while coupling products were observed at retention times t_R=3.5 min and t_R=5.9 min when 3-O-methyldopamine was used. Using isocratic conditions a 3-hydroxytyramine coupling product (t_R=21.6) was observed


mAU
A
WVL: 254 nm
L+Db+Tyramine
L+Db
Db+Tyramine
5 - 12,280
6 - 13,022
7 - 13,717
Cp
Cp
Retention time (min)

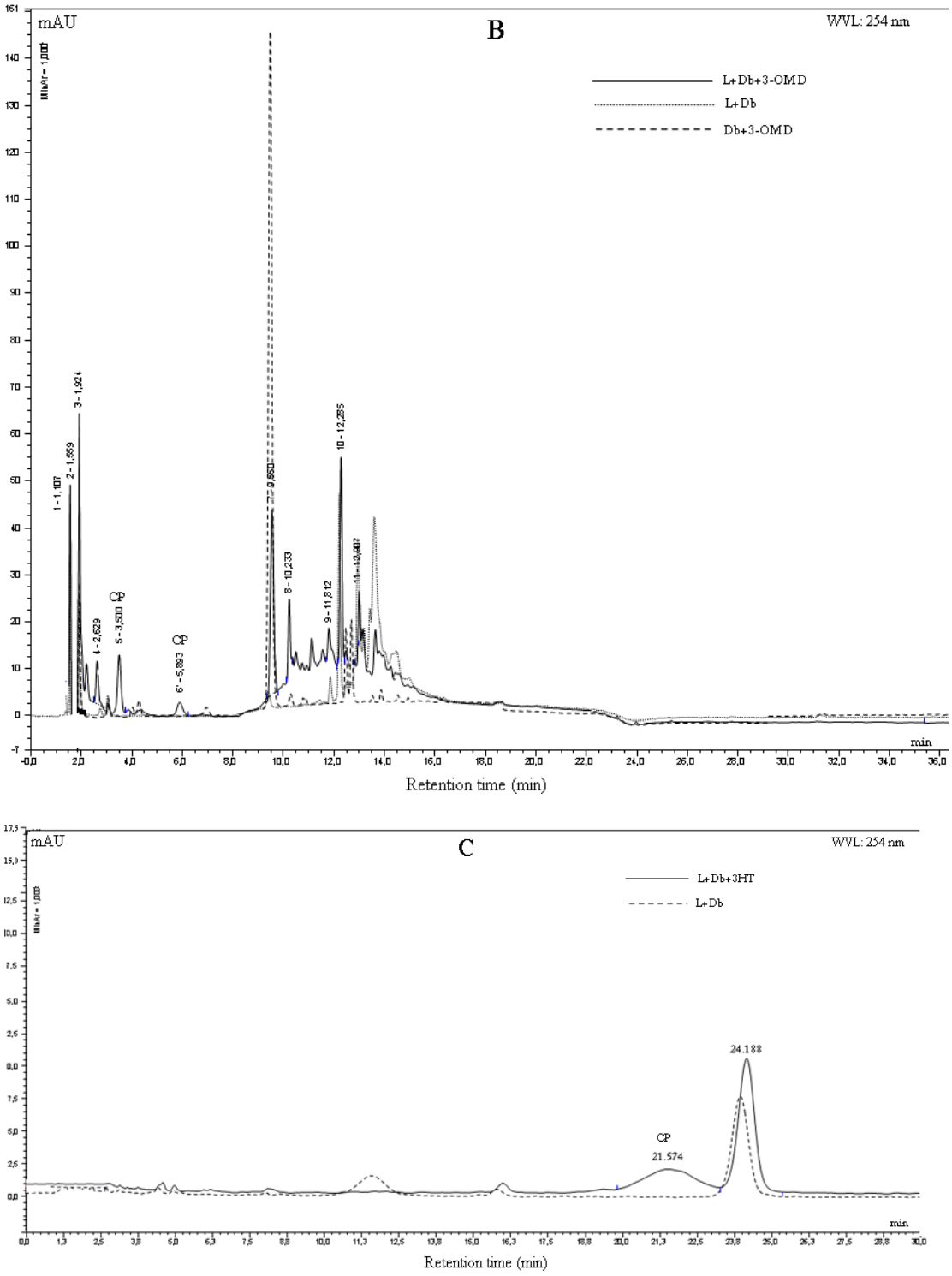


Figure 2. Laccase-mediated coupling of phenolic amines tyramine (A), 3-O-methyldopamine (3-OMD) (B) and 3- hydroxytyramine (3HT) (C) onto the lignin model compound dibenzodioxocin (Db)

Since tyramine was observed to be a non-laccase substrate, it was possible to quantify enzymatic coupling to lignin model compound using HPLC, based on the consumption of tyramine (Table 1). The percentage of tyramine coupling appeared to be related to the redox potential of the enzyme. Bacterial laccases have been reported to exhibit a lower redox potential of about 0.5 V [27] compared to 0.5 - 0.8 V for fungal laccases [28,29] and were consequently less efficient than the fungal laccases (redox potential 0.7- 0.8 V) used in this study.

Table 1. Coupling of tyramine (initial concentration 1 mM) to laccase-oxidized dibenzodioxocin and beech (*Fagus sylvatica*) wood

Enzyme	Tyramine transformation (%)*	
	Model	Beech wood
Trametes hirsuta laccase	13.6 ± 1.7	18.0 ± 2.1
Trametes villosa laccase	16.0 ± 2.0	20.9 ± 3.4
Bacillus SF laccase	6.8 ± 0.6	7.3 ± 2.5

*****All values are means of three replicates ± standard deviation

Coupling fluorophenols onto dibenzodioxocin

Previously fluorophenols have been chemically applied to materials as a way of increasing hydrophobicity [30,31]. In this study the fluorophenols, 4-fluoro-2-methylphenol and 4-[4-(trifluoromethyl)phenoxy]phenol were readily coupled enzymatically onto dibenzodioxocin. A new coupling product peak (t_R=14.9) was observed when 4-fluoro-2-methylphenol was used while in the presence of 4-[4-(trifluoromethyl)phenoxy]phenol, 4 coupling products were observed at retention times t_R=15.6, t_R=16.9 t_R=18.0 and t_R=19.0 (Fig 3). Enzymatic coupling of fluorophenols has practical significance since chemicals physically adsorbed are bonded by relatively weak Van der Waal forces and leach out over a long time due to continuous exposure to water [32]. This does not only leave the material hydrophilic and therefore prone to biodeterioration, but also exposes the environment to hazardous chemicals.

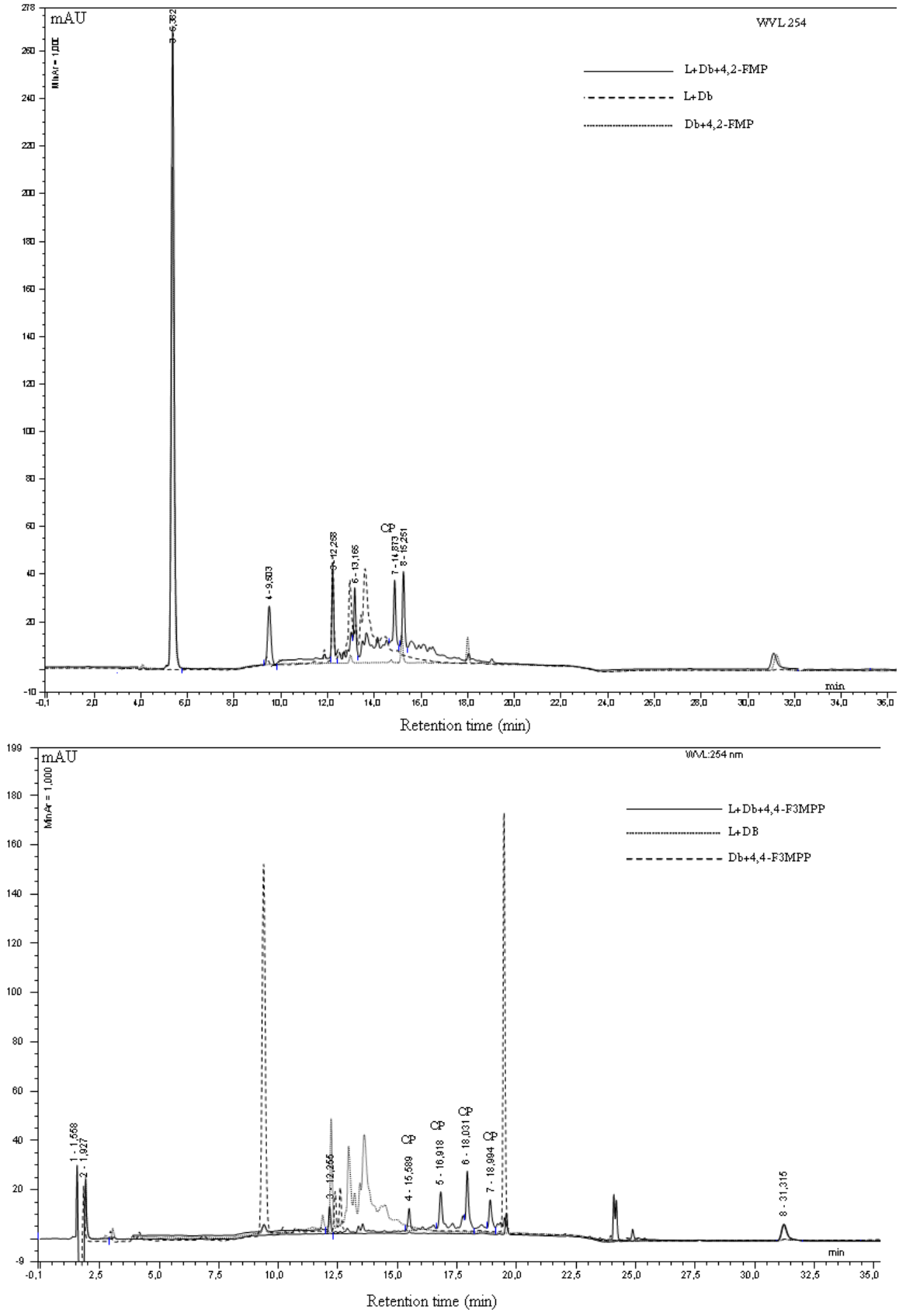


Figure 3. Laccase-mediated coupling of 4-fluoro-2-methylphenol (4,2-FMP) and 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP) onto the lignin model compound dibenzodioxocin (Db)

