UNIVERSITÀ DEGLI STUDI DELLA TUSCIA DI VITERBO

DIPARTIMENTO DI AGROBIOLOGIA E AGROCHIMICA

CORSO DI DOTTORATO DI RICERCA

BIOTECNOLOGIE VEGETALI - XXIII Ciclo

USE OF ENZYMATIC CATALYSIS IN BIOTECHNOLOGICAL APPLICATIONS FOR THE VALORISATION OF RENEWABLE RESOURCES

CHIM/06

Coordinatore: Prof. Stefania Masci

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Alla mia famiglia, presente e futura

ACKNOWLEDGEMENTS

Alla fine di questo lungo percorso, che si è rivelato molto impegnativo ma anche estremamente appassionante, alcuni sinceri ringraziamenti sono dovuti. Il mio primo pensiero va alla Professoressa Claudia Crestini di Tor Vergata, che è stata la mia vera guida per questi tre anni e si è dimostrata una persona di indubbio spessore scientifico, che mi ha insegnato materialmente il vero significato della parola ricerca, cioè tanto sacrificio, passione ma soprattutto soddisfazione. La ringrazio inoltre anche per il supporto umano e per aver saputo trovare sempre le parole giuste al momento giusto. I suoi 'non mollare!' mi sono stati di grandissimo aiuto nei momenti più difficili.

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ABSTRACT

Since the early 1990s the use of natural catalysts, enzymes, in the development of organic synthesis reactions has received a steadily increasing amount of attention, expecially in the frame of green chemistry and of and sustainable development concepts. In fact, due to the high chemical and energy efficiency of enzymatic transformation in water, biocatalysis is one of the greenest technologies for chemical syntheses. Among the main advantages in the use of enzymatic catalyst there is their high efficiency: compare to the nonenzymatic reactions, the rates of enzyme-mediated processes are accellerated by a factor of 10^8-10^{10}. Moreover the enzymes are biodegradable reagents that act in mild conditions both of pH and temperature . This minimises the problems of undesidered side reactions and make the processes environmentally friendly. On the contrary this aspect can lead to a limitations in large scale applications, especially if the reactions need extreme pH or temperature values to occur that can deactivate the enzyme. Finally, the costs of isolation and purification make the biocatalysts still unconvenient for industrial applications. One approach to overcome this constrains is the use of immobilisation.

The immobilisation is achieved by fixing the enzyme to or within an inert and insoluble material.[1] In this form, it is possible a more convenient handling of the enzyme and its easy separation from the product, minimising or eliminating protein contamination of the product. Moreover the immobilisation can facilitates the efficient recovery and reuse of costly enzymes and allows to use them in multienzyme and chemoenzymatic cascade processes. A further benefit is often the enhanced stability towards denaturation by heat or organic solvents. Nevertheless, compared with the free enzyme, the immobilised enzyme has usually its activity lowered and the Michaelis-Menten constant increased. These alterations result from structural changes introduced to the enzyme by the applied immobilisation procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution. In spite of these disadvantages, the creation of a microenvironment may allow to the enzyme to remain active at different temperatures or pHs than would be predicted for the enzyme when not immobilised, increasing the application possibilities.

During my PhD project, I developed new immobilised enzymes for the oxidative functionalisation of renewable materials with high added value but under-utilised

actually, lignin in particular. After cellulose, lignin costitutes the most abundant organic substance on the planet and is the residue of industrial processes, as for example paper production, but is also an agricultural and forestry residue. The use of lignin is nowadays still limited to thermovalorisation processes as filler in composites, component in binders and coatings, or, at a lower extent, surfactant/dispersant additives, whereas its potential as a source of valuable phenols in the production of high value-added biopolymers in alternative to petrol chemistry is largely unexploited.[2] For all these reasons the valorisation of this bioresource is considered a vey important task to achieve in the frame of the sustainable development. Oxidative enzymes are potential tools for the selective modification of lignin as well as for the simplification of its structure, but the industrial application is still limited to processes with free enzymes. Moreover, the mechanism of action of oxidation of lignin with these enzymes is still only partially understood.

Considering the chemical structure (*Figure* 1), lignin is a three-dimensional phenyl-propanoid polyphenol mainly linked by arylglycerol ether bonds between monomeric phenolic units, most of which are not readily hydrolysable even under severe experimental conditions.[3]

My study has been focused on two oxidative enzymes in particular, laccase from *Trametes versicolor*, and peroxidase from the plant *Armoracia rusticana* (HRP). The two biocatalysts have been choosen due to the fact that are among the enzymes most studied and utilised; fungal laccases are responsible for the wood degradation in nature, causing the white rot, HRP is the most used enzyme for biotechnological applications.

Among the different immobilisation techniques, I choose, to use a chemical method, the crosslinking with a multifunctional reagent, the glutaraldehyde, in the HRP immobilisation and for laccase I used both the chemical method and a physical immobilisation, the microencapsulation. Then, I choose to protect the catalysts from the denaturation using the layer by layer technique (LbL). This method allows multilayer assemblies to be obtained by the alternate deposition of polycations and polyanions in a cyclic way on a solid surface.[4]

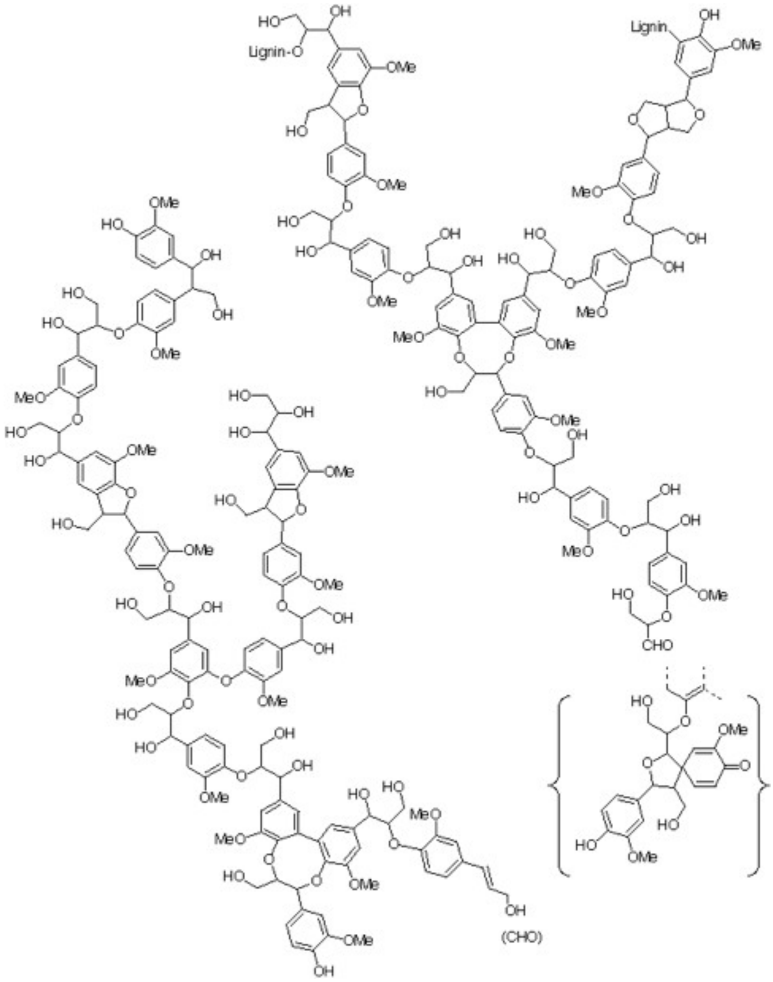
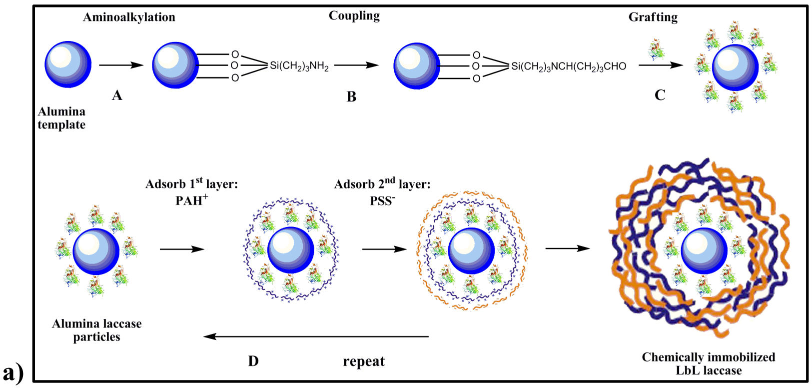


Figure **1- Lignin chemical structure.**

The polyelectrolyte films have the ability to protect proteins from high-molecular-weight denaturing agents or bacteria and to allow regulation of the permeability towards small substrates, which can enter the multilayer and react with the catalytic site (*Figure* 2). Immobilised laccases and HRP were used as biocatalysts for the oxidation of softwood lignins, using air as the primary oxidant in the case of laccase and hydrogen peroxide in the case of HRP.



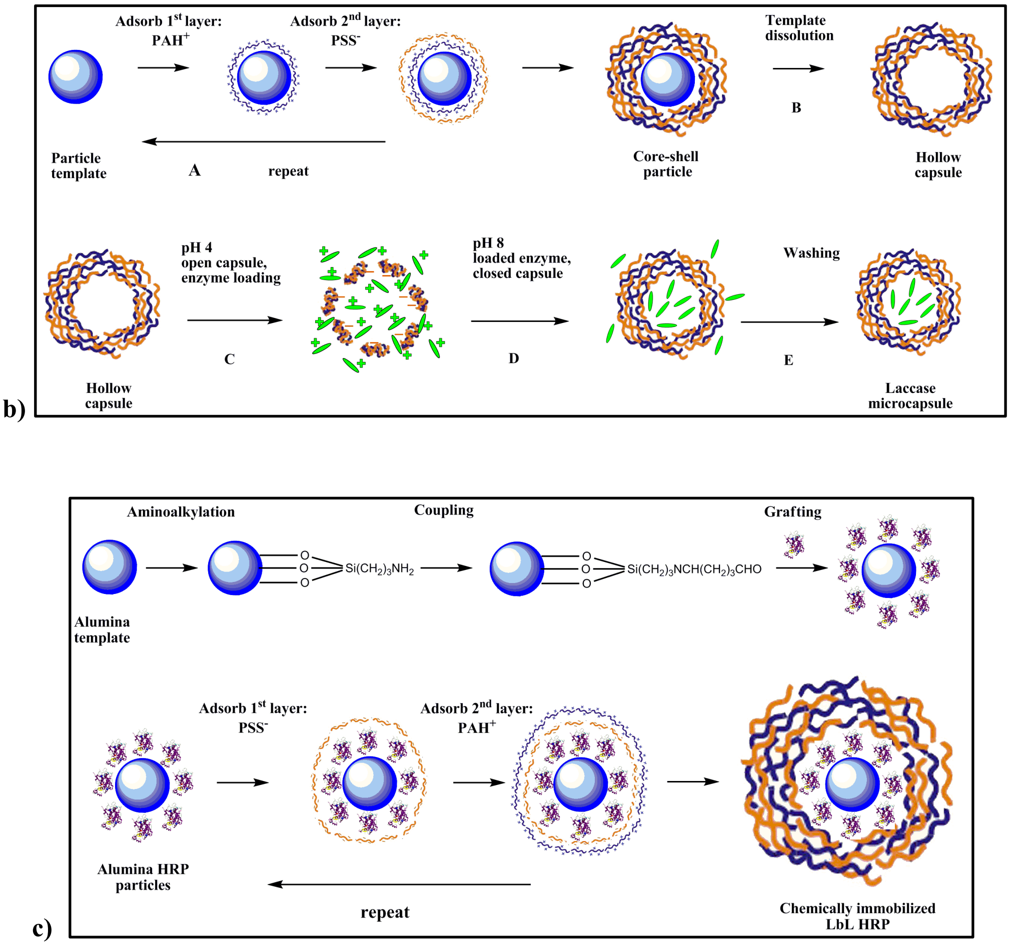


***Figure* 2- Immobilisation schemes of laccase and HRP: a) Chemical immobilisation of laccase; b) physical immobilisation of laccase; c) chemical immobilisation of HRP.**

With the aim of gaining further insight into the reaction pathway of oxidation of lignin with immobilised enzymes, the structure of enzymatically modified lignins was evaluated by means of nuclear magnetic resonance techniques based on the phosphorus nucleus (^{31}P NMR analysis). This advanced heteronuclear NMR technique allows the contemporary characterisation and quantification of all labile OH groups present in lignins such as the aliphatic OH groups, the different phenolic OH and carboxylic acids.[5]

In order to test the possibility of reuse the catalyst, both the free and the immobilised enzymes were allowed to react with ABTS for several successive 12 h batch reactions. All the enzymes retained their activity after 10 cycles, although in different proportions. The free enzymes retained about 20% of the starting activity, while the immobilised ones retained about 80% or more of the starting activity. The results obtained in this study show that the immobilised enzymes are more effective in lignin oxidation than native enzymes. The highest reactivity of immobilised laccases might be explained by the stabilisation effect of coating polyelectrolytes towards enzyme deactivation processes. Moreover, kinetic barriers for the approach of substrate to the active site of the enzyme are not operative. The strategy used for the laccase and HRP immobilisation is also an efficient tool to tune the selectivity of the enzyme. In fact, as evaluated by ^{31}P NMR analysis, the immobilised laccases follow a different reaction pathway than native enzyme, resulting in efficient depolymerisation without cross-linking reactions. The immobilised laccase performed side chain oxidation over possible alkyl-phenyl ether bond cleavage reactions, as shown by the decrease of aliphatic OH and increase of carboxylic groups. It is interesting to note that the presence of HBT and VA significantly enhanced the performance of immobilised laccases to yield lignins with lower amounts of aliphatic and condensed OH groups and a higher amount of guaiacyl OH group than native laccase. These data indicate the prevalence of depolymerisation reactions and the contemporary inhibition of repolymerisation processes by oxidative coupling cross-linking. In the oxidation with HRP, the COOH groups were found increased both by use of HRP and coated HRP particles. This indicates an efficient oxidation process. HRP and immobilised HRP behave differently from other oxidative enzymes as for example laccases. While laccases show a significant side-chain oxidation process, the HRP reactivity is selective toward the oxidation and cleavage of the aromatic ring.

From these data it was possible to conclude that immobilised laccase and HRP constitute new selective specific biocatalysts for the oxidative functionalisation and depolymerisation of lignin.

A manufacturing concept for converting plant biomass to energy and chemicals is the Biorefinery, that is based on the use of carbon molecules extracted from plant in order to substitute carbons from oil and gas.[6] Lignin costitutes the residue of these processes, in particular of bioethanol production. One of the major limitations of the ethanol production is the hydrolysis step, considered a critical step, where fermentable sugars are released from biomass using enzymes.[7] Enzymatic hydrolysis of cellulose has to be improved before commercialisation of the process, increasing the efficiency and decreasing the costs of cellulose hydrolysis. Lignin has a negative effect on the enzymatic hydrolysis of cellulose, and this is especially pronounced for softwood. It has been hypotized that the enzyme can bind unproductively on lignin surface, decreasing the catalytic activity. Moreover it has been reported[8] that surfactants are advantageous additives in cellulase production by increasing the cell wall permeability and releasing cell-wall bound enzymes to the liquid phase. During my Ph D project I tested the enzyme adsorption in enzymatic hydrolysis of various steam pretreated lignocelluloses (spruce, willow, hemp, corn stover, wheat straw, sweet sorghum bagasse) and its prevention by PEG addition. The adsorption pattern was investigated in a 72 h enzymatic hydrolysis reaction. As significant differences were observed among the enzyme binding capacities on different substrates, the structure of the steam pretreated lignins has been analysed in order to find a connection between enzyme adsorption, its decrease by PEG addition, and properties of lignin. In particular, the results suggest that the different degree of increased free cellulase activity obtained by PEG addition is based on the various structures of lignin in the substrates. It was concluded that phenolic hydroxyl groups exposed on the lignin surface interacts with PEG through hydrogen bonding, forming a layer of PEG on lignin surface, which prevents unproductive binding of cellulases on lignin.

Part of my project has been carried out in collaboration with North Carolina State University for the development of an innovative analitycal technique. In this technique, a high reactive free radical typically reacts with a double bond of a diamagnetic compound (the spin trap) to form a more stable radical (the spin adduct). Nitrones are the most frequently used compound as spin trapping agents, and the spin adducts being in this case a nitroxide. Recently it has been demonstrated that phosphorus-containing spin traps give rise to radical adducts that have longer half-lives compared to other spin traps. However, the presence of phosphorus within these systems allows for the use of phosphorus nuclear magnetic resonance (^{31}P NMR) spectrometry to investigate the detailed chemistry of radical reactions. This technique was termed "*NMR spin trapping*";[9] phosphorous NMR could be exploited to perform qualitative analyses since the chemical shift of the ^{31}P atom was found to be strongly dependent on the nature of the adducts, and quantitative analysis, in presence of a suitable internal standard.

In order to obtain a direct evidence of the radicals formation in the laccase and laccase-mediator system (LMS) catalyzed reactions, a spin trap technique could be applied. I tried to clarify the mechanism of action in the LMS of different mediators; in fact, to date, the mechanism involved in the system is only still partially understood, but in most of its aspect is unclear. It has been hypotised that the mediator improves the activity by changing the reaction pathway: from one electron oxidation to one atom hydrogen abstraction (*Figure* 3).[10]

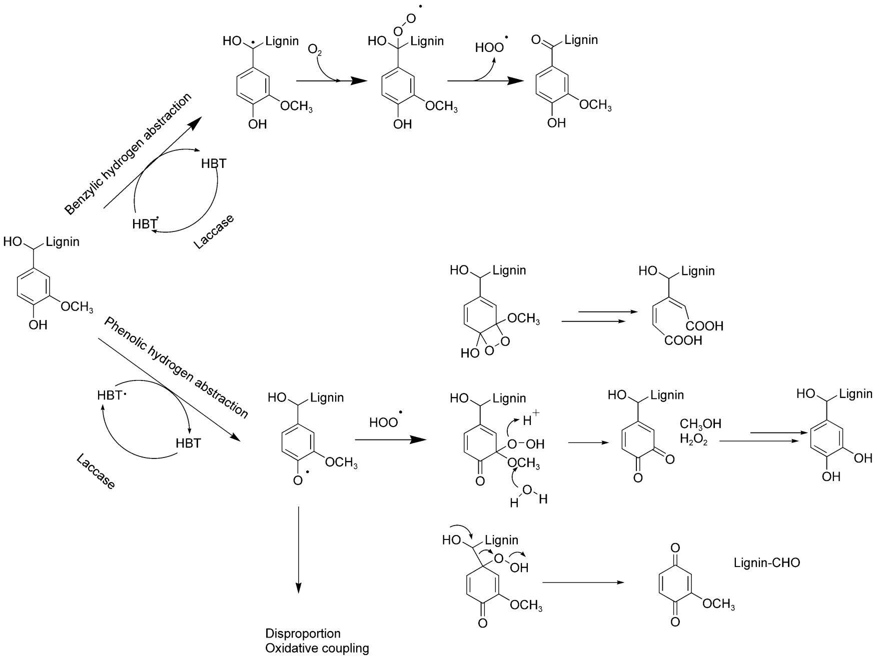


Figure **3- LMS, with HBT, in lignin degradation.**

To achieve this task I used the NMR spin trapping technique, that allows to determine and quantify all the radical intermediates involved in the reactions, both in presence and in absence of different chemical mediators and in different experimental conditions. In particular, the mechanism of action of three different mediators (HBT, ABTS, VA) was considered, moreover, to study in detail the radical chemistry of the LMS, the oxidation of phenolic and non phenolic substrates was carried out. Moreover, the reaction were performed in three different experimental conditions to understand the role of dioxygen in the laccase reactions. In fact, during its cycle laccase use air oxygen as primary oxidant and for this reason I choose to study the radical chemistry in presence of air, in a condition of local ipoxia and in complete anoxia. In all the cases, in presence of HBT, VA and ABTS, performing the reactions in local ipoxia or anoxia, it could not possible to detect any kind of radical adduct signals. Moreover it was demonstrated that a deeply difference, among the action mechanism of the mediators, occurs. In presence of laccase and VA or ABTS it was no possible to detect any signals at ^{31} P NMR analysis, even performing overnight reactions. The VA and ABTS, once that are oxidised, do not generate radical intermediates; from the NMR spin trapping analysis no signals was detected, even prolonging the reaction time until 12 hours. HBT, instead showed a different reaction mechanism; in fact after only 45 minutes (*Figure* 4), it was possible to detect radical adduct signals resulted from HBT oxidation (HBT•) and superoxide radicals (•OOH).

From this study, it was highlighted that the laccase-HBT mechanism proceeds through radical oxidation of the mediator, with the contemporary generation of superoxide. The basis mechanism, that confers a strong oxidative action to the laccase-HBT system, could be dued to the superoxide radicals production.

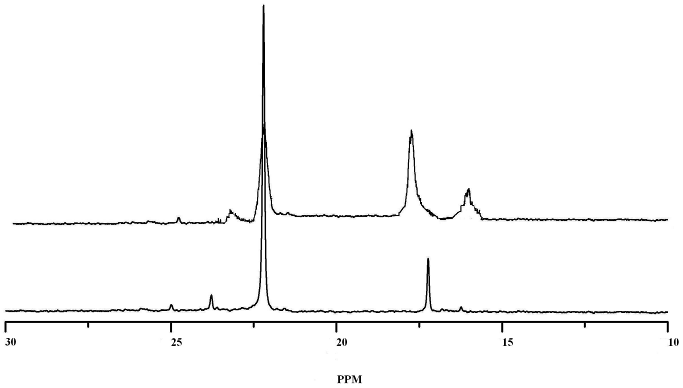


***Figure* 4- ^{31}P NMR spectra of: DIPPMPO and laccase in presence of HBT (above) and DIPPMPO and laccase in presence of HBT (below) with reaction time of 45 minutes and overnight, respectively.**

The NMR technique was also employed to study the lipoxygenase reactions. This enzyme catalyses dioxygenation of free and/or esterified PUFAs to their corresponding hydroperoxy derivatives. Due to the presence of unpaired electron, free radicals are highly reactive and tend to react with most biomolecules, including proteins, lipids and DNA, finally resulting in oxidative damage.[11]

In this case also, to understand the dioxygen role, the oxidation processes were performed in normoxic, ipoxic and anoxic conditions. From the quantitative analysis it appeard that the maximun amount of radical adduts were detected in anoxic condition; when the enzyme can complete its catalytic cycle, because there is a normal concentration of oxygen, the radicals are not released from the enzyme active site, but they are released only as hydroperoxides. In ipoxic conditions, the enzyme it's not able to complete the cycle and releases the radicals in solutions. The qualitative analysis demonstrated that three main radical species are formed: carbon radicals (R•), hydroxyl radicals (RO•) and peroxyl radicals (ROO•). It is possible to conclude that nder normoxic condition, lipoxygenase could not be considered a source of free radical; it is possible to trap low amount of ROO• radicals; at low oxygen concentration, lipoxygenase releases free radical in solution, the radical are trapped and detected as ROO• and R•; without oxygen, lipoxygenase could not complete its catalytic cycle and release free radical R• and RO• species in solution.

Both the study on laccase and lypoxygenase catalysed reaction, demonstrated that the ^{31}P NMR-spin trap system is the only analytical method that allows the detection and the quantification of radical spin adducts.

The last part of my Ph D project concerned the study of another highly complex polyphenolic polymer the melanin. To date its chemical structure it's only partially understood, studies on structural characterisation of this pigment are lacking, due to its no well definied spectral behaviour and to its insolubility. *Figure* 5 shows the melanin biosynthetic pathway.

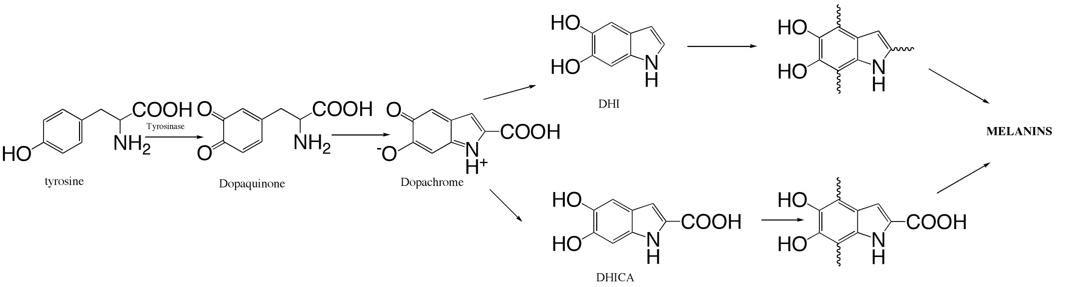


***Figure* 5- Melanin biosynthesis.**

The only studies on melanin chemical structured have been performed in solid state, there are only few informations on the funtional groups of this polymer; the characterisation and quantification of all the functional groups present on melanin can be considered an extremely relevant task to achieve, both for the biotechnological applications and for a detailed study of all the pigmentation disorders.[12]

In my study melanin from different origin were considered: the natural pigment from *Sepia officinalis;* melanins obtained by chemical or enzymatic reaction, starting by different precursors. After the complete solubilisation in Soluene-350, a solvent with a high capacity to dissolve all the cellular components, constituents melanin samples were characterised by their absorption in UV-Vis (290 nm<λ< 600nm). For all the melanins considered in this study, a typical UV-Vis spectrum was obtained, and in all the cases a maximum absorbance at 350 nm was recorded.

In an attempt at developing an innovative analytical technique for melanin structural characterization the attention was focused on the possible application of heteronuclear

^{31}P NMR. The rationale behind this approach consists in the selective phosphytylation of phenolic indolic, amino and carboxylic groups present in melanins. With the aim to develop a new and more detailed analytical protocol for the melanin characterisation in solution, a number of melanin model compounds were selected in order to be phosphytilated and submitted to solution ^{31}P NMR analysis. A series of different solvent systems were tested to find the best solubilisation conditions and the maximum reactivity. Once that these two parameters were solubilised, melanin model compounds first and successively, melanin samples, were subjected to ^{31}P NMR analysis.

From the study of different melanins by ^{31}P NMR it appears clearly that the chemical structure of melanin accepted to date should be revised on the basis of new acquired structural data. More specifically, the amount of phenolic hydroxyl groups is actually much lower than expected. Moreover, a low amount of phenolic hydroxyl groups is also supported by the Raper-Mason theory on melanogenesis.[13] In this theory is reported that, for the major part, the eumelanins origin from the polimerization of quinone and only in a smaller part from dihydroxyindolcarboxylic acid polimerization.

The rest of my study proposed a new approach, based on natural enzymes for melanin degradation. In particular, the use of natural enzymes and also of the LMS for melanin degradation, with further investigations, could be lead to the development of skin agents more effective and safer than the ones actually used, that are based on melanogenesis inhibition.

The three years of my Ph D project resulted in 5 international pubblications, 4 oral presentation and 10 poster presentation. Moreover 2 manuscripts are in preparation for the pubblication on international journals.

RIASSUNTO

Dall'inizio degli anni '90, con l'introduzione dei principi della green chemistry e dello sviluppo sostenibile, l'impiego degli enzimi su vasta scala nei processi industriali è stato estremamente favorito considerando il minimo impatto ambientale derivante dal loro utilizzo. Infatti, in quanto molecole di natura proteica, sono biodegradabili e operano in condizioni blande di pH, temperatura e a pressione atmosferica. L'utilizzo di catalizzatori enzimatici come integrativi o sostitutivi di molecole impiegate nella chimica tradizionale offre altri numerosi vantaggi. Infatti, rispetto ai classici catalizzatori chimici, gli enzimi presentano una specificità e selettività maggiore nei confronti del substrato, caratteristiche che danno come risultato la formazione di prodotti di reazione altamente specifici, senza la presenza di indesiderati prodotti secondari. Inoltre queste molecole sono in grado di aumentare la velocità di una reazione chimica di diversi ordini di grandezza maggiori rispetto ai corrispettivi catalizzatori chimici. Ad oggi comunque, l'impiego dei catalizzatori enzimatici su vasta scala nei processi di biotrasformazione industriale è ancora sfavorito dagli alti costi di isolamento e purificazione e dalla natura instabile degli enzimi quando vengono rimossi dai loro ambienti. Per superare questi limiti, uno degli approcci maggiormente utilizzato è l'immobilizzazione.

Il processo di immobilizzazione consiste nel fissare l'enzima sulla superficie o all'interno di supporti solidi inerti ed insolubili.[1] In questo modo, il sistema creato permette la separazione di catalizzatori e prodotti, minimizzando o eliminando la contaminazione di questi ultimi. Inoltre, l'immobilizzazione dà la possibilità di recuperare ed eventualmente riutilizzare, anche più volte, gli enzimi. Un ulteriore beneficio consiste nell'aumento della resistenza a processi di denaturazione causati per esempio da alte temperature, solventi organici, ecc. Questa metodica presenta comunque alcuni svantaggi. Infatti, rispetto alla sua forma nativa, l'enzima immobilizzato presenta attività più bassa e una più alta costante di Michaelis-Menten. Queste alterazioni derivano da cambiamenti nella struttura terziaria della proteina che vengono indotti dalla procedura di immobilizzazione applicata e dalla creazione di un microambiente all'interno del quale l'enzima svolge la sua attività che è differente rispetto alla soluzione. La creazione di un microambiente tuttavia ha anche un effetto protettivo nei confronti della molecola proteica, permettendo all'enzima di rimanere attivo a temperature o valori di pH differenti rispetto all'enzima nella sua forma nativa, estendendo le sue possibilità di applicazione. Parte del mio progetto di

dottorato è stato rivolto all'immobilizzazione di enzimi ossidativi per la funzionalizzazione di risorse rinnovabili ad alto valore aggiunto ma attualmente poco sfruttate, in particolare la lignina. Questo biopolimero, dopo la cellulosa, è considerato il materiale più abbondante della superficie terrestre e costituisce il residuo di lavorazioni agricole ma anche di molte lavorazioni industriali, come per esempio la produzione della carta. Questi materiali oggi hanno un impiego limitato, in particolare vengono utilizzati soprattutto nella termovalorizzazione e come filler nella produzione di compositi. In realtà la lignina presenta un notevole potenziale applicativo come fonte di sostanze fenoliche per la produzione di biopolimeri ad alto valore aggiunto in alternativa alle risorse non rinnovabili oggi utilizzate.[2] Proprio per questo la valorizzazione di questa risorsa può essere considerata un obiettivo di notevole importanza nell'ottica di uno sviluppo sostenibile. Gli enzimi ossidativi costituiscono potenziali strumenti per la modificazione selettiva della lignina, che possono essere impiegati al fine di modificare le caratteristiche chimiche del polimero, per arrivare alla produzione di nuovi materiali con prestazioni avanzate rispetto a quelli attualmente presenti sul commercio. Chimicamente la lignina è un polimero polifenolico altamente complesso (*Figura* 1). Deriva dalla via biosintetica dei fenilpropanoidi e viene generato da polimerizzazione radicalica casuale di monolignoli variamente sostituiti. Ne deriva una struttura priva di sequenza specifica e di unità ripetitive.[3]

Il mio studio si è focalizzato su due enzimi ossidativi in particolare: laccasi, dal fungo *Trametes versicolor*, e perossidasi dalla pianta *Armoracia rusticana* (HRP). Questi due biocatalizzatori sono stati scelti in quanto tra gli enzimi maggiormente studiati ed utilizzati nelle biotrasformazioni; le laccasi fungine sono coinvolte in natura nel processo di degradazione della lignina, dando luogo alla cosiddetta carie bianca del legno, mentre la perossidasi da rafano, simile nella sua attività alla lignina perossidasi, è stata considerata per la sua vastissima applicazione nei processi biotecnologici.

Tra le differenti metodiche di immobilizzazione degli enzimi, ho scelto di utilizzare nel caso della perossidasi un metodo chimico, cioè che prevede la formazione di legami di tipo chimico tra enzima e supporto, il crosslinking attraverso glutaraldeide, mentre la laccasi è stata immobilizzata sia attraverso crosslinking sia mediante l'impiego di un metodo fisico, la microincapsulazione, in cui sono coinvolte interazioni fisiche tra enzima e supporto. In entrambe le metodiche di immobilizzazione utilizzate, ho scelto di proteggere il catalizzatore dalla denaturazione attraverso la tecnica layer by layer (LbL). Questo metodo consente la creazione di film multistrato grazie alla interazione elettrostatica data dall'apposizione di strati di polielettroliti di carica opposta.[4]

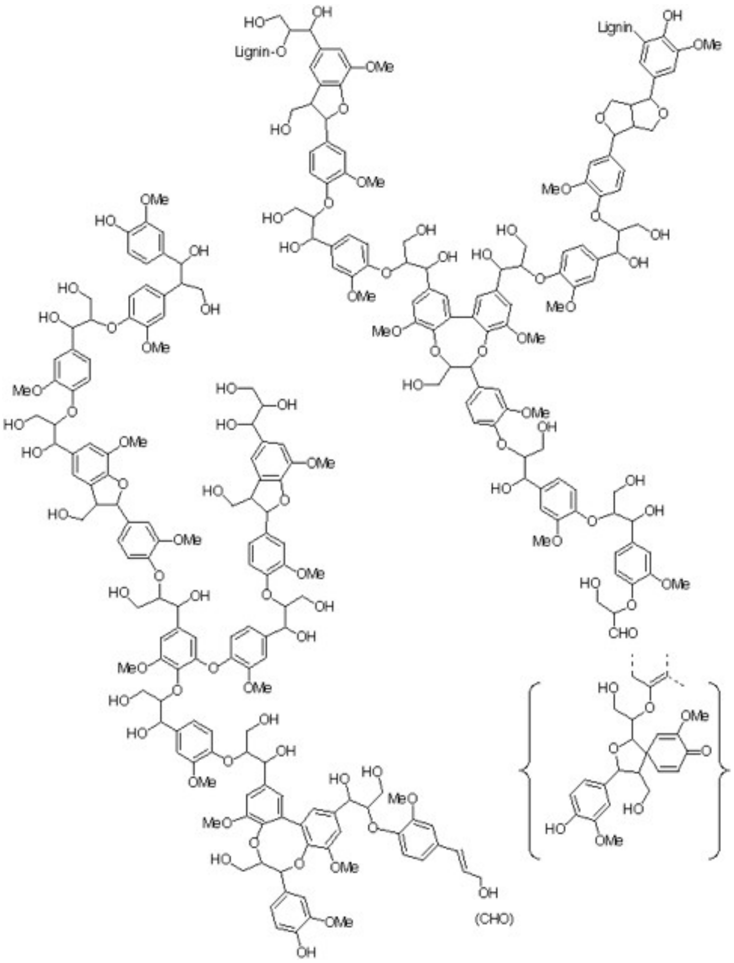
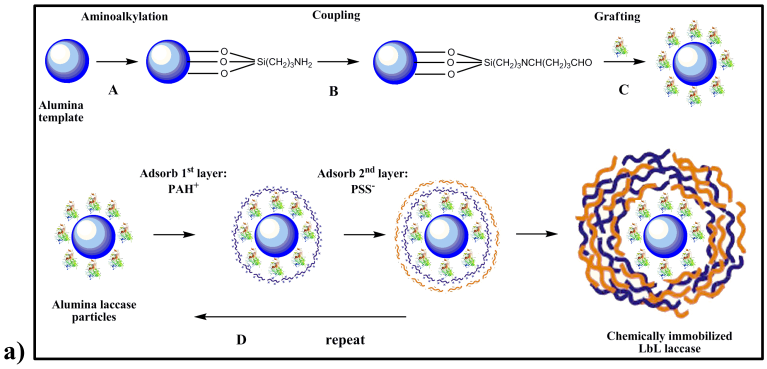


Figura **1- Struttura chimica della lignina.**

Nel mio caso il film di polielettroliti è stato creato ed apposto sulla superficie dell'enzima immobilizzato per creare una struttura protettiva nei confronti di possibili agenti denaturanti che fornisse maggiore resistenza all'enzima. Nella *Figura* 2 sono riportati gli schemi di immobilizzazione della laccasi e HRP, rispettivamente.