Coupling of wood preservatives 2 phenylphenol and triphenylphosphate onto dibenzodioxocin

Wood species are susceptible to decay and are also flammable which makes it imperative to use preservatives. Traditionally, wood treatments use high pressure in which the wood is impregnated in closed vessels. Unfortunately such methods are not only energy consuming but are associated with an increase in temperature which can negatively affect the wood or the preservative. Wood may shrink if it loses moisture during treatment while some preservatives may precipitate at elevated temperatures [33]. Both high pressure and low pressure treatment methods are generally not eco-friendly when used for volatile preservatives or those that leach out of the products which then easily escape into the environment and may affect plant and animal life. 2-Phenylphenol is a fungicide used in agriculture, on fibers and other materials and is a general disinfectant while triphenylphosphate and melamine are used as fire retardants in many materials including lignocellulosics. A coupling product between 2-phenylphenol and dibenzodioxocin was observed with retention time t_R=15.4 min while a coupling product (t_R=8.7) was observed with triphenylphosphate (Fig. 4). Coupling of melamine to dibenzodioxocin was demonstrated by LC MS (Fig. 5). Although a recent European Food Safety Authority (EFSA) Scientific Report lists 2-phenylphenol as biodegradable, the same report also considered the chemical as toxic to aquatic organisms and proposed its classification as R50 ("very toxic to aquatic organisms") [34]. Although solubility of 2-phenylphenol is less than 0.1 g/litre at 20 °C, its sodium salt is very soluble (122 g dissolve in 100 ml water) and is therefore easily leached into the environment. Triphenylphosphate is also generally regarded as biodegradable [35]. However, it easily enters aquatic systems via hydraulic fluid leakage as well as leaching and is known to be toxic to some aquatic organisms like fish and algae. Although melamine has low toxicity to both aquatic and soil organisms, release to water bodies should be avoided as its biodegradability in nature is considered poor. Consequently, it may accumulate in surface water or sludge. Laccase-mediated grafting covalently binds these molecules onto lignocellulose material thereby preventing leaching and release into the environment.

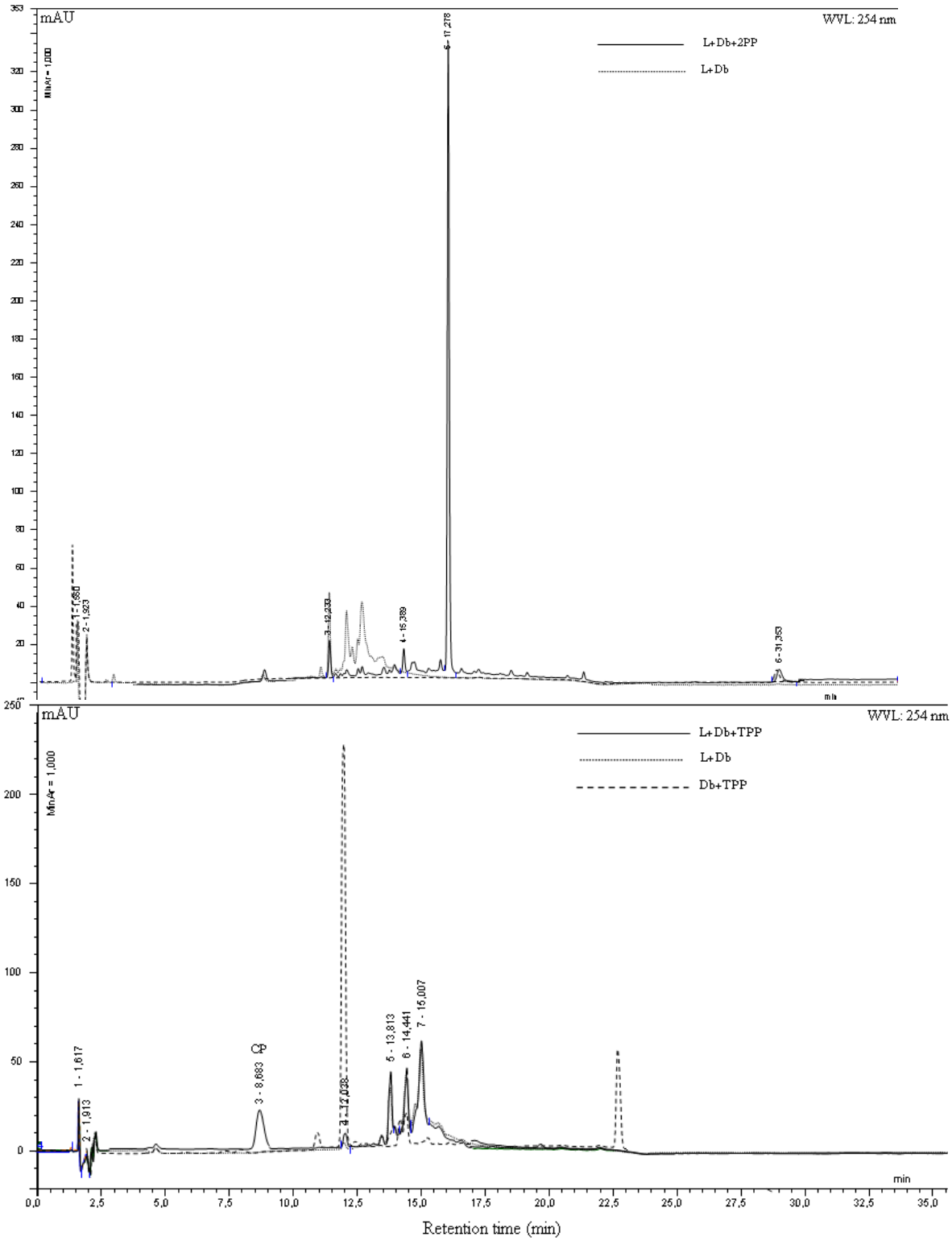


Figure 4. Laccase-mediated coupling of 2-phenylphenol (2PP) and triphenylphosphate (TPP) onto dibenzodioxocin (Db)

LC-MS analysis

The molecular mass of lignin model dibenzodioxocin is 484.4. LC-MS analysis in positive ion mode showed $[M+H]^+$ ion signals at *m/z* 620.5 (Db+Tyramine), *m/z* 637.4 (Db+3HT), *m/z* 650.5 (Db+3-ODM), *m/z* 636.6 (Db+4,3-HMB), *m/z* 609.5 (Db+4,2-FMP), *m/z* 737.6 (Db+4,4-F3MPP), *m/z* 609.3 (Db+melamine), and *m/z* 653.5 (Db+2PP) (Fig. 5), corresponding to molecular weights of 1:1 coupling products. According to existing knowledge preformed lignin oligomers usually results in units bonded through 5-5 and 4-O-5 linkages [36]. The hydroxyl group on the benzene ring is already known to be *ortho* or *para* directing [37,38,39] and molecules with free C-5 position usually cross-couple through 5-5 linkages due to stability of C-C bonds [40] as shown in most of the coupling products in Fig 6. This is supported by the observations that 5-5 bonds have a lower heat of formation than the other common lignin linkages [41]. Considering this literature, steric effects and the results from LC/MS analysis, structures of the coupling products were proposed (Fig. 6).

The demonstrated ability to couple different functional molecules onto dibenzodioxocin together with previous studies using other lignin model compounds [9, 11] shows the great potential applications of laccases in lignocellulose surface functionalizaion.

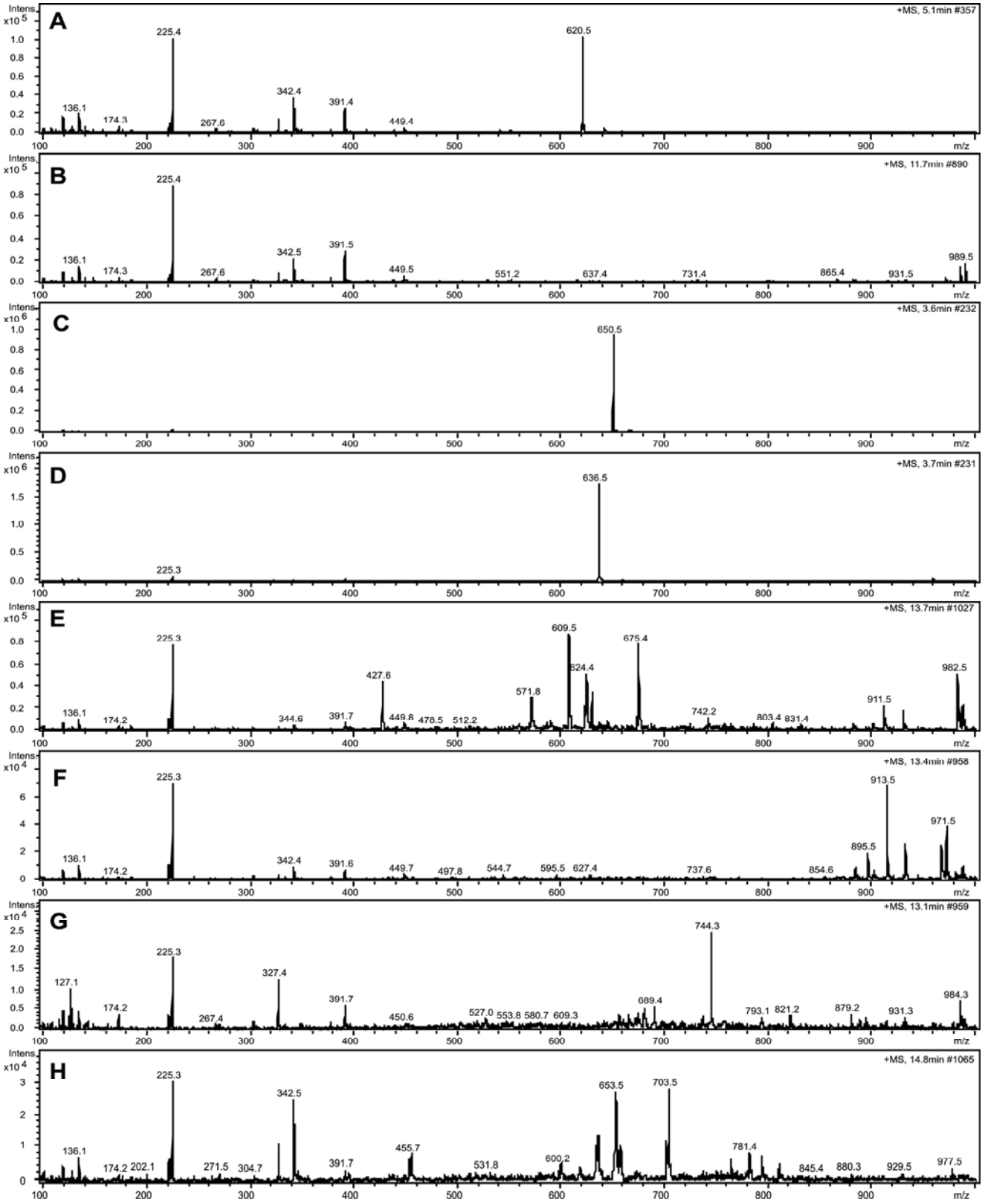
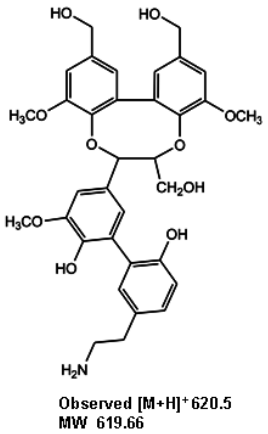
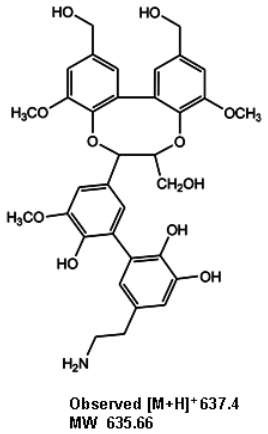


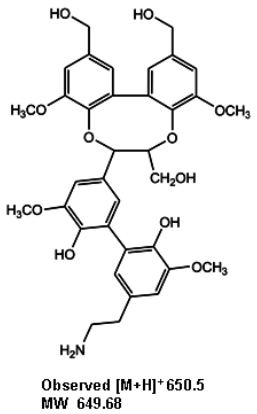
Figure 5. Mass spectra of coupling products between dibenzodioxocin and functional molecules, tyramine(**A**), 3 hydroxytyramine (**B**), 3-O-methyldopamine (**C**), 4-hydroxy-3-methoxybenzylamine (**D**), 4-fluoro-2-methylphenol (**E**), 4-[4-(trifluoromethyl)phenoxy]phenol (**F**), melamine (**G**) and 2 phenylphenol (**H**).



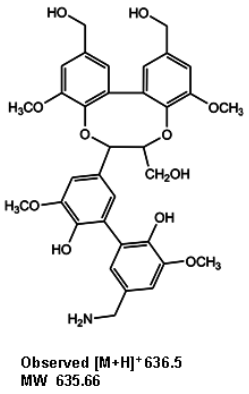
Db+Tyramine



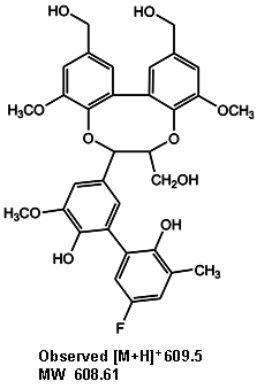
Db+3HT



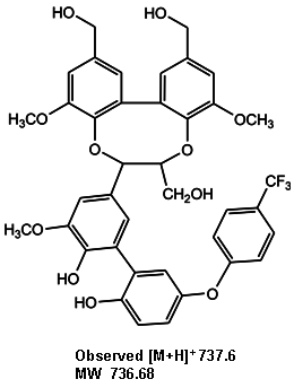
Db+3-OMD



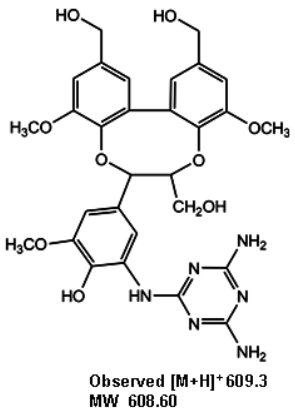
Db+4,3-HMB



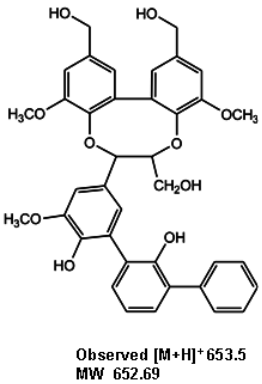
Db+4,2-FMP



Db+4,4-F3MPP



Db+melamine



Db+2PP

Figure 6. Proposed coupling structures between dibenzodioxocin (Db) and functional molecules

7.4 Conclusion

This study demonstrates, for the first time, coupling of different functional molecules (phenolic amines, fluorophenols and selected wood preservatives) onto the lignin model dibenzodioxocin. This study together with the previous investigations using other lignin models effectively demonstrate the ability of laccase to mediate the coupling of functional molecules onto the predominant lignin structures. Coupling of phenolic amines provides anchor groups onto which other molecules of interest can be grafted. The coupling of hydrophobicity enhancing fluorophenols and the preservatives (2-phenylphenol triphenylphosphate and melamine) covalently binds them to lignocellulose material so that they are not readily released into the environment. Since laccases work at ambient temperature using oxygen as electron acceptor and releasing water as the only by-product, this study therefore presents an eco-friendly model for functionalising lignocellulose material.

Acknowledgement

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7.5. References

[1] Friedman R. Principles of fire protection chemistry and physics. third ed.; Sudbury: Jones and Bartlett Publishers; 1998.

[2] Sjöström E. Wood Chemistry: Fundamentals and application. Academic Press. Orlando 1993.

[3] Karhunen P, Rummakko P, Sipila J, Brunow G, Kilpelinen I.. Dibenzodioxocins; a novel type of linkage in softwood lignins. Tetrahedron Lett (1995a); 36: 169-170.

[4] Karhunen P, Rummakko P, Sipila J, Brunow G, Kilpelinen I. The formation of dibenzodioxocin structures by oxidative coupling. A model reaction for lignin biosynthesis. Tetrahedron Lett (1995b);36:4501-4504.

[5] Boudet AM. Lignins and lignification: Selected issues. Plant Physiol Biochem 2000;38:81-96.

[6] Martínez AT, Rencoret J, Marques G, Gutiérrez A, Ibarra D, Jiménez-Barbero J and

del Río JC. Monolignol acylation and lignin structure in some nonwoody plants: A 2D NMR study. Phytochemistry 2008;69:2831-2843.

[7] Nyanhongo GS, Schroeder M, Steiner W, Gübitz GM Biodegradation of 2,4,6-trinitrotoluene (TNT):An enzymatic perspective. Biocatal Biotransform 2005;23: 1-17.

[8] Riva S Laccases: blue enzymes for green chemistry. Trends Biotechnol 2006; 24:219-225.

[9] Kudanga T, Prasetyo EN, Sipilä J, Nousiainen P, Widsten P, Kandelbauer A, Nyanhongo GS, Guebitz GM. Laccase-mediated wood surface functionalization. Eng Life Sci 2008;8: 297-302.

[10] Widsten P, Kandelbauer A, Laccase applications in the forest products industry : A review. Enzyme Microb Technol 2008;42:293-307.

[11] Kudanga T, Nugroho Prasetyo E, Sipilä J, Eberl A, Nyanhongo GS, Gübitz GM. Coupling of aromatic amines onto syringylglycerol β–guaiacylether using *Bacillus* SF spore laccase: a model for functionalization of lignin-based materials J. Mol. Catal. B: Enzym. 2009;61:143-149.

[12] Kukkola EM, Koutaniemi S, Gustafsson M, Karhunen P, Ruel K, Lundell TK, Saranpää P, Brunow G, Teeri TH, Fagerstedt KV. Localization of dibenzodioxocin substructures in lignifying Norway spruce xylem by transmission electron microscopy-immunogold labeling. Planta 2003; 217:229-37.

[13] Argyropoulos DS, Jurasek L, Krištofová L, Xia Z, Sun Y, Paluš E. Abundance and Reactivity of Dibenzodioxocins in Softwood Lignin. J. Agric. Food Chem 2002;50: 658–666.

[14] Karhunen P, Rummakko P, Pajunen A, Brunow G. Synthesis and Crystal Structure Determination of Model Compounds for the Dibenzodioxocin Structure Occurring in Wood Lignins. J Chem Soc Perkin Trans 1996; 1: 2303-2308.

[15] Brunow G, Kilpeläinen I, Sipilä J, Syrjänen K, Karhunen P, Setälä H, Rummakko P. Oxidative coupling of phenols and the biosynthesis of lignin. In: Lewis NG, Sarkanen S (eds) Lignin and lignan biosynthesis. ACS Symp Ser vol 697. American Chemical Society, Washington, DC, 1998; pp 131–147.

[16] Froass PM, Ragauskas, A J, Jianer J. Chemical structure of residual lignin from kraft pulp. J. Wood Chem. Technol. 1996;16:347-365.