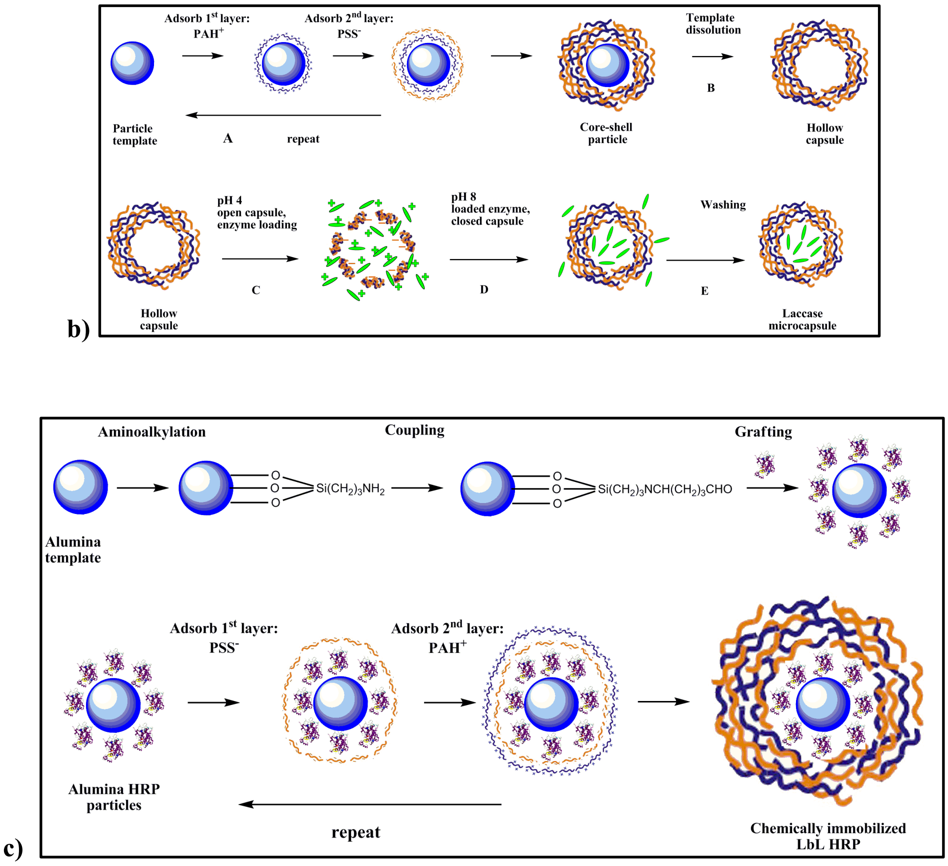


***Figura* 2- Schemi di immobilizzazione di laccasi e HRP: a) Immobilizzazione chimica di laccasi; b) immobilizzazione fisica di laccasi; c) immobilizzazione chimica di HRP.**

Gli enzimi immobilizzati sono stati utilizzati su una selezione di differenti lignine sofwood; dopo i trattamenti enzimatici, i cambiamenti strutturali avvenuti nel polimero sono stati valutati ed identificati attraverso l'uso dell' NMR del fosforo. Questa tecnica di risonanza magnetica eteronucleare è estremamente adatta per lo studio di polimeri altamente complessi, come la lignina, e costituisce uno strumento prezioso di analisi dei gruppi funzionali presenti sul polimero, sia a livello qualitativo che quantitativo.[5]

Una delle proprietà principali degli enzimi immobilizzati è la possibilità di essere riutilizzati più volte in differenti cicli di reazione. Al fine di testare questa possibilità, i catalizzatori ottenuti sono stati sottoposti ad esperimenti di riciclo. Questi hanno dimostrato che gli enzimi immobilizzati mantengono la loro attività catalitica anche dopo 10 cicli di reazione da 12 ore ciascuno, a differenza della bassissima attività residua dell'enzima libero. I risultati ottenuti dalle reazioni di ossidazione della lignina hanno dimostrato che l'utilizzo di entrambi gli enzimi nella forma immobilizzata ha portato ad una più efficiente degradazione del polimero della lignina rispetto ai corrispettivi enzimi nello stato nativo. Questo risultato può essere spiegato da un maggiore effetto di stabilizzazione dato dall'apposizione degli strati di polielettroliti attraverso la tecnica LbL; inoltre il sistema sviluppato non ha creato barriere cinetiche di alcun tipo all'approccio del substrato al sito attivo dell'enzima. L'analisi dei cambiamenti strutturali del polimero ha permesso anche lo studio del meccanismo di azione dei sistemi immobilizzati. Considerando le laccasi, l'analisi ^{31}P NMR ha rilevato che l'impiego degli enzimi immobilizzati ha prodotto un maggiore un'aumento di gruppi guaiacilici, rispetto all'enzima libero, fenomeno che indica un aumento dell'idrofilicità del polimero e la presenza di processi di rottura di legami alchil-aril etere, che danno luogo ad una forte depolimerizzazione. L'azione dell'enzima immobilizzato è stata poi potenziata dall'impiego dei mediatori, in particolare in presenza di acido violurico, che si è rivelato il mediatore di ossidazione migliore nella funzionalizzazione ossidativa della lignina. Considerando il trattamento con HRP, è stato osservato un aumento di gruppi carbossilici che è correlato con un'efficiente ossidazione; facendo un confronto in questo caso, a differenza della laccasi, la degradazione del polimero avviene principalmente attraverso reazioni di rottura dell'anello aromatico. In conclusione, dallo studio emerge che i sistemi enzimatici sviluppati hanno la capacità di mantenere una buona attività catalitica anche dopo 10 cicli di reazione. Inoltre gli enzimi immobilizzati non solo si sono dimostrati attivi nei confronti del polimero ma addirittura hanno presentato un'efficienza ossidativa maggiore nei confronti della lignina, portando ad una sua aumentata depolimerizzazione. Sulla base di questi dati, le laccasi e le perossidasi immobilizzate costituiscono nuovi e specifici biocatalizzatori per la funzionalizzazione ossidativa e la depolimerizzazione della lignina.

La lignina costituisce anche gran parte del residuo delle lavorazioni alla base dei processi cosiddetti di 'Biorefinery' che hanno come scopo la produzione di energia da fonti rinnovabili, come per esempio la produzione di bioetanolo da biomasse vegetali.[6] In questo processo la biomassa lignocellulosica viene sottoposta ad un pretrattamento iniziale per rendere la cellulosa accessibile da parte dell'enzima e poi ad idrolisi enzimatica con cellulasi.[7] Quest'ultimo processo è considerato il passaggio critico che deve essere migliorato ed ottimizzato per la produzione su vasta scala di bioetanolo. In questa fase, la lignina ha un ruolo cruciale, infatti questo polimero costituisce spesso un ostacolo al processo di idrolisi in quanto gli enzimi utilizzati possono essere adsorbiti improduttivamente sulla sua superficie, diminuendone l'attività catalitica. Recentemente è stato riportato che l'aggiunta di surfattanti può aumentare l'efficienza del processo di idrolisi.[8] Una parte del mio dottorato ha previsto lo studio del meccanismo alla base dell'aumento dell'attività cellulasica durante l'idrolisi dovuto all'aggiunta di polietilene glicol (PEG). In particolare l'effetto dell'aggiunta di PEG è stato considerato su substrati pretrattati di diversa origine, appartenenti sia ad Angiosperme che Gimnosperme. Dallo studio è emerso che l'effetto positivo sull'idrolisi dopo l'aggiunta di PEG è diverso a seconda della specie; tra tutte le specie considerate, l'abete si è dimostrata quella sul quale l'aggiunta era più efficace. Analisi cromatografiche al GC/MS e analisi ^{31}P NMR hanno evidenziato che l'effetto dell'aggiunta di PEG è determinato dalla diversa struttura della lignina dei substrati considerati. In particolare l'effetto del surfattante è strettamente connesso alla quantità di gruppi ossidrili fenolici che si trovano sulla superficie della lignina e che possono interagire con le molecole di PEG, presumibilmente attraverso legami idrogeno. Si viene così a formare uno strato di PEG sulla superficie della lignina che impedisce il legame improduttivo dell'enzima sul polimero. Maggiore è la quantità di gruppi ossidrili fenolici presenti sulla lignina del substrato, maggiore è la quantità di legami idrogeno che possono formarsi e più alto è l'effetto protettivo nei confronti dell'adsorbimento da parte dell'enzima.

Una parte del mio progetto di dottorato, che è stata svolta presso la North Carolina State University, ha previsto lo sviluppo di una tecnica analitica estremamente innovativa, l'NMR spin trapping, per l'analisi dei meccanismi alla base di reazioni catalizzate da enzimi. Questa tecnica abbina le proprietà del metodo spin trapping con le potenzialità dell'analisi ^{31}P NMR.[9] Nello spin trapping, un radicale libero altamente reattivo reagisce con un doppio legame di un composto diamagnetico, lo spin trap, dando vita ad un radicale più stabile, l'addotto di spin. Spin trap contenenti fosforo, danno luogo ad addotti radicalici che hanno emivita più lunga rispetto agli altri spin trap. Inoltre la presenza del fosforo consente l'uso dell'analisi ^{31}P NMR del prodotto diamagnetico generato dal decadimento della specie radicalica. Questa tecnica può essere utilizzata per effettuare sia analisi quantitative, attraverso l'uso di uno standard interno, sia analisi qualitative, in quanto il chemical shift del fosforo è fortemente dipendente dalla natura degli addotti. La tecnica NMR spin trapping è al momento l'unica in grado di misurare direttamente, sia qualitativamente che quantitativamente, tutti gli addotti radicalici che vengono generati in una reazione.

L'NMR spin trapping è stata utilizzata per chiarire i meccanismi d'azione alla base del sistema laccasi-mediatore (LMS). Infatti, le prestazioni delle laccasi, che presentano caratteristiche di grande interesse per le applicazioni biotecnologiche, possono essere sensibilmente aumentate dall'impiego dei mediatori di ossidazione. Queste molecole, una volta ossidate dall'enzima, generano un intermedio fortemente ossidante che si diffonde lontano dal sito attivo ed è in grado di ossidare ogni substrato che non è in grado di entrare direttamente a contatto con l'enzima, potenziando l'azione catalitica di quest'ultimo. Nonostante la vastissima applicazione del sistema LMS, anche in campo industriale, il meccanismo d'azione è ancora poco chiaro. Si ipotizza che i mediatori aumentino la reattività delle laccasi modificando il meccanismo di reazione, in particolare favorendo processi di astrazione di un atomo di idrogeno al posto di ossidazioni monoelettroniche (*Figura* 3).[10]

Nel mio studio sono stati considerati tre dei mediatori maggiormente utilizzati, HBT, acido violurico e ABTS. Le reazioni di ossidazione del mediatore sono state condotte in diverse condizioni sperimentali, in presenza di ossigeno, in condizioni di locale ipossia e di anossia completa. Svolgendo le reazioni in carenza o in completa assenza di ossigeno, in tutti e tre i casi non è stato possibile individuare la presenza di addotti radicalici di nessun tipo. Questo ha confermato il ruolo dell'ossigeno come ossidante primario nel ruolo catalitico delle laccasi. Inoltre è stata dimostrata una reale e profonda differenza nel meccanismo di azione dei tre diversi mediatori considerati.

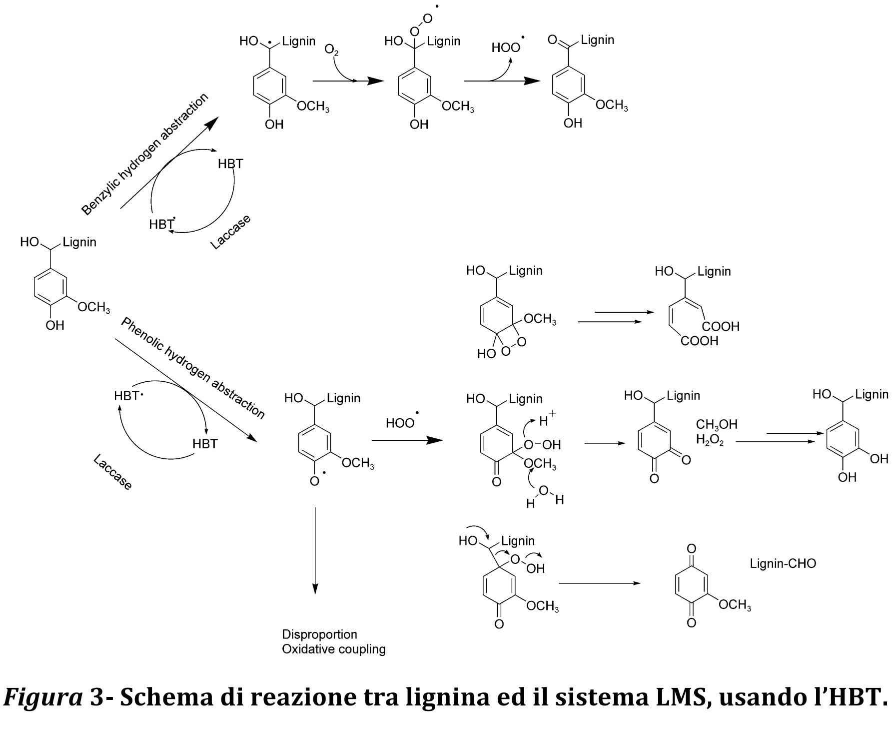


Figura 3- Schema di reazione tra lignina ed il sistema LMS, usando l'HBT.

L'acido violurico e l'ABTS una volta ossidati non generano intermedi radicalici; dall'analisi NMR spin trapping non è stato possibile individuare la presenza di addotti, neanche prolungando per 12 ore il tempo di reazione. L'HBT ha invece dimostrato un meccanismo diverso. Già dopo 45 minuti (*Figura* 4) è stato possibile osservare la presenza di sia di radicali derivanti dalla sua ossidazione (HBT•) che di radicali superossido (•OOH). Quindi il meccanismo laccasi-HBT procede attraverso ossidazione radicalica del mediatore e contemporanea generazione di superossido. Il meccanismo alla base della forte azione ossidante del sistema laccasi-HBT potrebbe essere dovuto alla produzione di radicali superossido.

La tecnica NMR spin trapping è stata poi utilizzata per l'analisi delle reazioni che vedono coinvolto un altro enzima, la lipossigenasi, che catalizza l'ossidazione di acidi grassi poliinsaturi ai loro corrispettivi idroperossidi. Le reazioni delle lipossigenasi sono considerate di estremo interesse in ambito medico in quanto possono generare radicali derivanti dai lipidi, considerati molto pericolosi in quanto altamente reattivi ed in grado di reagire con proteine e DNA.[11]

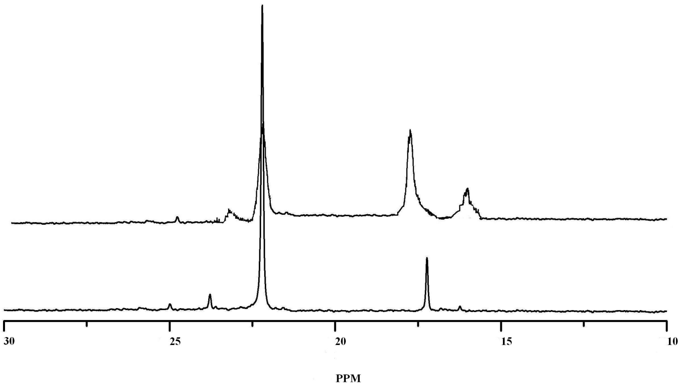


***Figura* 4- Spettri ^{31}P NMR della reazione tra DIPPMPO e laccasi in presenza di HBT per un tempo di reazione di 45 minuti (sopra) e overnight (sotto).**

Anche in questo caso, per evidenziare il ruolo dell'ossigeno, le reazioni di ossidazione sono state condotte in diverse condizioni sperimentali, in presenza di ossigeno, in condizioni di locale ipossia e di anossia completa. Dall'analisi quantitativa è emerso che la quantità maggiore di radicali si forma in condizioni di anossia; quando l'enzima è in grado di portare a compimento il suo ciclo, in quanto ha una quantità sufficiente di ossigeno, i radicali derivanti dagli acidi grassi non vengono rilasciati ma vengono trattenuti nel sito attivo dell'enzima e rilasciati solo quando si sono formati gli idroperossidi. In condizioni di ipossia, la lipossigenasi non può completare il suo ciclo e rilascia radicali. Dall'analisi qualitativa è emerso che i radicali che si formano maggiormente sono di tre tipi: radicali al carbonio (R•), radicali ossidrile (RO•) e radicali superossido (ROO•). In particolare in condizioni di normossia, la lipossigenasi non può essere considerata una fonte di radicali liberi in quanto è stato possibile individuare solo una bassissima quantità di ROO• ; in condizioni di ipossia la lipossigenasi rilascia radicali liberi in soluzione, soprattutto ROO• ed R•. In anossia la lipossigenasi può essere riconosciuta una fonte di radicali in quanto rilascia radicali R• ed RO•.

Entrambi gli studi sulla chimica dei radicali, in reazioni catalizzate da enzimi, dimostrano che l'efficacia della tecnica NMR spin trapping, unico strumento analitico che consente l'identificazione e la misura diretta di tutti gli addotti radicalici di una reazione.

L'ultima parte del mio progetto di dottorato ha riguardato lo studio e la caratterizzazione di un altro polimero fenolico altamente complesso e di grande rilievo dal punto di vista biologico, la melanina. Ad oggi la struttura chimica di questo pigmento è nota solo in parte, infatti la difficoltà nel suo studio è strettamente correlata alla sua insolubilità ed alla mancanza di un comportamento spettrale ben definito. Nella *Figura* 5 è schematizzato il processo della biosintesi della melanina. Finora le analisi della struttura chimica della melanina sono state effettuate solo allo stato solido, manca una caratterizzazione in soluzione di tutti i gruppi funzionali di questo polimero, aspetto estremamente rilevante sia per tutte le applicazioni biotecnologiche che per lo studio dettagliato di tutte le patologie ad esso connesse.[12]

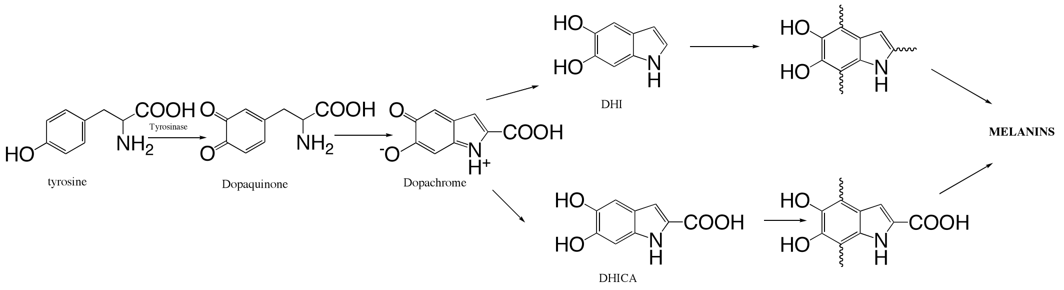


***Figura* 5- Biosintesi della melanina.**

Il mio studio è iniziato con una caratterizzazione del comportamento spettrale alle lunghezze d'onda UV-Vis di campioni di melanina sia naturali, provenienti da *Sepia officinalis*, che ottenuti attraverso polimerizzazione ossidativa enzimatica o chimica, partendo da differenti precursori. Dopo la completa solubilizzazione dei campioni di melanina grazie all'utilizzo del Soluene, una forte base organica in grado di dissolvere qualsiasi componente cellulare, l'analisi ha portato ad una caratterizzazione di tutti i campioni che ha dimostrato un massimo di assorbanza a 350 nm. Allo scopo di sviluppare un nuovo protocollo analitico che permettesse la dettagliata caratterizzazione strutturale della melanina in soluzione, sono stati studiati una serie di composti modello delle melanine. Questi sono stati sottoposti a reazioni di fosfitilazione con diversi reattivi in grado di marcare con un atomo di fosforo i gruppi funzionali ossidrilici, fenolici, carbossilici, amminici ed indolici caratteristici delle melanine. Sono stati testati allo scopo una serie di sistemi solventi compatibili con le reazioni di fosfitilazione ed in grado di solubilizzare le melanine. Una volta ottimizzati i parametri di reattività e solubilità i modelli delle melanine opportunamente marcati sono stati sottoposti ad analisi di spettroscopia di risonanza magnetica nucleare del fosforo 31. Questa tecnica NMR permette di discriminare tra i vari gruppi funzionali presenti sulle melanine in quanto la marcatura al fosforo è estremamente sensibile all'intorno chimico e presenta chimica shift significativamente diversi per ciascun gruppo funzionale marcato. E' stato così possibile effettuare una marcatura delle zone di assorbimento dei vari gruppi funzionali presenti nei modelli delle melanine. I dati ottenuti attraverso l'analisi ^{31}P NMR di diversi campioni di melanine di origine naturale e sintetica hanno mostrato per la prima volta aspetti finora sconosciuti della struttura chimica della melanina; in particolare emerge la presenza di una quantità più bassa di gruppi ossidrili fenolici rispetto a quanto ipotizzato in letteratura. Questo confermerebbe l'ipotesi di Raper e Mason[13] sulla melanogenesi, basata in gran parte sulla polimerizzazione di specie chinoniche e solo in piccola parte dalla polimerizzazione dell'acido diidrossindolcarbossilico.

Lo studio della melanina ha previsto anche la proposta di un nuovo approccio, del tutto naturale in quanto basato sugli enzimi, per la degradazione di questo polimero. In particolare l'enzima laccasi ed il sistema laccasi-mediatore è stato impiegato con successo per l'ossidazione della melanina. Il processo ha portato ad una decolorazione del pigmento, aprendo la strada a possibili applicazioni biotecnologiche in ambito medico e anche cosmetico.

Dagli studi effettuati durante i tre anni di dottorato di ricerca sono emerse 5 pubblicazioni su riviste internazionali, 4 comunicazioni orali in convegni internazionali e 10 presentazioni poster. Sono inoltre in stesura i manoscritti per 2 pubblicazioni su riviste internazionali. Segue l'elenco completo:

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10. Crestini C., **Perazzini R.**, Saladino R. "*Layer-by-layer immobilised laccases and peroxidases in the oxidation of lignins*" ISWFPC 2009, 15th International Symposium on wood , fiber and pulping chemistry (15-18/06/2009, Oslo, Norway, *oral presentation*).

11. Crestini C., Melone F., **Perazzini R.,** Saladino R. “Layer-by-layer coated oxidative enzymes in lignin oxidation” Italic 5, Italian meeting on lignocellulosic chemistry (1-4/09/2009, Varenna, Italy, *oral presentation*).
12. Crestini C., Saladino R., **Perazzini R.** “Layer-by-layer immobilised oxidative enzymes in lignin oxidation” The third annual workshop of COST FP0602 (2-4/09/2009, Varenna, Italy, *oral presentation*).
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15. Crestini C., **Perazzini R.**, Saladino R. *“Layer-by-layer immobilised laccases and peroxidases in the oxidation of lignins*” ISWFPC 2009, 15th International Symposium on wood , fiber and pulping chemistry (15-18/06/2009, Oslo, Norway, *poster presentation*).
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19. **Perazzini R.**, Saladino R., Crestini C. “*Oxidative functionalisation of lignin by layer-by- layer immobilised horseradish peroxidase”*. Lignobiotech One Symposium (28/03- 01/04/2010, Reims, France, *poster presentation*).
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"Research is to see what everybody else has seen,
and to think what nobody else has thought."

Albert Szent-Györgi

1 : INTRODUCTION

1.1 Principles of Green Chemistry

During the last century, the successful development of the chemical industry plays a key role in almost every aspect of modern society, providing a multitude of products that make an invaluable contribution to the quality of our lives. The greatest benefits were in medicine, with developments of pharmaceutical products as painkillers, antibiotics, heart drugs, etc., that were able to cure diseases that afflicted mankind throughout history. Chemistry contributed also in farming, with the development of crop protection and growth enhancement chemicals, that drastically increased the world food yields. Nevertheless, the extensive development in the chemicals industries in the twentieth century was at the cost of producing millions of tonnes of wastes, focusing the attention, especially from end of '80s, on the negative side effects of the chemical products on our natural environment and increasing the level of environmental awareness.[1,2]

At the same time another concept became worldwidely the focus of the attention both in industry and in society. In 1987 the United Nations Commission on Environment and Development recognised that the industrial and societal development must occurs on the basis of a fundamental principle: the sustainability. In particular, the sustainable development was defined as 'Development that meets the needs of the present without compromising the ability of future generations to meet their own needs'.[3] A decade later this concept was further elaborated and is now accepted by gorverments, industry and the public as a necessary goal for achieving the desired combination of environmental, economic and societal objectives. From the chemical and energy etic perspective, two of the key aspects of a sustainable development are: use of natural resources at rates that do not unacceptably deplete supplies over the long term and generation and dissipation of residues at rates no higher than can be assimilated by the natural environment.[1] It is recognised that the Earth does have a natural capacity for dealing with much of the waste and pollution which society generates; it is only when that capacity is exceeded that our lifestyle becomes unsustainable.[2] To follow a development that can be considered sustainable, it is necessary a radical change in the structure of the chemical industry, whereby the materials and energy input to a process are minimised and thus utilised at maximum efficiency. Moreover, the dispersion of harmful chemicals in the environment must be minimised or, preferably, completely eliminated.[1] To achieve these goals is necessary to create an innovative, sustainable chemistry based on green catalytic methodologies.[4]

During the early 1990s the US Environmental Protection Agency introduced the term Green Chemistry ‘To promote innovative chemical technologies that reduce or eliminate the use or generation of hazardous substances in the design, manufacture and use of chemical products’.[5] This concept is today recognised as both a culture and a methodology that efficiently utilises raw materials (preferably renewables), eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products.[6] Green chemistry is primary pollution prevention rather then waste remediation and is based on twelve principles, formulated by Anastas and Warner, that explain how this can be achieved:[5]

1. *Prevent waste*: Design chemical syntheses to prevent waste, leaving no waste to treat or clean up.
2. *Design safer chemicals and products*: Design chemical products to be fully effective, yet have little or no toxicity.
3. *Design less hazardous chemical syntheses*: Design syntheses to use and generate sudstances with little or no toxicity to humans and the environment.
4. *Use renewable feedstocks*: Use raw materials and feedstocks that are renewable rather than depleting. Renewable feedstock are often made from agricultural products or are the wastes of other processes; depleting feedstocks are made from fossil fuels (petroleum, natural gas or coal) or are mined.
5. *Use catalysts, not stoichiometric reagents*: Minimise waste by using catalytic reactions. Catalysts are used in small amounts and can carry out a single reaction many times. They are preferable to stoichiometric reagents, which are used in excess and work only once.
6. *Avoid chemical derivatives*: Avoid using blocking or protecting groups or any temporary modifications if possible. Derivatives use additional reagents and generate waste.
7. *Maximise atom economy*: design syntheses so that the final product contains the maximum proportion of the starting materials. There should be few, if any, wasted atoms.

8. *Use safer solvents and reaction conditions*: Avoid using solvents, separating agents, or other auxiliary chemicals. If these chemicals are necessary, use innocuous chemicals. If a solvent is necessary, water is good medium as well as certain eco-friendly solvents that do not contribute to smog formation or destroy ozone.
9. *Increase energy efficiency*: Run chemical reactions at ambient temperature and pressure whenever possible.
10. *Design chemicals and products to degrade after use*: Design chemical products to break down to innocuous substances after use so that they do not accumulate in the environment.
11. *Analyse in real time to prevent pollution*: Include in-process real-time monitoring and control during syntheses to minimise or eliminate the formation of byproducts.
12. *Minimise the potential for accidents*: Design chemical and their forms (solid, liquid or gas) to minimise the potential for chemical accidents including explosions, fires and releases to the environment.

Today and in the future the fundamental challenge for the chemical industry is to continue providing benefits to society, without overburdening or causing damage to the environment: sustainable development is the ultimate goal, green chemistry is a means to achieve it.

1.2 The role of biocatalysis

In recent years, the use of natural catalysts, enzymes, in the development of organic synthesis reactions has received a steadily increasing amount of attention, expecially in the frame of green chemistry. In fact, due to the high chemical and energy efficiency of enzymatic transformation in water, biocatalysis is one of the greenest technologies for chemical syntheses. Specifically, biocatalysis can prevent waste generation by performing catalytic processes with high stereo- and regio-selectivity, and by preventing or limiting the use of hazardous organic reagents.[7] Moreover, the enzymes are able to promote reactions under very mild reaction conditions of temperature, pH and pressure.

In biocatalysis the reactions are usually referred as biotransformations. A biotrasformation is a process that describes a reaction or a set of simultaneous reactions in which a pre-formed precursor molecule is converted using enzyme and/or whole cells, or combinations thereof, either free or immobilised.[8] Numerous industrial biotrasformations are in operation worldwilde, in many areas, with the production of different type of compounds (*Figure* 1.1).

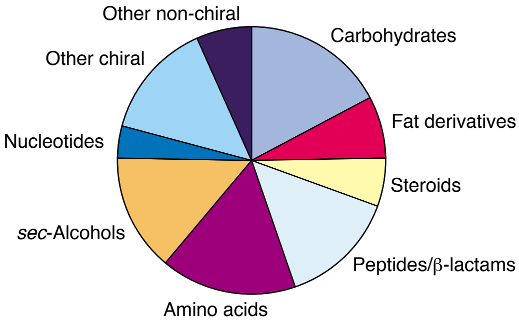

Figure 1.1- The type of compounds produced using biotransformation processes, based on 134 industrial processes (from Straatohf *et al.,* 2002).

Most of the enzymes for commercial application are obtained from the microorganisms, including bacteria, fungi and yeast, only some are obtained through plant source (*Table* 1.1).[9]

Table 1.1- Plant source of enzymes.

Source	Enzyme	Application
Jack bean (*Canavalia* spp.)	Urease	Diagnostic
Papaya (*Carica papaya* L.)	Papain	Baking, tanning, dairy
Pineapple (*Ananas comosus* L.)	Bromelain	Baking
Horseradish (*Armoracia rusticana* Gaert., Mey et Sch.)	Peroxidase	Diagnostic
Wheat (*Triticum* spp.)	Esterase	Ester hydrolysis
Barley (*Hordeum vulgare* L.)	ß-amylase	Baking, maltose syrup
Soybean (*Glycine max* L.)	ß-amylase	Baking, maltose syrup

Biocatalysis can be still considered an emerging field, in many cases the classical chemical technologies are preferred to the industrial biotransformation, mainly due to the lack of sufficient commercially available biocatalysts.[10] Moreover, the use of biocatalysis sometimes requires longer times both in the design and in the process phase. Many of these issues are now being addressed. With the continued expansion in the number of microorganisms whose genomes have been sequenced, the application of bioinformatic methodologies is leading to a rapid expansion in the number of commercially available enzymes. Furthermore the advances in genetic engineering techniques are expanding the number of commercially available enzymes.[11]

1.2.1 Advantages and disadvantages of biocatalysts

In the *Table* 1.2 the advantages and the disadvantages in the use of enzymes for catalysis processes are summarised.

Table 1.2- Comparison of advantages and disadvantages of the biocatalysis.

Advantages	Disadvantages
Very efficient catalysts	Present in nature in only one enantiomeric form
Environmentally benign reagents	Require narrow operation parameters
Action in mild reaction conditions	Display their highest catalytic activity in water
Compatibility	In some cases are bound to their cofactors
Catalysis of a broad spectrum of reactions	Are prone to inhibition phenomena
Selectivity	May cause auto-immune responses including allergies
Valuable resource for green chemistry	High costs of purification

Among the main advantages in the use of enzymatic catalyst there is their high efficiency: compared to the nonenzymatic reactions, the rates of enzyme-mediated processes are accelerated by a factor of 10^8-10^{10}. Moreover the enzymes are biodegradable reagents that act in mild conditions both of pH (usually in the range 5-8) and temperature (in the range of 20-40 °C). This minimises the problems of undesidered side reactions and make the processes environmentally friendly. On the contrary this aspect can lead to a limitations in large scale applications, especially if the reactions need extreme pH or temperature values to occur that can deactivate the enzyme. Finally, the costs of isolation and purification make the biocatalysts still unconvenient for industrial applications, especially if are necessary also cofactors, molecule relatively unstables and prohibitively expensive to use in stoichiometric amounts.

In the following sections the three enzymes, that are been used in this Ph D study for biotechnological applications, will be presented: laccase, horseradish peroxidase and lipoxygenase.

1.3 Laccase

1.3.1 Distribution in nature

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) belongs to a group of polyphenol oxidases containing copper atoms in the catalytic center and usually called multicopper oxidase. Other members of this group are the mammalian plasma protein ceruloplasmin and ascorbate oxidases of plants. Laccase performs the reduction of dioxygen to water accompanied by one electron oxidation of a substrate, typically phenolic systems. It was first discovered by Yoshida in 1883 in the exudates of *Rhus vernicifera*, the Japanese lacquer tree, and a few years later it was also demonstrated in fungi. In fact, laccases are typically found in plants and fungi, more recently, proteins with features typical of laccases have been also identified in insects and prokaryotes.[12,13] Among the plant laccases the most studied is from *Rhus vernicifera*, data on other plant laccases are limited, in particular laccases from *Rhus succedanea*,[14] *Acer pseudoplatanus*,[15,16] *Pinus taeda*,[17] *Populus euroamericana*,[18] *Liriodendron tulipifera*,[19] *Nicotiana tobacco*,[20] *Lolium perenne*[21] and *Zea mays*[22] are only partially characterised. The majority of laccases characterised were isolated from fungi; up to date more than 100 laccases have been purified from the wood rotting fungi belongs to the genera *Cerrena*, *Coriolopsis*, *Lentinus*, *Pleurotus*, *Trametes*.[23]

The physiological role of these biocatalysts, which can be intracellular or extracellular, is different in the various organisms but they all catalyse polymerisation or depolymerisation processes. It has been proposed that laccases are involved in cuticle sclerotisation in insects[24] and in the assembly of UV-resistant spores in *Bacillus* spp. Plant laccases partecipate in the radical-based mechanisms of lignin polymer formation and is thought that can also protect fungal pathogens from toxic phytoalexins and tannins, thus they are an important virulence factor in many fungal diseases.[25]

1.3.2 Structural and catalytic properties

All the plant laccases are extracellular monomeric proteins with a range of glycosylation between 22-45%. Most fungal laccases are monomeric proteins, containing about 500 amino acids and with an average weight of 60-70 kDa. Several laccases, however, exhibit a homodimeric structure, the enzyme being composed of two identical subunits with a molecular weight typical for monomeric laccase.

Moreover, like the plant proteins, they are glicosylated, but generally to a lessere extent (10-25%).[26] Considering the three-dimensional structure, laccases show a characteristic fold that comprises three cupredoxin domains, with a mononuclear copper centre located in between the first and the third domains (*Figure* 1.2).[27]

The four copper atoms costitute the enzyme catalytic core and are classified in three different classes, based on their spectroscopic features. The type 1 (T1) mononuclear copper centre shows an intense absorption band at 600 nm, which is responsible for the blue colour of the protein, and is due to the ligand-to-metal charge transfer between the cysteine sulphur and the copper atom.

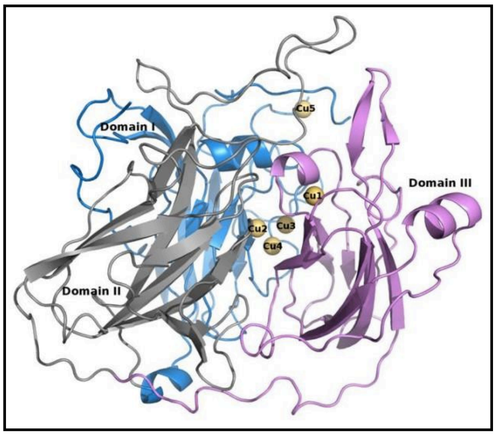

Figure 1.2- Three-dimensional structure of *Bacillus subtilis* laccase with the three different cupredoxin domain; domain I in blue, domain II in gray and domain III in violet. Copper atoms are represented by the spheres in yellow (from Bento *et al.*, 2010).

This site also shows a characteristic EPR signal that is due to the high covalency at the copper site. The type 2 (T2) copper site, located in the trinuclear centre, also exhibits a characteristic EPR signal, but no observable bands in the absorption spectra. The pair of type 3 (T3) copper ions are also localised in the trinuclear centre and are EPR silent, that is attributed to their antiferromagnetic coupling in the presence of a bridging ligand, normally assumed to be hydroxyl. The T3 site also shows an absorption band at 330 nm that has been attributed to the charge transfer between an hydroxyl bridging group and the copper atoms.[27]

To date, the three-dimensional structures have been determined for various fungal laccase from *Cerrena maxima*,[28] *Coprinus cinereus*,[29] *Lentinus tigrinus,*[30] *Melanocarpus albomyces,*[31] *Pycnoporus cinnabarius,*[32] *Trametes Trogii*[13] and *Trametes versicolor.*[33] Among the bacteria the three-dimensional structure of laccase from *Bacillus subtilis*[34] and *Streptomyces coelicolor*[35] has also recently been published.

Laccase catalyses one-electron oxidation processes, and four molecules of substrate are oxidised in order to reduce a dioxygen molecule to two water molecules. This cycle is composed by three major steps. In the first one, the sustrate oxidation at the mononuclear T1 centre occurs, with the reduction of the copper atom. Then the electrons are transferred to the trinuclear T2/T3 cluster where, finally, the molecular oxygen is reduced to water.[36] In *Figure* 1.3 is shown the catalytic cycle of laccase.[37] The dioxygen molecule interacts with the completely reduced trinuclear cluster (T2/T3) via a $2e^-$ process to produce the peroxide intermediate which contains the dioxygen anion. One oxygen atom of the dioxygen anion is bound with the T2 and T3 copper ions and the other oxygen atom is coordinated with another copper ion of T3 (*Figure* 1.3*a*).

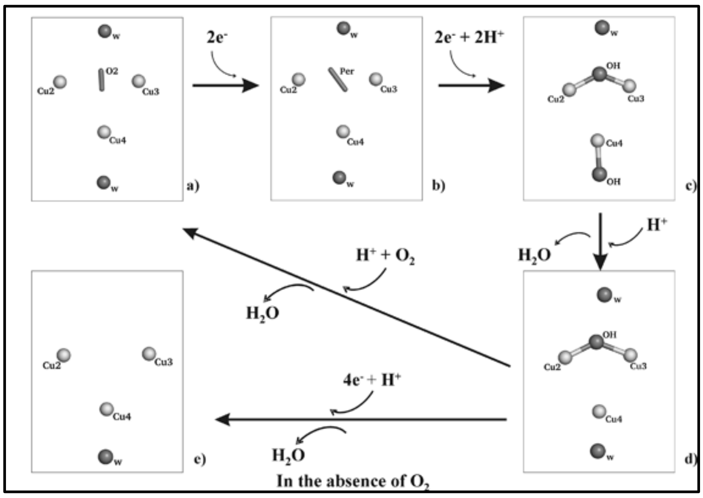

Figure 1.3- Schematic view of laccase catalytic cycle: a) the resting state of the enzyme with a dioxygen molecule bound between the type 3 copper ions and interacting with the type 2 copper; b) the peroxide intermediate; c) the native intermediate formed from the peroxide moiety splitting into two hydroxyl groups; d) the molecule with one bridging hydroxyl group; e) the enzyme in its fully reduced state (from Bento *et al.*, 2005).