[17] Held C, Kandelbauer A, Schroeder M, Cavaco-Paulo A, Guebitz GM. Biotransformation of phenolics with laccase containing bacterial spores. Environ Chem

Lett. 2005;3:74-77.

[18] Almansa E, Kandelbauer A, Pereira L, Cavaco-Paulo A, Guebitz GM, Influence of Structure on Dye Degradation with Laccase Mediator Systems. Biocat Biotrans 2004; 22(5):315-324.

[19] Nugroho Prasetyo E, Kudanga T, Steiner W, Murkovic M, Nyanhongo GS, Guebitz GM Antioxidant activity assay based on laccase-generated radicals. Anal. Bioanal.Chem. 2009; 93:679–687.

[20] Guerra A, Mendonça R, Ferraz A, Lu F, Ralph J. Structural Characterization of Lignin during Pinus taeda Wood Treatment with Ceriporiopsis subvermispora. Appl Environ Microbiol 2004;70:4073–4078.

[21] Thurston F. The structure and function of fungal laccases, Microbiology 1994;140: 19–26.

[22] Buchert J, Mustranta A, Tamminen T, Spetz P, Holmbom B. Modification of Spruce Lignans with Trametes hirsute Laccase. Holzforschung 2002;56:579–584.

[23] Paice MG, Bourbonnais R, Reid ID. Bleaching kraft pulps with oxidative enzymes and alkaline hydrogen peroxide. Tappi J. 1995;78:161–169.

[24] Takahama U. Oxidation of hydroxycinnamic acid and hydroxycinnamyl alcohol derivatives by laccase and eroxidase-interactions among p-hydroxyphenyl, guaiacyl and syringyl groups during the oxidation reactions. Physiol Plantarum 1995;93:61- 68.

[25] Grönqvist S, Rantanen K, Raimo A, Mattinen M, Buchert J, Viikari L. Laccase-catalysed functionalization of TMP with tyramine. Holzforschung 2006;60:503–508.

[27] Melo EP, Fernandes AT, Dura P, Martins LO. Insight into stability of CotA laccase from the spore coat of Bacillus subtilis. Biochem Soc Trans 2007;35:1579-1582.

[28] Kumar S, Phale P, Durani S, Wangikar P. Combined sequence and structure analysis of the fungal laccase family. Biotechnol Bioeng 2003;83:386-394.

[29] Kersten PJ, Kalyanaraman B, Hammel KE, Reinhammar BKirk TK. Comparison of lignin peroxidase, horseradish peroxidase and laccase in the oxidation of methoxybenzenes. Biochem J 1990;268:475–480.

[30] Zhang L, Huang WY. Synthesis of polyfluorinated aromatic polyesters. J. Fluor Chem (2000);102:55-59.

[31] Tsukada Y, Iwamoto K, Furutani H, Matsushita Y, Abe Y, Matsumoto K, Monda K, Hayase S, Kawatsura M, Itoh T. Preparation of novel hydrophobic fluorine-substituted-alkyl sulfate ionic liquids and application as an efficient reaction medium for lipase-catalyzed reaction. Tetrahedron Lett 2006;47:1801–1804.

[32] Razzaque MA, The effect of concentration and distribution on the performance of water repellents applied to wood. University of Wales, Ph.D. Thesis1982.

[33] Ibach RE, Wood preservation. In Forest Products Laboratory. Wood handbook—Wood as an engineering material. Gen. Tech. Rep. FPL–GTR–113. Madison, WI: U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 1999.

[34] EFSA Scientific Report. Conclusion on pesticide peer review regarding the risk assessment of the active substance 2-phenylphenol. EFSA Scientific Report 2008; 217: 1-67.

[35] Seager VW, Hicks O, Kaloy RG, Michael PW, Mieure JP, Tuker ES. Environmental fate of selected phosphate esters. Environ Sci Technol 1979;13:840–844.

[36] Boerjan W, Ralph J, Baucher M. Lignin biosynthesis. Annu Rev Plant Biol 2003; 54:519–46.

[37] Suparno O, Covington AD, Phillips PS, Evans CS. An innovative new application for waste phenolic compounds: Use of kraft lignin and naphthols in leather tanning. Resources Conservation and Recycling. 2005;45:114-127.

[38] Schultz A, Jonas U, Hammer E, Schauer F. Dehalogenation of chlorinated hydroxybiphenyls by fungal laccase. Appl Environ Microbiol 2001;67:4377–4381.

[39] Jonas U, Hammer E, Haupt ETK, Schauer F. Characterisation of coupling products formed by biotransformation of biphenyl and diphenyl ether by the white rot fungus Pycnoporus cinnabarinus. Arch Microbiol 2000;174:393-398.

[40] del Río JC, Gutiérrez A.. Rapid assessment of the lignin composition of lignocellulosic materials commonly used for paper pulp manufacturing by analytical pyrolysis. 18th Int Symp Anal Appl Pyr P99 2008 p. 237.

[41] Valencia PJS, Bolívar Marinez LE. Lignin in the restoration of the cultural heritage. Revista Colombiana de Física 2005; 37:106-109.

8

Chemo-enzymatic functionalisation of lignocellulose materials using oxiranes

A new non-selective chemoenzymatic process using oxiranes for functionalisation of lignocellulose materials has been developed. Using a lipase and hydrogen peroxide, soybean oil and linoleic acid were epoxidised resulting in a 52.7 % and 92.4 % relative conversion to oxirane, respectively. A commercial oxirane, 1,2 epoxyoctane and oxiranes generated from epoxidation of oils (using lipase and hydrogen peroxide) were successfully coupled onto long chain alkylamines while the oxirane was also successfully coupled to lignin molecules. Treatment of beech veneers with dodecylamine using this process resulted in a 90 % increase in hydrophobicity. Since oxiranes react directly with hydroxyl groups and other functional groups this method may provide a new enzymatic non-targeted approach for the functionalization of various polymeric materials.

Submitted as Kudanga et al. to *Biotechnology and Bioengineering*

Introduction

The use of wood and wood-based products is an integral part of human life. Among the major wood product categories used for different purposes are sawn timber, wood-based panels, woodchips, paper and paper products, poles and railway sleepers (Nugroho Prasetyo et al., 2009). Traditionally a number of physico-chemical methods have been used to improve properties of wood, especially focussed at improving strength properties, aesthetic properties and reducing biodeterioration. Some of these methods have been summarised by a number of authors (Schultz et al., 2007; Evans, 2003; Ibach 1999; Kumar, 1994; Rowell and Banks, 1985). However, in recent years there has been a sudden shift in world policy towards green processes preferably using renewable materials. In this regard a number of studies are starting to emerge describing processes for enzymatic modification of lignocellulose materials (Petri and Kandelbauer, 2008; Mikolasch and Schauer, 2009). Most of these biologically based methods of wood surface functionalization have targeted lignin. However, lignin only comprises 18 – 35 % of lignocellulose material thereby leaving at least 65 % of the surface unmodified.

In this study, a novel approach to functionalize the surface of lignocellulose material has been developed. This is based on the chemo-enzymatic generation of oxiranes from unsaturated oils. The oxiranes are highly reactive molecules which bind none selectively to structural components of the lignocellulose material. The generation of oxiranes from unsaturated oils has been studied partly due to their importance as intermediates in the synthesis of polymers and as products resulting from the production of peroxy acids which are used as additives of polymers, lubricants and detergents (Wöhlerś et al., 1958; Rangarajan et al., 1995; Sinadinovic-Fiser et al., 2001; Campanella and Baltanás, 2005; Goud et al., 2006a, 2007). Normally their production is achieved using strong mineral acids like concentrated sulfuric acid as catalysts. In essence the Prileshajev epoxidation of unsaturated plant oils has for a long time been considered the only commercial source of epoxidized oils. In this process, peroxy acids such as peracetic acid are generated (preferably in-situ to avoid danger of handling peroxy acids) from the corresponding acid and hydrogen peroxide in the presence of a strong mineral acid (Formo, 1982). Unfortunately, the process is limited by the potential danger of handling peroxy acids, acid facilitated corrosion of equipment (Rüsch gen Klaas and Warwel, 1999), and lower selectivity due to a considerable number of side reactions (oxirane ring opening to diols, hydroxyesters, estolides and other dimmers) (Rangarajan et al., 1995).

However, a lipase [Novozym 435 R (EC 3.1.1.3; from *Candida antarctica*)] has been shown to catalyze the formation of peroxy acids from fatty acids in the presence of hydrogen peroxide (Björkling et al., 1990, 1992). In the presence of unsaturated plant oil, the lipase generated peroxy acids epoxidize the C=C-bonds. The reaction product is a mixture of epoxidized tri-, di- and monoglycerides, glycerol, and epoxy fatty acids. A general scheme of chemoenzymatic formation of oxiranes is shown in Figure 1.