In this structure all the copper ions will be in the oxidised +2 state; the blue color of the enzyme is certainly consistent with the type 1 copper being oxidised.[37] Then the formation of a peroxide intermediate occurs; two electrons are transferred to dioxygen, each of the type 3 transferring one electron to an oxygen atom of the bound dioxygen (*Figure* 1.3*b*). After partecipating in the transfer process, the type 3 copper ions are in the +2 state. Then, the peroxide intermediate undergoes a second $2e^-$ and the peroxide O-O bond is splitted to produce a native intermediate which is a fully oxidised form with the three copper centers in the trinuclear site mutually bridged by the product of full O_2 reduction (*Figure* 1.3*c*). The following stages involve the addition of a proton to the hydroxyl group bound to Cu4 and the release of the first water molecule into the outlet channel. This leaves a structure with only a single hydroxyl moiety bridging the two type 3 copper ions (*Figure* 1.3*d*). This hydroxyl moiety thens migrate to the far side of Cu4, becomes protonated and is finally released as the second water molecule. The enzyme can then bind a second dioxygen molecule for a re-iteraction of the cycle. Alternatively, in the absence of dioxygen the assimilation of four electron leaves the enzyme in the fully reduced state, ready to bind dioxygen as soon it becomes available (*Figure* 1.3*e*).[14,37,38]

Fungal laccases typically exhibit pH optima in the range from 3.5 to 5.0, when the substrates are hydrogen atom donor compounds; the optimum pH for phenolic compounds can actually increase at higher pH to a limit that results from the balance between the redox potential difference the substrate and the inhibition of the T2/T3 copper site by the binding of OH^- ion.[39] Furthermore, a wide spectrum of compounds has been described to inhibit laccase. These inhibitors include small inorganic anions such as azide, cyanide, fluoride and hydroxide. These ions bind with the T2/T3 site and inhibits the enzymatic activity.[40]

1.3.3 Laccase in organic synthesis

Due to their catalytic and electrocatalytic properties, laccases have received much attention in the last decades for the possibility of a wide application in several industrial and biotechnological processes. Moreover, laccases can also be used in fine organic synthesis for the ability to oxidise a great variety of compounds. The redox potential of laccase is in the range of 0.5 to 0.8 V versus NHE.[40] In the reactions where the substrate to be oxidised has a higher redox potential than laccase or when the substrate is too large to penetrate into the enzyme active site, the presence of so-called ‘chemical mediator’ may facilitate the reaction. First, the mediator reacts with the laccase to form a strongly oxidizing intermediate. Then, this oxidised mediator interacts with the bulky or high redox-potential substrate. The mediators that are widely used are N-hydroxybenzotriazole (HBT, redox potential 1.13 V),[41] 2,2’-azinobis-(3-ethylbenzylthiozoline-6-sulphate) (ABTS, redox potential 0.55 V),[42] violuric acid (VA, redox potential 0.97 V),[41] 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO, redox potential 0.75 V)[43] (*Figure* 1.4). It has been demonstrated that, depending on their chemical structure, mediators follow three different mechanisms of oxidation: compounds such as ABTS follow the electron-transfer mechanism; N–OH compounds (e.g. HBT, VA) prefer the radical hydrogen transfer route; and, on the contrary, TEMPO undergoes two electron oxidations.[25] The laccase-mediator system (LMS) finds potential application in many different areas, such as in delignification and biobleaching of pulp;[44] treatment of wastewater from industrial plants;[45] enzymatic modification of fibers and dye-bleaching in textile and dye industries;[46] enzymatic crosslinking of lignin-based materials to produce medium density fiberboards;[47] detoxification of pollutants and bioremediation;[48] detoxification of lignocellulose hydrolysates for ethanol production by yeast;[49] enzymatic removal of phenolic compounds in beverages, wine and beer stabilization, fruit juice processing[50] and construction of biosensors and biofuel cells.[51]

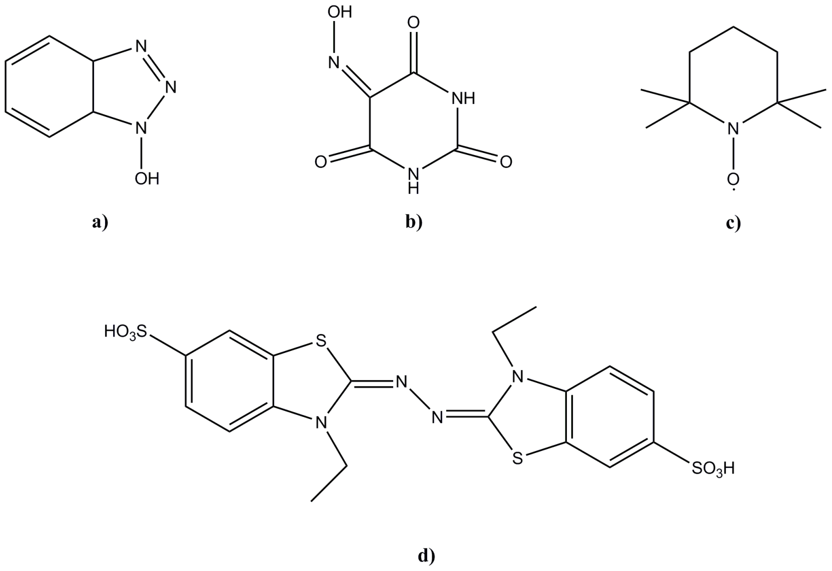

Figure 1.4- Examples of laccase mediators: a) N-hydroxybenzotriazole (HBT); b) Violuric acid (VA); c) 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO); d) 2,2'-azinobis-(3-ethylbenzylthiozoline-6-sulphate) (ABTS).

1.4 Peroxidase

Peroxidases (EC 1.11.1.7) are a group of enzymes that are widely distribuited in nature; they can be found in bacteria, fungi, plants and animals. They are among the first enzymes discovered, Schönbein was the first to observe the oxidation of certain organic compounds by hydrogen peroxide as early 1855.[52] They are involved in many biological functions; plant peroxidases are responsable for the degradation of the anthocyanin group which forms pigments ranging from pink and red to blue[53] where in fungi partecipate in lignin breakdown.[54] Peroxidases are generally classified in three different superfamilies: plant peroxidases, animal peroxidases and catalases. The plant peroxidases superfamily can be further divided into three classes, based on structural similarities and in a suspected common evolutionary origin:[55]

- *Class I*: Intracellular peroxidase which acts as an antioxidant to prevent damage to mitochondria caused by H_2O_2;
- *Class II:* Peroxidase secreted by fungi which partecipate in the degradation of lignin in wood;
- *Class III*: Peroxidases secreted by plants which allows to the plant to adapt to seasonal change and prevent stress to plant physiology and biosynthesis.

Horseradish peroxidase (HRP) and yeast cytochrome c peroxidase (CcP) are some of the most intensively studied peroxidases. In 1976, Welinder determined the first complete primary structure of a peroxidase, HRP. In 1980 Yonetani pubblished the primary sequence of CcP.[55]

1.4.1 Horseradish peroxidase: properties and catalytic cycle

HRP is a glycoprotein enzyme containing a heme prosthetic group that utilises hydrogen peroxide to oxidise a wide variety of organic and inorganic compounds. It is isolated from the roots of horseradish (*Armoracia rusticana* Gaert., Mey et Sch., Cruciferae) that contain a number of distinctive peroxidase isoenzymes of which the C isoenzyme is the most abundant.[56] The isoenzymes have many functions in plant physiology such as crosslinking of cell wall polymers, lignification and resistance to intracellular infection. Actually, the production of peroxidase from horseradish roots occurs on a large scale because of the commercial uses of the enzyme, for example as a component of clinical diagnostic kits and for immunoassay. [57]

Horseradish peroxidase isoenzyme C comprises a single polypeptide of 308 amino acid residues with a glicosylation between 18-22%. HRP C contains two types of metal centre, iron(III) protoporphyrin IX and two calcium atoms. Both are essential for the structural and functional integrity of the enzyme.[56,57] The heme prosthetic group, a protoporphyrin IX, is made of four pyrrole rings joined by methene bridges with iron(III) centring the molecule. In nature, iron is usually six-coordinated, but in native enzymes, the iron is five-coordinated. In HRP, positions 1-4 are occupied by the four pyrrole nitrogen atoms, position 5 is located on the proximal side of the heme and is occupied by the imidazole side chain of a histidine residue. The two calcium binding sites are located at positions distal and proximal to the heme plane and are linked to the heme-binding region by a network of hydrogen bonds.[56] The enzyme three dimensional structure is largely α–helical although there is also a small region of β–sheet. There are two domains, the distal and proximal, between which the heme group is located (*Figure* 1.5).[57]

The mechanism of catalysis of HRP and in particular, the C isoenzyme, has been investigated extensively; the catalytic cycle is summarised in *Figure* 1.6.

The HRP catalytic cycle is composed by three steps, of which two mono-electron oxidations that lead the enzyme, by using the H_2O_2, to convert the substrate into a free radical. In the first step of the cycle, the iron(III) resting state of the enzyme (*Figure* 1.6*a*) reacts with H_2O_2 to generate the compound I, a high oxidation intermediate comprising an iron(IV) oxoferryl centre and a porphyrin-based cation radical (*Figure* 1.6*b*). Then, the first one-electron reduction step requires the partecipation of a reducing substrate and leads to the generation of compound II, an iron(I) oxoferryl specie (*Figure* 1.6*c*). Both compound I and II are powerful oxidants, with redox potentials estimated to be close to +1 V. Finally, in the second one-electron reduction step the compound II returns to the resting state of the enzyme.[57,58]

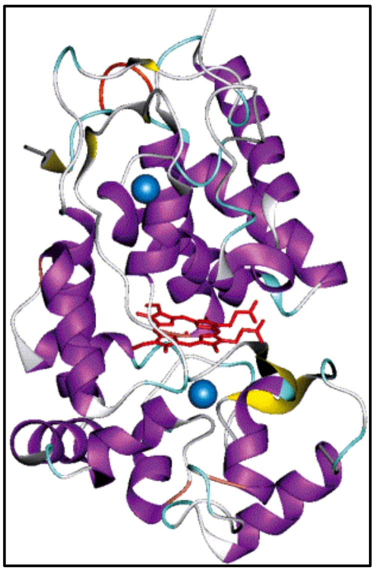

Figure 1.5- Three-dimensional structure of horseradish peroxidase isoenzyme C. The heme group is coloured in red, the calcium atoms are shown as blue spheres. α–helica and β–sheet regions of the enzyme are shown in purple and yellow, respectively (from Veitch and Smith, 2001).

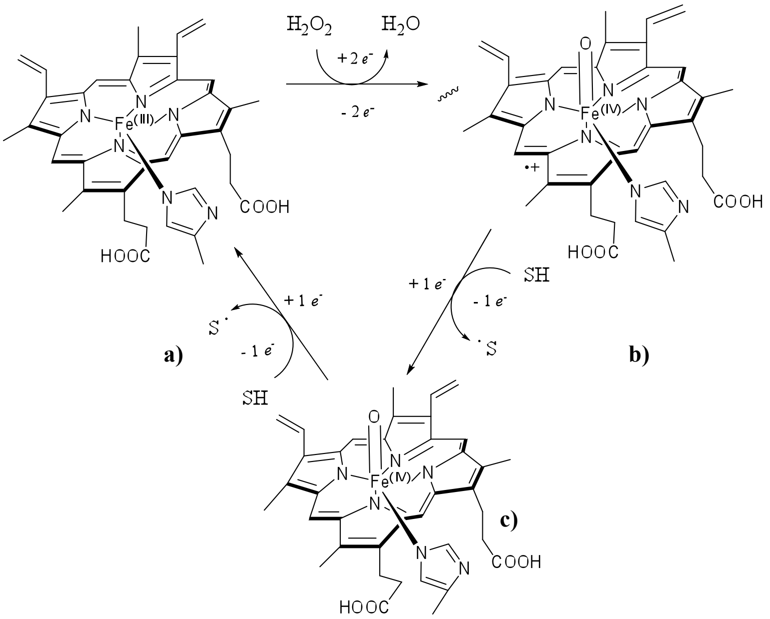

Figure 1.6- Schematic view of HRP catalytic cycle: a) enzyme resting state; b) formation of intermediate compound I; c) formation of intermediate compound II.

The radicals then undergo coupling to produce dimers. Successive oxidation, coupling, and a transfer reaction eventually results in the formation of a polymer. The generation of radicals in this cycle is enzyme-dependent. However, radical-radical coupling and transfer are controlled exclusively by phenoxyl radicals and solvent chemistry.[59]

1.5 Lipoxygenase

1.5.1 Lipid peroxidation

The formation of oxygenated fatty acids is considered one of the main reactions in lipid alteration.[60] In particular, in the process are formed the so-called oxylipins, a class of compounds of several hundred distinct oxidised lipophilic molecules.[61] The initial formation of hydroperoxides may occur either by chemical oxidation or by the action of enzymes.[62] The enzymatic oxygenation can be achieved by two main enzymatic systems: fatty acids dioxygenases with catalytic non-heme iron (lipoxygenases or LOX) and dioxygenases with a heme group (ciclooxygenases or COX in mammals; α-dioxygenases or α-DOX in plants).

Plant oxylipins are derived from the oxidation of the most abundant polyunsatured fatty acids, or PUFAs, in plants, such as linoleic acid (LA, 18.2ω6) and α-linoleic acid and include hydroperoxy-, hydroxy-, oxo- and epoxy-fatty acids, divinyl ethers, volatile aldehydes and the hormone, jasmonic acid.[63,64] This great structural variety reflects the different biological roles of plant oxylipins as second messengers and antimicrobial, anti-insecticidal as well as antifungal compounds.[65] In animals LOX and COX products commonly act by signaling through G-protein–coupled receptors and appear to be involved in cellular homeostasis, proliferation, and differentiation, and also in pathophysiological processes (inflammation and cancer).[66]

1.5.2 Lypoxygenase: occurrence and classification

LOXs (linoleate:oxygen oxidoreductase, EC 1.13.11.12) constitute a large and heterogeneous family of lipid peroxidising enzymes that catalyse dioxygenation of free and/or esterified PUFAs to their corresponding hydroperoxy derivatives.[67] A first scientific report on a lipoxygenase was presented in 1932, when it was described oxidation of lipids in soybeans by an enzyme named lipoxidase.[68] Lipoxygenases were considered exclusively plant enzymes until the mid 1970's, when 12-LOX activity was discovered in human and bovine platelets.[69] Today it is known that this group of enzymes occur ubiquitously in plants and mammals, and only recently has been detected in coral, moss, fungi and a number of bacteria as well.[61]

Plant LOXs are classified based on their positional specificity in introducing molecular oxygen into LA, the most common substrate. The oxygenation can occur either at carbon atom 9 (9-LOX) or at carbon 13 (13-LOX) of the hydrocarbon backbone.[66] These different specificities lead to the formation of the corresponding hydroperoxides, the 9-hydroperoxy and the 13-hydroperoxy derivatives of the substrate.[62]

An alternative and more comprehensive classification of plant LOXs, based on comparison of their primary structure, has been proposed.[70] According to their overall sequence similarity, plant LOXs can be grouped into two gene subfamilies. Those enzymes harboring no plastidic transit peptide and that have a high sequence similarity (>75%) have been designated type 1-LOXs. The other group includes enzymes that carry a plastidic transit peptide sequence and show only a moderate overall sequence similarity (~35%). They have been classsified as type 2-LOXs. Plant LOXs in some cases are costitutive, but most of them are often activated by pathogen attack[71] or used as storage proteins during the vegetative growth.[72] Moreover, since

LOXs are normally present in seeds, they are involved in the mobilisation of storage lipids during germination.[73]

The animal LOXs are classified with respect to their positional specificity of arachidonic acid (AA, 20.4ω6) oxygenation as 5-LOXs, 8-LOXs, 11-LOXs, 12-LOXs and 15-LOXs. However, this classification is not optimum since AA, the most common substrate in mammalians, is not a good substrate for many non-mammalian LOXs and with other substrate fatty acids their reaction specificity can be quite different.[74] Considering the biological functions in mammalians, 5-LOXs seem to be associated with pulmonary hypertension[75], renal ischemia-reperfusion injury[76] and cancer;[77] 12-LOXs are supposed to have a role in regulation of immunological and haemodynamic processes.[78]

In fungi there is an interesting correlation beetween pathogenicity and lipoxygenases; the plant pathogen interactions may induce secretion of lipoxygenases. *Magnaporthe grisea*, the rice blast fungus, can induce the expression of a rice lipoxygenase as an early response of the host to the pathogen.[79]

1.5.3 LOXs structural and catalytic properties

LOXs are non-heme iron enzymes consisting of a single polypeptide chain that is folded into two domains, the mostly α-helical catalytic domain and the N-terminal β–barrel domain.[66] The C-terminal domain contains the substrate-binding pocket and the catalytic non-heme iron, where the N-terminal is important for membrane binding and regulates the catalytic activity.[74] Molecular mass of plant LOXs is of about 95-100 kDa; soybean lipoxygenase-1 is a 839 residue protein closely related to mammalian lipoxygenase.[80] Animal LOXs are proteins with an average molecular mass of 75-80 kDa and are usually shorter than plant sequences by 150 to 200 residues. Comparing the sequences, the identity between plant and mammalian lipoxygenases is highest in the regions of the catalytic domain.[81]

LOXs catalises the regio- and stereo-specific dioxygenation of PUFAs containing a (1*Z*,4*Z*)-pentadiene unit.[62] The non-heme iron exists in two oxidation state: Fe(II) and Fe(III); the native enzyme is inactive and in the Fe(II) form. When treated with an equimolar amount of product, the iron is oxidized to the Fe(III) form, resulting in an active enzyme. The ferric form can then catalyze the abstraction of a hydrogen from the bisallylic carbon atom of the substrate from 11 position of the substrate, in a stereospecific manner, yielding a pentadienyl radical complexed with the ferrous enzyme. Bimolecular oxygen is then inserted to the pentadienyl radical, through a channel in the lipoxygenase, which leads to the formation of the hydroperoxide product and the reoxidation of the cofactor to the ferric form (*Figure* 1.7).[82,83]

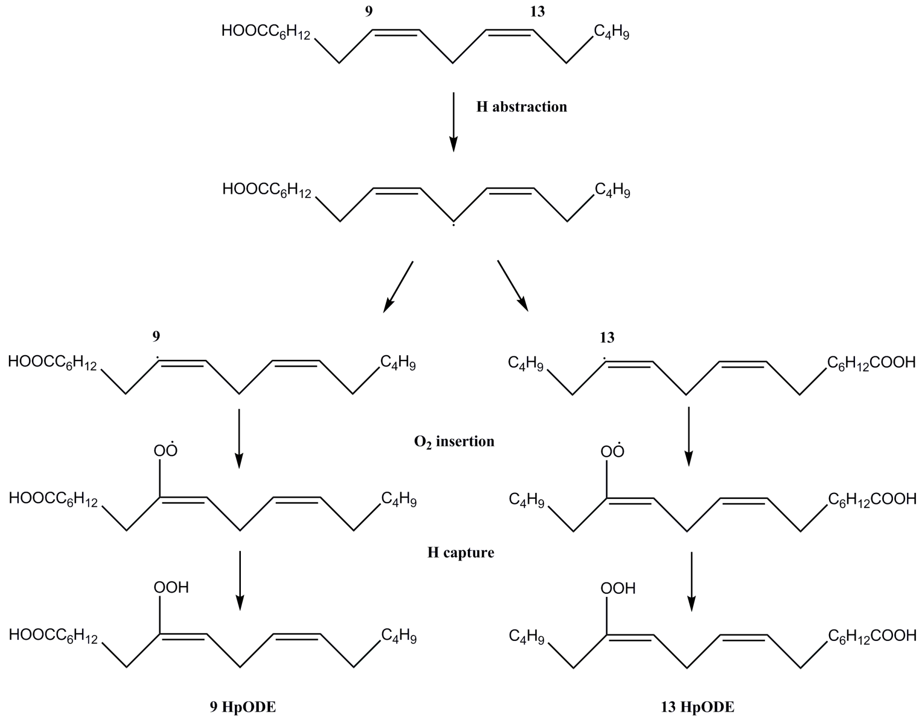

Figure 1.7- Radical mechanism of the reaction between LOX and LA, the most common substrate in plants.

Summarising, the LOX reaction consists of four consecutive steps:

1. stereo-selective hydrogen abstraction from a bisallylic methylene forming a carbon-centered fatty acid radical;
2. rearrangement of the fatty acid radical, which is bound at the active site as planar pentadienylic intermediate or, more likely, as nonplanar allylic radical;
3. stereo-specific insertion of molecular dioxygen forming an oxygen-centered hydroperoxy radical;
4. reduction of hydroperoxy fatty acid to the corresponding product anion.

1.6 Enzyme immobilisation

Industrial biotechnology, also known as white biotechnology, is the application of modern biotechnology to the sustainable production of chemicals, materials, and fuels from renewable sources, using living cells and/or their enzymes. Much interest has been generated in this field mainly because industrial biotechnology is often associated with reduced energy consumption, greenhouse gas emissions, and waste generation, and also may enable the paradigm shift from fossil fuel-based to bio-based production of value-added chemicals.[84] The biotransformation, that play a key role in the industrial biotechnology, notwithstanding the many benefits, is often impeded by low operational stability and shelf-life in addition to the difficult recovery and re-use of the enzymes. Although these problems can be alleviated by *in vitro* evolution, another approach to rendering enzymes more robust and recyclable is to immobilise them.[85] The immobilisation is achieved by fixing the enzyme to or within an inert and insoluble material. In this form, it is possible a more convenient handling of the enzyme and its easy separation from the product, minimising or eliminating protein contamination of the product. Moreover the immobilisation can facilitates the efficient recovery and reuse of costly enzymes and allows to use them in multienzyme and chemoenzymatic cascade processes.[86] A further benefit is often the enhanced stability towards denaturation by heat or organic solvents.[87] Finally the possibility to use the enzyme in a greater variety of bioreactor designs. Nevertheless, compared with the free enzyme, the immobilised enzyme has usually its activity lowered and the Michaelis-Menten constant increased. These alterations result from structural changes introduced to the enzyme by the applied immobilisation procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution.[88] In spite of these disadvantages, the creation of a microenvironment may allow to the enzyme to remain active at different temperatures or pHs than would be predicted for the enzyme when not immobilised, increasing the application possibilities.[89]

Enzymes may be immobilised by a great variety of methods, that can be classified as physical and chemical. The physical methods are based on weak interactions between support and enzyme, where in the chemical immobilisation covalent bonds are formed with the enzyme. However, no single method and support is best for all enzymes and their applications. This is because of the widely different chemical characteristics and composition of enzymes, the different properties of substrates and products, and the different uses to which the product can be applied; all of the methods present advantages and disadvantages.[88] Nevertheless, for the choice of the immobilisation, some aspects have to be considered. The method of attachment have to prevent loss of enzymatic activity, the knowledge of the catalytic site will avoid reaction with the essential binding site group of the enzyme. Alternatively, the active site can be protected during attachment as long as the protective groups can be removed later on without loss of enzyme activity. The surface on which the enzyme is immobilised play an important role in retaining of tertiary structure in the enzyme by hydrogen bonding or the formation of electron transition complexes. The retenction of tertiary structure is crucial in maximising the thermal stability. The microenvironment of surface and enzymes has an anionic or cationic nature that can cause a shift in the optimum pH of the enzyme. This shift can be up to 2 pH units, extending or modifing the region in which the enzyme can work effectively.[90] The main physical and chemical immobilisation techniques are shown in *Figure* 1.8 and described below.

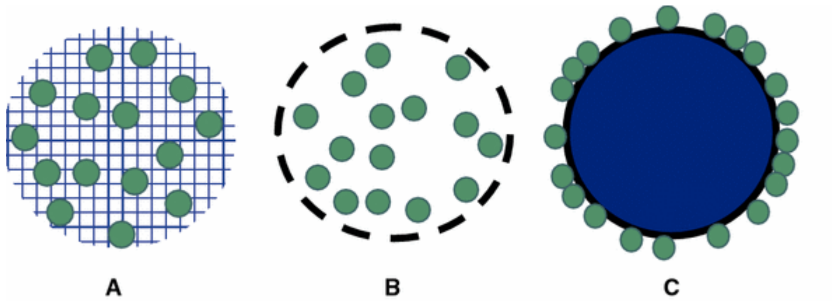

Figure 1.8- Physical enzyme immobilisation techniques: A) entrapment; B) encapsulation; C) solid support (adsorption, covalent binding or ionic binding) (from Brady and Joordan, 2009).

1.6.1 Physical methods

Entrapment

This technique is based on the inclusion of an enzyme in a polymer network, such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule (*Figure* 1.8A). The physical restrains usually are too weak to prevent enzyme leakage entirely, additional covalent attachment is often required.[86] Entrapment protects enzymes by preventing direct contact with the environment, minimising the effects of gas bubbles, mechanical sheer and hydrophobic solvents, but has the disadvantage of mass transfer limitations and low enzyme loading.[91] One of the most common method of entrapment is through use of silica sol-gel matrices formed by hydrolytic polymerisation. By adjusting the polymerisation conditions, the polymer porosity, network structure, surface functionalities and particle size can be modified.[92]

Encapsulation

This method, similar to entrapment, has the aim to protect the catalyst from the external environment, forming a layer around the enzyme with a certain permeability (*Figure* 1.8B). It has a limited application for the biocatalysis of large substrates as they are prone to mass transfer limitations.[91] The most common used materials for enzyme encapsulation are amino polymer, such as polyethyleneimine. The microcapsules formation involves enclosing the enzymes within semi-permeable polymer membrane. The preparation of enzyme microcapsules requires extremely well-controlled conditions.

Adsorption

This technique involves the enzyme physical adsorption onto the backbone or support material, often a polymer matrix (*Figure* 1.8C). It is a reletively easy because typically involves bathing the support in a solution of the enzyme and incubating to allow time for the physical adsorption to the surface to occur.[89] Unfortunately, this method is troublesome, because it allows leaching of the enzyme while reacting, thereby, contamining the substrate.[93] Physical adsorption can also denaturate the enzyme depending on the surface chemistry of the support material.

1.6.2 Chemical methods

Covalent binding to a water-insoluble matrix

In this technique a strong interaction between support and enzyme occurs, in particular the catalyst is covalently bounded to a water-insoluble carrier via functional group of the enzyme that are not essential for catalytic action (*Figure* 1.8C). Usually the bound involve the amino group of a particular aminoacid, typically (but not exclusively) a lysine. Epoxide groups are used on the support for linkage as they are relatively stable, can bind lysine, and react with protein under very mild conditions.[94] The covalent binding may alter the conformational structure and active center of the enzyme, resulting in loss of activity and/or changes of the substrates.

Ionic binding

Enzymes can be bound, by ionic binding, to water-insoluble carriers containing ion-exchange residues (*Figure* 1.8C). Polysaccharide as dextran, agarose and chitosan, are usually used as carriers.[92] The ionic binding cause only little changes in the conformation and the enzyme active site, therefore in mostly of the cases the enzymatic activity is not altered. The main difference between ionic binding and physical adsorption is that the linkages are much stronger for ionic binding although weaker than in covalent binding.

Cross-Linking

The immobilisation occurs by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix. Generally this method is used in conjunction with one of the other immobilisation method and the use of a carrier inevitably leads to 'diluition activity', especially at high enzyme loadings.[95] The most common reagent used for cross-linking is glutaraldehyde.

1.6.3 Layer-by-layer (LbL)

The layer-by-layer (LbL) technique, first introduced by Decher *et al.*,[96] it's a powerful, versatile and simple method for the controlled fabrication of multimaterial surface coatings on a large variety of surfaces. By the introduction, it has found applications in a multitude of areas: nonlinear optical materials formation,[97,98] patterning,[99] separations,[100-103] biosensing,[104,105] biocatalysis,[106] and others.

In LbL, film growth proceeds through electrostatic interaction by alternatively exposing a solid substrate to polycationic and polyanionic solutions.[107] Each exposure results in an overcompensation of charges and could be continued over many layers, allowing an unprecedented level of control over the composition of multilayers assemblies.[96] In *Figure* 1.9 the main steps in the LbL technique are schematically represented. In particular, in the LbL assembly, a sequential adsorption of positive or negative charged species, by alternatively dipping into the solutions, occurs. The excess or remaining solution after each adsorption step is washed with solvent and thus it is possible to obtain a thin layer of charged species on the surface ready for next adsorption step.

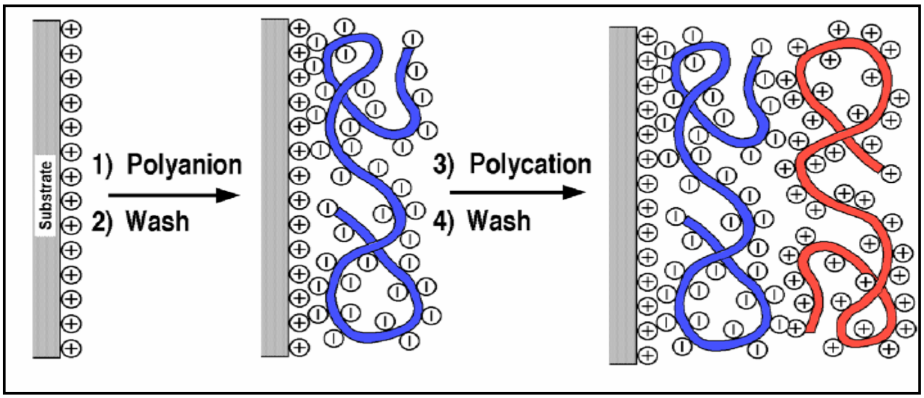

Figure 1.9- The LbL deposition process.

The major advantages of LbL technique are that many different materials can be incorporated in individual multilayer films and that the film architectures are completely determined by the deposition sequence.[96]

For biocatalysis, since enzymes are multiply charged molecules, the electrostatic adsorption seems to be one of the simplest ways to perform the physical immobilisation[106] but also to embedd the enzyme with a polyelectrolite film that can protect the catalyst from the denaturation. Several reports on the use of such LbL assembly of enzymes showed that proteins or enzymes embedded within polyelectrolyte multilayer films retain their biological activities,[107-109] indicating that the secondary conformation of the enzymes is conserved.

1.6.4 Advances in enzyme immobilisation

Current advancements in biotechnology have prompted the use of immobilised enzymes for a wide range of applications and several patents have been published pertaining to the immobilisation of biocatalysts using different procedures and materials. In the synthesis of chemicals, has been presented a method for the production of hydrogen, catalysed by the enzyme hydrogenase immobilised in mesoporous titanium dioxide film.[110] This is of a particular interest, because of the relative instability of hydrogenase and the large application and need for hydrogen production in the world economy. Another process utilises lipase enzymes immobilise onto a hydrophobic microporous surface for the production of biodiesel.[111] This method is an improvement over the previous method of biodiesel production, which was through the transesterification of vegetables oils and animal fats by a strong base, because the lipase enzyme can effectively catalyse a reaction with crude vegetable oil, not just refined, and the glycerol byproduct of the reaction is easily removable. In biosensors field, a method is based on the enzyme glucose oxidase, immobilised onto a thick and porous substrate in the detection of glucose present in blood.[112] In another case an immobilised enzyme in a breathalyserlike sensor, can be used to detect the acetone level that is considered a metabolic indicator for diabetics.[113]

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2: NATURAL POLYPHENOLS AS SUBSTRATES FOR BIOTECHNOLOGICAL APPLICATIONS

2.1 Occurrence and classification of phenolic compounds

Phenolic compounds have a wide distribution in nature; they can be found in fungi, such as Badiomycetes, in animals, for example in insects and crustaceans, but the largest part of phenolics is produced by plants. In fact, the phenolic compounds are secondary metabolites of plants and are generally involved in defence against ultravioler radiation or aggression by pathogens.[1]

Phenols and polyphenols constitute a large group of compounds; to date more than 8000 phenolic compounds have been identified in various plant[2] species that may be classified according to different criteria. Harborne and Simmons (1964) classified these compounds into groups based on the number of carbons in the molecule.[3] This classification is shown in *Table* 2.1.

Table 2.1- Classification of phenolic compounds, according to Harborne and Simmons.

Structure	Class
C_6	simple phenolics
C_6-C_1	phenolic acids and related compounds
C_6-C_2	acetophenones and phenylacetic acids
C_6-C_3	cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C_6-C_3	coumarins, isocoumarins and chromones
C_{15}	chalcones, aurones, dihydrochalcones
C_{15}	flavans
C_{15}	flavones
C_{15}	flavanones
C_{15}	flavanonols
C_{15}	anthocyanidins
C_{15}	anthocyanins
C_{30}	biflavonyls
C_6-C_1-C_6	benzophenones, xanthones
C_6-C_2-C_6	stilbenes
C_6,C_{10},C_{14}	quinones
C_{18}	betacyanins
$(C_6$-$C_3)_2$	lignans, neolignans (dimers or oligomers)
$(C_6$-$C_3)_n$	lignin (polymers)
$(C_6)_n$	catechol-melanin (polymers)
$(C_6$-C_3-$C_6)_n$	tannins (oligomers or polymers)

One of the most important biological aspects of these compounds is their low redox potential. Oxidation of phenolic compounds can results in the browning of tissues, such as in fruits, or leads to the formation of metabolites that are toxic to animals and plants or that inhibit pathogenic microorganisms.[4] The oxidation can occurs by different processes; two common type are the auto-oxidation and the enzymatic oxidation.

The term auto-oxidation is referred to the formation of cross-linked structures as a result of exposure to light and oxygen. Under the influence of light, oxygen can abstract a proton, thereby generating a radical. This is particularly likely to occur if the proton is adjacent to a double bond, because the radical electron can be delocalised, thus lowering the energy. Given their aromatic nature, phenolic compounds are easily auto-oxidised. The radical that is generated can subsequently react with other radicals to form a dimer. Since the radical electron is delocalised, several structures can be formed depending on the precise location of the radical electrons at the time of the reaction.

An alternative mechanism for the oxidation of phenolic compounds is enzyme-catalysed oxidation. Several classes of enzymes can catalyse this reaction, in particular the ones that belong to the class of oxidoreductases (EC 1) and, among these, the oxidoreductase that use oxygen as electron acceptor (EC 1.10.3, *for details* Chapter 1.3), the peroxidases (EC 1.11.1, *for details* Chapter 1.4), and monophenol monooxygenase (EC 1.14.18.1).[4]

2.2 Phenolic polymers

2.2.1 Lignin: general features and ecological aspects

After cellulose, lignin costitutes the most abundant organic substance on the planet and is one of the three structural polymers present in all the vascular plants. Lignin is derived from the Latin term lignum, which means wood.[5] Anselme Payen (1838) was the first to recognise the composite nature of wood and referred to a carbon-rich substance as the 'encrusting material' which embedded cellulose in the wood. Schulze (1985) later defined this encrusting material as lignin. This macromolecule plays a vital role in providing mechanical support to plants; without lignin, plants would never have moved from the aquatic to the terrestrial environment during Carboniferous. Lignin is a characteristic chemical and morphological component of the vascular tissues of the higher plants (gymnosperms and angiosperms), specialized for liquid transport and mechanical strength, the xylem. Lignin seals the water conducting system against the hydraulic pressure drop produced by the transport of water from the soil to the leaves and needles. Indeed it is lignin that reinforces the plants to withstand the forces of gravity and wind. Moreover, this cell-wall reinforcing process provides in assisting the means for re-orientating stems and branches in response to changes in mechanical stressed and light levels.[6] Finally, lignin plays an important function in plant natural defence against degradation by impeding penetration of destructive enzymes through the cell wall.[7]

The amount of lignin present in different plants is quite variable (in the range 20-40%), among the angiosperms, the monocotyledons are less lignified.[8] Additionally the distribution of lignin within the cell wall and the lignin contents of different organs in plants are not uniform. For example, high lignin contents are characteristic of the lowest, highest and inner parts of the stem; the lignin contents of needles and leaves are described inconsistently as high or low, possibly depending on their state of development.[9]

Lignin plays an important role as it is being recycled in the natural ecology: it serves the soil as a complexing agent for minerals and as a moisture-retention aid, since it is fundamental in the constitution of humus. Plant litter materials provide the primary sources for organic matter formation in soil. The amount of plant litter, its composition and its properties are essential controlling factors for the formation of soil organic matter and the humification processes in terrestrial ecosystems. Essentially, two types of plant tissues reach the soil for decomposition: parenchymatic tissue and woody tissue. Parenchymatic cells are found in the living green tissue of leaves and cortex of young twigs and fine roots and are composed of cellulose walls, the protoplast, rich of protein and vacuoles. Woody tissues from the woody part (xylem) and supporting tissue (sclerenchym) of stems, leaf epidermis, leaf ribs and barks contain lignin. The plants components that reach the soil to be degraded are: proteins, chlorophyll and pigments, cellulose, non-cellulosic polysaccharides, lignin, tannins, other polyphenols, lipids, cutin and suberin.

The humification process consists in general in the degradation of the organic matter into small molecules and the successive polymerization and resynthesis of longer molecules. The decomposition of organic matter in natural environment integrates collective activities of microorganisms within the soil and sediment. This process is considered as a prolonged stabilization of organic substances against biodegradation and give important structural and chemical properties to the soil. Moreover the humification is one of the most important processes that permit the full recycling of carbon in the environment, ensuring closure of the carbon cycle.[10]

Despite the many important roles of lignin, many uncertainties remain in our understanding of the lignin structure and the process of formation.

2.2.2 Lignin biodegradation

Lignin resists attack by most microorganisms; in nature only a few organisms, belonging to the group of basidiomyceteous white-rot fungi, are able to degrade lignin efficiently, by an enzymatic process that originated in the Upper Devonian period, in parallel with the evolution of vascular plants.[11] Some white-rot basidiomycetes preferentially attack lignin more readily than hemicellulose and cellulose in wood tissue. This process of selective delignification leaves cellulose-enriched white material (*Figure* 2.1). These is considerable interest in the industrial application of these fungi, because many uses of wood involve preferentially removing lignin, such as in biopulping.[12] Examples of this group of fungi include *Phlebia* spp. and *Pleorotus* spp. Many white-rot fungi, however, exhibit a pattern of simultaneous decay characterised by degradation of all cell wall components with formation of radial cavities. Examples of this group of fungi include *Trametes versicolor*, *Heterobasidium annosum* and *Irpex lacteus*.[13] In contrast to white-rot fungi, which are more commonly found on angiosperms wood, brown-rot fungi grow primarily on conifers and represent 7% of wood-rotting basidiomycetes. This group of basidiomycetes can degrade wood polysaccharides after only a partial modification of lignin, resulting in a brown material consisting of oxidised lignin, which represents a potential source of aromatic compounds for the stable organic matter fraction in forest soils.[14] Examples of this group of fungi include *Gloeophyllum trabeum*, *Laetiporus portentosus*, *Piptoporus betulinus*, *Postia placenta* and *Serpula lacrimans*.[12]

White-rot fungi secrete a pool of oxidative enzymes that are involved in degradation of cell-wall components. The four major groups of enzymes for the degradation of lignin are lignin peroxidase (EC 1.11.1.14), manganese dependent peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16) and laccase (EC 1.10.3.2).

Figure 2.1- White rot process in wood.

2.2.3 Biosynthetic pathway

Lignin is considered an amorphous, phenolic material arising from an enzyme-mediated dehydrogenative polymerization of the three phenylpropanoid monomers: coniferyl, sinapyl and *p*-hydroxycinnamyl alcohols.[7] According to the relative content of these three monomeric precursors, lignin polymers are classified as: guaiacyl lignins (or G-lignins), that are predominantly polymerizates of coniferyl alcohol and distinctive of gymnosperms (softwood); guaiacyl-syringyl lignins (GS-lignins) typical of hardwood, which are composed by varying parts of the aromatic moiety guaiacyl (the aromatic nucleus deriving from coniferyl alcohol) and syringyl (deriving from synapyl alcohol); guaiacyl-syringyl in addition to small amount of *p*-hydroxyphenyl units (deriving from *p*-hydroxycinnammyl alcohol) which are distinctive of grass (GSH-lignins).[15]

The biosynthesis of lignin starts with glucose deriving from photosynthesis. Glucose is converted to shikimic acid, the most important intermediate substance of the so-called *shikimic acid pathway*. Then different steps leads to the formation of the lignin precursors (*Figure* 2.2).[16]

The two aromatic amino acids L-phenylalanine and L-tyrosine are formed as the final compounds of the shikimic acid pathway by reductive amination of phenylpyruvic acid, through the intermediate prephenic acid. On the other hand they are the starting substances ("amino acid pool") for the enzymatic phenylpropanoid metabolism (*cinnamic acid pathway*) leading not only to the three cinnamyl alcohols via activated cinnamic acid derivatives, but also to extractive components like flavonoids or stilbenes. Indeed the aminoacids are deaminated by deaminases (for instance PAL, phenylalanine ammonia lyase) to their corresponding cinnamic acid. The dominant further steps are hydroxylation of the aromatic nuclei followed by partial methylation leading to *p*-coumaric acid, caffeic acid, ferulic acid, 5-hydroxy-ferulic acid, and sinapic acid. The cinnamyl alcohols are finally formed by enzymatic activation and reduction of the corresponding acids *via* coenzyme-A thioester and aldehyde intermediates.