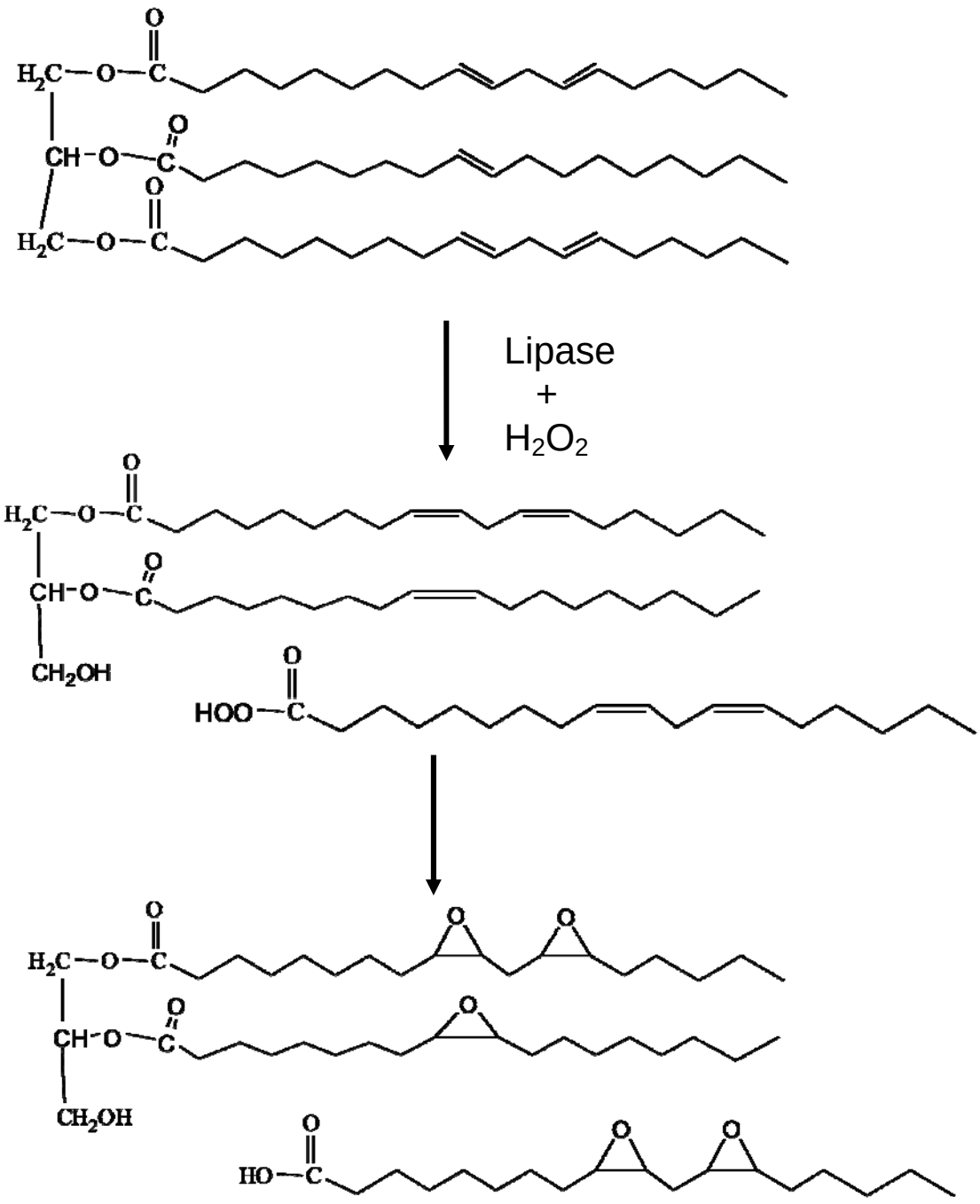


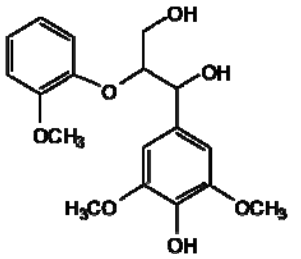
Figure 1. General scheme of the chemoenzymatic formation of oxiranes

Excellent yields of epoxidized plant oils have been obtained using this mild approach (Rüsch gen Klaas and Warwel, 1996). For example, after reaction of rapeseed oil with Novozym 435 R and hydrogen peroxide, only 1% of the double bonds remained and oxirane-oxygen content was 5.3 %, which represented 91 % yield of the theoretical value (Rüsch gen Klaas and Warwel, 1996). Today soybean oil is the most widely epoxidised oil hence its production has increased to about 200,000 t/year (Rüsch gen Klaas and Warwel, 1999). Since oxiranes easily react with lignocellulose materials and functional molecules carrying for example -OH, -NH or -SH groups (Nishikubo et al., 1998; Wing et al., 1970), this work presents this method as a new approach for functionalisation of lignocellulose materials.

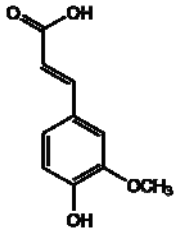
Materials and Methods

Oils, chemicals and enzyme

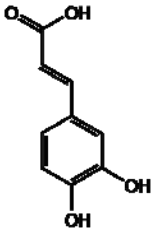
Candida antarctica lipase (≥10,000 U/g) immobilised on macroporous acrylic resin and all chemicals including soybean oil (SBO), linoleic acid (LA), dodecylamine (DA) and the model epoxide (1,2 epoxyoctane) used for modelling coupling reactions, were purchased from Sigma Aldrich. The lignin model compound syringylglycerol β-guaiacyl ether was synthesised as described by Sipilä and Syrjänen (1995). The structures of some of the molecules used in this study are shown in Fig. 2.



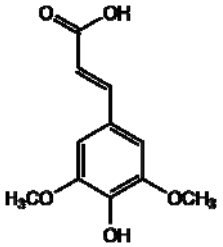
Syringylglycerol β-guaiacyl ether



Ferulic acid

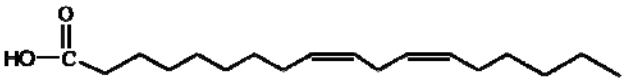


Caffeic acid

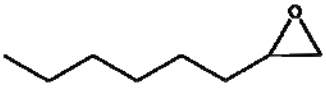


Sinapic acid

Dodecylamine (DA)



Linoleic acid (LA)



1,2-epoxyoctane

Figure 2. Structures of selected molecules used in this study

Veneers

The wood veneers used in the present investigation were prepared from European beech (*Fagus sylvatica*) and measured 10 mm x 10 mm x 1 mm. Prior to their use in grafting experiments, they were Soxhlet-extracted with acetone overnight to remove lipophilic extractives which could interfere with the designed reactions and also affect analysis of modified surface (Nzokou and Kamdem, 2004).

Oxidation reactions

Oxidation reactions were carried out as broadly described by Rüsch gen. Klaas and Warwel (1999) with some modifications. Soybean oil (5 g) or linoleic acid (0.5 g) was dissolved in 5 ml acetone and the lipase (10 mg; ≥ 100 U) was added. After stirring for 10 minutes, 54 µl of 35 % H_2O_2 was added. The mixture was incubated at 40 ^oC while shaking at 150 rpm. Every 15 min, the addition of H_2O_2 was repeated until 540 µl H_2O_2 was added and the stirring was continued for a further 16 h. It has been reported that exposure of the enzyme to high concentrations of aqueous hydrogen peroxide resulted in complete deactivation of the lipase (Bjorkling et al., 1990). Therefore, the hydrogen peroxide was added to the reaction media in small aliquotes. Afterwards the lipase was removed by filtration. More experiments (as shown in Table II) and the relevant controls were set up and run in a similar manner.

Determination of iodine value and oxirane oxygen

The percentage of oxirane oxygen was determined by the direct method with hydrobromic acid solution in acetic acid. The iodine value was obtained using the Wijs method (Paquot, 1979).

From the oxirane content values, the relative fractional conversion to oxirane was calculated from the following expression:

Relative conversion to oxirane = (OOe/OOt) ×100

where, OOe is the experimentally determined oxirane oxygen; OOt is the theoretical maximum oxirane oxygen, which was determined from the following expression (Petrović, 2002):

$$OOt = \{(IV_0/2Ai)/[100+(IV_0/2Ai)Ao]\} \times Ao \times 100 \qquad \text{(Equation 1)}$$

where, Ai (126.9) and Ao (16.0) are the atomic weights of iodine and oxygen, respectively and IV_0 is the initial iodine value of the oil sample.

Coupling of 1,2-epoxyoctane to dodecylamine and to lignin molecules

The reaction mixture contained 1,2-epoxyoctane (5.0 mM) and 5.0 mM of the lignin model molecules in 50 mM ammonium acetate buffer (pH 4.5). Reactions were carried out at 37 ºC while shaking at 700 rpm using a thermomixer (Thermomixer Comfort Eppendorf AG, Hamburg Germany) for 2 h. A second set of reactions was set up in a similar manner except that 1.0 mM dodecylamine was used instead of the lignin model molecules. The coupling products were analyzed by HPLC-MS.

HPLC-MS analysis of reaction products

To the incubation mixtures an equal volume of methanol was added. The mixture was centrifuged for 15 minutes at 14000 g and 600 µl aliquots were transferred into vials. HPLC analysis was performed using a system from Dionex equipped with a P580 pump, an ASI-100 autosampler and a PDA-100 photodiode array detector while monitoring elution at 254 nm. Separation of coupling products was achieved by reversed phase HPLC RP-C18 column (Eurospher 100-5, C18, 150 x 4.6 mm with precolumn, Knauer GmbH, Berlin, Germany) using a linear gradient of acetonitrile (solvent B) and 0.1 % formic acid (solvent A) as solvent at a flow rate of 1 ml min^{-1}, an injection volume of 10 µl and an oven temperature of 30 °C. The gradient was set as shown in Table I.

Table I. Gradient set up for HPLC analysis

Time (min.)	Initial	5	20	21	35	35.1	45(end)
Solvent A %	80	80	25	10	10	80	80
Solvent B %	20	20	75	90	90	20	20

The MS spectra were acquired with an Agilent Ion Trap SL (Palo Alto, CA, USA) equipped with an electrospray ionisation coupled to the Dionex HPLC-UVD-system as described above and initial separation of coupling products was carried out using the same protocol. The coupling products were measured in positive ion mode and the electrospray voltage was set to + 3500 V. Dry gas flow was set to 10 l min^{-1} with a temperature of 350 °C, nebulizer set to

50 psi. Maximum accumulation time was fixed to 300 ms and the loading of the trap was controlled by the instrument with an ICC of 30000.

Identification of the product of the chemo-enzymatic reaction **9** (Table II) was done by mass spectrometry using direct infusion method and the instrument was set up as described above. Reaction **9** was chosen for easy interpretation of results since the molecular weights of linoleic acid and the dodecylamine are known. Therefore results from this reaction can be used to demonstrate both oxirane formation and coupling of the amine to the oxirane. Samples for analysis were prepared by dissolving the products from the reaction in cyclohexane. Aliquots of this mixture were then combined with methanol in a ratio of 1:1 before analysis. The sample was introduced into the mass spectrophotometer at a rate of 5 µl/min.