The biosynthesis process, which consists essentially of radical coupling reactions, sometimes followed by the addition of water or of primary, secondary and phenolic hydroxyl groups to quinonemethide intermediates, leads to the formation of a three dimensional polymer that is not formed by a genetically prescribed, regular mechanism, but by a random coupling of lignols to a non linear polymer. For this reason, lignin is viewed not as a constitutionally defined compound, but as a composite of physically and chemically heterogeneous materials whose structure may be represented by models.

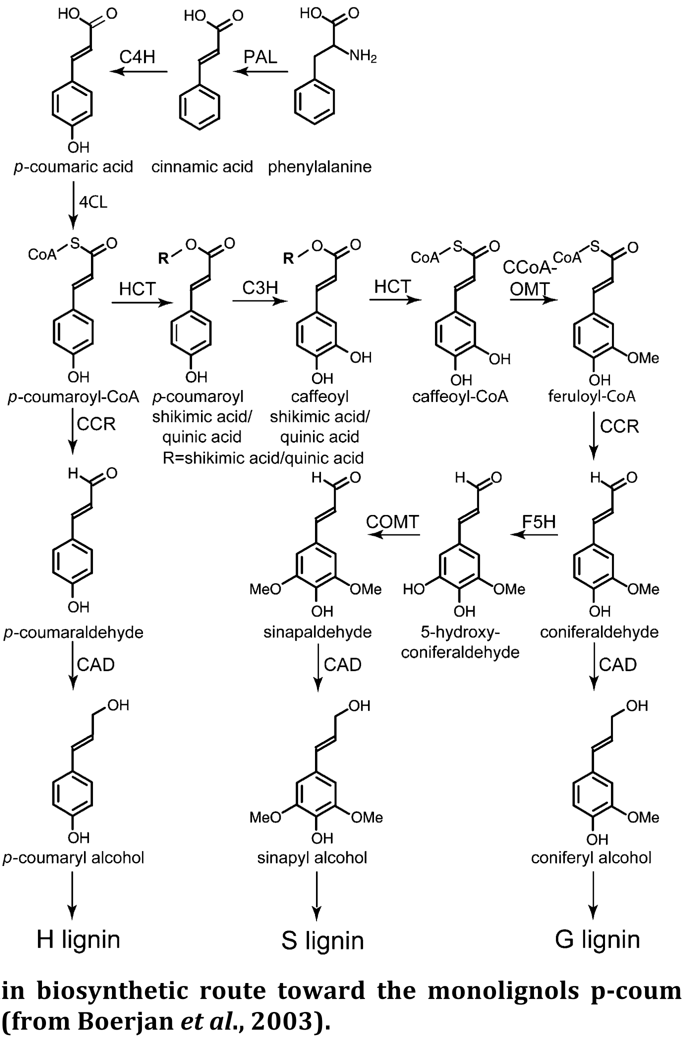

Figure 2.2- The main biosynthetic route toward the monolignols p-coumaryl, coniferyl, and sinapyl alcohol (from Boerjan *et al.*, 2003).

From a morphological standpoint the growing lignin molecules are forced to fill up the space between the preformed polysaccharidic fibrillar elements in the cell wall. Terashima *et al.*[17] postulated a comprehensive structural model of cell wall lignification where the individual cell controls the deposition of polysaccharides and lignin. The primary cell wall (*Figure* 2.3) is the first layer deposited and consists of cellulose microfibrils and hemicelluloses. Subsequently, secondary thickening inside the primary wall forms the secondary cell wall. X-ray crystallography studies revealed that the orientation of the cellulose microfibrils appears to be random in the primary cell wall but fixed in the outer (S_1), middle (S_2), and inner (S_3) layers of the secondary cell wall.[18] The intercellular layer, which separates the walls of adjoining cells, is called the middle lamella (ML).

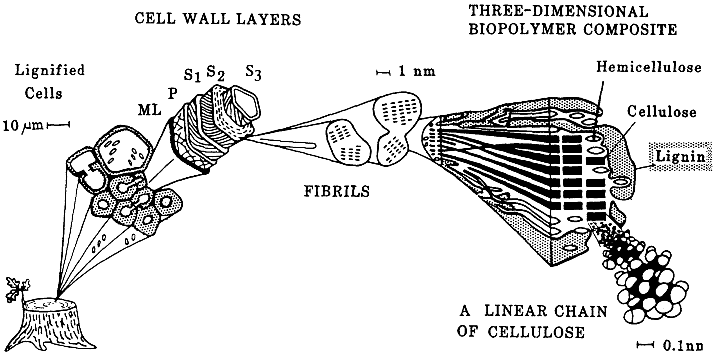

Figure 2.3- Schematic representation of a lignified cell wall.

The first stage of lignification starts at the cell corners and middle lamella after the deposition of carbohydrates in the primary cell wall and just before the formation of S_1 begins.[18] The thickness of the middle lamella is more than 100 nm and the lignin formed in this region is a three-dimensional gel which is non-crystalline and optically inactive.[17] During the second stage, cellulose microfibrils are deposited in the S_2 layer and subsequently lignification proceeds while the S_3 layer is formed. Because of geometric constrains imposed by the cellulose microfibrils, lignin in the secondary wall is probably a non-random two-dimensional network polymer of about 2 nm thick.[17] The deposition of precursor monomers changes with type, age and morphological region of the cell, generally in the order of *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol.

It is generally accepted that lignin is not simply deposited between the cell wall polysaccharides, but it is linked and associated with at least part of them. According to Freudenberg[15] the presence of carbohydrates is even a prerequisite for the formation of lignin macromolecules in plant cell walls.. The phenomenon of the intimate association between the polysaccharide and lignin part to the cell wall is described by the terms Lignin-Polysaccharide Complex (LCC). In a more practical sense the term describes the fact that the components cannot be totally separated by selective chemical treatments or special separation and purification techniques. Even in highly purified cellulose there will remain some residual lignin.

The knowledge about the covalent linkages existing between lignin and polysaccharides is mainly derived from degradation experiments, mostly performed by means of mild alkaline, acid or enzymatic hydrolysis. The most frequently suggested bond types are ether linkages, ester linkages as well as glycosidic bonds, as reported in *Figure* 2.4.[19,20]

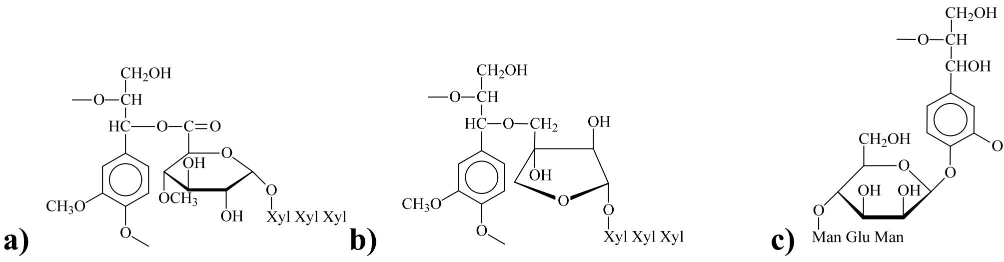

Figure 2.4- Lignin glycosidic bonds: a) benzyl ester linkage; b) benzyl ether linkage; c) phenyl glycosidic linkage.

2.2.4 Lignin structure

Lignin is one of the most difficult polymers to describe, due to its structure and heterogeneity; after many years of study, the structure of native lignin still remains unclear. Numerous analytical studies of model compounds, synthetic lignins and isolate lignins were necessary to yield what is believed to be an accurate representation of lignin structure.

Lignin models are not uniform with regard to molecular weight; in addition they ignore the role of carbohydrates in the lignification process. Isolated lignins suffer from degradation and changing effects, impurities and difficulty of isolating reproducible and identical lignin samples from wood. Finally it is still questionable whether carefully isolated lignins are representative of the total lignin in wood.[9] Anyway, the investigations of model compounds, synthetic lignins and isolated lignins are fundamental in the structural studies of lignin and have elucidated structural features such as the dominant linkages between the phenylpropane units and their abundance, as well as the abundance and frequency of functional groups. These studies revealed that the lignin macromolecule cannot be described as a simple combination of few monomeric units by few types of linkages.

The first lignin model was designed by Freudenberg in 1968:[15] it is based on the dehydrogenative polymerization concept and fulfills all analytical data available at that time; later on, Adler gave a structural scheme for spruce lignin comprising 16 prominent C_9-units, mainly derived from the results of oxidative degradation experiments.[21] Nevertheless the largest softwood lignin structural model was evaluated by simulating the lignin formation by computer. The original model reported by Glasser[22] was mainly based on the simulation of radical coupling reactions of the *p*-hydroxycinnamyl alcohols, and was extended and improved in details.[23] One of this structure comprises 94 units, corresponding to a total molecular weight of more than 17000, and is based on a wide array of analytical information obtained from investigation of lignin from loblolly pine (*Pinus taeda*).

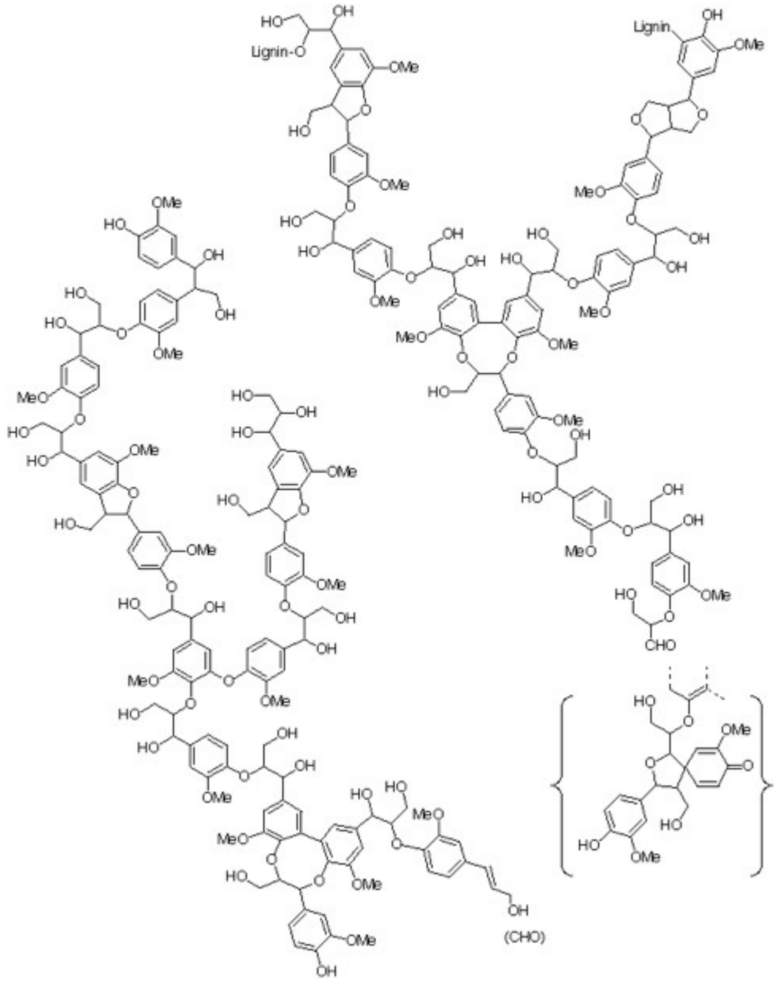

Figure 2.5- Lignin structure as proposed by Brunow *et al.*, 2008.

Three-dimensional computer modeling of middle lamella, secondary wall and DHP lignin models was first introduced in 1992 by Gravitis,[24] who used also fractal geometry to develop a new approach[21] in the study of lignin geometry which has been subsequently used by Jurasek *et al.*[25]

Later on, Brunow[26] discovered the presence of a new type of inter-monomer linkage in softwood lignin representing an 8-membered ring (dibenzodioxocin) and in 1998 proposed a structural scheme for softwood lignin (*Figure* 2.6).[27]

Linkages between the phenylpropane units and the various functional groups on these units give lignin a unique and very complex structure; *Figure* 2.7 shows some of the common linkages found in softwood lignin. Furthermore in *Table* 2.2 and *Table* 2.3 are reported the percent abundance of the linkages and the frequency of some common functional groups found in softwood lignin, respectively.[28]

Table 2.2- Linkages found in softwood lignin.

Lignin interunit bonding	**Number per 100 C_9 units**
β-O-4	44,7 ± 0,9
β-5	10,6 ± 2,0
β-β	3,19 ± 0,01
β-1	1,25 ± 0,34
5-5’-O-4	3,07 ± 0,05
SD	0,24 ± 0,05

Table 2.3- Functional groups in softwood lignin per 100 phenyl propane units.

Functional Group	**Abundance per 100 C9 units**
Phenolic hydroxyl (free)	15-30
Methoxyl	92-96

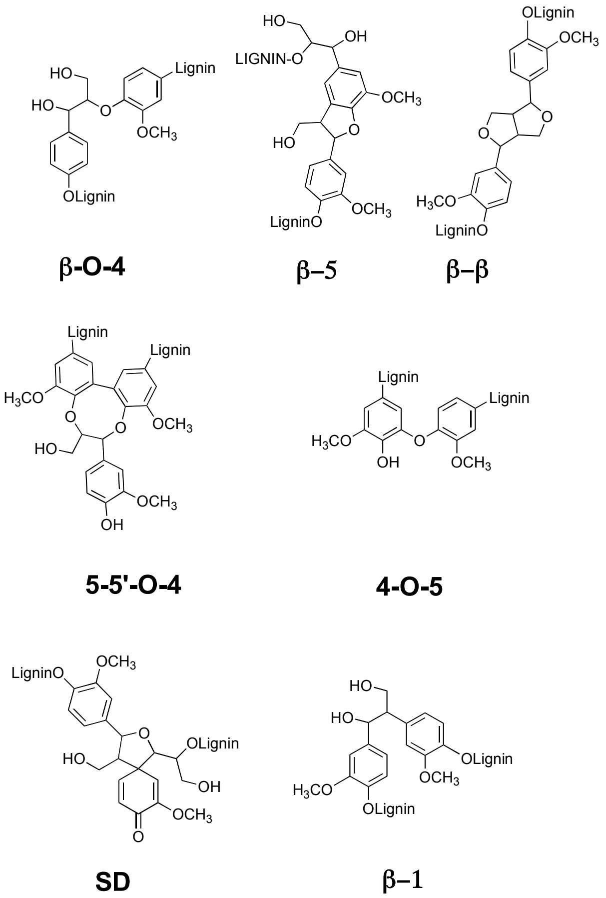

Figure 2.7- Common phenylpropane linkages in lignin

2.2.5 Isolation and characterisation methods

The structural complexity of lignin has continually challenged the researchers to develop suitable methods for its isolation and characterisation.

During the years, the use of isolated lignins gave a great contribution in the study of its biochemistry, especially in the understanding the basic mechanism of microbial degradation. There are different extractive lignins, that can be obtained by different isolation processes. The main preparations are:[28]

Acidolysis lignin: Lignin is extracted from plant tissues by using a mild acid hydrolysis (0.2 M HCl in aqueous dioxane) at RT. This lignin is reported to have only few carbohydrate impurities.

Cellulase enzyme lignin (CEL): It is prepared from milled wood after the removal of polysaccharides using a commercially available cellulase-hemicellulase mixture. The enzyme mixture usually cannot remove all of the carbohydrates, so the lignin preparation may have 12-14% carbohydrate contamination.

Milled wood lignin (MWL): This lignin is obtained by Björkman's procedure. Briefly, it is purified from the aqueous *p*-dioxane extract of finely milled wood, which has been first extracted with organic solvent to remove extraneous components. Only minor changes are supposed to occur in the structure of lignin during this procedure, therefore is the most used preparation, it is considered to be representative of the original lignin. [29] Notwithstanding this, milled wood lignin is not considered to be representative of whole lignin in wood, it originates primarily from the secondary wall of the plant cell tissue.

Braun's native lignin: The lignin is prepared by the extraction of lignocellulosic material using 95% solution of ethanol in water. The obtained yield is very low because the bulk of the lignin is insoluble in ethanol and only a small portion is soluble in ethanol-water. The solubilised lignin contains only very low molecular fractions and is generally considered not representative of the bulk of lignin in the extracted tissues. Except for its molecular weight, the structure of Braun's native lignin is similar to that of MWL and therefore may be used as the preliminar lignin substrates, and successful investigations can then move to use other more representative isolated lignins.[29]

Klason lignin: Klason lignin refers to the material left after cold, 72% sulfuric acid treatment of lignocellulosic material. The extreme conditions under which this lignin is prepared causes the material to be highly condensed and resinous. This treatment is used as a quantitative method for measuring the acid-insoluble lignin content of lignocellulose material.

Kraft lignin and lignosulfonates: These lignins are readily available because are the residue of chemical pulping processes in paper production. Kraft lignin, obtained by the kraft process, is highly modified, it conatins non-lignin derived materials and is lower in molecular weight, has a higher phenolic content and a lower methoxyl content and undergone extensive side-chain reactions with respect to the other preparations. Lignosulfonate is the sulfonated lignin removed form wood by sulfite pulping and has a higher molecular weight than kraft lignin. Hydrolysis reactions have occured and sulfonation can be extensive. These lignin preparations are not suitable for studies modeling the behaviour of native lignin, but they are important as industrial byproducts, and the research is warranted on their bioconversion.[29]

In this PhD study, MWLs have been especially used, for its similarity with the native lignin, and RKL, for the possibility to convert an industrial byproducts highly available and actually under-utilised. Moreover, to estimate the lignin content of lignocellulose material, the Klason lignin has been used.

The investigations on the structure and reactivity of lignin exposed the inadequacy of many of the methods currently in use and underscored the ever-continuing need to develop new methods capable of solving the analytical problems associated with lignin. Such methods should be selective, sensitive, accurate, suitable for quantitative measurements and capable of being applied directly to the lignin samples.

In the lignin study it is possible to distinguish between two different approaches:

- a classic chemical one where lignin is derivatised, modified and chemically degraded;
- an instrumental approach where lignin is analysed in solid state or in solution.

A very useful strategy has been found in the combination of these two approaches: to date, the instrumental analysis of the chemically degraded lignin.

In the earliest studies on lignin, when the classic chemical approach was the favorite one, the most important analysis, carried out on woody materials, was the lignin detection, that was carried out by using different methods based on color reactions with different reagents such as aliphatic compounds, phenols and aromatic amines.[30]

The studies on lignin detection were followed by its quantitative determination. Lignin determination is performed routinely for characterising lignocellulosic material, obtained by different procedures that can be divided into two main subgroups: the direct and the indirect methods. The most important direct method is the Klason method; the indirect methods, comprises the procedures based on: the oxidant consumption; the determination of the kappa number; the nitrosation method and some spectrophotometric methods such as UV-microspectrophotometer and UV-microscopy.[31]

An important example of classical chemical approach is the functional groups analysis, that is realised through chemical derivatization reactions such as acetylation, aminolysis, periodate oxidation.

With the introduction of chemical degradation methods such as acidolysis, nitrobenzene and cupric oxide oxidation, permanganate oxidation, thioacidolysis, hydrogenolysis, nucleus exchange reaction and ozonation, the instrumental analysis became more and more important. At the beginning the Gas Chromathographic (GC) technique had a fundamental role in the detection of the lignin chemical degradation products, then the advanced techniques such as Gas Chromatography coupled with

Mass Spectrometry (GC/MS) became essential in parallel to the increasing in specificity and sensitivity requirements. The techniques utilised in the molecular weight and size distribution determination were the Gel Permeation Chromatograph (GPC), Light Scattering, Vapor Pressure Osmometry (VPO) and Ultrafiltration.

In this PhD study advanced applications of the Nuclear Magnetic Resonance, Mass Spectrometry and Gel Permeation Chromatography techniques in the studies lignins have been considered.

2.2.5.1 NMR spectroscopy

The Nuclear Magnetic Resonance (NMR) is a powerful spectroscopic technique based on the capacities of some atomic nuclei of absorbing a radio frequency if subject to a magnetic field. The radio frequency absorbed, related to a wavelength between 1 and 10^3 m, corresponds to the low energy zone of the electromagnetic field and cause a little energetic transition.

A lot of atomic nuclei are suitable for NMR studies. Actually some atomic nuclei have a nuclear spin (I), and the presence of a spin makes these nuclei behave such as bar magnets. In presence of an applied magnetic field they can orient themselves in 2I+1 ways. Those nuclei, with odd mass number, have nuclear spin of 1/2, or 3/2, or 5/2 and so on. In the application of NMR spectroscopy the most important nuclei are those with an I value of 1/2, which corresponds to two possible orientations, a low energy orientation aligned with the applied field and a high energy orientation opposed to the applied field. The difference in energy is given by the *Equation* 2.1**:**

$$\Delta E = h\gamma B_0/2\pi$$

Equation 2.1- Difference in energy, where g is the magnetogyric ratio (a proportionality constant, differing for each nucleous, which essentially measures the strength of the nuclear magnets) and B_0 is the strength of the applied magnetic field.

The number of nuclei in the low energy state and the number of nuclei in the high energy state will be determined by the Boltzmann distribution.

When a radio frequency signal is applied to the sample subjected to a magnetic field some nuclei are promoted from the low energy state to the high energy state: this is known as resonance frequency. During relaxation, when the nuclei from the high energy state come back to the low energy state, they emit a radio frequency.

The atomic nuclei interested in our study are shown in *Table* 2.4.

Table 2.4- NMR characteristic of ^{13}C, ^{1}H and ^{31}P atoms.

Isotope	**Spin**	**Natural abundance**	**Magnetogyric ratio**
^{1}H	½	99.985	26.752
^{13}C	½	1.108	6.728
^{31}P	½	100.000	10.841

^{1}H and ^{13}C NMR are usually used in the organic chemistry applications because they respectively give information about the hydrogens present in the molecule, in particular about the functional groups, and give information about the carbon structure too. The spectrum obtained is called *1-Dimensional spectrum* where the spectrum has 2 axes, plotting frequency against the intensity of the signals. Some of these techniques, often employing complex pulse sequences to obtain specific information, are still very useful in determining structures from spectra.

The common ^{13}C NMR spectra are usually complicated by the for the ^{13}C-^{1}H spin coupling phenomena, and are generally recorded through decoupling to collapse the C signal to a single peak. This phenomena can be useful, since they give additional structural information such as spatial relationships inside a molecule. The spectrum obtained is called *2-Dimensional spectrum*.

When we talk about a 2-Dimensional spectrum, we mean a spectrum in which both axes show chemical shifts, with the intensity of the peaks constituting a third dimension. This gives a contour plot. From a practical point of view, the most useful 2-D spectra are those that show either ^{1}H-^{1}H correlations or ^{1}H-^{13}C correlations.

In our studies great importance was also given to the ^{31}P NMR, a powerful technique selected to label functional group in lignin because of its sensitivity, availability of suitable derivatizing reagents, and ease of obtaining quantitative derivatization under mild conditions. Phosphorous-31, in fact, is a nucleus that largely meets these criteria, because it is 100% naturally abundant. This technique, as it is reported in detail later on, allows the study of particular functional groups in lignin with increasing resolution capacities.[32,33] In fact, it permits the discrimination between the different phenolic hydroxyl groups contents (syringyl, guaiacyl and *p*-hydroxyphenyl), the determination of hydroxyl aliphatic groups, the distinction between the condensed forms 4-O-5’, 5-5’ and diphenylmethoxy and, finally, the carboxylic groups content. The analysis requires the derivatization of hydroxyl groups with the phosphitylation reagent, 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane. The phosphitylation reaction is reported in *Figure* 2.7.

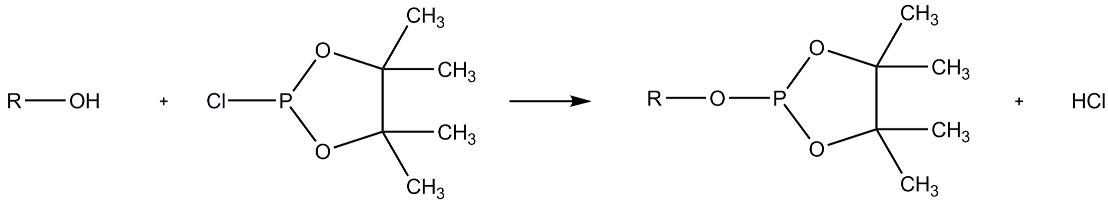

Figura 2.7- Phosphitylation reaction of hydroxyl groups.

This reagent allows the overall distribution of hydroxyl groups present in a lignin sample to be determined. It has been found to be particularly useful in the discrimination between the condensed structures, in which the ligninic bond involves the phenolic hydroxyl, and the uncondensed phenolic units, and in resolving the primary and secondary hydroxyls.[30,31]

The presence of four methyl groups in the 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane reagent affects the spin-lattice relaxation profiles of the phosphorus atoms attached on lignin and increase the stability of the phosphitylated lignin: thus the analysis is reproducible also some hours after the derivatization reaction. Usually, the analysis is realized in the presence of the relaxation reagent chromium(III) acetylacetonate. With the use of an internal standard it is possible to make a quantitative analysis to quantify the spin-lattice relaxation of the various hydroxylic functional groups present in lignin.[32-34] In *Table* 2.5 and in *Figure* 2.8 the ^{31}P chemical shift of phosphorus nuclei labeling hydroxyl groups in different environments in lignin and a typical lignin ^{31} P NMR spetrum are reported:

Table 2.5- ^{31}P NMR attribution (from Argyropoulos *et al.*)

OH groups	Chemical shift
Aliphatic	149.0-146.0
Diphenilmethane	144.27-142.78
4-O-5'	142.78-141.24
5-5'	141.72-140.24
Guaiacylic	140.24-138.8
p-OH phenylic	138.8-137.4
COOH	135.5-134.0

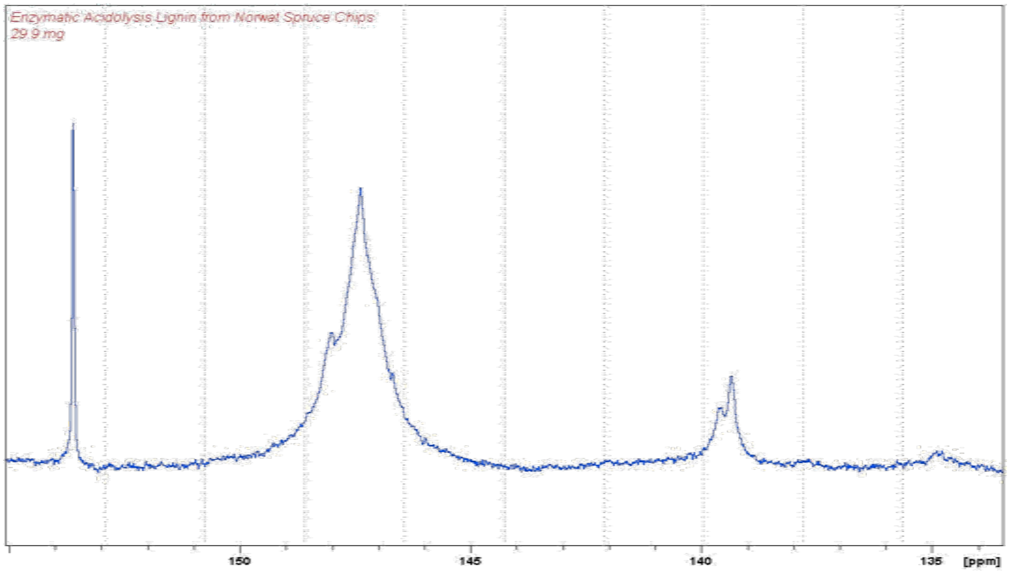

Figure 2.8- ^{31} P NMR spectrum obtained by lignin isolated from Norway spruce.

The ^{31}P NMR has been used to study the structures of both MWL and RKL, before and after the enzymatic treatment, and, in an innovative way, also for melanin characterisation. Moreover it has been applied successfully also in the studies of radical species formed by enzymatic reactions.

2.2.5.2 GPC

Gel permeation chromatography (GPC), sometimes also referred to as a gel filtration chromatography (GFC) or size exclusion chromatography (SEC), entails the cheromatographic fraction of macromolecules according to molecular size.[35] In this technique gels, having varying pores sizes are used as the stationary phase. When swollen in water, such gels form a three-dimensional network that acts as a molecular sieve. When an aqueous solution of macromolecules is allow to move through a column containing the gel, a chromatographic separation takes place; molecules of low molecular weight are able to penetrate into the gel particle pores but large molecules are excluded from the pores and pass directly through the column. Consequently, the largest molecules elute first and the smallest last.[36]

For the GPC analysis, the sample should be completely soluble in THF, so in the case of lignin, the complete solubilisation can be reached by acetylation or methylation. The acetylation is based on the overnight reaction of lignin with pyridine-acetic anhydride at RT and allows complete recovery of the sample with a minimum of added impurities. For the methylation, lignin is first dissolved in a mixture of dioxane-methanol and then the diazomethane is added to the solution. Another procedure that can be used in order to make the lignin samples soluble in THF is the acetobromination. By dissolving a lignin sample in acetyl bromide diluted with glacial acetic acid, the primary alcoholic and the phenolic hydroxyl groups are acetylated, while the benzylic α-hydroxyls are displaced by bromide.[37] This method displays some advantages. First, it allows a more seletive lignin acetylation, that concern only the phenolic OHs, moreover the overall procedure can be carried out without at RT. Finally the use of pyridine is avoided. In *Figure* 2.9 is shown a typical GPC of lignin obtained after acetobromination.

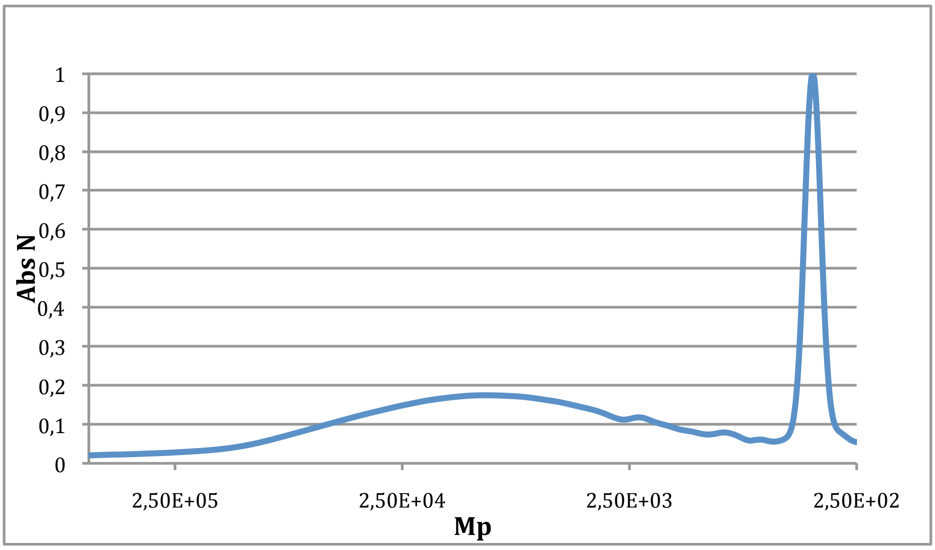

Figure 2.9- Gel permeation chromatography of black spruce lignin previously acetobrominated.

The major problem that can be encountered with this technique in lignin analysis, is that different solvent systems may swell the gel to different degrees with the result that pore size distribution is altered. Analysis of polyelectrolytes such as lignins, which contain acidic group of various type constitutes a special problem.[36] Thus, chromatographic separation may be affected by expansion of the molecules stemming from electrostatic repulsion of the charged groups and solvation. Such effects lead to a separation behavior that is strongly dependent on the ionic strenght of the eluant. Moreover there is a problem concerning the standards. To date, the commercially available standards for the calibration curves construction are synthetic polymers, usually polystyrene, that may have a different behaviour respect to lignin polymer. This can lead to a difference in the separation processes.

In this thesis the GPC analysis has been used to study the change in lignin structure before and after the enzymatic treatments (*Chapter* 4).

2.2.6 Lignin as bioresource

The perspective of fossil fuel depletion, the concerns of energy security and global warming are the main drivers of alternative and renawable material promotion by public authorities in industrialized countries. Cellulosic biomass provides a low cost and abundant resource, that has the potential to support large-scale production of fuels and chemicals. It is the most abundant organic source on Earth, with an annual production in the biosphere of about 170 billion metric tons.[38] Moreover lignocellulosic biomass includes materials considerated wastes, such as agricultural residues, forestry residues, portion of municipal solid waste and various industrial wastes.

A manufacturing concept for converting plant biomass to energy and chemicals is the Biorefinery, that is based on the use of carbon molecules extracted from plant in order to substitute carbons from oil and gas.[39] In this view, plant biomass is separated into a number of compounds and energy is recovered and the used strategy leads to reduce as many as possible waste and by-products. Utilisation of these heterogeneous bonded-together mixtures is the key to the future sustainable supply of chemicals and energy. In fact, utilising renewable carbon eliminates an additional fossil-derived carbon dioxide burden to the environment and reduces the Green-House Gas driven global climate change.[40] *Figure* 2.10 illustrates how is it possible to convert lignocellulosic material into liquid fueld, electricity and other products, now obtained from nonrenewable carbon.[41]

Still current technologies for the chemical utilisation of wood use a ‘destructive strategy’ to obtain a single component, usually cellulose. Other fraction, such as lignin, that constitutes about one third of wood, can be considered an under-utilized source of chemical energy. In fact, the use of lignin is nowadays still limited to thermovalorisation processes as filler in composites, component in binders and coatings, or, at a lower extent, surfactant/dispersant additives, whereas its potential as a source of valuable phenols in the production of high value-added biopolymers in alternative to petrol chemistry is largely unexploited. Thus, novel processing methods and product concepts are required to extend the role of lignin for future biomass and biofuel applications. The chemical heterogeneity of lignin is one of the main reasons for the lack of valorisation of lignin residues that emerge from pulp and paper, and also from modern saccharification processes that lead to the production of biofuels such as bioethanol. The possible strategies of lignin valorisation are focused into two main directions, namely the selective functionalisation of the lignin polymer in order to improve its compatibility and performance in composite and copolymer materials, or in alternative, in its oxidative polymerization to get polyfunctional monomeric compounds to be used as feed-stocks for polymer industry as an alternative to fossil fuels derived building blocks.[42]

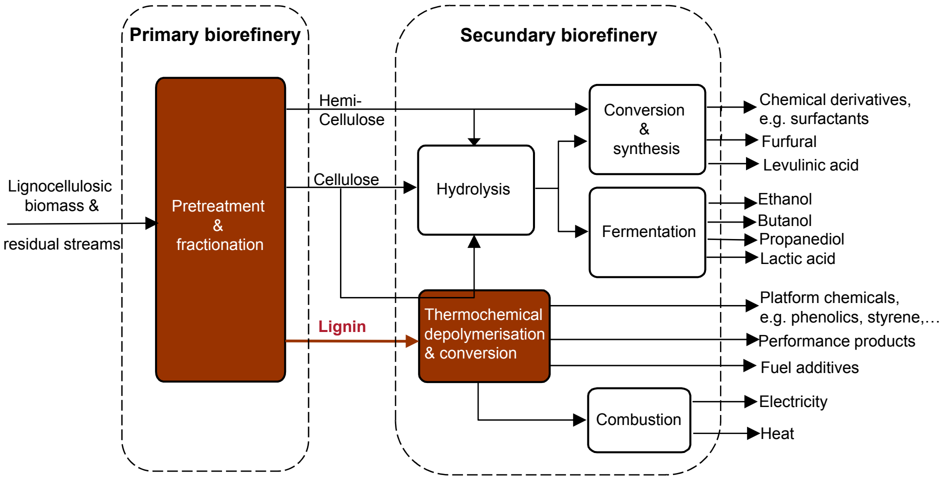

Figure 2.10- Schematic view of biorefinery processes.

The biorefining philososphy provides an alternative approach for utilisation of lignocellulosic biomass which allows higher value recovery. Lignocellulosic biomass can be fractionated into the main components by sequential treatments to give separate streams that may be used for different product applications, allowing a maximum of the benefits of a renewable and complex resource that will become increasingly important as fossil sources become more constrained or expensive.[39]

Bioethanol is projected to be one of the dominating renewable biofuels in the transportation sector within the coming 20 years, and has already been introduced on a large scale in Brasil, USA and some European country. However, almost all bioethanol today is produced from sugar- or starch-based agricultural crops, using so-called first-generation technologies. Although this ethanol is produced at a competitive cost, the raw material supply will not be sufficient to meet the increasing demand for fuel ethanol, and also the reduction of greenhouse gases resulting from the use of sugar- or starch-based ethanol is not as high as desirable. One of the most promising options to meet this challenge is the production of bioethanol from lignocellulose feedstocks, such as agricultural residues (e.g. wheat straw, sugar cane bagasse, corn stover) and forest residues (e.g. sawdust, thinning rests), as well as from dedicated crops (salix, switch grass) using second-generation technologies. These raw materials are sufficiently abundant and generate very low net greenhouse gas emissions, reducing environmental impacts.[41]

Ethanol production from lignocellulosic biomass comprises the following main steps: hydrolysis of cellulose and hemicellulose, sugar fermentation, separation of lignin residue and, finally, recovery and purifying the ethanol (*Figure* 2.10). The task of hydrolysing lignocellulose to fermentable monosaccharides is still technically problematic because the digestibility of cellulose is hindered by many physico-chemical, structural and compositional factors. Owing to these structural characteristics, pretreatment is an essential step for obtaining potentially fermentable sugars in the hydrolysis step. The aim of the pretreatment is to break down the lignin structure and disrupt the crystalline structure of cellulose for enhancing enzymes accessibility to the cellulose during hydrolysis step.[43,44]

In *Chapter* 7 is presented a possible strategy to improve the enzymatic hydrolysis, that is considered the most critical step in bioethanol generation from lignocellulosic biomasses.

2.2.7 Melanin: general properties and biosynthetic pathway

Melanin is an important class of pigments that have attracted attention from a wide range of scientists.[45-47] In fact, this term describes a large family of natural and synthetic phenolic-quinonoid pigments of diverse origin, such as bacteria, fungi, plants and animals, and chemical nature. Natural melanins are usually differentiated by their origin, e.g. bovine eye melanin, melanoma melanin and sepia melanin. Synthetic melanins are named after the compound from which they were prepared via chemical or enzymatic oxidation.[48] In addition, melanins in human are classified in two distinct types: eumelanins, responsible for the pigments from black to brown and pheomelanins, responsible for the pigments from yellow to red.[49,50] From a chemical point of view, eumelanins are insoluble where pheomelanins are alkali-soluble polymers. In higher animals, eumelanins in the hair, skin and epithelial cells of the retina are derived from either L-tyrosine or L-DOPA metabolism in specialised organelles of melanocytes, the melanosomes.[51,52] Neuromelanins, present in the cells of substantia nigra and locus ceruleus of the human brain, are produced in catecholamine-producing neurons from L-DOPA, dopamine, norepinephrine, epinephrine and 5-hydroxytryptamine.[53-55]

The proposed biological functions of melanin are quite diverse, including photoprotetion, photosensitization, metal ion chelation, antibiotic, thermoregulation and free radical quencing.[45,56,57] Not all of its effects are positive in regard to human health, there are data that demonstrate that melanin induces DNA damage through its own photoreactions following ultraviolet excitation.[58] Moreover melanin is believed to be responsible for the remarkable resistane of human melanoma cells to therapeutic radiation and chemotheraphy.[59,60]

Despite their biological importance, natural melanins have defied chemical and structural analysis; these materials are amorphous, heterogenous, insoluble and resistant to crystallization. For instance, the insolubility of brown or black nitrogen-containing eumelanins precludes their study with solution-state hydrodynamic, spectroscopic, and light scattering techniques.[61]

Most of the present knowledge on the chemistry of eumelanins derives from model studies on their biosynthesis by tyrosinase-catalyzed oxidation of tyrosine.[62] The initial stage for the melanin formation involves the formation of dopaquinone which is subsequently converted into dopachrome by oxidative intramolecular cyclization. In turn dopachrome undergoes a slow rearrangement reaction leading to 5,6-dihydroxyindol-2-carboxylic acid (DHICA) and after decarboxylation to 5,6-dihydroxyindole (DHI) (*Figure* 2.11).

It is possible to make synthetically pure DHI and DHICA melanin. Natural melanin, however, are comprised of a DHI/DHICA copolymer and there can be great variability in the relative amounts of these two precursor in the macroscopic pigment.[63-66]

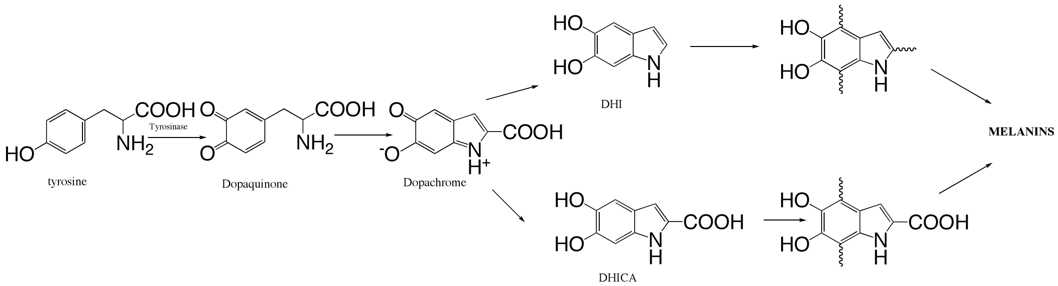

Figure 2.11- Melanin biosynthesis.

Pheomelanin is a polymer formed via cysteinyldopas through reactions of dopaquinone with cysteine or glutathione.[67] Pheomelanin is more photolabile than eumelanin. Irradiation of pheomelanin may lead to the formation of potentially toxic hydroxyl radicals, which are thought to play a role in carcinogenesis.[68,69] They may contribute to the higher incidence of melanoma in red-haired individuals.

2.2.7.1 Characterisation studies

The first studies on the chemical nature of started in 1970's and melanins are described as both insulator and semiconductors,[70,71] ion-exchange resins,[72] redox polymers[73,74] and free-electron scavengers.[75]

From the mid of 1990's started a number of studies, using scanning electron microscopy (SEM) to clarify its supramolecular structure. In particular the attention was focused on the more ubiquitous pigments, the eumelanins. It has been suggested that eumelanins consist of protomolecular structures approximately 15 Å in size made up of 4-5 planar sheets of four-to-eight 5,6-dihydroxyindole units each stacked in the z-plane with a graphite-like stacking spacing of 3.4 Å.[76-79] A natural eumelanin that is used as a human model system can be isolated from the ink sacs of *Sepia officinalis*. It is one of the most studied, and most of the available data in literature on this pigment are on *Sepia* melanin. In this eumelanin, a sequence of aggregation steps has been suggested to account for the apparent three levels of stuctural organisation (*Figure* 2.12).[80] In fact, first SEM images on Sepia melanin suggested that the pigment is an aggregated structure consisting of subunits that have a lateral dimension on the order of 100-200 nm.[81] Later was observed smaller particles, of about 10-20 nm, that adhering to the larger subunits.[82] Finally wide-angle X-ray diffraction measurements of dried eumelanins have led to the proposal of a "fundamental aggregate" that is a graphite-like layered structure 4-5 layers thick in which each layer is composed of a small, oligomeric structure.

Numerous studies using scanning tunneling microscopy (STM),[78] studies using atomic force microscopy (AFM),[83] X-ray diffraction,[84] mass-spectrometry,[85] NMR[86] and advanced quantum chemical calculations [87,88] have addressed eumelanin structure, and thought most of them appear to support the stacked aggregate pitcure, but this model has yet to see definitive proof.[80]

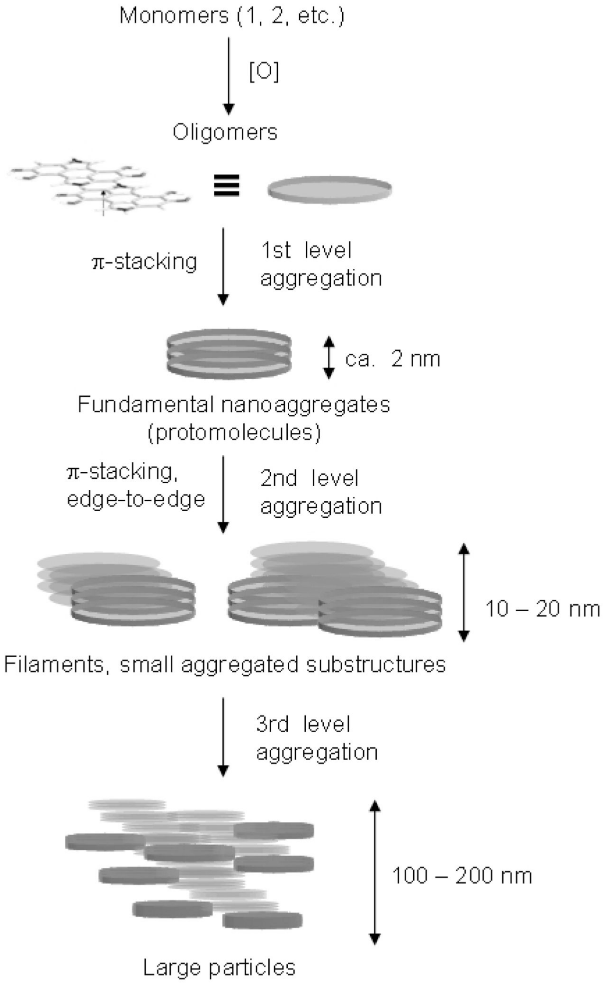

Figure 2.12- A schematic view of the hierarchical aggregate structure proposed for sepia eumelanin (from d'Ischia *et al.* 2009).

Considering the molecular structure, in 1992 a titrimetric method for the quantification of moderately acidic and weakly acidic groups in sepia melanin was reported, although it was suitable for the determination of functional groups present on the melanin surface only.[89] However, these techniques do not allow a rapid and quantitative identification and evaluation of melanin substructures.

The structures of synthetic and natural melanins were examined by solid-state NMR using cross polarization magic angle spinning and high–power proton decoupling. Resonances consistent with known pyrrolic and indolic structures and signals due to aliphatic residues were detected in all the samples examined.[90]

Despite the broad array of experiment carried out during the last years a direct evidence for the for an extensive indolyl and/or pirrolic melanin substructure is to date not available.

2.2.7.2 Melanin degradation and melanogenesis control

Today there is an increasing attention in the development of skin agents that improve disorders of hyperpigmentation, such as melasma, freckles and age spots, but also in the understanding the processes at the basis of pathologies such as depigmentation and vitiligo. Many approaches to achieving the control of the processes involved in melanogenesis already exist, but they are not really effective and totally safe.

In the study of melanin formation, the enzyme tyrosinase is considered to have a key role. In particular, the tyrosinase inhibition has been the aim of most of the scientific literature since 2000 and many studies have considered inhibitors by extraction from natural sources, mostly of Asian origin, such as the higher plants *Anacardium occidentale*,[91] *Artocarpus gomezianus*,[92] *Morus alba*,[93] *Pulsatilla cernua,*[94] *Sophora flavescens*[95] and various lichen species.[96,97] The inhibitors contained in these sources can be categorized into two main subgroups: polyphenols and aldehydes.

Among the polyphenols some potent tyrosinase inhibitory flavanoids are kaempferol,[98] quercetin,[99] kurarinone and kushnol F.[100] Many studies aimed to establish the relationship between the inhibitory activity of this compounds and their structure, but today a clear explanation is still lacking. Some evidence demostrate that all flavanoids inhibit the enzyme due to their ability to chelate copper in the active site.[101] In other cases relationships seem to occur between presence or the absence of some functional groups in the structure and inhibitory activity. For example in cardol derivatives the addition of a hydroxy group increase the inhibitory activity, where addition of a methyl group decrease it.[102] Moreover, strong tyrosinase inhibitory activity was reported by oxyresveratrol, due to the presence of a maximum number of hydroxy groups in the ring.[103] Furthermore, certain substituted phenol have a depigmenting action due to their ability to acts as substrate for tyrosinase, resulting in the generation of quinones, has lead to the examination of this system as a possible targeted antimelanoma therapeutic strategy in the case of disseminated melanoma.[104]

Among the aldehydes, a large number of compounds were isolated and characterised as tyrosinase inhibitors, such as trans-cinnamaldehyde,[105] anisaldehyde,[100] cuminaldehyde and cumic acid.[106] As the aldehyde group is known to react with biologically important nucleophilic groups such as sulfhydryl, amino and hydroxy groups, it has been proposed that its inhibitory effect is due to the formation of a Schiff base with the primary amino group of the enzyme.[100]

Even if a number of tyrosinase inhibitors are reported from natural sources, only a few of them are used as skin agents, primarily due to various safety concern. Actually, the polyphenols arbutin and aloesin are the most used compounds in the cosmetic industry because they show strong inhibition toward the tyrosinase. However, the agents proposed for melanin control are all based on melanogenesis inhibition, that can lead to indesiderable and uncontrollable collateral effect. An alternative approach could consist in the control of the final product, the melanin, acting on its degradation. The action on the final product of the metabolic pathway can lead to the development of safer skin products and also to a better comprehension of the main pigmentary disorders.

In this thesis, in *Chapter* 8, a new technique that allow to clarify the basic overall melanin chemical structure is presented and moreover a new approach based on the melanin degradation obtaining by use of natural oxidative enzymes coupled with a mediator is proposed.

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3: IMMOBILISED ENZYMES FOR LIGNIN OXIDATIVE FUNCTIONALISATION: LACCASE

3.1 Introduction

Laccase catalyses the reduction of molecular oxygen by various organic compounds to water without the step of hydrogen peroxide formation.[1] The industrial interest in the application of this enzyme lies in the low substrate specificity, high value of catalytic constants, use of air oxygen as the primary oxidant and high thermal resistance. However, its efficiency in bleaching pulps is poor, while when used in the presence of radical (natural or synthetic) chemical mediators, which are low molecular weight phenols, or N-hydroxy derivatives such as 1-hydroxybenzotriazole (HBT), a general increase in laccase reactivity has been shown.[2,3,4]

In recent years, the laccase-mediator system has been used for many different biotechnological applications such as pulp delignification, oxidation of organic pollutants and development of biosensors or biofuel cells.[5,6] Nevertheless, the industrial application of laccases is limited since their stability and catalytic activity are considerably affected by a wide variety of environmental conditions.[7] One approach to overcome these constraints is the use of immobilised laccases. In fact, immobilised enzymes allow easy recovery of products, multiple reuse of the biocatalyst, plug flow processes, rapid termination of reactions and a greater variety of bioreactor designs. Immobilised laccases have been extensively reported in the archival scientific literature.[8-12]

The layer-by-layer (LbL) technique, first introduced by Decher *et al.* allows multilayer assemblies to be obtained by the alternate deposition of polycations and polyanions in a cyclic way on a solid surface.[13,14] Encapsulation is an effective means for the immobilisation of enzymes since the polyelectrolyte films have the ability to protect proteins from high-molecular-weight denaturing agents or bacteria and to allow regulation of the permeability towards small substrates, which can enter the multilayer and react with the catalytic site.[13] More specifically, immobilisation of laccases based on the LbL technique and the LbL stabilisation has recently been reported.[7,14,15] There are also reports on the preparation of laccase immobilised in microcapsules for sensor development.[16,17]

To date, the continuous reduction of fossil fuel feedstocks as well as the requirement of environmentally friendly processes have prompted the development of novel processes based on alternative renewable materials. In this context, lignin, the second most abundant natural polymer on the planet, represents an invaluable resource. Oxidative enzymes, such as laccases, are potential tools for the selective modification of lignin as well as for the simplification of its structure, but the industrial application is still limited to processes with free enzymes. Moreover, the mechanism of action of oxidation of lignin with these enzymes is still only partially understood.[18]

In this regard, I spent part of my Ph.D. project in the development of novel immobilised laccases into polystyrene sulphonate and polyallylamine microcapsules based on the LbL technology as compared to chemically immobilised laccases coated by the same polyelectrolytes, the evaluation of their reaction pathway with respect to the native enzyme and their use in the oxidative functionalisation of lignin. More specifically, we applied the LbL procedure to improve the stability of laccase, to achieve higher resistance in the lignin oxidation reaction conditions and to develop processes suitable for eventual industrial scale-up. By means of quantitative ^{31}P NMR spectroscopy, a detailed study of the chemical selectivity of differently immobilised laccases was performed.

3.2 Results and discussion

3.2.1 Immobilised laccases

3.2.1.1 Preparation of cLbL-laccase

We applied the LbL technology in two different biocatalytic systems. In the first case, laccase from *Trametes versicolor* was chemically bonded onto functionalised alumina pellets, which were selected as the carrier material due to their known mechanical resistance at high values of pH and reaction temperature.[19] The yield of immobilised laccase was evaluated by analysing the residual enzymatic activity in the waste waters after the reaction of the enzyme with activated alumina particles and successive washings. An immobilisation yield of 89% with respect to the starting enzyme was observed, denoting a high efficiency for the process. This result was comparable with previously reported results for the chemical immobilisation of laccase and other enzymes on functionalised alumina pellets under similar experimental conditions.[15,19,20]

The alumina-laccase particles were successively stabilised by sequential deposition of the alternatively charged polyelectrolytes PSS (which carried a negative charge) and PAH (which carried a positive charge). The deposition procedure started with PAH because this polymer was characterised by an opposite charge with respect to alumina-laccase particles at that value of pH. The procedure was repeated several times to achieve three layers of differently charged polyelectrolytes (*Figure* 3.1).

No bleaching of laccase was observed during the sequential deposition of polyelectrolytes as evaluated by enzymatic assays on the coated enzyme. The activity of isolated cLbL-laccase was determined spectrophotometrically using ABTS as the substrate.[21] Under these experimental conditions, cLbL-laccase retained about 73% of activity with respect to native laccase.

Figure 3.2 shows the SEM photographs of the immobilised enzyme before (*a*-left) and after PSS/PAH coating (*b*-right). As can be seen from *Figure* 3.2*b*, the ultrathin coating layers show the presence of micropores of the average dimension of 200–300 nm.

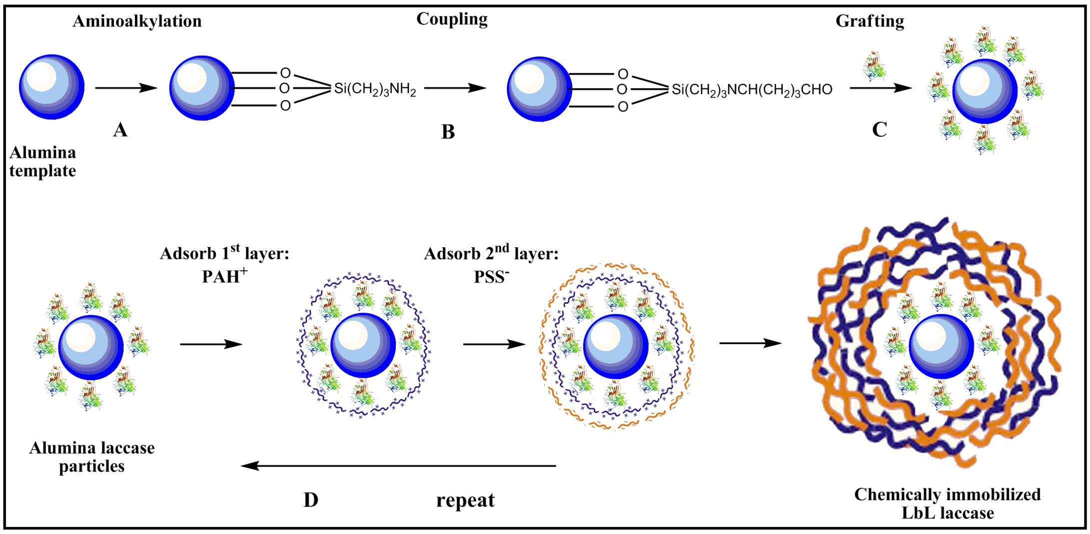

Figure 3.1- Scheme of preparation of PSS/PAH-coated laccase/alumina particles: (A) support silanization; (B) coupling with the cross-linker; (C) laccase cross-linking with the support; (D) layer-by-layer coating of supported laccase.

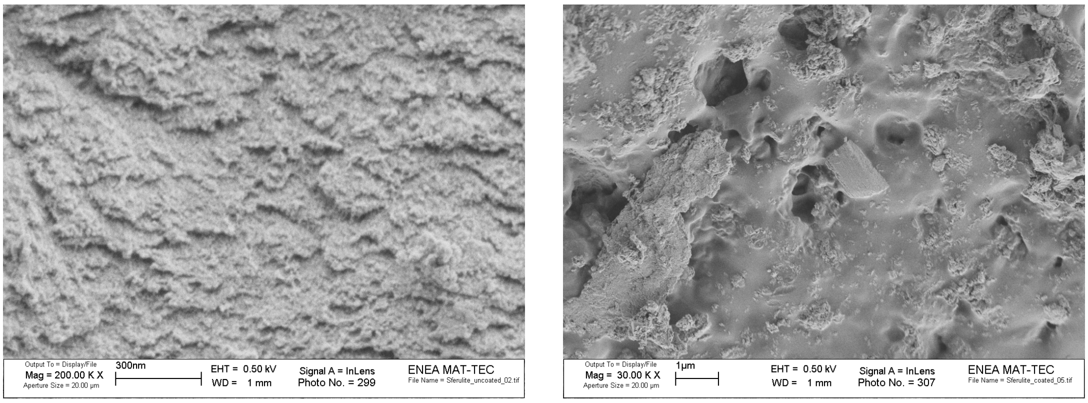

Figure 3.2- SEM images of laccase/alumina particles before (*a*-right) and after (*b*-left) polyelectrolyte coating.

3.2.1.2 Preparation of microencapsulated laccase (mLbL-laccase)

Next, we evaluated the effect of the LbL procedure on the immobilisation of laccase by a physical approach in which no covalent bonds were formed between the enzyme and the support. We studied the physical entrapment of laccase inside microcapsules produced by the Coulombic interaction between PSS and PAH polymers. Microcapsules of PSS and PAH were produced by the stepwise assembly of polymeric multilayers on preformed $MnCO_3$ particles. The number of polyelectrolyte layers is important when planning a microcapsule immobilisation. More specifically, the number of polyelectrolyte layers should be high enough to constitute a robust microcapsule that can efficiently protect the enzyme from leaching. Nevertheless, the microcapsule should present on its surface, pores wide enough to allow the enzymatic reaction to occur. On the basis of these considerations, we decided to rely on the previous work of Nayak and McShane[22] which optimised the number of polyelectrolyte layers on peroxidase microcapsules. Thus, after the deposition of 8 alternate polyelectrolyte layers, the $MnCO_3$ core was dissolved and the loading of laccase on PSS/PAH capsules was successively performed at pH 5. At this value of pH, some channels were opened in the PSS/PAH particles, thus favouring the diffusion of the enzyme from the bulk of the solution to the core of the capsules.

Polyelectrolyte microcapsules and multilayers typically show a pore length scale in the range of 100–500 nm as shown by SEM images.[23] *Figure* 3.3*a* shows an SEM image of laccase microcapsules. It can be seen that the dimensions of the capsules are of about 1–3 mm.

Sukhorukov et *al*. reported the results of a study on the pH-dependent swelling of LbL PSS/PAH microcapsules.[24] In the open state, these capsules were shown to be able to load fluorescein isothiocianate-labelled dextran of molecular weight 2000 kD and labelled bovine serum albumin.

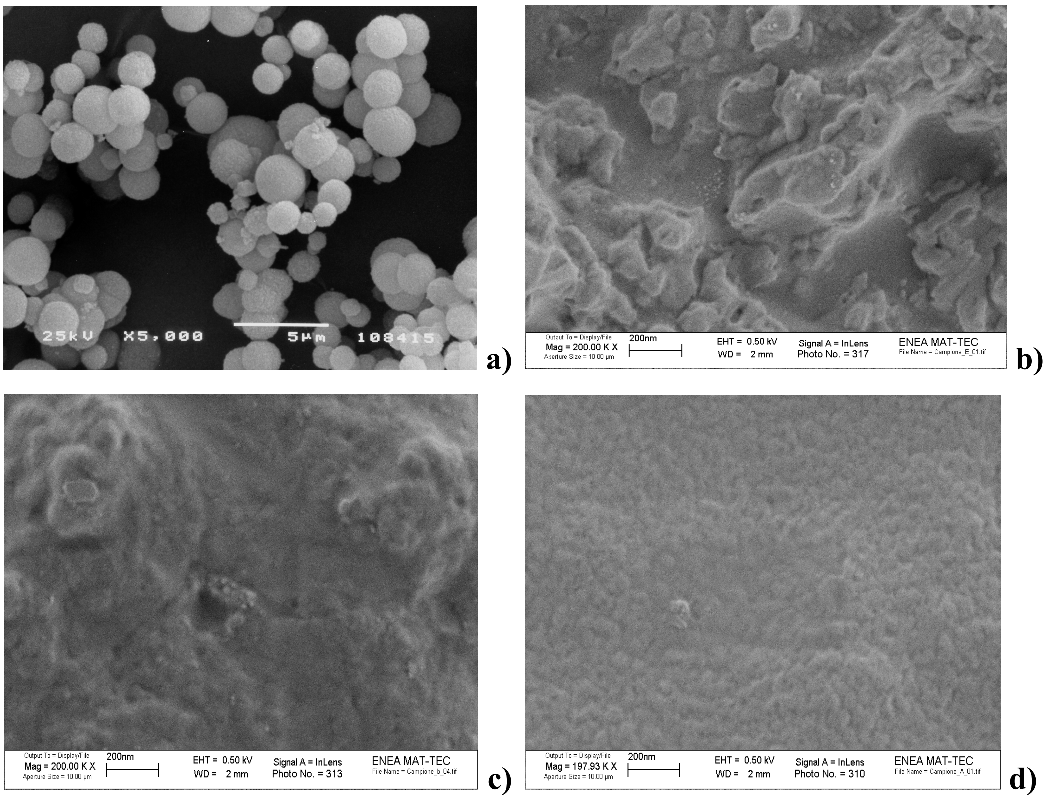

Figure 3.3- SEM images of laccase in PSS/PAH multilayer microcapsules: (a and b) pH 8; (c) pH 4; (d) pH 2.8.

Figure 3.3 shows how laccase microcapsules assume variable pore dimensions according to pH modification of the medium. SEM images of the microparticles at pH 8, 4 and 2.8, shown in *Figure* 3.3b-d, respectively, show that their number increased upon decreasing pH. The pores dimensions range from 30–40 to 150 nm.

The yield of immobilised laccase was evaluated by analysing the residual enzymatic activity in the water solution and washings after the loading of the enzyme. *Figure* 4 shows the scheme for Layer by Layer microcapsules laccase (mLbL- laccase) preparation.

A value of immobilisation yield of 75% with respect to the starting enzyme was observed, denoting a high efficiency for the process. The activity of isolated mLbL-laccase was determined spectrophotometrically using ABTS as the substrate.[21] Under these experimental conditions, mLbL-laccase retained about 60% of activity with respect to parent laccase. In order to examine potential enzyme diffusion from the microcapsules to the bulk of the solution, activity assays were performed with the buffer solution 2 h after microcapsule suspension and removal. They clearly showed that the activity in the buffer solution after microcapsule removal was absent.

From our data, it appears that Layer by Layer coated laccase (cLbL-laccase) is more active than mLbL-laccase, but both the immobilised laccase forms are valuable for the oxidative functionalisation of lignin.

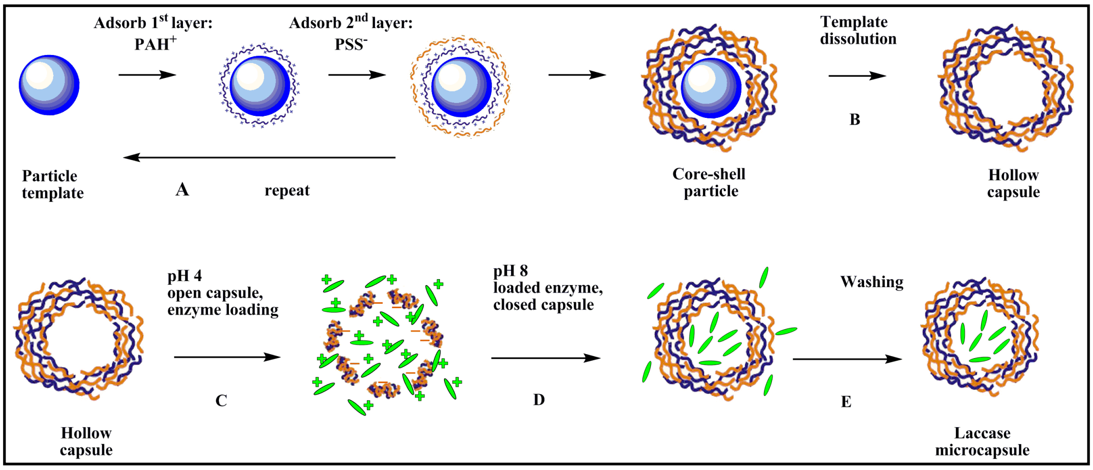

Figure 3.4- Scheme of preparation of layer-by-layer PSS/PAH microcapsule immobilised laccase: (A) PSS/PAH deposition onto a core particle template; (B) core dissolution; (C) capsule swelling and enzyme loading; (D) loaded microcapsule closure; (E) washing.

3.2.2 Catalyst recycle

Both the free and the coated laccases were allowed to react with ABTS for several successive 12 h batch reactions. All the laccases retained their activity after 10 cycles, although in different proportions.

Figure 3.5 shows the percentage of retained activity versus catalytic cycles. The free laccase retained 22% of its activity, while the cLbL- and mLbL-laccases retained 84% and 68% of their activity, respectively. The percentage of retained activity was calculated with respect to the initial activity for each case. Therefore, the layers preserved the laccase activity both on immobilised laccase and in microcapsules.

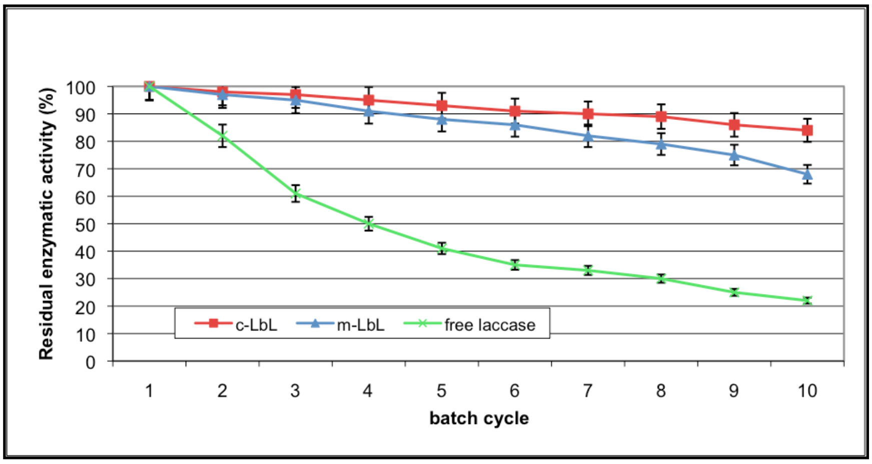

Figure 3.5- Laccase, cLbL-laccase and mLbL-laccase residual activity (%) as a function of the number of catalytic cycles. The laccases were allowed to react with ABTS after 10 successive 12 h batch reactions.

3.2.3 Lignin oxidation with laccase and immobilised laccases

Immobilised laccases, cLbL-laccase and mLbL-laccase, were used as biocatalysts for the oxidation of two representative lignins, namely a cypress MWL and a residual lignin isolated from kraft pulp, using air as the primary oxidant. MWL was chosen because has a structure close to the native lignin in wood. Softwood lignin was chosen due to its known recalcitrant properties to both depolymerisation and oxidative functionalisation, mainly due to concomitant formation of cross-links between phenyl propanoid units. The study of the oxidation of softwood lignin thus allows the evaluation of competitive lignin oxidative functionalisation versus condensation processes.

MWL is a network polymer with a high degree of polydispersity. The values of Mn and Mw of the lignin studied here are 13,000 and 24,000 g/mol, respectively. As such, lignin, or at least its low molecular weight fractions are able to approach the enzyme's active site through the micropores present on the LbL coatings both in coated particles and microcapsules. In this system, the lignin oxidation can thus be carried out not only by means of oxidation mediators, but also by direct diffusion through the polyelectrolyte shell. The role of low molecular weight lignin fractions as mediators in the laccase oxidation of higher molecular weight fractions cannot be ruled out. This is in accord with literature data that show the high efficiency of natual phenolic compounds as oxidation mediators.[2-4,25] RKL is a highly modified lignin with respect to the native one produced as waste during pulp and paper manufacture. This lignin contains a high amount of phenolic condensed and guaiacyl aromatic groups which stabilise the polymer towards oxidation. As a general procedure, the oxidation of MWL and RKL (80 mg) was carried out with cLbL- laccase, mLbL-laccase and free laccase as reference (60U of enzyme in each case). The reactions were also performed in the presence of selected low molecular weight compounds, such as HBT and VA, which act as mediator for the laccase, modifying the oxidation pathway of lignin. Specifically, laccase first oxidises the mediator by formation of the corresponding radical intermediate. In turn, the radical species undergoes a hydrogen atom abstraction process on lignin either at the phenolic (usual site for laccase oxidation) or benzylic position. This latter transformation opens the route to a wide array of novel oxidative functionalisation/depolymerisation reactions.[25,26] The extent of the oxidation was evaluated as conversion of lignin after the treatment (g of solubilised lignin/100 g of starting lignin), which is the extent of oxidation increases with the conversion yield. In fact, it is known that an efficient oxidative functionalisation (which can also involve a depolymerisation process) changes the chemical properties of lignin, which becomes more hydrophilic and soluble in water.[25] The data are summarised in *Table* 3.1.

Table 3.1- Conversion of MWL and RKL after treatment with free laccase, cLbL-laccase and mLbL-laccase in the presence or absence of mediators for the oxidation.

Entry	Lignin[a,b]	Biocatalyst	Yield[f] (%)
1	MWL	laccase	54.4
2	MWL	laccase-HBT[c]	51.2
3	MWL	laccase-VA[c]	48.9
4	MWL	cLbL	91.9
5	MWL	cLbL[d]/HBT	94.0
6	MWL	cLbL/VA	93.9
7	MWL	mLbL	91.3
8	MWL	mLbL[e]/HBT	93.5
10	MWL	mLbL/VA	93.7
11	RKL	laccase	45.0
12	RKL	laccase/VA	68.8
13	RKL	cLbL	77.7
14	RKL	cLbL/VA	80.0

[a] MWL = cypress milled wood lignin; RKL = residual kraft lignin. [b] Lignins were oxidised with laccase or immobilised laccase (60 U) in acetate buffer (40 mL) at 40 °C.[c] In some cases, the reactions were performed in the presence of mediators of the oxidations (1.0 mM) under similar experimental conditions. HBT = 1-hydroxybenzotriazole; VA = violuric acid. [d] cLbL-laccase = chemically immobilised layer-by-layer laccase. [e] mLbL-laccase = microencapsulated layer-by-layer laccase. [f] Conversion is defined as gram of residual lignin/100 gram of starting lignin.

It is worth noting that, irrespective of the experimental conditions, cLbL-laccase and mLbL-laccase were more efficient biocatalysts than free laccase in the oxidation of

MWL (*Table* 3.1, entries 4 and 7 versus entry 1) under otherwise identical experimental conditions. In particular, it was possible to recover only 8% of the starting material by oxidation of MWL with immobilised laccases, while the conversion yield for the native one was about 54%. Thus, the immobilised laccases did not exhibit significant specific kinetic barriers to the approach of lignin at their multicopper catalytic site. When the oxidations with both cLbL-laccase and mLbL-laccase were performed in the presence of HBT and VA, a significant increase of the conversion was observed, thus confirming a role of the mediators in the increase of the efficiency of the reaction (*Table* 3.1, see for example entries 4 and 7 versus entries 5 and 8). The same effect was not observed with native laccase (*Table* 3.1, entry 1 versus entries 2 and 3). A similar behaviour was observed in the oxidation of RKL, where cLbL-laccase was more efficient than native laccase (*Table* 3.1, entry 13 versus entries 11 and 12). Again, the reactivity of cLbL-laccase was found to be increased in the presence of VA (*Table* 3.1, entry 14). On the basis of these data, it is reasonable to suggest that the immobilisation procedure can stabilise laccase, enhancing its performance in the oxidative functionalisation of lignin. cLbL- and mLbL-laccases showed comparable reactivity in the presence of oxidation mediators. In both cases, a similar lignin conversion increase was found, irrespective of the nature of the oxidation mediator. This suggests that the polyelectrolyte coatings on cLbL- and mLbL-laccases behave in a similar way towards mediator diffusion through the surface micropores.

3.2.4 ^{31}P NMR characterisation of lignin after treatment with laccase and immobilised laccases

With the aim of gaining further insight into the reaction pathway of oxidation of lignin with immobilised laccases, we evaluated the structure of enzymatically modified MWL and RKL by means of nuclear magnetic resonance techniques based on the phosphorus nucleus (^{31}P NMR analysis). The oxidative modification of the structure of MWL and RKL recovered after treatment with cLbL-laccase and mLbL-laccase was determined using ^{31}P NMR spectroscopy after in situ phosphytylation of the samples.[24,27-30] Results were compared with data recovered with free laccase. This advanced heteronuclear NMR technique allows the contemporary characterisation and quantification of all labile OH groups present in lignins such as the aliphatic OH groups, the different phenolic OH and carboxylic acids. In particular, the samples of residual lignins were phosphitylated in quantitative yield with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane in pyridine/deuterated chloroform mixture (1.6:1.0, v/v ratio) and then subjected to quantitative ^{31}P NMR analysis in the presence of N-hydroxynaphthalimide as internal standard. The assignment of the different OH signals was carried out on the basis of the comparison with the chemical shift of selected models as reported in previous works.[28,29,31] To date, this is a widely applied technique to evaluate lignin modifications. *Figure* 3.6*a–c* shows the spectra of laccase, cLbL-laccase and mLbL-laccase-treated lignins, respectively.

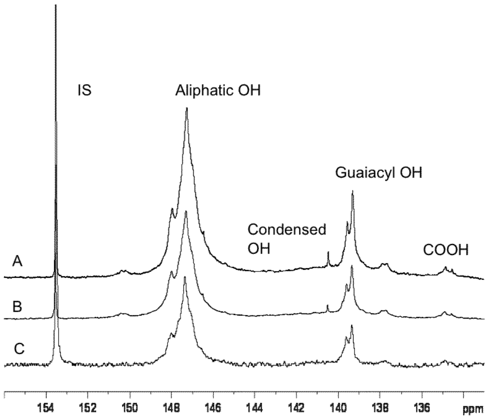

Figure 3.6- ^{31}P NMR spectra of lignins treated with laccase (A), cLbL-laccase (B) and mLbL-laccase (C) and successively phosphytylated with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane.

Table 3.2 summarises the data for the oxidation of MWL and RKL. In *Figure* 3.7, the possible lignin oxidation pathways are reported. Free laccase showed a low conversion yield with respect to immobilised ones; nevertheless, it was able to modify the lignin structure, although not to the extent to solubilise it. More specifically, treatment of MWL with free laccase decreased the amount of aliphatic OH and increased the guaiacyl and condensed OH and COOH groups (*Table* 3.2, entry 2 versus entry 1) (*Figure* 3.7 routes A and B). These results are in accordance with previously reported data.[28,32] The decrease of aliphatic OH and the increase of COOH groups are attributed to the occurrence of side chain oxidation processes (*Figure* 3.7 route A), while the increase of condensed OH is due to the occurrence of oxidative coupling reactions on phenolic end groups in lignins (*Figure* 3.7 route B). A similar behaviour was observed in the presence of immobilised laccases (*Table* 3.2, entries 3 and 4 versus entry 1). As a general trend, the increase in guaiacyl OH groups suggests an increase of hydrophilicity of the polymer and the occurrence of alkyl-phenyl ether bond cleavage reactions which finally result in lignin depolymerisation (*Figure* 3.7 route C). On the other hand, the increase of condensed phenolic OH groups showed that oxidative coupling processes were also present. This effect was more significant in the case of free laccase with respect to both cLbL-laccase and mLbL-laccase (*Table* 3.2, entries 3 and 4 versus entry 2).