Contact angle measurements

After washing-off unbound alkylamines from the veneers and conditioning to equilibrium moisture content, the sessile contact angle of water was measured in the grain direction five seconds after drop deposition according to the Young-Laplace method using a DataPhysics OCA 35 goniometer and SCA20 software. The drop volume was 1.5 µl. For each veneer, the contact angle was measured on five spots and the results averaged. For each treatment triplicate samples were measured.

XPS measurements

Binding of the dodecylamine to veneers was determined using X-ray photoelectron spectroscopy (XPS). XPS measurements were performed at Joanneum Research, Graz, Austria, with a multiprobe UHV surface analysis system from Omicron Nanotechnology. Equipment and parameters: x-ray source: DAR400 aluminium-anode; quartz-crystal monochromator XM 500; x-ray excitation energy: 1486.70eV (Al Kα1-line); monochromated x-ray line width (FWHM): 0.3 eV; energy analyzer: hemispherical analyzer, type EA 125; signal-detection with pulse-counting channeltron (five channels for count-rate enhancement); total energy resolution (x-ray source + hemispherical analyzer): 0.45 eV for detailed scans at 10 eV pass-energy (+ lifetime broadening of the electronic state); charge neutralization for non-conducting samples: electron emission gun for low-energy electrons (0.1–500 eV): FG 15/40 (SPECS). The analyzed surface area was ~ 1 mm^2; five spots were analyzed per sample and the results averaged.

Results and Discussion

The feasibility of generating oxiranes chemoenzymatically has been extensively demonstrated (Björkling et al., 1990, 1992, Rüsch gen Klaas and Warwel, 1999; Warwel and Rüsch gen Klaas, 1995; Ankudey et al., 2006). The highly reactive epoxides generated are widely known to react with OH-groups, a major functional group on lignocellulose structural components and also to a number of functional groups which may be found on other polymers and molecules of interest. Our interest in this technology is therefore in its potential for functionalising lignocellulose materials. It is clear from Table II that the chemo-enzymatic reaction resulted in a reduction in the content of double bonds, as shown by the decrease in the iodine values of the treated substrates. For soybean oil a 51-60 % iodine conversion was measured whereas for linoleic acid almost complete iodine conversion was seen. The process has also been shown to be readily catalysed by acids (Mungroo et al., 2008; Dinda et al., 2008; Petrović et al., 2002; Goud et al., 2006b). It is therefore plausible that epoxidation of linoleic acid was facilitated by self catalysis hence an almost 100 % conversion of the double bonds**.**

To confirm that the reduction in content of double bonds was a result of formation of oxiranes, the concentration of epoxy oxygen was determined. The results showed that there was a significant build up of epoxy oxygen in all the experiments where the potential epoxidation reagents (lipase and H_2O_2) were added (Table II). It is interesting to note that the relative fractional conversion to oxiranes correlates with the reduction in levels of unsaturation. Thus, soybean oil (SBO) which showed a 51-60 % iodine conversion showed a 52.7 % relative conversion to oxirane whereas linoleic acid (LA) was more readily epoxidised with a 92.4 % relative conversion to oxirane. Similarly, Warwel and Rüsch gen Klaas (1995) observed a 91 % relative conversion to oxirane when they used LA under similar conditions but at room temperature. Although there was an almost 100 % iodine conversion when LA was used, this was not accompanied by a 100 % relative conversion to oxirane possibly due to side reactions (Petrović et al., 2002).

Selectivity (S) is a measure for the amount of side reactions occurring during epoxidation; it gives the relative yield of epoxides and was calculated as:

$$S = (EOe/EOt)/([IV_0–IV_1]/IV_0)$$

where EOe (%) is the experimentally determined content of epoxy oxygen in 100 g of oil, IV_1 is the iodine value, IV_0 is the initial iodine value and EOt (%) is the theoretical content of epoxy oxygen in 100 g of oil at complete conversion, calculated from equation 1.
For IV_0 =180.5, EOt = 10.216.

Despite the possibility of side reactions, the selectivity value for our experiments (0.93 and 0.9), are within the range 0.9 – 1.0 which indicate a relatively low level of side reactions (Petrović et al., 2002).

An interesting observation was the reduction in the amount of epoxy oxygen when wood, dodecylamine or both were included in the reaction mixture, despite the fact that the iodine values were fairly constant. This could be explained by the reaction of oxiranes to the hydroxyl groups on the wood and/or to the amine group on dodecylamine (Nishikubo et al., 1998; Wing et al., 1970). This argument is strengthened by the fact that the epoxy oxirane level is lower in the presence of both wood and the amine.

Table II. Iodine values and oxirane formation during chemo-enzymatic epoxidation of soybean oil (SBO) and linoleic acid (LA)

Reaction	Iodine value (g/100g oil)[a]	Iodine conversion (%)[b]	Epoxy oxygen (%)[a]	Relative conversion to oxirane (%)[c]
1. SBO	130.2 ± 3.63	-	0	0
2. SBO+H_2O_2+lipase	59.2 ± 4.22	54.52	4.0 ± 0.51	52.7
3. SBO+ H_2O_2+lipase wood	51.4 ± 4.77	60.49	3.9 ± 0.17	nd
4. SBO+ H_2O_2+lipase+DA	58.2 ± 5.44	55.28	3.8 ± 0.40	nd
5. SBO+ H_2O_2+lipase+DA+wood	63.2 ± 5.92	51.46	3.6 ± 0.54	nd
6. LA	180.5 ± 3.26	-	0	0
7. LA+ H_2O_2+lipase	1.0 ± 0.12	99.43	9.4 ± 0.66	92.4
8. LA+ H_2O_2+lipase wood	0.3 ± 0.03	99.81	5.9 ± 0.69	nd
9. LA+ H_2O_2+lipase+DA	0.2 ± 0.03	99.88	5.1 ± 0.73	nd
10. LA+ H_2O_2+lipase+DA +wood	0.1 ± 0.02	99.93	3.3 ± 0.53	nd

[a]All values are means of three replicate determinations ± standard deviation

[b]Iodine conversion expressed as a % of the initial iodine value

[c]Relative conversion rate expressed as a % of the theoretical epoxy oxygen assuming complete epoxidation (7.586 for SBO and 10.216 for LA) calculated from equation 1 using the determined iodine values.

SBO - soybean oil; LA - linoleic acid; DA -dodecylamine

nd – not determined

Product identification by mass spectrometry

The findings above were supported by results from LC-MS analysis. Mass spectra acquired in positive mode for the reaction between 1,2-epoxyoctane and dodecylamine showed $[M+H]^+$ions at *m/z* 314. 4 (Fig. 3a) which suggests a 1:1 coupling of the oxirane with the amine (exact mass [M]=313.4). Similarly $[M+H]^+$ ions at *m/z* 482.4 (Fig. 3b) acquired from product of reaction **9** (Table II) suggest coupling of the amine (MW 185) to epoxidised linoleic acid (MW 296) (exact mass of product, [M]=481.45). Reaction of 1,2 epoxyoctane with syringylglycerol β-guaiacyl ether yielded a product with molecular ions at *m/z* 479.3 which suggest coupling of the oxirane to the lignin model (exact mass 478.26) (Fig. 3c). Also, 1,2 epoxyoctane was readily coupled onto the phenolic molecules caffeic acid (observed $[M+H]^+$ ions at *m/z* 309.2, exact mass [M] 308.16), ferulic acid (*m/z* 323.2; exact mass 322.18), and sinapic acid (*m/z* 353.2; exact mass 352.19) (Fig. 3d-f). Based on the known reactions of oxiranes (Nishikubo et al., 1998; Wing et al., 1970), the structures of products between oxiranes and alkylamines or lignin model molecules, were proposed and are embedded in the MS spectra figures (Fig. 3). Since it was possible to couple the functional molecule DA to oxirane and also to couple the oxirane to lignin molecules, this approach could potentially be used to functionalise lignocellulose materials and similar molecules.