Table 3.2- ^{31}P NMR analysis of MWL and RKL before and after laccase, cLbL-laccase and mLbL- laccase oxidation.[a]

Entry	Lignin	Biocatalyst	Aliphatic OH	Condensed OH	Guaiacyl OH	COOH
1	MWL	none	6.82	0.21	0.92	0.08
2	MWL	laccase	6.78	0.60	1.18	0.21
3	MWL	cLbL-laccase	6.09	0.44	1.11	0.25
4	MWL	mLbL-laccase	6.31	0.55	1.34	0.14
5	MWL	laccase-HBT[b]	6.30	0.18	1.28	0.24
6	MWL	laccase-VA[b]	6.47	0.16	1.52	0.13
7	MWL	cLbL-laccase/HBT	4.87	0.10	1.19	0.13
8	MWL	cLbL-laccase/VA	5.83	0.15	1.46	0.18
9	MWL	mLbL-laccase/HBT	6.03	0.15	1.78	0.11
10	MWL	mLbL-laccase/VA	6.15	0.10	1.75	0.12
11	RKL	none	2.45	1.40	1.53	0.52
12	RKL	laccase/VA	1.63	0.95	0.99	0.36
13	RKL	cLbL-laccase/VA	1.30	0.69	0.70	0.17

[a] Amount (mmolg^{-1}) of aliphatic, phenolic and carboxylic OH groups present on residual MWL before and after treatment with laccase, cLbL-laccase and mLbL-laccase as evaluated by quantitative ^{31}P-NMR analysis. The ^{31}P-NMR data reported in this regard are averages of three phosphytylation experiments followed by quantitative ^{31}P-NMR acquisition. The maximum standard deviation of the reported data was 2x10^{-2} mmol/g, while the maximum standard error was 1x10^{-2} mmol/g. [b] HBT = 1-hydroxybenzotriazole; VA = violuric acid.

A different selectivity was observed in the oxidation of MWL with cLbL- or mLbL-laccase. In fact, cLbL-laccase selectively performed side chain oxidation over possible alkyl-phenyl ether bond cleavage reactions, as shown by the decrease of aliphatic OH and increase of carboxylic groups (*Figure* 3.7 route A). Instead, mLbL-laccase mainly catalysed the alkyl-phenyl ether bond cleavage, resulting in a pronounced increase in phenolic OH groups.

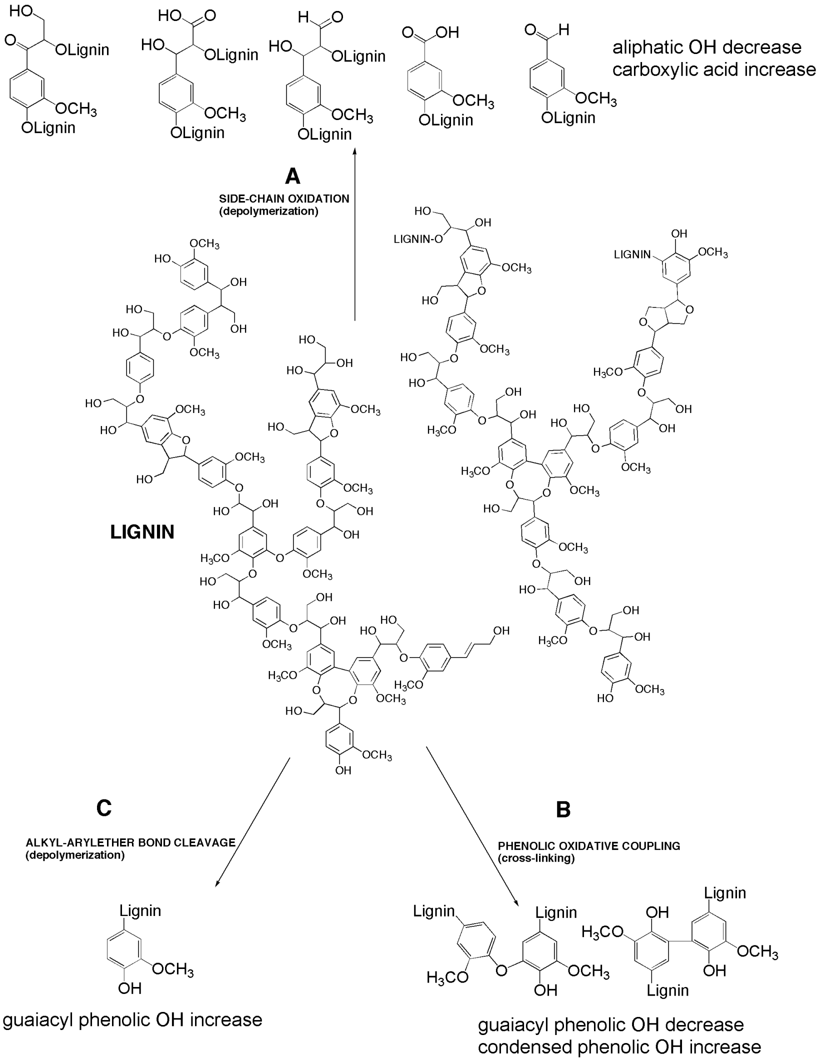

Figure 3.7- Lignin oxidation pathways.

Next, the role of the mediators in the reaction pathway was evaluated. When the oxidation of MWL was performed with native laccase in the presence of HBT or VA, the residual polymer showed a decrease in aliphatic and in condensed OH and an increase in guaiacyl OH and in COOH groups, in accordance with data previously reported (*Table* 3.2 entries 5 and 6).[25] It is interesting to note that the presence of HBT and VA significantly enhanced the performance of immobilised laccases to yield lignins with lower amounts of aliphatic and condensed OH groups and a higher amount of guaiacyl OH group than native laccase (*Table* 3.2, entries 7–10 versus entries 5 and 6). These data indicate the prevalence of depolymerisation reactions (*Figure* 3.7 routes A and C) and the contemporary inhibition of repolymerisation processes by oxidative coupling cross-linking (*Figure* 3.7 route B). The oxidation of

MWL with immobilised laccases in the presence of HBT and VA also confirms the general reactivity pattern described above: while cLbL-laccase preferentially undergoes side chain oxidation/cleavage processes, mLbL-laccase favours alkyl-phenyl ether bond cleavage reactions (*Table* 3.2, entries 7 and 8 versus entries 9 and 10). Table 3.2 also reports the quantitative data obtained by ^{31}P NMR analysis for RKL oxidation with laccase and cLbL-laccase systems in the presence of VA as representative examples (entries 11–13). cLbL-laccase oxidised RKL more efficiently than native laccase. More specifically, cLbL-laccase showed the highest decrease in aliphatic and in condensed OH groups, suggesting the occurrence of efficient side chain oxidations. In this latter case, significant coupling processes were not observed.

Other oxidation strategies have been developed for lignin selective oxidation with varying degrees of efficiency.[33-39] As an example, synthetic metalloporphyrins immobilised onto clays have been developed as biomimetic systems for lignin peroxidase.[33-37] In this case, the conversion yields for both lignin model compounds and lignins were found to be lower than those obtained using the laccase-mediator system. The oxidative pattern of lignin oxidation in the presence of hydrogen peroxide/immobilised porphyrins consisted of the reduction of aliphatic OH groups and no significant variation in condensed phenolic groups and a reduction of guaiacyl units, which indicated a less hydrophilic final lignin structure than that obtained in the presence of laccases. On the contrary, the use of methyltrioxorhenium (MTO)[38] and immobilised MTO[39] onto polystyrene or polyvinylpyridine resulted in higher conversion yields for both lignin model compounds and lignins than those obtained with laccases. The structure of residual lignins after oxidation showed a similar trend to laccase-mediator catalysed reactions. However, in the presence of MTO, the poor mass balances obtained indicated an extensive overoxidation of both lignins and lignin model compounds. This drawback has ruled out the possibility of using MTO in the oxidation of lignin for functionalisation purposes.

3.3 Conclusion

The results obtained in this study show that cLbL-laccase and mLbL-laccase are more effective in the oxidation of MWL and RKL than native laccase. The highest reactivity of immobilised laccases might be explained by the stabilisation effect of coating polyelectrolytes towards enzyme deactivation processes. Moreover, kinetic barriers for the approach of substrate to the active site of the enzyme are not operative. The strategy used for the laccase immobilisation is also an efficient tool to tune the selectivity of the enzyme. In fact, as evaluated by ^{31}P NMR analysis, the immobilised laccases follow a different reaction pathway than native enzyme, resulting in efficient depolymerisation without cross-linking reactions. The cLbL-laccase performs mainly side chain oxidations, while mLbL-laccase favours alkyl-phenyl ether bond cleavage. Irrespective of the experimental conditions, the efficacy of the oxidation of MWL and RKL is enhanced in the presence of HBT and VA as redox mediators able to diffuse from the catalytic site of the enzyme to the bulk of the solution. On the basis of these data, cLbL-laccase and mLbL-laccase constitute new selective specific biocatalysts for the oxidative functionalisation and depolymerisation of lignin.

3.4 Experimental

3.4.1 Lignin isolation

RKL and MWL were isolated from cypress (*Cupressus* spp.). RKL was obtained by using a slightly modified acidolysis procedure as previously reported.[40,41] The yield was 38%, and purity was confirmed by UV and Klason lignin content standard measurements as reported in the literature.[42] MWL was prepared from ultraground extractive-free powder according to Bjorkman's procedure with some modifications.[43] Extractive-free powders, prepared by milling the samples at 40 mesh followed by exhaustive extraction with acetone:water (9:1, v/v), were ultraground for 3 weeks in a rotatory ball mill. The MWL fractions were then extracted with dioxane:water (96:4, v/v). The residues were concentrated under reduced pressure and freeze-dried. Purification ensued by dissolving the lignins in 90% acetic acid. The solution was then added dropwise to stirred water. The precipitated lignins were centrifuged and freeze-dried. They were dissolved again in a mixture of 1,2-dichloroethane:ethanol (2:1, v/ v) and precipitated in diethyl ether. The resulting products were about 82% lignins as evidenced from UV lignin content measurements.

3.4.2 Enzyme immobilisation

3.4.2.1 Immobilisation of laccase on alumina pellets and coating with polyelectrolyte layers

Laccase was treated with activated alumina pellets bearing glutaraldehyde linkers previously introduced by the reaction of crude particles with γ-aminopropyltriethoxysilane and glutaraldehyde.[9,20] At the end of the reaction, laccase was immobilised on the support.[20] The adopted immobilisation technique consisted of four different stages. In the first one, *aminoalkylation*, alumina pellets were silanised with 2% (v/v) γ-aminopropyltriethoxysilane in acetone at 45 °C for 20 h. The silanised supports were washed once with acetone and silanised again for 24h. They were then washed several times with deionised water and dried through air. In the second stage, the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50%, v/v) for 2h at room temperature, washed again with deionised water and dried through air. In the third stage, *grafting*, 150g of support was put in contact with the enzyme, by immersion, for 48 h at room temperature, in 250 mL of laccase (5000 U/L) solution obtained by dissolving the enzyme in 100 mM citrate buffer pH 5 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution. In the final stage, LbL coating, the pellets were first washed three times with 0.1 M NaCl and then the sequential deposition of polyelectrolyte (PAH^+, PSS^-) layers onto the alumina particles was performed. Polyelectrolyte solutions (0.01 M) with 0.5 M NaCl were prepared and the supports were immersed inside each solution for 20 min. Since the alumina pellets with immobilised enzyme were negatively charged, microcapsules consisting of three layers were created, starting with the positively charged polyelectrolyte (PAH^+, PSS^-, PAH^+). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl. The red particles of chemically immobilised LbL laccase (cLbL-laccase) were obtained by simple filtration from the reaction mixture.

3.4.2. Laccase microencapsulation

The laccase microencapsulation was performed by stepwise assembly of alternatively charged polyelectrolyte layers on preformed manganese carbonate ($MnCO_3$) particles.[44-46] $MnCO_3$ particles (5.5 mm diameter) were synthesised as previously described.[44] To a mixture of 0.016 M $MnSO_4$ and 0.16 M NH_4HCO_3 in equal volumes and under vigorous stirring, was added ethanol until a final ethanol concentration of 5% (v/v). The material obtained was incubated at 50 °C for 1 h and then the particles were centrifuged and washed several times. The LbL coating was performed by immersion of the particles in positively charged polyelectrolyte solution (PAH^+, 2 mg/mL) with 0.2 M NaCl for 20 min, followed by three washings in water. The deposition of oppositely charged polyelectrolyte (PSS^-) was then performed, followed by three washings. After deposition of four polyelectrolyte bilayers (PAH+, PSS^-), the particles were treated with 0.1 M HCl solution for 1 min to degrade the particle cores. The hollow spheres were thoroughly washed with water and were then loaded with laccase by pH tuning as follows.[22] The microcapsule suspension (200 mL) was incubated with a 200 mL laccase solution (20 U/mL) in 0.1 M acetate buffer pH 5 for 24 h at room temperature. Under acidic conditions, the permeability of microcapsules increases dramatically and allows enzyme loading.[44] After the enzyme loading, the pH was adjusted to 8 by adding 0.1 M phosphate buffer; washings in slightly alkaline solution regenerated the wall making it again impermeable to high molecular weight compounds, entrapping in this way the majority of the enzyme.[45] Finally, after 1 h, the solution was centrifuged and washed several times to remove the enzyme that had not been encapsulated.

High resolution scanning electron microscopy was performed by a FE-SEM LEO1530 apparatus. The samples were fixed on an adhesive graphitic support.

3.4.3 Enzyme activity assay

Free laccase activity was determined spectrophotometrically using ABTS as the substrate. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate pH 5 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 415 nm for 1 min for the free enzyme and for 3 min for the immobilised enzyme. One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min.[21] The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

3.4.4 Laccase treatment of lignin

The treatments were carried out in both the presence and absence of a mediator, namely HBT or VA. Lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) with or without the mediator (1 mM) and treated at 40 °C with the enzyme (60 U) under vigorous stirring to optimise the contact of the solution with air. After 12 h, the mixture was cooled, acidified at pH 3 and centrifuged. The residue was washed with water three times to eliminate solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analysed by ^{31}P NMR.

3.4.5 Quantitative ^{31}P NMR

Derivatisation of the lignin samples with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane was performed as previously described.[27-29,47] Samples of lignin (30 mg), accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio (0.4 mL). Tetramethylphospholane (0.1 mL) was then added, followed by the internal standard and the relaxation reagent solution (0.1 mL). The NMR spectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.[29,30] To improve resolution, a total of 256 scans was acquired. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2 x 10^{-2} mmol/g, while the maximum standard error was 1 x 10^{-2} mmol/g.[27,28]

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4: IMMOBILISED ENZYMES FOR LIGNIN OXIDATIVE FUNCTIONALISATION: HRP

4.1 Introduction

HRP is one of the most used peroxidases due to its versatile and wide applicability in numerous and different areas such as analytical, environmental and clinical fields.[1] In fact the enzyme shows many interesting characteristics for application purposes (in reaction specificity and stability and availability in pure form at reasonable costs).[2] Improvements to these desiderable qualities such as its relatively good stability in aqueous and non-aqueous solvent systems are actively sought.[3] In general the enzymes exhibit a number of features that make their use advantageous as compared to conventional catalysts; however there are a number of constraints in the use of the enzymes; the common perception is that enzymes are sensitive, unstable and have to be used in water, features that are not ideal for a catalyst and undesirable in most syntheses.[4] Several approach have been proposed to overcome these limitations; among them immobilisation is generally considered favourable for industrial scale applications since it allows for continuous processes.[5] Many examples of immobilised HRP have been reported in literature.[6,7,8,9]

The layer-by-layer (LbL) adsorption technique, introduced by Decher *et al.*,[10] is a general and versatile tool for the controlled fabrication of multimaterial surface coatings on a large variety of surfaces.[11-14]

Today the rising energy consumption, the depletion of fossil fuel feedstocks and the increased environmental concerns have focused the attention on the use of alternative renewable materials and on the development of environmentally friendly processes that operate in mild reaction conditions. Among the most important renewable materials there is lignin, the second most abundant natural polymer on the planet. Oxidative enzymes are potential tools for lignin oxidative modification. HRP has been previously used in the preparation of synthetic lignins (DHP) since HRP catalyses the oxidation of monolignols to the corresponding phenoxy radicals.[15] To our knowledge the oxidation pathway of lignin by HRP has never been studied in detail. After the laccase immobilisation my Ph D prompted to the design and the development of novel immobilised HRP. More specifically the enzyme was chemically immobilised on alumina particles and then coated by polyelectrolytes bilayers, based on the LbL technique, and used for the oxidative functionalisation of lignin. The aim of this part of my project was to study the reaction pathway of HRP and immobilised HRP on lignins from different origins. The chemical modifications on lignin after the treatments with the immobilised HRP were determined by means of ^{31}P NMR spectroscopy and gel permeation chromatography (GPC).

4.2 Results and discussion

4.2.1 Preparation of immobilised HRP

For the immobilisation of HRP we applied a chemical method and then we chose to coat the immobilised catalyst with polyelectrolytes layers using the LbL adsorption technique. Thus HRP from *A. rusticana* was chemically immobilised onto alumina pellets, a material that was chosen for its well known mechanical resistance at high pHs and temperatures.[16] The supports were previously functionalised with glutaraldehyde that covalently links to the enzymes via available amino functions.[17,18] Enzyme cross-linking was avoided by washing several times the supports before adding the enzyme to remove all excess glutaraldehyde. An immobilisation yield of 95% with respect to the starting enzyme was obtained, highlighting the high efficiency of the process. Then the alumina-HRP particles were coated by a sequential deposition of alternatively charged polyelectrolytes, in particular the procedure started by the deposition of polystyrene sulphonate (PSS), with a negative charge, followed by the deposition of polyallylamine hydrochloride (PAH), with a positive charge, until the formation of three layers. The deposition started by a negative charge because at neutral pH the alumina-HRP particles were positive charged (*Figure* 4.1). Under these experimental conditions, the immobilised enzyme retained about 68% of its activity with respect to native laccase, as determined by the activity assay, measured spectrophotometrically using ABTS as substrate.[19] This result is comparable with previously obtained results for HRP immobilised on supports activated with glutaraldehyde. [20,21]

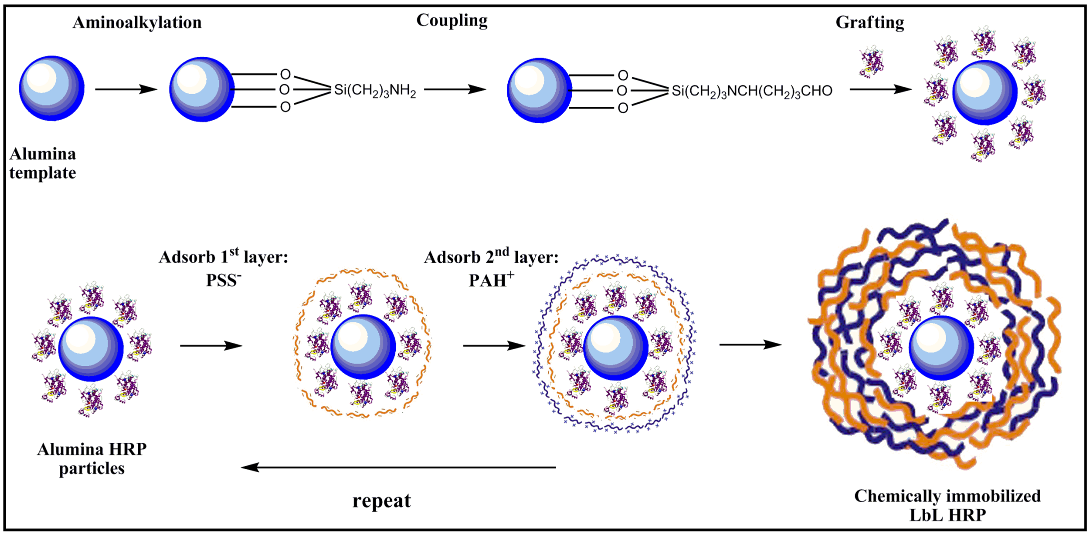

Figure 4.1- Scheme of preparation of PSS/PAH/PSS-coated HRP/alumina particles: a)support silanisation; b) coupling with glutaraldehyde; c) enzyme cross-linking with the support; d) layer-by-layer coating of supported HRP.

Figure 4.2 shows the scanning electro microscopy of the immobilized and the coated immobilized HRP, *left* and *right* respectively. The coated HRP shows pores on its surface that allow substrate approach to the enzyme active site.

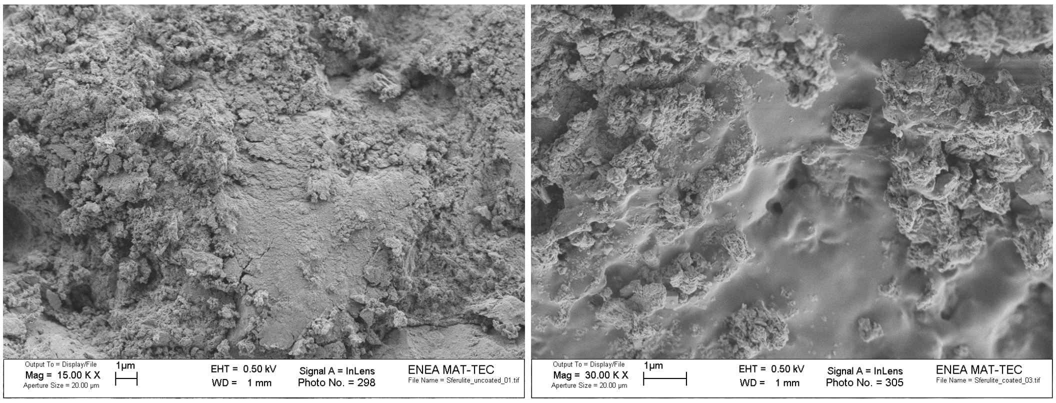

Figure 4.2- SEM images of HRP/alumina particles before (left) and after (right) polyelectrolyte coating.

4.2.2 Catalyst recycle

In order to test the possibility of reuse the catalyst, both the free and the immobilised enzymes were allowed to react with ABTS for several successive 12 h batch reactions. In *Figure* 4.3 the percentage of retained activity versus catalytic cycle is shown. All the HRPs retained their activity after 10 cycles, although in different proportions. The free enzyme retained 20% of its activity, while the immobilised one retained about 82% of its activity. The percentage of retained activity was calculated with respect to the initial activity for each case. Therefore, it is evident that, in the case of the immobilised enzyme, the polyelectrolytes layers preserved the HRP activity.

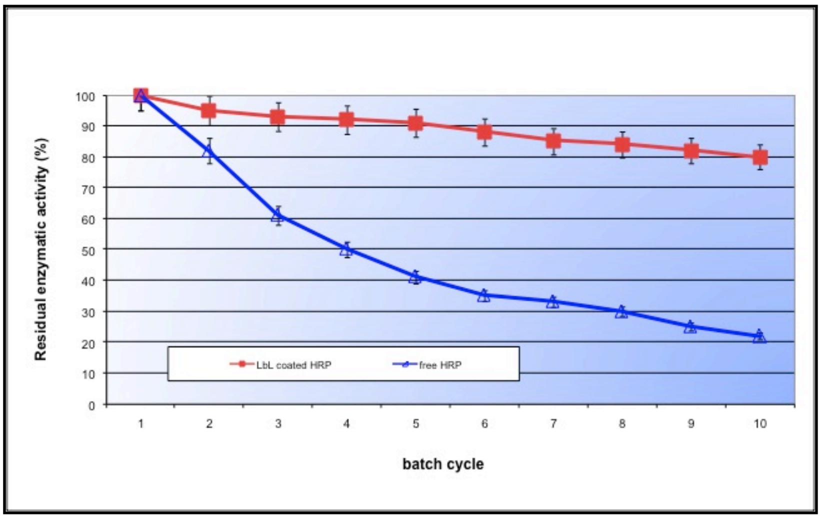

Figure 4.3- HRP free and coated LbL-HRP residual activity (%) as a function of the number of catalytic cycles. The enzymes were allowed to react with ABTS after 10 successive 12 h batch reactions.

4.2.3 Lignin oxidation with native and immobilised HRP

HRP and the immobilised HRP were used as biocatalyst for the oxidation of two different lignin samples, a black spruce MWL and a residual lignin isolated from softwood kraft pulp. Hydrogen peroxide was used as primary oxidant. MWL is considered the lignin that is more similar to the native one, it is extracted using a mixture of dioxane and water and this procedure is supposed to cause only minor changes in the polymer structure.[22] The Mw of these lignin preparations range between of 15,000–20,000.[23] We chose to use a softwood lignin because of its major resistance to the enzymatic treatments. In fact it has been suggested that guaiacyl lignins, typical of softwoods, restricts fibre swelling and thus accessibility more than the syringyl lignin in the hardwood due to its higher degree of branching.[24] The analysis of this lignin allowed to us to clarify the extent of the oxidative functionalisation versus condensation processes. With respect to the native one, RKL is a highly modified lignin that is produced by chemical pulping processes. It shows a low molecular weight, a higher phenolic content and a lower methoxyl content and has undergone extensive side-chain oxidation.[25]

After the HRP treatments on MWL and RKL (80 mg) with immobilised and free enzyme (60 U) were performed, the conversion yield of lignin (g of solubilised lignin/100 g of starting lignin) was calculated (*Table* 4.1). In fact, the oxidation increased with the conversion yield due to the changes in the chemical properties of lignin, that became more hydrophilic and soluble in water.[26] Under the same experimental conditions the immobilised enzyme was found more efficient in the oxidation than the free enzyme both on MWL (*Table* 4.1, entry 2 *vs* entry 1) and on RKL (*Table* 4.1, entry 4 vs entry 3). More specifically it was possible to recover only about 10% of the starting material using the immobilised HRP in both the lignins, where using the native HRP the recoveries were up to 40%. From these results it is evident that the active site of HRP did not show kinetic barriers to approach the substrate, performing a more extensive oxidative action on lignin with respect to the native enzyme. It can be hypothesized that the immobilisation process confers a stabilisation effect to the enzyme, improving the oxidative performances.

Table 4.1- Conversion of MWL and RKL after treatment with free and immobilised HRP.

Entry	**Lignin[a,b]**	**Biocatalyst**	**Yield[c] (%)**
1	MWL	HRP	53.8
2	MWL	Immobilised HRP	89.8
3	RKL	HRP	53.1
4	RKL	Immobilised HRP	88.1

[a] MWL = black spruce milled wood lignin; RKL = softwood residual kraft lignin. [b] Lignins were oxidised with HRP or immobilised HRP (60 U) in acetate buffer (40 mL) at 40 ^{0}C. [c] Conversion is defined as gram of residual lignin/100 gram of starting lignin. Reaction time: 12 hours.

4.2.4 ^{31}P NMR characterisation of lignin after treatment with native and immobilised HRP

In order to extensively clarify the reaction pathway after the HRP treatments both with the native and with the immobilised enzyme, we used an advanced heteronuclear magnetic resonance analysis, the ^{31}P NMR.[27-29] In *Table* 4.2 the quantitative data, for the different functional groups, obtained for MWL and RKL before and after enzymatic treatments, are reported. Both MWL and RKL were deeply modified upon HRP and coated HRP particles treatments. *Figure* 4.4 shows possible lignin oxidation pathways. The COOH groups were found increased both by use of HRP and coated HRP particles (*Table* 4.2). This indicates an efficient oxidation process.

The aliphatic OH groups were not significantly modified, meaning that the side chains were not extensively oxidised (*Figure* 4.4 route A). HRP and supported HRP behave differently from other oxidative enzymes as for example laccases. While laccases show a significant side-chain oxidation process,[30] the HRP reactivity is selective toward the oxidation and cleavage of the aromatic ring as shown in *Figure* 4.4 route B. Lignins treated with HRP and coated HRP particles showed increase of condensed OH groups due to oxidative coupling reactions (*Figure* 4.4 route C) on phenolic end groups. As a general trend, the increase in guaiacyl OH groups observed after all the HRP treatments, suggests an increase of hydrophilicity of the polymer and the occurrence of alkyl–phenyl ether bond cleavage reactions which finally result in lignin depolymerisation (*Figure* 4.4 route D). Overall the oxidised insoluble lignins were characterized by a higher content in hydrophilic groups, the treatment with coated HRP particles being more efficient both on MWL and RKL.

Table 4.2- ^{31}P NMR analysis of MWL and RKL before and after the treatments with free and immobilised HRP.

Entry	Lignin	Biocatalyst	Aliphatic OH	Condensed OH	Guaiacyl OH	COOH
1	MWL	none	6.82	0.21	0.92	0.08
2	MWL	HRP	3.32	0.94	1.47	0.50
3	MWL	Immobilised HRP	6.72	0.68	1.13	0.21
4	RKL	none	2.40	1.38	1.48	0.45
5	RKL	HRP	2.45	1.40	1.53	0.52
6	RKL	Immobilised HRP	4.94	6.36	2.68	3.62

[a] Amount (mmolg^{-1}) of aliphatic, phenolic and carboxylic OH groups present on MWL and RKL before and after the enzymatic oxidation, as obtained by quantitative ^{31}P NMR analysis. The ^{31}P NMR data reported in this regard are averages of three phosphytylation experiments followed by quantitative ^{31}P-NMR acquisition. The maximum standard deviation of the reported data was 2x10^{-2} mmol/g, while the maximum standard error was 1x10^{-2} mmol/g. [b] MWL = black spruce milled wood lignin; RKL = softwood residual kraft lignin. [c] Lignins were oxidised with free or immobilised HRP (60 U) in acetate buffer (40 ml) at 40 °C for 12 h.

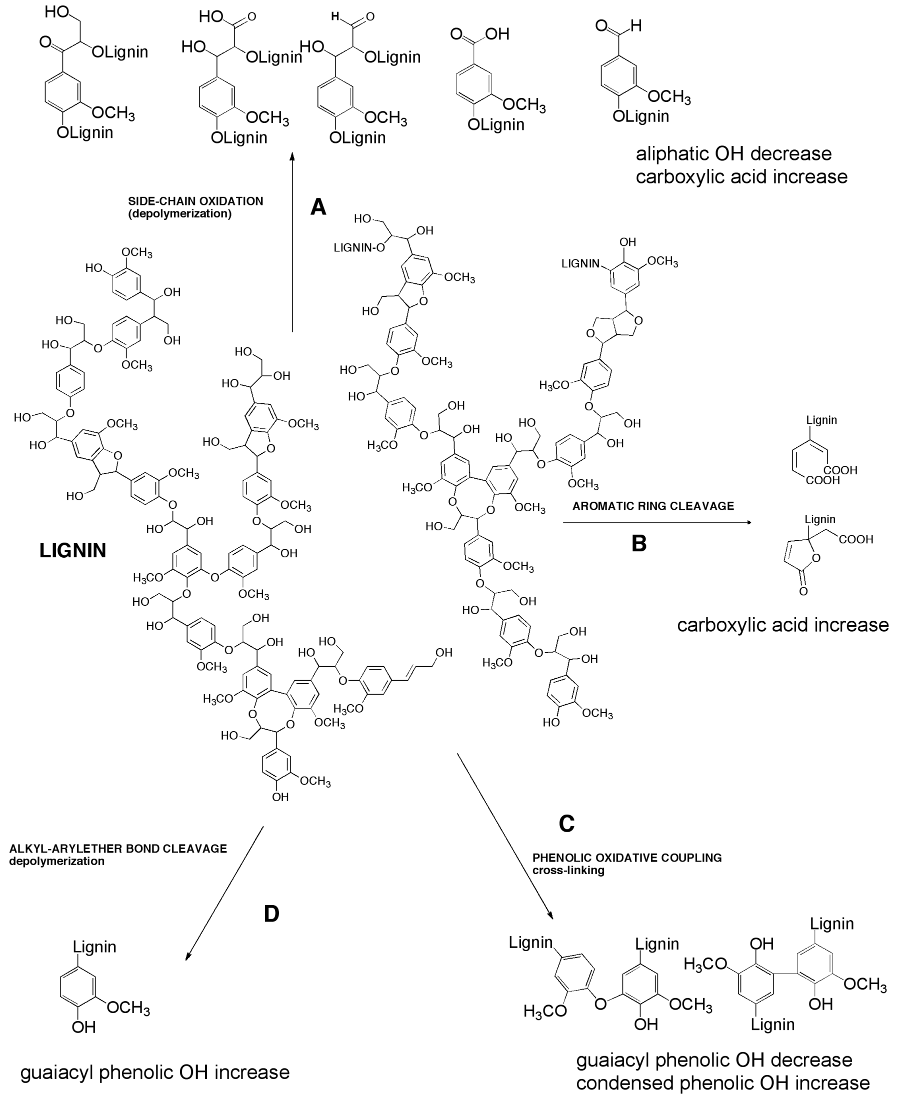

Figure 4.4- Lignin oxidation pathways.

4.2.5 GPC analysis of lignin after treatment with native HRP and coated HRP particles

The lignins isolated after treatments with the soluble and coated HRP particles were submitted to acetobromination according to a previously reported procedure and successively analysed by gel permeation chromatography in order to investigate the effect of the oxidative treatments on lignin molecular weight distribution. GPC was carried out using a system of columns connected in series calibrated against monodisperse polystyrene standards, monomeric and dimeric lignin model compounds. More specifically 4-(1-hydroxyethyl)-2-methoxyphenol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde were used as monomeric and dimeric lignin standard, respectively. *Figure* 4.5 shows the GPC analyses before and after the enzymatic treatments. It is evident that, as expected by previous studies on monolignols polymerisations, the Mn and MW values of the treated lignins were increased by the different HRP treatments. More specifically, the treatment with the coated HRP particles resulted in a more pronounced effect on lignin molecular weight increase with respect to the soluble HRP (*Table* 4.3). The GPC analysis of the soluble fractions obtained from lignin oxidation reactions (material not shown), showed only the presence of low molecular weight fragments, while the polymeric portion was entirely localized in the insoluble isolated lignin.

Table 4.3- Weight-average (M_w) and number average (M_n) molecular weights and polydispersity (M_w/M_n) of lignin samples before and after the enzymatic treatments.

Sample	M_w	M_n	M_w/M_n
Black spruce	190,500	26,200	7.27
Black spruce + free HRP	92,500	17,100	5.41
Black spruce + imm. HRP	81,400	22,700	3.59

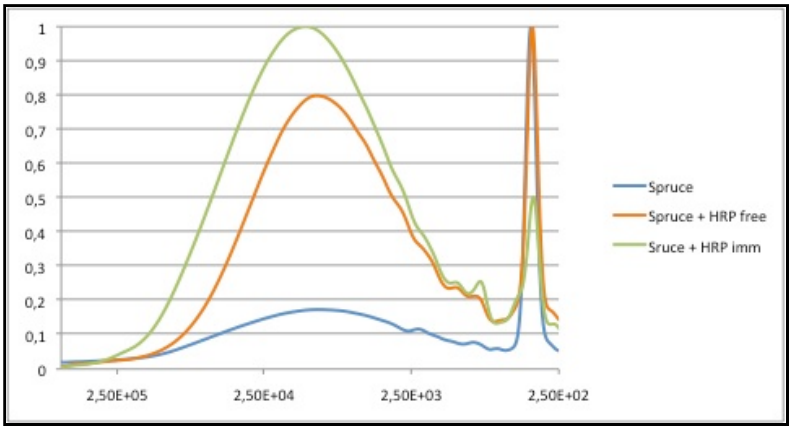

Figura 4.5- Gel permeation chromatography of black spruce lignin before and after the treatment with free and immobilized HRP. All lignins were previously acetobrominated.

4.3 Conclusion

A new immobilised HRP, supported onto alumina particles and coated using the LbL absorption technique was developed. The immobilised enzyme showed an increased stability with respect to the native one.

Unexpectedly, the oxidation of both MWL and RKL by HRP showed about 50% lignin conversion, meaning that about 50% of the starting polymer was solubilised and reduced in low molecular weight fragments (i.e., dimers, trimers mainly). The remaining lignin showed a higher molecular weight distribution meaning that a recondensation/ repolymerisation process occurred upon HRP treatment. The oxidised lignins showed a higher content in phenolic OH groups and carboxylic acids. When coated HRP particles were used for lignin oxidative functionalisation the lignin conversion was about 90%. Thus coated HRP particles are more reactive toward lignin depolymerisation with respect to the native enzyme. The higher reactivity might be due to the protection effect of coating polyelectrolytes toward enzyme deactivation. Moreover the immobilised enzyme does not show kinetic barriers to the approach of the polymeric substrate, this is probably due to the irregularity of the polyelectolytes surface, in which pores can be formed. The insoluble lignin recovered after the treatments showed in this case a higher content in aliphatic, phenolic OH groups and carboxylic acids. Overall the process of lignin oxidation by the HRP/ hydrogen peroxide system seems to proceed through both depolymerisation and recondensation of the lignin molecules. Thus we could hypothesize that the depolymerisation process proceeds together with the oxidative coupling slowly depleting the insoluble lignin and increasing the molecular weight of the residual polymer. The constant content of aliphatic OH groups in all treated lignins seems to indicate the occurrence of preferential condensation reactions on the aromatic rings. This is also supported by the increase of carboxylic acids content that could be due to the occurrence of aromatic ring cleavage processes.

On the basis of these data, HRP and coated HRP particles constitute new selective specific biocatalysts for the oxidative functionalisation and depolymerisation of lignin.

4.4 Experimental

4.4.1 Isolation of residual kraft lignin (RKL) and milled wood lignin (MWL)

MWL was isolated from black spruce. RKL was obtained by using a slightly modified acidolysis procedure as previously reported.[31,32] The yield was 38%, and purity was confirmed by UV and Klason lignin content standard measurements as reported in the literature.[43] MWL was prepared from ultraground extractive-free powder according to Bjorkman’s procedure with some modifications.[44]

4.4.2 Enzyme immobilisation

The enzyme was treated with activated alumina pellets bearing glutaraldehyde linkers previously introduced by the reaction of crude particles with γ-APTS and glutaraldehyde.[35,36] Then, at the end of the reaction, HRP was immobilised on the support.[37] The immobilisation process was carried out according to the following steps. Alumina pellets were silanised with 2% (v/v) γ-APTS in acetone at 45 °C during 20 h. The silanised supports were washed once with acetone and silanised again during 24 h. They were then washed several times with deionised water and dried through air. In the coupling stage the alumina pellets were treated with 2% (v/v) aqueous glutaraldehyde (50%, v/v) during 2 h at room temperature, washed again with deionised water and dried through air. During the grafting, 150 g of support was put in contact with the enzyme, by immersion, for 48h at room temperature, in 250 mL of HRP (5000 U/L) solution obtained by dissolving the enzyme in 100 mM citrate buffer pH 7 with 100 mM NaCl. The particles were then washed several times with 0.05 M phosphate buffer (pH 7) until no enzymatic activity was found in the washing solution. In the final stage, LbL coating, the pellets were first washed three times with 0.1 M NaCl and then the sequential deposition of polyelectrolyte (PSS⁻, PAH+) layers onto the alumina particles was performed. Polyelectrolyte solutions (0.01 M) in 0.5 M NaCl were prepared and the supports were immersed inside each solution during 20 min. Since the alumina pellets with immobilised enzyme were positively charged, microcapsules consisting of three layers were created, starting with the negatively charged polyelectrolyte (PSS-, PAH+, PSS-). After each layer, the excess of polyelectrolyte was removed by washing with 0.1 M NaCl. The particles of immobilised LbL HRP were obtained by simple filtration from the reaction mixture.

4.4.3 Enzyme activity assay

Free HRP activity was determined spectrophotometrically using ABTS as the substrate. The assay mixture contained 0.02 M ABTS, 0.01 M H_2O_2, 0.1 M phosphate buffer pH 7 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 405 nm for 2 min for the free enzyme and for 6 min for the immobilized enzyme, as previously reported.[18] One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min. The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

4.4.4 Enzymatic treatments of lignin

Lignin (80 mg) was suspended in acetate buffer pH 6 (40 mL) with 0.5 mM H_2O_2 and treated at 40 °C with the enzyme (60 U) under vigorous stirring to optimise the contact of the solution with air. After 12 h, the mixture was cooled, acidified at pH 3 and centrifuged. The residue was washed with water three times to eliminate solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analysed by ^{31}P NMR.

4.4.5 Quantitative ^{31}P NMR

Derivatisation of the sample with 2-chloro-4,4',5,5'-tetramethyl-1,3,2-dioxaphospholane was performed as previously described.[14,27,28] Samples of lignin (30mg), accurately weighed, were dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1.0 (v/v) ratio (0.4 mL). Tetramethylphospholane (0.1mL) was then added, followed by the internal standard and the relaxation reagent solution (0.1 mL). The NMRspectra were recorded on a Bruker 300 NMR spectrometer using previously published methods.[27,28] To improve resolution, a total of 256 scans was acquired. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2 x 10^{-2} mmol/g, while the maximum standard error was 1 x 10^{-2} mmol/g.[27,28]

4.4.6 GPC analysis of lignins

Acetobromination of lignin samples for GPC analysis was carried out following the procedure described previously.[37] Briefly, 10 mg of lignin is suspended in acetic acid glacial/acetyl bromide mixture (2.5 ml of 92:8 v/v) and stirred at room temperature. After 2 h the solvent is evaporated under reduced pressure and then the residue is dissolved in 5 ml THF. The GPC analyses were performed using a Shimadzu LC 20AT liquid chromatograph with a SPD M20A ultraviolet diode array (UV) detector set at 280 nm. The sample (20 μl) is injected into a system of columns connected in series (Varian PL gel MIXED-D 5 μm, 1–40 K and PL gel MIXED-D 5 μm, MW 500–20 K) and the analysis is carried out using THF as eluent at a flow rate of 0.50 ml min^{-1}. The GPC system has been calibrated against polystyrene standards (molecular weight range of 890-1.86 x 10^{6} mol^{-1}) and lignin monomers and model dimers. In particular apocynol and (3-methoxy-4-ethoxy-2-phenyl)-2-oxoacetaldehyde were synthesized according to literature procedure[38,39] and used as monomer and dimer lignin standard, respectively.

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5: NMR SPIN TRAPPING AT THE SERVICE OF LACCASE-CATALYZED REACTIONS

5.1 Introduction

Laccase, *p*-diphenol:dioxygen oxidoreductase, catalyses the reduction of molecular oxygen by various organic compounds, typically phenolic systems, to water, through two one-electron oxidation steps.[1] The oxidation proceeds through an outer-sphere electron-transfer process that generates a radical cation, which after fast proton abstraction generates a phenoxyl radical. In particular mechanism studies on the laccase-catalyzed oxidation[2] showed that one mole of oxygen reacts with four moles of phenols giving phenoxy radicals, as shown on *Figure* 5.1:

$$4\,\mathrm{PhOH} + \mathrm{O_2} \xrightarrow{\textbf{laccase}} 4\,\mathrm{Ph\dot{O}} + 2\mathrm{H_2O}$$

Figure 5.1- Laccase reaction.

In nature, this radicals are the intermediate species in modifying the lignin structure; during the years many studies have been focused on the elucidation of laccase rection mechanism. First Kleinert (1967)[3] reported the direct observation of stable radicals in milled wood lignin oxidised by peroxidase, although no detailed chemical structure could be detected. Then Caldwell and Steelink[4] (1969) reported peroxidase-catalyzed formation of phenoxy radicals on a number of lignin monomers and dimers with typical half-lives of about 30 min. Later, Felby *et al.*[5,6] (1997) found a high concentration of stable radicals with a half-life of more than 2 weeks on beech wood fibers subjected to laccase-catalyzed oxidation and detected phenoxy radicals by electron-spin resonance (ESR) spectrometry. Other type of radicals that may be associated with laccase activity are the partly reduced oxygen species superoxide and hydroxyl radicals, such as oxygen radicals have been detected during fungal degradation of lignin.[7-9]

In biotechnological applications, laccase is often used with the addition of so-called ‘oxidation mediators’, which are low molecular weight phenols such as violuric acid (VA) or N-hydroxy derivatives such as 1-hydroxybenzotriazole (HBT), that act as intermediate substrates for the laccase, whose oxidized forms are able to interact with the bulky or high redox-potential substrate and perform the oxidation of all the substrates that could not be oxidized directly from laccase.[10-13]

Many efforts have been made to understand the mechanism of the laccase-mediator system; there are some evidences that oxidation mediators are able to increase the enzyme reactivity by the modification of the reaction mechanism from the one electron oxidation to a hydrogen atom abstraction process.[14]

For many years spin traps have been used to increase the stability of free radicals in order for them to be identified and detected by Electron Paramagnetic Resonance (EPR) spectrometry and thereby allowing the acquisition of abundant information on the production of such species in biological, biochemical and chemical systems.[15] In this technique, a high reactive free radical typically reacts with a double bond of a diamagnetic compound (the spin trap) to form a more stable radical (the spin adduct) which can be detected by EPR spectrometry.[16-20] Nitrones are the most frequently used compound as spin trapping agents, and the spin adducts being in this case a nitroxide. Recent accounts[21] have demonstrated that phosphorus-containing spin traps give rise to radical adducts that have longer half-lives compared to other spin traps. This fact can be used to expand the capability of EPR spectrometry. Anyway, these radical adducts degrade with time, becoming diamagnetic and, therefore, EPR-undetectable. Moreover a given set of EPR parameters may not always clearly characterize a particular spin adduct, and the EPR-spin trap techniques essentially allow to determine the general type of radical trapped. However, the presence of phosphorus within these systems allows for the use of phosphorus nuclear magnetic resonance (^{31}P NMR) spectrometry to investigate the detailed chemistry of radical reactions. This technique was termed "*NMR spin trapping*" by Khramtsov *et al.*,[22] phosphorous NMR could be exploited to perform qualitative analyses since the chemical shift of the ^{31}P atom was found to be strongly dependent on the nature of the adducts,[23,24] and quantitative analysis, in presence of a suitable internal standard.

Various different spin trap species have been investigated; [22,25] recently another novel phosphorous-containing spin nitroxide, the 5-diidopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO), was investigated.[26] It has been demonstrated that the DIPPMPO/$^{\bullet}$OOH adduct is more stable than other traps toward superoxide (O2$^{\bullet-}$) and that ^{31} P NMR signals for DIPPMPO/$^{\bullet}$OOH and DIPPMPO/$^{\bullet}$OH are distinguishable.[15]

In order to obtain a direct evidence of the radicals formation in the laccase and LMS catalyzed reactions, a spin trap technique could be applied. Using the 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO) as spin trap, the presence of phosphorus within this system allows the use of phosphorus nuclear magnetic resonance (^{31}P NMR) spectrometry to investigate the detailed chemistry of the radical reaction. ^{31}P NMR could be exploited to perform quantitative analyses, in presence of a suitable internal standard,[15] and qualitative analyses since the chemical shift of the ^{31}P atom was found to be strongly dependent on the nature of the adducts.[23,24]

During my PhD project, I tried to clarify the mechanism of action in the LMS of different mediators; in fact, to date, the mechanism involved in the system is only still partially understood, but in most of its aspect is unclear. To achieve this task I used the NMR spin trapping technique, that allows to determine and quantify all the radical intermediates involved in the reactions, both in presence and in absence of different chemical mediators and in different experimental conditions. In particular, the mechanism of action of three different mediators (HBT, ABTS, VA) was considered; moreover, to study in detail the radical chemistry of the LMS, the oxidation of phenolic and non phenolic substrates was carried out.

5.2 Results and discussion

For the study I chose to use a laccase from *Trametes versicolor* L., a Basidiomycete that belongs to the family of Poliporaceae, since is one of the most studied fungus for its ability of causing the white rot, a plant pathology that is responsible for the wood degradation. Moreover the reaction were performed in three different experimental conditions to understand the role of dioxygen in the laccase reactions. In fact, during its cycle laccase use air dioxygen as primary oxidant and for this reason I chose to study the radical chemistry in presence of air, in a condition of local ipoxia and in complete anoxia.

In the spin trapping method, the general radical specie (R·) reacts with the double bond of the nitrone spin trap DIPPMPO to form the paramagnetic adduct. This nitroxide paramagnetic species is very unstable and rapidly decays via different reactions (unimolecular or/and bimolecular) such as oxidation, reduction, dismutation and rearrangement. In many cases the products of the decomposition reactions are the diamagnetic corresponding hydroxylamine and nitrone. These species are more stable and suitable detected using ^{31}P NMR; in fact the chemical shift of the phosphorous atom is strongly dependent from the nature of the radical being trapped. Moreover hydroxylamine and nitrone species can be accurately quantified using a phosphorus containing internal standard. In Table 5.1 are reported the chemical shifts for various radical adducts species as, previously reported in literature.[15,22,23]

Table 5.1- Chemical shifts of the different radical adducts detected by ^{31}P NMR analysis.

Radical adduct	Chemical shift
DIPPMPO/·OH	25.0
DIPPMPO/·OOH	17.3 / 16.8
DIPPMPO/·HBT	23.8
DIPPMPO/PhO·	25.2

In the study of the laccase catalytic mechanism no reaction occurred between DIPPMPO and the laccase enzyme. This confirmed that the laccase was not able to oxidize the spin trap since the ^{31}P NMR spectrum showed a single signal at 22.2 ppm due to the starting unreacted DIPPMPO (*Figure* 5.2*,* above). An analogous result has been obtained also in presence of the mediator HBT, performing the reaction in a closed vessel with the head space filled by argon (*Figure* 5.2, below). In this experimental conditions it couldn't be detected any kind of radicals adducts, there was the presence of the spin trap signal at 22.22 ppm only. This indicates that the radical adducts observed during the oxidation reactions are generated from the enzymatic reaction, that can't occur at low oxygen concentrations because the laccase needs the presence of oxygen to complete its catalytic cycle. In *Table* 5.2 are summarised all the experimental data on spin trap reactions with DIPPMPO in presence of laccase obtained by ^{31} P NMR analysis.

Table 5.2 -^{31}P MNR signals for DIPPMPO reaction adducts of HBT with the two generating systems.

Substrates	Generating system	Chemical shift (ppm)
-	-	-
-	Laccase	-
VA	-	-
VA	Laccase	-
ABTS	-	-
ABTS	Laccase	-
HBT	-	-
HBT	Laccase	23.8/17.3/16.8
HBT	CAN	23.8/17.3/17.7/16.8

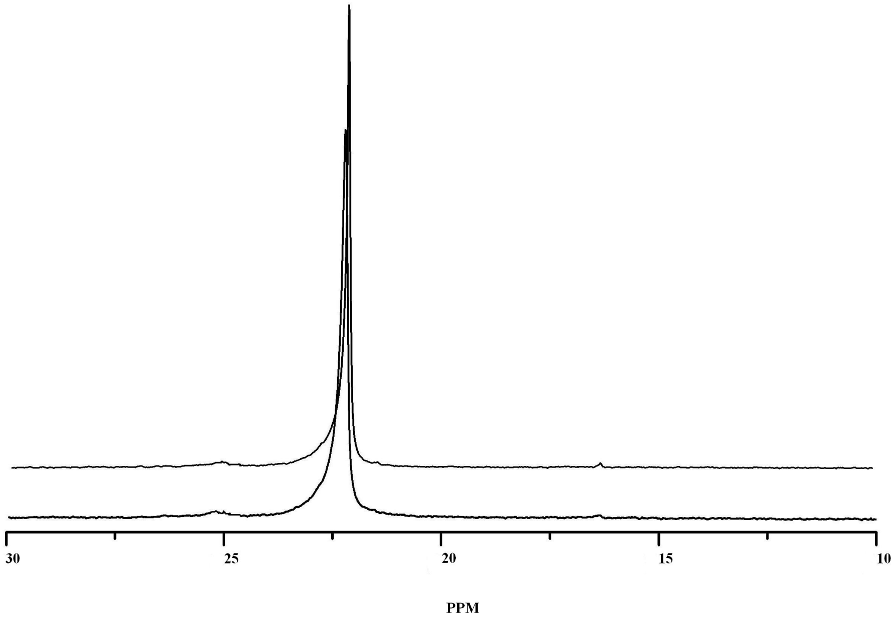

Figure 5.2- ^{31}P NMR spectra of: DIPPMPO and laccase in atmosphere with air bubbling (above); DIPPMPO and laccase in presence of HBT in Argon head space (upon).

In the study of laccase-catalyzed reaction, to clarify the mechanism of action of LMS, I considered three of the most used mediators: VA, ABTS and HBT. In presence of laccase and VA or ABTS it was no possible to detect any signals at ^{31} P NMR analysis, even performing overnight reactions. In both the cases, the presence of the mediator do not generate radicals, neither from dioxygen or from the mediator. Only in presence of HBT it was possible to detect different signals. In *Figure* 5.3 is reported the typical ^{31}P NMR spectra obtained in atmosphere with air bubbling after the DIPPMPO trapping reaction of laccase with HBT after 45 minutes reactions and overnight reactions, respectively.

In the presence of the mediator HBT, the ^{31}P NMR spectrum showed that the DIPPMPO was able to trap and create two different radical adducts. In *Figure* 5.4 is reported the general trapping mechanism of HBT radicals generated by laccase with DIPPMPO.

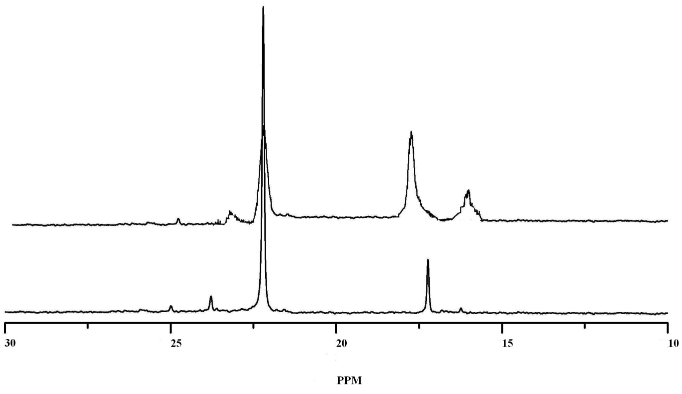

Figure 5.3- ^{31}P NMR spectra of: DIPPMPO and laccase in presence of HBT (above) and DIPPMPO and laccase in presence of HBT (below) with reaction time of 45 minutes and overnight, respectively.

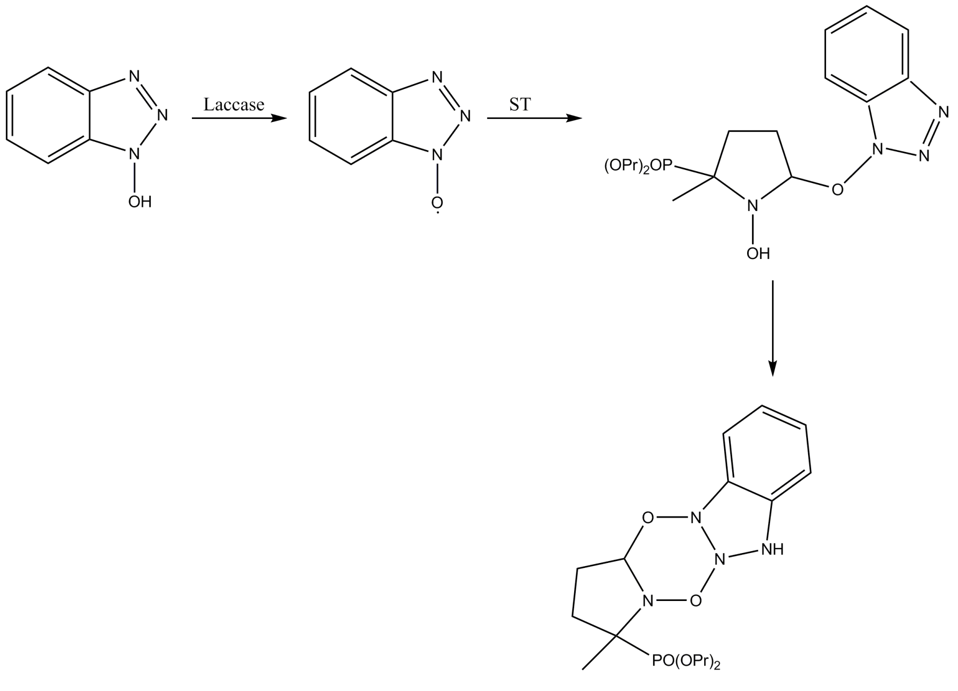

Figure 5.4- Mechanism of trapping HBT radical generated by laccase with DIPPMPO.

In presence of HBT, it was possible to detect signals at 23.8 and at 17.3 ppm, together with another at 16.8 ppm. The signal at 17.3 and 16.8 are due to •OOH radicals.[15] The signal at 23.8 ppm is a direct consequence of adduct formation of the HBT radical with DIPPMPO (*Figure* 5.4) as reported in literature. In particular, *Zoia and Argyropoulos*[23,24] described the presence of two different signals, at 23.5 and 17.9 ppm, in the HBT oxidation by HRP in D_2O. The difference in the chemical shift for our data are probably due to the solvent used, $CDCl_3$ in our case. Moreover this kind of signals are time-dependent, extending the reaction time from 45 minutes to one night their intensity was found increased.

In order to compare the ^{31}P NMR signals obtained from the enzymatic oxidation of HBT, we generated HBT derived radicals with a different oxidation system. As reported by Brandi *et al.*[27] the aminoxyl radical BTNO (benzotriazole-N-oxyl), besides hydroxyl radicals, can be generated by the oxidation of 1-hydroxybenzotriazole with a CeIV salt in MeCN at room temperature (*Figure 5.5*).

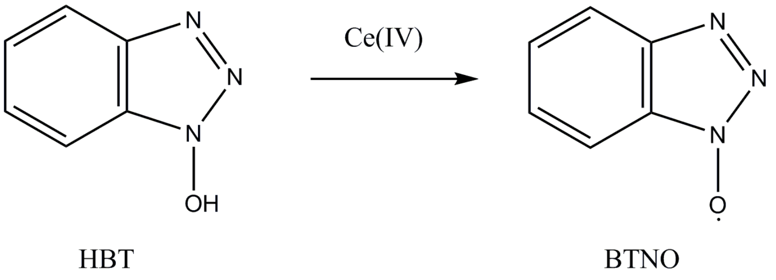

Figure 5.5- HBT radical generation by chemical reaction.

In this case, the DIPPMPO could trap a much higher amounts of radicals after only 5 minutes from the reaction beginning. More specifically, different adducts were observed, giving rise to ^{31}P NMR signals at 25.5-25.1 ppm, at 23.8/17.7 ppm and at 17.3/16.8 ppm. The signal around 25 ppm and at 16.8 and 17.3 ppm are due to •OH and •OOH respectively.[23] Differently from the enzymatic oxidation, in the chemical oxidation the signal intensity is maximum after 5 minutes and start to decrease just after 45 minutes. These data univocally confirm that the signals recorded after radical trapping in the HBT laccase reaction are due to the formation of the HBT radical species.

In order to understand in detail the LMS in presence of HBT, experiments with two different substrates were performed. In particular, the oxidation of a phenolic and non phenolic substrates, guaiacol and benzyl alcohol, was carried out. In the non phenolic compound oxidation, it was detected only the signals due to •OOH/DIPPMPO and HBT•/DIPPMPO radicals adducts, indicating that no radicals were directly formed from the substrate. In the guaiacol oxidation, in presence of the HBT-laccase system (*Figure* 5.6), it was possible to detect signals at 25.2, 23.8, 17.3 and 16.8 ppm. The signals at 17.3 and 16.8 are due to •OOH radicals and the signal at 23.8 ppm is due to the HBT• . Moreover, the signal at 25.2 ppm is due to the phenoxy radical adduct generated by guaicol enzymatic oxidation, as previously described.[23]

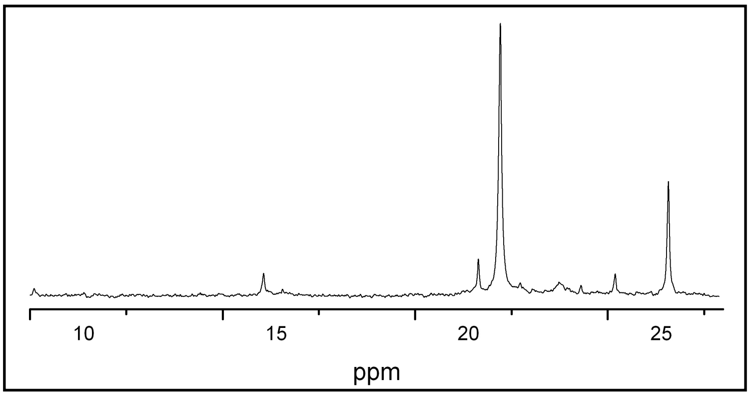

Figure 5.6- ^{31} P NMR spectrum obtained by guaicol oxidation.

The advanced heteronuclear NMR technique allows not only a qualitative analysis but also the quantification, using a suitable internal standard. From the quantitative data obtained (not shown) it is evident that the enzymatic and the chemical reactions have two different kinetics. In fact, the laccase reactions are slower than the chemical ones; using the enzyme, the radical adducts formation start after 45 minutes and reach the maximum values after one night, where the radical adducts formation for the chemically starts after 5 minutes and decreases in overnight reactions.

5.3 Conclusion

In this last year of my PhD project I used the DIPPMPO/^{31}P NMR-spin trap system, that allows the detection and the quantification of radical spin adducts, for studying and elucidate the action mechanism in the laccase-mediator system. The use of the mediator, coupled with the enzyme, improves the catalytic performance and that is actually largely used for industrial applications. From the study it is evident that the mediators follow different mechanism of action. In all the cases, in presence of HBT, VA and ABTS, performing the reactions in local ipoxia or anoxia, it could not possible to detect any kind of radical adduct signals. Moreover it was demonstrated that a fundamental difference, among the action mechanism of the mediators, occurs. In presence of laccase and VA or ABTS it was no possible to detect any radical signal at ^{31}P NMR analysis after DIPPMPO spin trapping, even performing overnight reactions. On the contrary, HBT showed a different reaction mechanism; in fact after only 45 minutes, it was possible to detect radical adduct signals resulted from HBT oxidation (HBT•) and superoxide radicals (•OOH). From this study, it was highlighted that the laccase-HBT mechanism proceeds through radical oxidation of the mediator, with the contemporary generation of superoxide. The basis mechanism, that confers a strong oxidative action to the laccase-HBT system, could be due to the superoxide radicals production. In guaiacol oxidation in presence of HBT, signal related to phenoxy radical have been detected.

This study demonstrated that this innovative technique is a powerful tool to understand the radical intermediates involved in laccase-catalyzed reactions, such as in lignin chemistry. For the future it could be interesting to study the radicals intermediates involved in the oxidation of phenolic lignin model compound to clarify the mechanism of lignin degradation in nature by oxidative enzymes. Moreover it could be also interesting to clarify the reaction mechanism and the radical intermediates involved using immobilised enzymes.

5.4 Experimental

5.4.1 Synthesis of DIPPMPO

DIPPMPO (5-diisopropoxyphosphoryl-5-methyl-1-pyrrolineN-oxide) was synthesized according to a modified two step procedure, in which the catalytic amount of the Lewis acid, boron trifluoride diethyl etherate, was added to shorten the reaction time of diisopropyl-(2-methyl-1-pyrrolidin-2-yl) phosphonate from 12 days to 3 days in high yield (96%).[28] It was then oxidized with H_2O_2 using catalytic amounts of Na_2WO_4. The product, after the dissolution in chloroform (250 mg/ml), was purified by Flash chromatograph (Biotage with column 25+M, flow rate 25 ml/min, UV detection 254 nm). First the column was equilibrated with 36 ml of chloroform methanol (90:10) and then a gradient was established in which the methanol starts to elute from 10% and end a 25% with a total volume of 250 ml. The purity of different collection was checked by ^{31}P NMR: the spectra showed a single resonance at 22.2 ppm, in agreement with literature. The chemical shifts as well as the multiplicities for the proton resonances were: δH (400.13 MHz; $CDCl_3$; Me_4Si) 6.822 (1H, q, $J_{H,P}$ 2.8, $J_{H,H}$ 2.8, HC = N), 4.81 (1H, d sept., $J_{H,P}$ 0.6, $J_{H,H}$ 6.3, $OCHMe_2$), 4.72 (1H, d sept., $J_{H,P}$ 1.1, $J_{H,H}$ 6.3, $OCHMe_2$), 2.65-2.81 (2H, m, CH_2), 2.44–2.55 (1H, m, CH_2), 1.92-2.08 (1H, m, CH_2), 1.60 (3H, d, $J_{H,H}$ 14.79, CH_3), 1.306 (3H, dd, $J_{H,P}$ 0.24, $J_{H,H}$ 6.3, CH_3), 1.286 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.282 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.277 (3H, d, $J_{H,H}$ 6.3, CH_3). EI Mass Spectrum m/z (%): 263 (M+,5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21). The spin trap was stored under argon at -78 °C.

5.4.2 Laccase purification and enzymatic assay

In order to remove impurities or contaminants, laccase powder was purified before using, by an Amicon Ultra 15 Centrifugal filter (Millipore) with a molecular cut off of 30 KDa, then stored in acetate buffer 0.1 M and used within 48 hours. Free laccase activity was determined by a Hewlett Packard 8453E spectrophotometer using ABTS as the substrate. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate pH 5 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 415 nm for 1 min.[29] One activity unit was defined as the amount of enzyme that oxidised 1 µmol ABTS/min.

5.4.3 Mediators oxidation by laccase

The mediators oxidation (HBT, ABTS, VA) was carried out at room temperature in two different experimental conditions: in atmosphere with air bubbling and magnetic stirring and in a closed vial tube with the head space filled by argon. Briefly, in a glass vial tube with screw cap, 0.15 mM of HBT were dissolved in 12 ml of 0.1 M acetate buffer (pH 5.0). In order to completely dissolve the substrate, 2-3 drops of dimethylformamide were added. For the spin trapping experiments, 5 mg of DIPPMPO were added to the mixture. Finally an amount laccase was added (usually 1/10 w/w respect to the amount of the mediator). After 5, 45 minutes or one night the solution was acidified with a 1 M HCl solution until pH 4 and extracted 3 times with chloroform. Then the organic layer was recovered, dried with Na_2SO_4 and evaporated under reduce pressure. The residual was dissolved in $CDCl_3$ for ^{31}P NMR analysis.

5.4.4 BTNO radicals generation

The BTNO radical species was generated in MeCN as previously described.[19] Briefly to a 0.5 mM solution of CAN was added a 0.5 mM solution of HBT and immediately was checked the radical species formation by spectroscopic analysis at 474 nm. Then the spin trapping experiments were performed as described above.

5.4.5 Substrates oxidation by laccase

The substrate oxidation (guaiacol and benzyl alcohol) was carried out at room temperature in a glass vial tube with screw cap, 0.5 mM of substrate, in presence or absence of a mediator were dissolved in 12 ml of 0.1 M acetate buffer (pH 5.0). In order to completely dissolve the substrate, 2-3 drops of dimethylformamide were added. For the spin trapping experiments, 5 mg of DIPPMPO were added to the mixture. Finally an amount laccase was added (usually 1/10 w/w respect to the amount of the substrate). After one night the solution was acidified with a 1 M HCl solution until pH 4 and extracted 3 times with chloroform. Then the organic layer was recovered, dried with Na_2SO_4 and evaporated under reduce pressure. The residual was dissolved in $CDCl_3$ for ^{31}P NMR analysis.

5.4.6 ^{31} P NMR spectra

^{31}P NMR spectra were recorded on a Bruker-300 spectrometer (operating at 121.49 MHz). The chemical shifts reported are relative to external orthophosphoric acid (85%). All spectra were acquired with proton decoupling. To improve resolution, a total of 256 scans was acquired with an acquisition time of 1.60 s. Trimethylphosphate was used as the internal standard for quantification and added to the sample prior to measurement. The ^{31}P NMR data reported in this regard are averages of three oxidation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2×10^{-2} mmol/g, while the maximum standard error was 1×10^{-2} mmol/g.

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6: THE USE OF NMR SPIN TRAPPING TECHNIQUE IN THE STUDY OF LIPOXYGENASE CATALYZED REACTIONS

6.1 Introduction

Lipoxygenases (LOXs) are enzymes that catalyse the oxygenation of polyunsatured fatty acids (PUFAs) to hydroperoxy fatty acids. Although the LOX-reaction involves the formation of radical intermediates it may not be considered an effective source of free radicals as most of the intermediate remain enzyme bound. However under certain condition a considerable portion of radicals may escape the active site leaving the enzyme in the inactive form Fe(II).[1,2] These observation were based on the change in plant LOXs regiospecificity at different oxygen concentration. In fact, under most condition, soybean lipoxygenase-1 is highly specific for the position at which the dioxygen is inserted (position 13). However this marked specificity can be greatly influenced by the oxygen concentration.[2]

A free radical is defined as an unstable form of any atom or molecule capable of indipendent existence (hence the term "free") that contains one or more unpaired electrons.[3] Free radicals can be formed in many different ways such as by the loss of a single electron from a stable molecule, by the gain of an electron to a molecule or also by the break of covalent bonds throught homolitic fission. The free radical's life is very short (10^{-10} sec) and when it is formed, the radical tries to transform in a stable state. In biological systems, free radicals are produced in a wide range of physiological processes by electron transfer reactions, which can be accidentally or deliberately mediated by enzymatic or nonenzymatic reactions. Due to the presence of unpaired electron, free radicals are highly reactive and tend to react with most biomolecules, including proteins, lipids and DNA, finally resulting in oxidative damage. This process is involved in pathology of atherosclerosis, cancer as well as neurodegenerative diseases and the aging processes.[4] The most biologically significant free radicals are the radical derivatives of oxygen, better known as Reactive Oxygen Species (ROS). These include superoxide ($O_2\cdot^-$), hydroxyl radical ($\cdot OH$), nitric oxide ($NO\cdot$), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl).[5]

In order to obtain a direct evidence of the increase in radical escaping from the enzyme active site at different dioxygen concentration, the spin trap technique could be applied. Using the 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide

(DIPPMPO) as spin trap, the presence of phosphorus within this system allows for the use of phosphorus nuclear magnetic resonance (^{31}P NMR) spectrometry.

In the present study we tried to determine and explain the influence of dioxygen concentration on soybean lipoxygenase-1 radical generation. The dioxygen concentration in a reaction solution is a function of two parameters: the initial dioxygen concentration and the rate of consumption. Thus, we varied initial and continuos oxygenation condition (Argon head-space, air bubbling). To determine and quantify the radical intermediates involved in the different experimental conditions, we utilized the DIPPMPO spin trap coupled with the ^{31}P NMR.

The present work has been carried out in collaboration with prof. D.S. Argyropoulos and Dr. Luca Zoia at the North Carolina State University were I spent a six month period of my Ph.D. course.

6.2 Results and discussion

Soya bean lipoxygenase-1 has high sequence identity with all mammalian lipoxygenases, and also its X-rays crystal structure reveals similar details. For this reason, soya bean lipoxygenase-1 has been commonly used as an analogue of the mammalian enzyme.[6-8] Qian *et al.*[9-11] examined the free radical generation in the reaction between soya bean lipoxygenase-1 and linoleic acid by using nitrone spin trapping including DMPO (5,5'-dimethyl-1-pyrroline-N-oxide) and POBN (α-(4-pyridyl-1-oxide)-N-t-butylnitrone). Koshiishi *et al.*[12] have studied the same reaction in presence of CmP (3-carbamoyl-2,2,5,5-tetramethyl-pyrrolinidine-N-oxyl), a well-examined and convenient nitroxyl radical as an indicator for redox balance and free radical reactions. In the present study, we used DIPPMPO (5-diisopropyloxy-phosphoryl-5-methyl-1-pyrroline-N-oxide) as spin trap system to study the radicals involved in the linoleic acid oxidation catalyzed by lipoxygenase.

6.2.1 Identification of lipid-derived radical adducts

As previously described in *Chapter* 5, the general radical specie (R•) reacts with the double bond of the nitrone spin trap DIPPMPO (a) to form the paramagnetic adduct (b). The nitroxide paramagnetic species (b) decays with time via different reactions (unimolecular or/and bimolecular) such as oxidation, reduction, dismutation and rearrangement. In many case the products of the decomposition reactions are the diamagnetic corresponding hydroxylamine (c) and nitrone (d). These species are suitable detected and accurately quantified using ^{31}P NMR in the presence of phosphorus containing internal standard (*Figure* 6.1). Moreover the chemical shift of the phosphorous atom has been shown to be related to the nature of the radical being trapped.

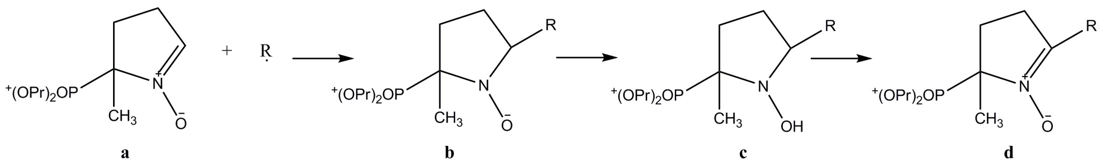

Figure 6.1- The general trapping chemistry of DIPPMPO spin trap system.

In *Figure* 6.2 is reported a series of typical ^{31}P NMR spectra obtained after the DIPPMPO trapping reaction of lipid-derived radicals from linoleic acid/lipoxygenase system at different oxygenation condition. The experiments were run: A) in a screw vial with air satured buffer and head space filled with argon, B) in air without stirring, C) with air bubbling and stirring. The initially oxygen concentrations (approximately 260 μM) were in any case smaller than substrate concentration (642 μM). In the condition A the oxygen is limiting, assuming a stoicometric of 1:1 oxygen and substrate consumption, and untransformed substrate remains on the solution. The amount of the resulting hydroperoxides, detected by UV, correlates with the initially dissolved oxygen content in the reaction mixture (data not show). In the condition B, the oxygen concentration at some point of the reaction becomes limiting due to the low oxygen dissolution rate in absence of stirring respect the consumption by the reaction.[2] In the condition C the oxygen concentration is not limiting relative to the substrate concentration, due to the continuous bubbling throughout the reaction. In this case, a complete depletion of the substrate was detected and all the linoleic acid was converted in the corresponding hydroperoxide.

The peak at 22.2 ppm is related to the original and unreacted spin trap. In the case of linoleic acid oxidation at condition A, the ^{31}P NMR spectrum showed the presence of different adducts at 23.2 and 29.5 ppm (*Figure* 6.2-A). On the basis of the chemical shift values, the adducts have been interpreted as carbon-centered radicals.

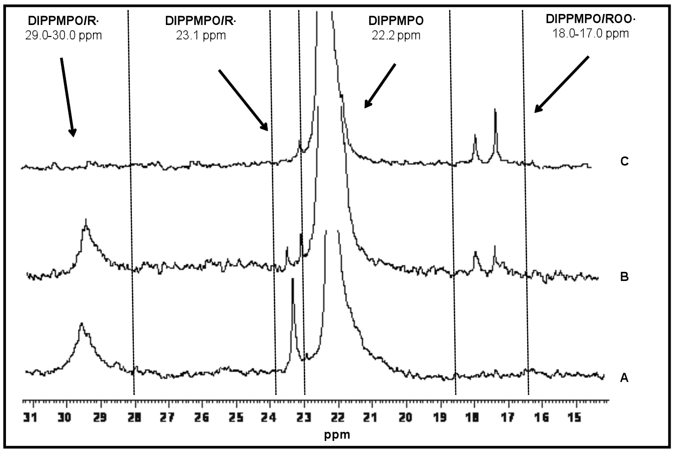

Figure 6.2- ^{31}P NMR spectra of DIPPMPO after reaction with linoleic acid in presence of lipoxygenase: A) in a screw vial with air satured buffer and head space filled with argon; B) in air without stirring; C) with air bubbling and stirring.

As reported by Khramtsov *et al.*,[13] during the trapping of methyl radical with DEPMPO, the ^{31}P NMR spectrum showed the presence of three radical adducts decomposition products: 24.5, 30.8 and 32.3 ppm. The peak at 24.5 due to the similarity with the original spin trap (23.7 ppm) was related to the nitrone product decay, while the peaks at 30.8 and 32.3 ppm were related to the two stereoisomeric hydroxylamines. For the methyl radical trapping with DIPPMPO, the nitrone was detected at 23.1 ppm.[14] In view of this data, the peak at 23.2 ppm in the linoleic/lipoxygenase oxidation was related to the nitrone form of the radical adducts decay product (*Figure* 6.3). In fact the nitrone is shifted 1.0 ppm downfield from the parent spin trap, as it was observed for the methyl radical (0.8 for DEPMPO and 0.9 ppm for DIPPMPO). In the same manner the peak observed at 29.5 ppm was related to the hydroxylamine degradation product. The difference between the adducts and the parent spin trap (7.3 ppm downfield) matches with the difference between the DEPMPO parent spin trap and the hydroxylamine of the methyl radical adduct (7.1 and 8.6 respectively for the two stereoisomers). Moreover the peak related to the hydroxylamine appears broad, indicating maybe the presence of different diastereoisomers.