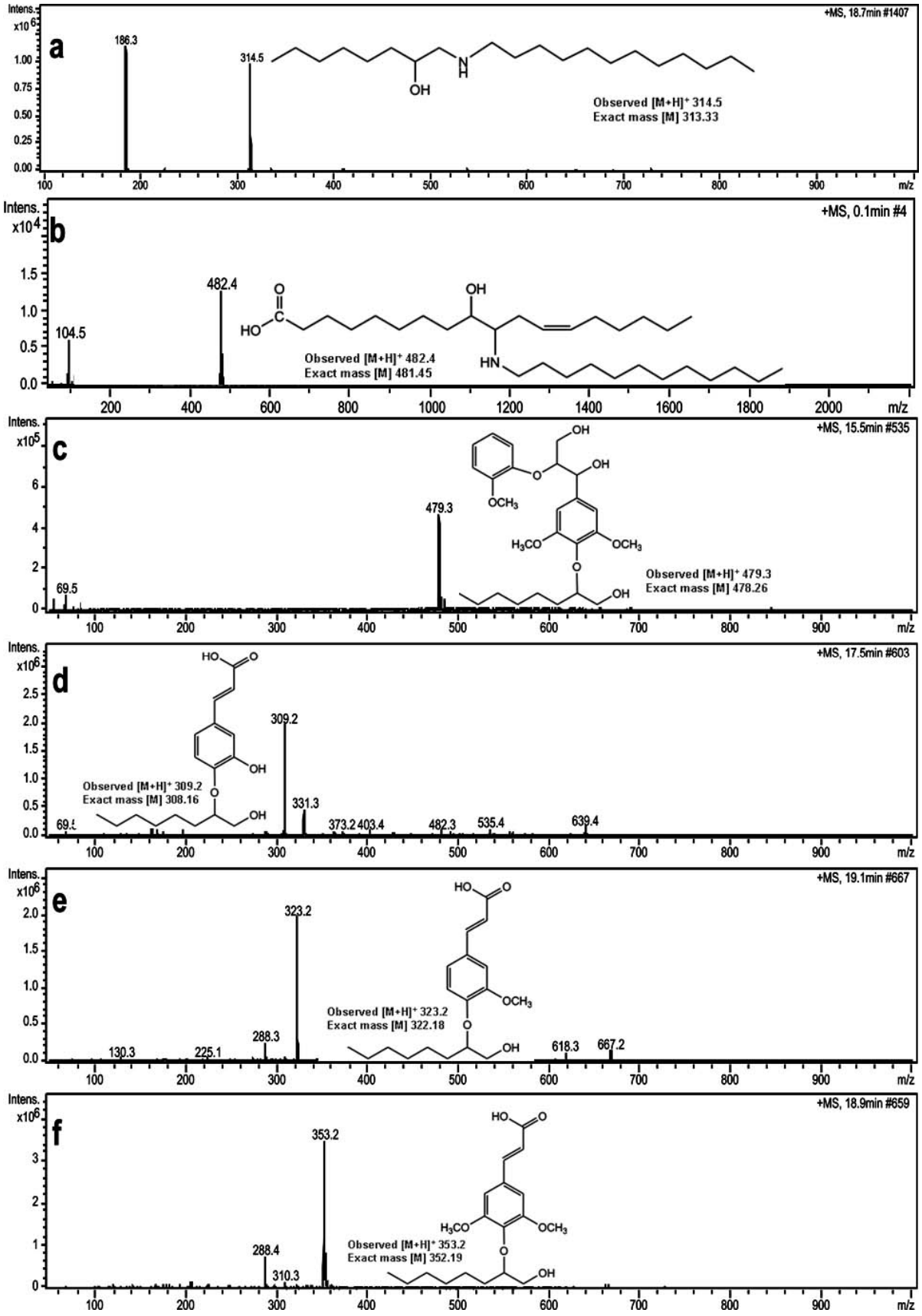


Figure 3. Mass spectra of coupling products between (a) 1,2 epoxyoctane and dodecylamine, (b) epoxidised linoleic acid (LA) and dodecylamine (DA) d) 1,2 epoxyoctane and syringylglycerol β-guaiacyl ether e) 1,2 epoxyoctane and caffeic acid f) 1,2 epoxyoctane and ferulic acid, and g) epoxyoctane and sinapic acid

Contact angle and XPS measurements

In order to demonstrate potential application of the chemo-enzymatic process to lignocellulose materials, the contact angles of wood treated with dodecylamine using this approach, were measured. The results in Figure 4 show that the contact angle of the beech veneers increased from 66.3^{o} to 125.8^{o} (approximately 90 % increase) as a result of the attachment of alkylamines through the oxirane ligands. There was also an increase in contact angle by simply epoxidising oil and reacting with wood possibly due to oxirane-mediated binding of the alkyl chain of the fatty acids. However, when LA was used, the increase in contact angle was smaller probably because a low (10× lower) concentration of LA was used. Our interest in using LA was to mechanistically demonstrate oxirane formation and coupling of the amine using LC-MS. XPS studies showed an O/C ratio of 0.18 in wood treated with DA using the chemoenzymatic process, a huge decrease when compared to 0.54 in untreated controls. This can be explained by the incorporation of the carbon-rich but oxygen-free DA. Previously we have used the method of direct grafting of DA to lignin moieties using laccase and the contact angle only increased to 107.4^{o} (Kudanga et al., 2010) while in similar studies using fluorophenols as hydrophobicity enhancing molecules, only a 65 % increase in hydrophobicity was observed (Kudanga et al., 2010). This method is therefore a useful alternative to the current enzymatic methods which are mainly based on radical coupling and only target certain components of lignocellullose materials especially lignin moieties. In addition the methods which are based on radical formation require meticulous optimisation as radicals can easily be quenched before attachment of molecules of interest (Grönqvist et al., 2006). Although this method appears cumbersome due to the need to titrate with small amounts of hydrogen peroxide as the reaction proceeds (to prevent inactivation of lipase), recently, a complex urea-hydrogen peroxide, an anhydrous form of hydrogen peroxide, has been successfully used and has the potential of releasing hydrogen peroxide in a controlled manner (Ankudey et al., 2006). This avoids the need to add the aqueous hydrogen peroxide slowly to the reaction mixture. Alternatively, we have previously shown that hydrogen peroxide could be generated in-situ from cellulose by using cellobiose dehydrogenase (Pricelius et al., 2009).

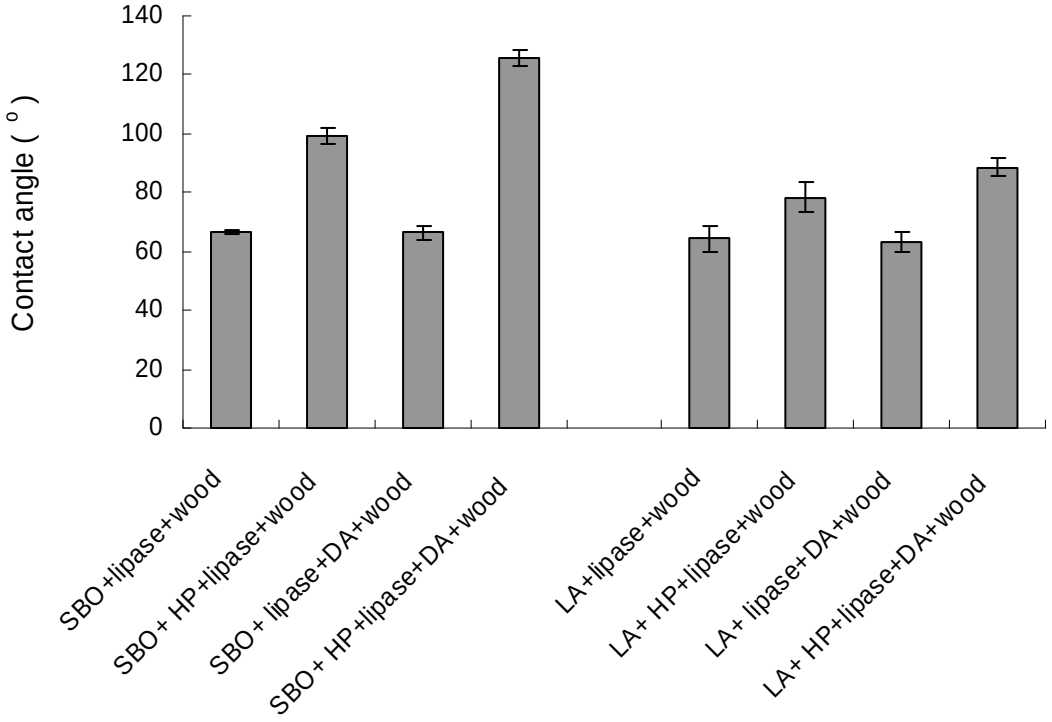


Figure 4. Increase in hydrophobicity as a result of treatment of beech veneers with DA using oxiranes

*All values are means of three replicates ± standard deviation

SBO – soybean oil; LA - linoleic acid; DA – dodecylamine; HP – hydrogen peroxide

Conclusion

This work has demonstrated for the first time the possibility of using oxiranes generated by chemo-enzymatic epoxidation to functionalise lignocellulose materials. The generation of oxiranes has been demonstrated using standard methods while the possibility of attachment to lignocellulose functional groups and functional molecule (dodecylamine) has been demonstrated by LC-MS. Furthermore, evidence of potential application has been demonstrated by the increase in hydrophobicity of beech veneers treated with dodecylamine using this chemoenzymatic process. Unlike the current enzymatic processes, this method is relatively non-selective and can be applied to all the major constituents of lignocellulose materials making it a comparatively more effective way of lignocellulose material functionalisation. As the oxiranes can be generated in good yields and react with a number of functional groups, this method can be a credible alternative to the current enzymatic approaches in lignocellulose materials functionalisation.

Acknowledgements

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References

Ankudey E, Olivo HF, Peeples TL. 2006. Lipase-mediated epoxidation utilizing urea–hydrogen peroxide in ethyl acetate. Green Chem 8:1–5.

Björkling F, Godtfredsen SE, Kirk O. 1990. Lipase-mediated formation of peroxycarboxylic acids used in catalytic epoxidation of alkenes. J Chem Soc, Chem Commun 1301 – 1303.

Björkling F, Frykman H, Godtfredsen SE, Kirk O. 1992. Lipase catalyzed synthesis of peroxycarboxylic acids and lipase mediated oxidations. Tetrahedron 48:4587–4592.

Campanella A, Baltanás MA. 2005. Degradation of the oxirane ring of epoxidized vegetable oils in liquid-liquid systems: I. Hydrolysis and attack by H_2O_2. Lat Am Appl Res 35:205-210.

Dinda S, Patwardhan AV, Goud VV, Pradhan NC. 2008. Epoxidation of cottonseed oil by aqueous hydrogen peroxide catalysed by liquid inorganic acids. Biores Technol 99:3737–3744.

Evans P. 2003. Emerging technologies in wood protection. Forest Prod J 53(1):14-22.

Formo MW. 1982. Miscellaneous oil and fat products. In: Swern D, editor. Bailey's industrial oil and fat products, 4th ed. New York: Wiley. p. 366–371.

Frihart CR. 2005. Wood Adhesion and Adhesives In: Rowell RM, editor. Handbook of wood chemistry and wood composites New York: CRC Press. p 215 – 279.

Goud, VV, Patwardhan AV, Pradhan NC. 2006a. Studies on the epoxidation of mahua oil (Madhumica indica) by hydrogen peroxide. Biores Technol 97:1365–1371.

Goud VV, Pradhan NC, Patwardhan AV. 2006b. Epoxidation of Karanja (*Pongamia glabra*) Oil by H_2O_2. JAOCS 83(7):635-640.

Goud VV, Patwardhan AV, Dinda S, Pradhan NC. 2007. Epoxidation of karanja (*Pongamia glabra*) oil catalysed by acidic ion exchange resin. Eur J Lipid Sci Technol 109:575–584

Grönqvist S, Rantanen K, Raimo A, Mattinen M, Buchert J, Viikari L. 2006. Laccase-catalysed functionalization of TMP with tyramine. Holzforschung 60:503–508.

Ibach RE. 1999. Wood Preservation. In Forest Products Laboratory. Wood handbook – Wood as an engineering material. Gen. Tech. Rep. FPL-GTR-113. Madison, WI: U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 463 p.

Kudanga T, Nugroho Prasetyo E, Widsten P, Kandelbauer A, Jury S, Heathcote C, Sipilä J, Weber H, Nyanhongo GS, Guebitz GM. 2010. Laccase catalyzed coupling of fluorophenols to lignin increases wood surface hydrophobicity. Biores Technol 101:2793–2799.

Kudanga T, Nugroho Prasetyo E, Sipilä J, Nyanhongo GS, Guebitz GM. Reactivity of long chain alkylamines to lignin moieties: implications on wood hydrophobicity. J. Biotechnol, in press.

Kumar S. 1994. Chemical modification of wood. Wood and Fiber Sci 26(2):270-280.

Mikolasch A, Frieder Schauer. 2009. Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials. Appl Microbiol Biotechnol 82:605–624.

Mungroo R, Pradhan, NC, Goud VV, Dalai AK. 2008. Epoxidation of Canola Oil with hydrogen peroxide catalyzed by acidic ion exchange resin. J Am Oil Chem Soc 85:887–896.

Nishikubo T, Kameyama A, Kawakami S. 1998. A novel synthesis of poly(ester-*alt*-sulfide)s by the ring-opening alternating copolymerization of oxiranes with γ-thiobutyrolactone using quaternary onium salts or crown ether complexes as catalysts. Macromolecules 31:4746-4752.

Nugroho Prasetyo E, Kudanga T, Eichinger R, Nyanhongo GS, Guebitz GM. 2009. Laccase catalysed coupling of functional molecules to lignocelluloses: Polymers derived from coupling of lignin onto silanes. Proceedings of the 6th International Conference on textile and Polymer Biotechnolog, Ghent Belgium. p.76 – 82.

Nzokou P, Kamdem DP. 2004. Influence of wood extractives on moisture sorption and wettability of red oak (*Quercus rubra*), black cherry (*Prunus serotina*), and red pine (*Pinus resinosa*). Wood and Fiber Sci 36(4): 483-492.

Paquot C. 1979. Standard methods for the analysis of oils, fats, and derivatives, Part 1, sixth ed. Oxford: Pergamon. 170 p.

Petrović ZS, Zlatanić A, Lava CC, Sinadinović-Fišer S. 2002. Epoxidation of soybean oil in toluene with peroxoacetic and peroxoformic acids – kinetics and side reactions. Eur J Lipid Sci Technol 104:293–299.

Pricelius S, Ludwig R, Lant N, Haltrich D, Guebitz G. 2009. Substrate specificity of *Myriococcum thermophilum* cellobiose dehydrogenase on mono-, oligo-, and polysaccharides related to in situ production of H_2O_2. Appl Microbiol Biotechnol 85:75-83.

Rangarajan B, Havey A, Grulke EA, Culnan PD. 1995. Kinetic parameters of a two-phase model for in-situ epoxidation of soybean oil. J Am Oil Chem Soc 72:1161-1169.

Rowell RM, Banks WB. 1985. Water repellency and dimensional stability of wood. Gen. Tech. Rep. FPL-50. Madison, WI, U.S. Department of Agriculture, Forest Service, Forest Products Laboratory. 24 p.

Rüsch gen Klaas M, Warwel S. 1996. Chemo-enzymatic epoxidation of unsaturated fatty acids esters and plant oils. J Am Oil Chem Soc 73:1453–1457.

Rüsch gen Klaas M, Warwel S. 1999. Complete and partial epoxidation of plant oils by lipase-catalysed perhydrolysis. Ind Crops Prod 9 (2):125–132.

Schultz TP, Nicholas DD, Preston AF. 2007. A brief review of the past, present and future of wood preservation. Pest Manag Sci 63:784–788.

Sinadinović-Fišer S, Janković M, Petrović ZS. 2001. Kinetics of in situ epoxidation of soybean oil in bulk catalyzed by ion exchange resin. J Am Oil Chem Soc 78:725-731.

Sipilä J, Syrjänen K. 1995. Synthesis and ^{13}C NMR spectroscopic characterization of six dimeric arylglycerol-β-aryl ether model compounds representative of syringyl and *p*-hydroxyphenyl structures in lignins. On the aldol reaction in β-Ether preparation. Holzforschung 49:325-331.

Warwel S, Riisch gen. Klaas M. 1995. Chemo-enzymatic epoxidation of unsaturated carboxylic acids. J Mol Catal B: Enzym 1:29-35.

Widsten P, Kandelbauer A. 2008. Laccase applications in the forest products industry: A review. Enzyme Microb. Technol. 42(4):293-307.

Wing RE, Doane WM, Rist CE. 1970. Oxirane ring-opening reactions of *3- 0-(2,3-*epoxypropyl)-l ,2:5,6-di-o-isopropylidene-α-D-glucofuranose. Carbohyd Res 12:347-353.

Wöhlerś HC, Sack M, LeVan HP. 1958. Yield in Epoxidation Reactions. Ind Eng Chem 50:1685-1686.

9

General Conclusions and Future perspectives

Novel enzymatic applications and processes for surface modification of lignocellulose materials based on direct grafting of functional molecules or use of anchor groups as binding sites have been described. Using the method of direct grafting, three fluorophenols: 4-fluoro-2-methylphenol (4,2-FMP), 4-[4-(trifluoromethyl)phenoxy]phenol (4,4-F3MPP) and 4-(trifluoromethoxy)phenol (4-F3MP) have been successfully grafted onto lignocellulose materials using laccase, as demonstrated by XPS studies and supported by positive application to increase wood hydrophobicity. The increase in hydrophobicity was shown to be directly related to the amount of fluorophenol incorporated. The fluorophenols were covalently grafted leaving the fluorine molecule free, a condition necessary for the hydrophobic properties as proved by mechanistic studies using LC-MS and NMR. A similar novel application based on the demonstration of laccase-catalysed reactivity of alkylamines to small lignin molecules, complex lignin models and to lignocellulose materials, was developed. Although DHA showed modest increase in hydrophobicity, dodecylamine performed better than all the fluorophenols used in the study. Although most of the mechanistic studies on direct grafting were based on lignin models guaiacylglycerol β-guaiacyl ether (erol) and syringylglycerol β-guaiacyl ether (G-S-β-ether), laccase-mediated coupling of functional molecules (phenolic amines, fluorophenols, and selected wood preservatives) has been demonstrated for the first time using the recently discovered lignin model, dibenzodioxocin.

Direct grafting of hydrolysable tannins, particularly tannic acid, and cationic mimosa tannin significantly inhibited the growth of *S. aureus* on wood veneer and pulp and also show some antibacterial effect on *E. coli*, while condensed (mimosa) tannin imparted less resistance against these bacteria. The growth inhibition in veneers and most pulps was greater when laccase was present during the tannin treatment of wood, possibly due to better retention of tannin on the substrate surface. This was supported by the semi-quantitative colour measurements and confirmed by studies with tannins and lignin model molecules which showed covalent coupling only in the presence of laccase. The structures of a number of coupling products proposed based on findings from LC-MS and existing literature, showed 5-

5, 4-*O*-5 and *β*-*O*-4 as the predominant linkages. The effectiveness of the different tannins as antibacterial agents is in line with their minimum inhibitory concentrations (MIC) for *S. aureus* and *E. coli* and their reactivity toward laccase. The MICs were lower for the tannins than for the tannin-related monomeric phenols which confirm the assertion that oligomeric phenols have higher efficacy in comparison with monomeric phenols. Tannins therefore need to be seriously considered as a credible alternative in development of hygiene lignocellulose surfaces e.g. in packaging materials. However, more work is required to fully understand the anti-microbial mechanism of tannins. Although a number of novel applications have been developed based on direct grafting of functional molecules, more work is needed to provide quantitative information on the extent of laccase-induced coupling which should provide a basis for optimisation of the processes.

Since direct coupling or grafting reactions are negatively affected by quenching of radicals, a new method has been developed in which reactive amines are first grafted to lignocellulose where the phenolic amines act as anchor groups, creating a stable reactive surface which counteract negative effects of radical quenching during subsequent coupling/grafting reactions. Mechanistic studies using lignin models have shown that the amines are successfully covalently coupled leaving the -NH_2 group free for further attachment of functional molecules. This method facilitated coupling of antifungal agents by up to 58 %, when compared to direct laccase-mediated coupling. Coupling of aromatic amines to lignin model molecules has also been demonstrated using *Bacillus* spore laccase. The *Bacillus* spore laccase was active within the neutral to alkaline conditions (pH 7- 8.5) and was more resistant to common laccase inhibitors than fungal laccases, demonstrating potential application under alkaline pH and extreme conditions which normally inhibit fungal laccases.

Laccase-catalysed binding of functional molecules targets the lignin molecule. Since, lignin only comprises 18 – 35 % of lignocellulose material, this processes leaves at least 65 % of the surface unmodified. A novel process has been developed in which lipase-generated oxiranes act as anchor groups. The reactivity of oxiranes to putative lignin monomers and models and to functional molecule dodecylamine has been demonstrated. Since oxiranes are known to react with hydroxyl groups – effectively with all the structural elements of wood, and also with a number of functional groups, this method is non-targeted. Using this method to bind dodecylamine, we achieved an increase in hydrophobicity which is superior to laccase-mediated hydrophobicizing with the same molecule. However, there is a possibility of disrupting hydrogen bonding in structural fibres. As such more work is needed to determine the effect of this process on mechanical properties of lignocellulose materials.

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