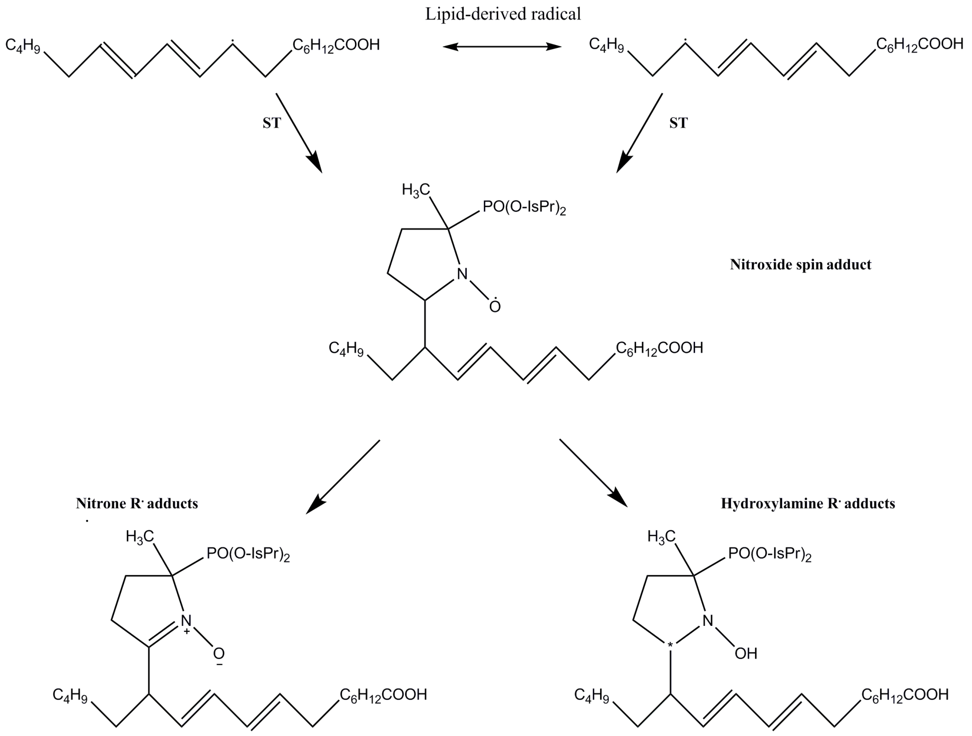

Figure 6.3- Trapping reaction with DIPPMPO of the lipid-derived carbon-centered radicals from linoleic acid/lipoxygenase system and relative decomposition reaction of the spin adducts. For clarity reasons, the structure of the adducts are reported only for the 13 regioisomers.

During linoleic acid oxidation catalyzed by lipoxygenase, the enzyme reacts with the substrate by hydrogen abstraction from the bisallylic methylene position, as reported by literature. The enzyme/substrate radical complex (LOX-R·) may react with molecular dioxygen to form enzyme/peroxyl radical complex (LOX-ROO·). This complex is stabilized during the catalytic cycle via intracomplex electron transfer, which reduces the radical to the corresponding anion (ROO^-). Alternatively, at low oxygen concentration, the LOX-R· could decay liberating the inactive enzyme and the substrate radical R·. The radical, as showed in *Figure* 6.3, is trapped by DIPPMPO and the nitroxide radical adduct, after decomposition reactions, is ^{31}P NMR detected.

During the reaction in condition B (air without stirring) we detected similar radicals adducts at 23.2 and 29.5 ppm. Moreover additional peak were detected at 17.0 and 18.0 ppm (Figure 6.2-B). We interpret these adducts by the trapping of oxygen-centered radicals. In fact, also the LOX-ROO· complex, during the lipoxygenase cycle, could decay releasing the peroxyl radical ROO· in solution. The peroxyl radical could be trapped by DIPPMPO. The peroxyl radical could be also generated in solution by stereo random dioxygenation of the radical R·, escaped from the lipoxygenase active site. As reported by Argyropoulos *et al.*,[14] the HOO· radical adducts was trapped with DIPPMPO and detected by ^{31}P NMR: the chemical shift has been found at 16.9 and 17.1 ppm. A possible trapping reaction is showed in *Figure* 6.4.

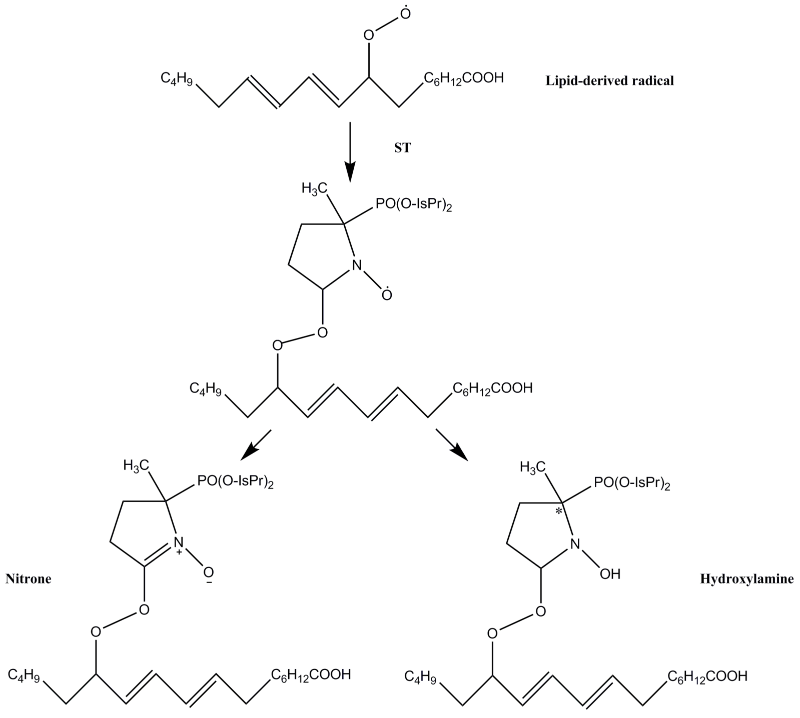

Figure 6.4- Trapping reaction with DIPPMPO of the lipid-derived oxygen-centered radicals from linoleic acid/lipoxygenase system and relative decomposition reaction of the spin adducts. For clarity reason, the structure of the adducts are reported only for the 13 regioisomers.

Similar radical adducts have been reported by Reis *et al.* and detected by LC-MS.[15,16] The authors have studied the radicals involved in Fenton system oxidation of linoleic acid using DMPO as spin trap. By LC-MS they have been able to observe the formation of carbon- and oxygen-centered radical of linoleic acid.

Partial confirmations of our conclusion were found on the ^{31}P NMR spectrum of the reaction in condition C (Figure 6.2-C). Under continuos air bubbling and stirring, the spin trap DIPPMPO was able to trap only oxygen-centered radicals.

6.2.2 Quantification of radical adducts

Using the possibility to quantify the radical trapped by DIPPMPO with ^{31}P NMR, we are exploring how the experimental conditions applied determine the amount of free radical in solution. Initially we compare the amount of radical trapped by DIPPMPO in the LOX/linoleic acid system in the A, B and C experimental condition above described. The data reported in *Figure* 6.5 showed how these simple and different experimental conditions affect the amount of the total trapped radicals.

Therefore we applied the ^{31}P NMR spin trapping system in order to understand how the oxygen concentration affect the lipoxygenase reactivity. We compare the amount of radical adducts formed in two different condition of sample oxygenation. The first serie of experiments were run on atmosphere with air bubbling and vigorous stirring. This system should permit to maintain a constant oxygen concentration (water satured in air, roughly 260 μM) all along the experiment. The second serie of experiments were conducted with the medium satured of air but in sealed vial with the head space filled with argon. While in the first serie of experiment the oxygen has not been limiting and the reaction occurred till the complete depletion of the substrate (checked by UV-Vis measurements), in the second serie the oxygen limited the consumption of linoleic acid.

Thus depending from the starting linoleic acid concentration, the condition reached at the end of reaction varied from normoxic (linoleic acid 160 μM) to anoxic (linoleic acid 642 μM). The data from trapping experiments are reported in *Figure* 6.6. The amount of radicals trapped by DIPPMPO in the lipoxygenase/linoleic acid system for the first serie (gray bars) was in any case lower that the second serie (black bars). Moreover increasing the linoleic acid concentration, the amount of trapped radical increased in significant way.

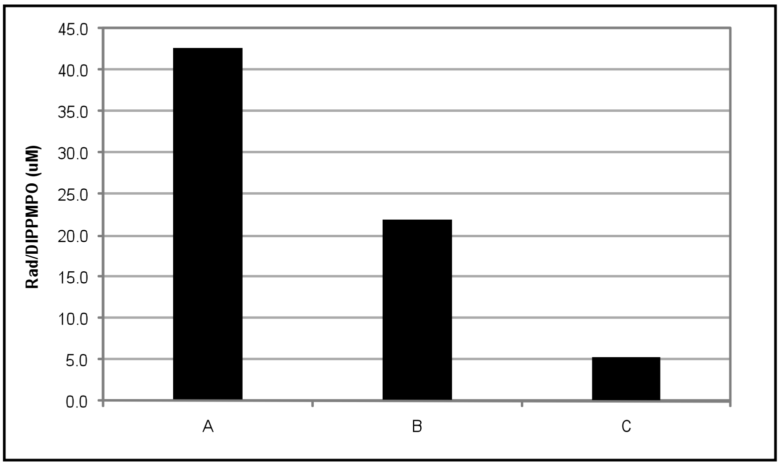

Figure 6.5- Effect of the different experimental condition on the amount of radical adducts: A) in a screw vial with air satured buffer and head space filled with argon; B) in air without stirring; C) with air bubbling and stirring.

As reported from Berry,[2] when the oxygen is not limiting respect the substrate, the specificity of lipoxygenase oxidation reaction is high: the hydroperoxy-octadecadienoic acid (HPOD) 13:9 ratio was close to the generally reported 95:5. When the reaction buffer was bubbled with N_2 before the reaction, LOX catalyzed the oxygenation reaction with a specificity lower than the observed in higher oxygen concentration (13-HPOD:9-HPOD = 55:45). It has been hypothesized that the complex enzyme-linoleic acid radical could dissociate instead of associating with O_2, leading to a release of radical in solution. At normal oxygen concentration (air bubbling), the amount of radicals released is low. As reported in *Figure* 6.6, the amount of radicals trapped was linearly correlated with the amount of starting linoleic acid, and the maximum radical adducts measured was 8.6 µM. The enzyme cycle is complete, with the formation of 13-HPOD as major oxidation product. The percentage of radical adducts respect the initial linoleic acid is a constant value around 1.5 %. The data are in agreement with the observed specificity of 95:5. When the argon head-space conditions were applied, the amount of trapped radical increased in significant way (*Figure* 6.6).

At 160 µM the amount was similar in air bubbling and Argon head-space. At 247 µM it was slightly higher, while at 311 and 642 µM the amount of trapped radical was significantly higher. Assuming a stoichiometric ratio of 1:1 between oxygen and linoleic acid consumption, during the reaction with 160 µM and 247 µM of starting material in presence of air satured medium (O_2, 260 µM), the oxygen was not limiting during the reaction. At 311 and 642 µM during the reaction the oxygen started to be limiting. In this condition the amount of radical released in solution increased. At the end of reaction the amount of radicals trapped were measured to be around the 12 % of the starting substrate. This is in agreement with the observed reduction of specificity at low oxygen concentration.

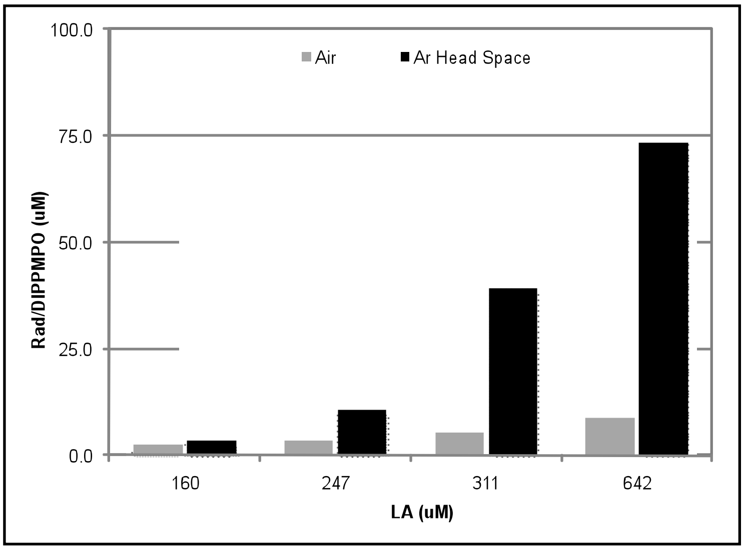

Figure 6.6- Quantification by ^{31}P NMR of radical adducts formation. Condition: 1) atmosphere with air bubbling and stirring (gray bars); 2) Argon head space (Black bar).

Moreover, in qualitative way, we observed on the ^{31}P NMR spectra, that the radical adducts trapped in air bubbling condition were mainly identified as oxygen-centered radicals (LOO·) while in Argon head space condition at high linoleic acid concentration they were mainly carbon-centered radicals. All the results are in agreement with the hypothesized mechanism: at normal oxygenation condition the LOX system is a weak radical generator (low amount of L· could escape from the active site and it react with oxygen in non regiospecific way leading to the formation of oxygen-centered radicals trapped by DIPPMPO). When the oxygen starts to be limiting the amount of radicals escaping the active site is higher and they are trapped mainly as carbon-centered radicals due to the low concentration of oxygen.

Moreover, the same type of experiments were conducted at constant LOX concentration (17.5 nM). The results are shown in *Figure* 6.7.

As in the previous serie of data the amount of radical trapped increased from low linoleic acid concentration (160 and 247 µM) to high linoleic acid concentration (311 and 642 µM). In this case the radicals adducts are not related with the initial starting material concentration. When the oxygen is not limiting the amount of radical trapped is around 7.0 µM, while when at the end of reaction the oxygen is depleted the radicals trapped are roughly constant (38.0 and 35.0 µM respectively). The data indicated that the amount of radical trapped seems to be related to the concentration of the complex enzyme-L·.

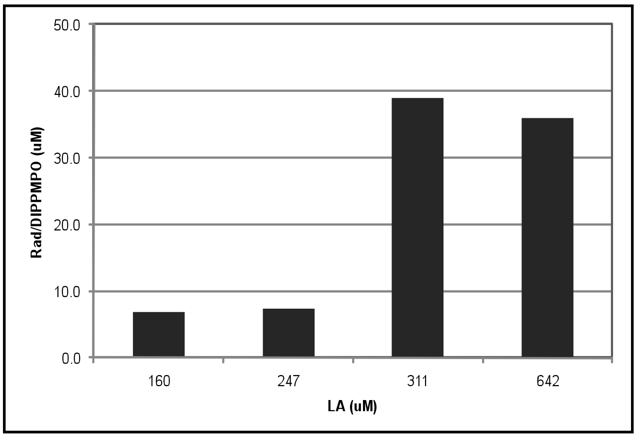

Figure 6.7- Trapped radical in Argon head-space condition at different starting linoleic acid concentration and at constant LOX concentration.

In order to confirm this hypothesis, we run a serie of experiments in argon head-space condition at high concentration of starting linoleic acid (642 µM) to reach anoxic condition during the reaction, with different LOX concentration. The data are reported in *Figure* 6.8. The data indicated that the amount of radicals trapped was linearly correlated with the LOX concentration.

Taking into account all the data shown and reporting the total amount of radical trapped by DIPPMPO related with the LOX concentration on the experiment we can observe a linear correlation. More specifically, if we consider in separated way the data from the experiments where the oxygen was not limiting throughout the reaction vs the experiments where the oxygen was complete depleted, the correlations showed two different angular coefficient (*Figure* 6.9).

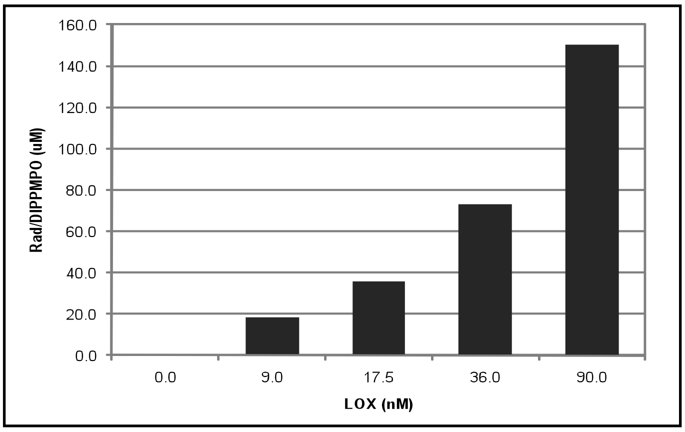

Figure 6.8- Trapped radical in Argon head-space condition at constant starting linoleic acid concentrations and at different LOX concentrations.

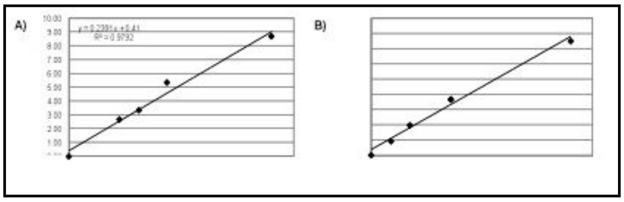

Figure 6.9- Linear correlation between the amount of radical trapped with DIPPMPO and the LOX concentration in the experiment with Argon head space (A) and in air with stirring (B).

The results are rationalized considering that during the linoleic acid oxidation the enzyme exists as a complex with the allyl radical. In normal condition the oxygen is inserted in 13 position leading to the formation of the linoleic acid hydroperoxide. Part of the complex could release the radical in solution. This natural decay is describe quantitatively by the factor 0.24. When the oxygen concentration is low, the release of the radical become more important as reaction pathway. This reactivity is quantitatively describe by the coefficient 1.65.

Moreover it is known in the literature [17] that the enzyme when release the radical in solution became inactive in the form Fe(II). Thus the enzyme need to be reactivated by formation of Fe(III) and this could happen by reaction with an hydroperoxide. To confirm this hypothesis we run this experiment: linoleic acid was oxidized with lipoxygenase in air until the depletion of the starting material (checked by UV-Vis). The reaction was purged with Argon and keep under argon bubbling. An aliquot of linoleic acid was added with the spin trap. After 2 hours we determined the amount of radicals trapped. Qualitatively the radical were of the same type than the previous experiments. Quantitatively the results are showed in *Figure* 6.10 were we put in comparison the amount of radical trapped in air stirring oxidation, in Argon head space and during the anoxic reactivation cycle.

The experiment demonstrated the radical generation from the linoleic acid/lipoxygenase cycle in complete absence of oxygen. Thus in the experiments when initial oxygen concentration are smaller than substrate concentration, the reaction could show three distinct phases in term of radical generation. An oxygen consumption phase due the enzymatic reaction, where the oxygen concentration is not limiting and the radicals generated by the system are low. In this condition the DIPPMPO is able to trap a limited amount of oxygen-centered radicals in agreement with the observed high lipoxygenase specificity. A second phase where the oxygen concentration becomes limiting and the amount of radical trapped increase. This higher amount of released radicals (mainly carbon-centered) is related to the decrease in the enzymatic specificity. A third phase where the oxygen concentration is ~ 0, and the enzyme in presence of either product and residual substrate is able to generate radicals until the complete deactivation.

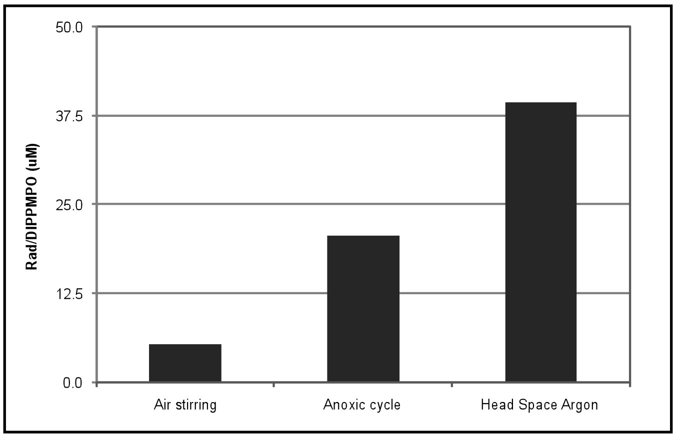

Figure 6.10- Amount of radical trapped in different conditions.

6.3 Conclusion

The present set of preliminary experiments have shown the power of the DIPPMPO spin trapping system toward understanding the chemistry and the intermediate radicals involved in the linoleic acid/lipoxygenase reaction. The spin trap system permit to quantify the radical involve and to correlate them to the experimental condition. We report that under certain condition, the LOX system may generate a considerable amount of free radical and the release of these intermediates may increase the multiplicity of LOX-induced secondary reaction.

6.4 Experimental

6.4.1 Synthesis of DIPPMPO

DIPPMPO (5-diisopropoxyphosphoryl-5-methyl-1-pyrrolineN-oxide) was synthesized according to a modified two step procedure, in which the catalytic amount of the Lewis acid, boron trifluoride diethyl etherate, was added to shorten the reaction time of diisopropyl-(2-methyl-1-pyrrolidin-2-yl) phosphonate from 12 days to 3 days in high yield (96%).[18] It was then oxidized with H_2O_2 using catalytic amounts of Na_2WO_4. The product, after the dissolution in chloroform (250 mg/ml), was purified by Flash chromatograph (Biotage with column 25+M, flow rate 25 ml/min, UV detection 254 nm). First the column was equilibrated with 36 ml of chloroform/methanol (90:10) and then a gradient was established in which the methanol starts to elute from 10% and end a 25% with a total volume of 250 ml. The purity of different collection was checked by ^{31}P NMR: the spectra showed a single resonance at 22.2 ppm, in agreement with literature. The chemical shifts as well as the multiplicities for the proton resonances were: δH (400.13 MHz; $CDCl_3$; Me_4Si) 6.822 (1H, q, $J_{H,P}$ 2.8, $J_{H,H}$ 2.8, HC = N), 4.81 (1H, d sept., $J_{H,P}$ 0.6, $J_{H,H}$ 6.3, $OCHMe_2$), 4.72 (1H, d sept., $J_{H,P}$ 1.1, $J_{H,H}$ 6.3, $OCHMe_2$), 2.65-2.81 (2H, m, CH_2), 2.44–2.55 (1H, m, CH_2), 1.92-2.08 (1H, m, CH_2), 1.60 (3H, d, $J_{H,H}$ 14.79, CH_3), 1.306 (3H, dd, $J_{H,P}$ 0.24, $J_{H,H}$ 6.3, CH_3), 1.286 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.282 (3H, d, $J_{H,H}$ 6.3, CH_3), 1.277 (3H, d, $J_{H,H}$ 6.3, CH_3). EI Mass Spectrum m/z (%): 263 (M+,5), 221 (23), 179 (25), 162 (13), 144 (10), 98 (100), 82 (20), 80 (21). The spin trap was stored under argon at -78 °C.

6.4.2 Enzymatic assay

The enzyme activity was determined by direct measurement of the product formation following the increase of absorbance at 234 nm using a Hewlett Packard 8453E spectrophotometer and found to be 127000 units/mg. One activity unit correspond to the oxidation of 0.12 µmole of linoleic acid.

6.4.3 Linoleic acid oxidation by lipoxygenase

The linoleic acid oxidation was carried out at room temperature in three different experimental conditions: in atmosphere with air bubbling and magnetic stirring, in atmosphere without stirring and in a closed vial tube with the head space filled by argon. Briefly, in a glass vial tube with screw cap (inner volume 70 ml), different amounts of linoleic acid (3.25, 5.0, 6.3 and 13 µL) were dissolved in 65 ml of 0.1 M carbonate buffer (pH 9.0) containing 0.01 M of EDTA (ethylendiaminetatracetic acid). In order to completely dissolve the substrate, 2-3 drops of NaOH 5 % were added. For the spin trapping experiments, 5 mg of DIPPMPO were added to the mixture. Finally different amounts of soya bean lipoxygenase-1 were added (usually 1/10 w/w respect to the amount of the substrate). After 30 minutes the solution was acidified with a 1 M HCl solution until pH 4 and extracted 3 times with chloroform.

Then the organic layer was recovered, dried with Na_2SO_4 and evaporated under reduce pressure. The residual was dissolved in $CDCl_3$ for ^{31}P NMR analyses.

6.4.4 LOX reaction analysis

The LOX reaction was followed spectrophotometrically by measuring the increase in absorbance at 234 nm. For photometric measurements a Hewlett Packard 8453E spectrophotometer was used.

6.4.5 ^{31}P NMR spectra

^{31}P NMR spectra were acquired on a Bruker-300 spectrometer (operating at 121.49 MHz). The chemical shifts reported are relative to external orthophosphoric acid (85%). All spectra were acquired with proton decoupling. The total number of scans for all experiments was 256-1024 with an acquisition time of 1.60 s. Trimethylphosphate was used as the internal standard for quantification and added to the sample prior to measurement.

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7: USE OF LIGNOCELLULOSIC BIOMASSES FOR BIOETHANOL PRODUCTION: IMPROVING THE ENZYMATIC HYDROLYSIS STEP

7.1 Introduction

Production of ethanol and other low-priced products from lignocellulosic biomass has currently great interest. One of the major limitations of the second generation (cellulosic) ethanol production is the hydrolysis step, where fermentable sugars are released from biomass using enzymes.[1] Enzymatic hydrolysis of cellulose has to be improved before commercialisation of the process, increasing the efficiency and decreasing the costs of cellulose hydrolysis. This goal can be achieved in several ways, such improving the pretreatment techniques,[2,3] optimising cellulase enzyme comlex composition,[4] genetic modification of cellulolytic microorganisms,[5] and tailoring enzymes for better performance.[6]

Several studies have focused on the addition of extra enzyme components,[7,8] other kinds of proteins (such as bovine serum albumin, BSA),[9] surfactants[10-13] or other chemicals (such as polymers).[14,15] Surfactants, in particular, have already been recognised as advantageous additives in cellulase production in the late '60s by increasing the cell wall permeability and releasing cell-wall bound enzymes to the liquid phase.[16] It was found later[17] that surfactants prevent cellulases from surface inactivation; in fact the most important enhancing effect of surfactants on lignocelluloses is based on the fact that enzymes can absorb unproductively to the surface of lignin.

Eriksson *et al.*[11] reported a study in which a number of surfactants (such as Tween 20, Tween 80, Triton X-100, Agrimul and SDS) were tested for their ability to improve cellulose hydrolysis. Non-ionic surfactants were found to be the most effective. The addition of Tween 20 was found to be advantageous on the ethanol production in the case of simultaneous saccharification and fermentation of steam-pretreated spruce (SPS).[18] When the structure of the surfactants was further investigated,[14] it was found that the enhancing effect was increased when the ethylene oxide (EO) chain (hydrophilic tail) of the surfactant was longer. As the effect was rather dependent of the length of hydrophilic EO tail[14] instead of the hydrophobic part,[10] pure poly(ethylene oxide) polymer (PEO) and poly(ethylene glycol) (PEG) were also studied, and were found to increase the hydrolysis of SPS. (PEG and PEO polymers are chemically similar, with polymers above 20,000 Da molecular weight called PEO and those below 20,000 Da, PEG.) In a study by Kristensen *et al.*[13] different non-ionic surfactants and PEG 6000 were screened in hydrolysis of wheat straw pretreated with various techniques and PEG was found to be efficient in the increase of cellulose conversion and increase the free endoglucanase concentration after hydrolysis. PEG was further investigated to find the optimal concentration and chain length to achieve the maximal enhancement of the hydrolysis of lignocellulose. PEG prevented enzyme inactivation effectively at greater than 2.5g/l concentration and 4000Da (n=91) molecular weight.[14]

When investigating the effect of PEG addition on the hydrolysis, lignin should get special attention. Lignin has a negative effect on the enzymatic hydrolysis of cellulose, and this is especially pronounced for softwood.[19] When lignocellulosic biomass is steam pretreated the lignin melts and dense agglomerations occur in the cell wall.[20] When PEO polymers were tested for fractionation of kraft pulps by flocculation,[21] the main adsorption sites on lignin were identified as phenolic lignin residues where protons could form hydrogen bonds with the ether oxygens of PEO. PEG 4000 was also tested as an additive in water and dilute-acid pretreatment of corn stover.[22] PEG had little impact on the removal of lignin during hydrolysis in contrast with Tween 80, while it had positive effect on the glucan conversion in enzymatic hydrolysis of the pretreated material.

The aim of the this part of my research, was to test the enzyme adsorption in enzymatic hydrolysis of various steam pretreated lignocelluloses (spruce, willow, hemp, corn stover, wheat straw, sweet sorghum bagasse) and its prevention by PEG addition. The adsorption pattern was investigated in a 72 h enzymatic hydrolysis reaction. As significant differences were observed among the enzyme binding capacities on different substrates, the structure of the steam pretreated lignins has been analysed in order to find a connection between enzyme adsorption, its decrease by PEG addition, and properties of lignin.

This research was carried out in collaboration with Budapest University of Technology and Economics. Hydrolysis treatments and relative measurements were carried out by Bálint Sipos at Department of Applied Biotechnology and Food Science. Lignin structural characterization by ^{31}P NMR was carried out at the Dipartimento di Scienze e Tecnologie Chimiche, Tor Vergata University, Rome, Italy.

7.2 Results and discussion

7.2.1 Composition of various steam pretreated substrates

The compositions of the different steam pretreated lignocellulose substrates showed large variations, especially regarding their residual hemicellulose and lignin content (*Table* 7.1). Substrates from perennial plants had lower lignin content, with wheat straw as an exception, while woody materials and wheat straw had remarkably higher lignin content. The amount of hemicellulose present in the substrate may give information about the severity of the pretreatment, as the more severe the pretreatment is, the better the hemicellulose solubilisation in acid catalysed steam pretreatment.

Table 7.1- Composition of steam pretreated substrates. Mean values of triplicate measurements and standard deviations are presented.

Sample	Cellulose	Hemicellulose	Lignin
spruce (SPS)	62.20±0.17	3.23±0.03	35.57±1.13
willow (SPW)	60.11±2.46	4.36±0.18	30.54±0.29
wheat straw (SPWS)	55.66±0.90	2.54±0.07	44.38±0.20
hemp (SPH)	65.24±0.83	7.99±0.06	24.68±0.20
sweet sorghum bagasse (SPSSB)	48.24±0.59	15.24±0.23	20.33±0.27
corn stover (SPCS)	58.31±0.67	13.73±0.35	19.52±0.27

7.2.2 Cellulose conversion

To test the theoretical maximum in enzymatic hydrolysis were carried out preliminar experiments on SPS taking samples at different time (after 5 min and 3, 6, 24, 48 and 72 h). The effect of PEG on enzymatic hydrolysis of SPS was investigated at 50 °C and 2% of dry matter (DM) substrate concentration. The average hydrolysis rate in the presence of PEG was significantly higher compared to the experiment without PEG (*Table* 7.2). The main difference in the hydrolysis rates was observed during the first 3 h, and the amount of glucose released was nearly twofold higher when PEG was present than when it was not (2.30 and 1.36 mg/ml/h, respectively). Between the 3rd and the 6th hour, the hydrolysis rates were similar (0.30 and 0.33 g/l/h). The hydrolysis rates decreased rapidly after 6 h in both cases, and no significant additional glucose release occurred. In both experiments, the final conversion (*Figure* 7.1) was reached after 48 h, with 80.7% and 95.4% conversion values without PEG addition and in the presence of 2.5g/l PEG, respectively.

Table 7.2- Average hydrolysis rates (liberated sugar between two sampling times) in g/l/h. SPS (2% DM) was hydrolysed at pH 4.8 at 50 °C using 20 FPU/g DM Celluclast 1.5L, supplemented with Novozym 188 to 20 IU/g DM. 2.5 g/l PEG 4000 was added.

Average Hydrolysis rates (g/l/h)						
Hydrolysis intervals	0-0.17 h	0.17-3 h	3-6 h	6-24 h	24-48 h	48-72 h
Hydrolysis without PEG	0.91	1.36	0.30	0.07	0.02	0.00
Hydrolysis with PEG	0.90	2.30	0.33	0.03	0.01	0.00

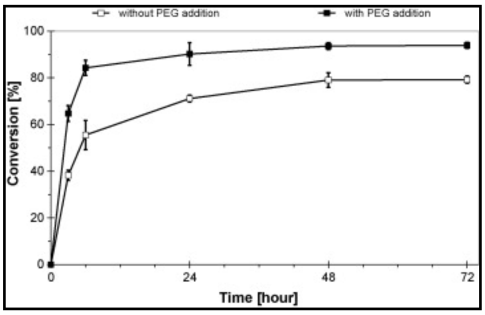

Figure 7.1- Enzymatic conversion of SPS with and without addition of 2.5 g/l PEG 4000. SPS (2% DM) was hydrolysed at pH 4.8, 50 °C using 20 FPU/g DM Celluclast 1.5L, supplemented with Novozym 188 to 20 IU/g DM. Experiment were performed in triplicate, and the mean values and standard deviations are presented.

The 72 h cellulose conversions achieved in hydrolysis experiments for all the six samples are shown on *Figure* 7.2. Significant increase due to PEG addition was observed in case of SPW (p=0.022), SPH (p=0.032) and SPS (Cel) (p=0.002), while there was absolutely no effect of PEG addition in case of SPCS (p=0.228), SPWS (p=0.860) and SPSSB (p=0.120). The increase in case of SPS (Cel+Nov) was rather pronounced, but the statistical evaluation showed no significance (p=0.135) due to the high standard deviation of the 'without PEG' case data.

The positive effect of addition of surfactants on the enzymatic conversion of pretreated lignocelluloses has already been reported to various extent[10,11, 13, 23, 24, 25] and the degree of the conversion enhancement depends on several parameters, such as the nature of the substrate,[13,26] temperature of hydrolysis[15] or enzyme loading[25,27]. In our experiments, the conversion increasing effect of PEG was not pronounced, as relatively high enzyme loading was applied due to the planned adsorption measurements and low (2% DM) substrate concentration because of mass transfer considerations. The different degree of conversions among various substrates is probably determined by the recalcitrance of the substrate or the pretreatment efficiency. When SPS was hydrolysed using Celluclast alone or with combination of Novozym 188 (β-glucosidase supplementation), the addition of either PEG 4000 or Novozym 188 resulted the same order of magnitude increase in cellulose conversion (66% for SPS (Cel), 85% for SPS (Cel) + PEG, 88% for SPS (Cel+Nov)), therefore regarding the cellulose conversion, β-glucosidase supplementation and PEG addition alone had an effect with similar magnitude. When no β-glucosidase supplementation was applied, significant amount of cellobiose was also present in the hydrolysis products, which counted for an additional 15% and 9% cellulose conversion for SPS (Cel) and SPS (Cel) + PEG, respectively. The combination of PEG and Novozym 188 has resulted 93% conversion (SPS (Cel+Nov) + PEG).

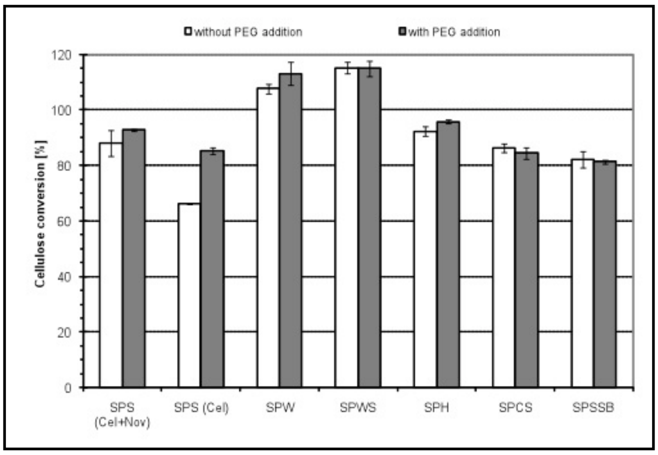

Figure 7.2- Cellulose conversion as percentage of the theoretical maximum in enzymatic hydrolysis (72 hours) of steam pretreated substrates.

7.2.3 Protein adsorption during hydrolysis

Protein concentrations measured in the 72 h hydrolysis supernatants are presented on *Figure* 7.3. Significant increase in protein concentration in the hydrolysis supernatant in case of SPS (Cel+Nov) (p=0.0002), SPS (Cel) (p=0.0002), SPH (p=0.002), SPW (p=0.00005) and SPWS (p=0.002) was observed. For SPCS the difference between the ’with PEG’ and ‘without PEG’ cases was not significant (p=0.156), while for SPSSB a significant (p=0.002) but revers trend was observed. The most pronounced increase in protein concentration due to PEG addition was observed for SPS substrate (both SPS (Cel) and SPS (Cel+Nov)). The decrease in protein concentration can be regarded as adsorption. As most of the differences occurred during the first 24 h of the hydrolysis, changes in protein concentration are presented in that period for SPS (*Figure* 7.4). In hydrolysis of SPS, the protein concentration decreased rapidly from 0.13 to 0.02mg/ml, while in the case of PEG addition, the protein concentration was 0.03 mg/ml after 3 h. From the 3–24-h period, in case of PEG addition, higher free protein concentration could be observed in contrast to the base case, where the decrease in protein concentration was continued at a lower rate.

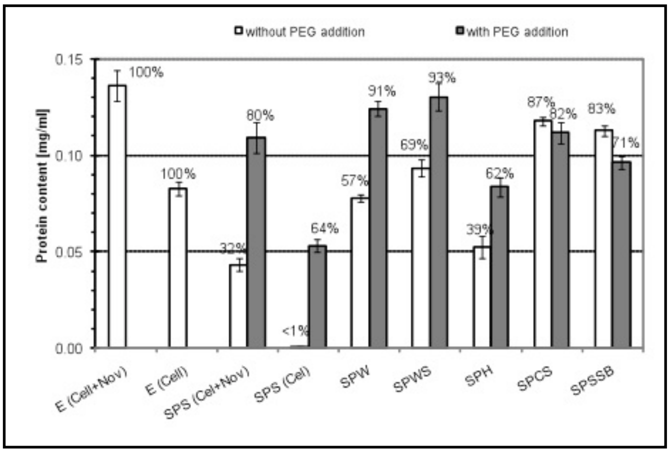

Figure 7.3- Protein concentration in 72 h hydrolysis supernatants. The ratio compared to the originally loaded is indicated in the top of each column. E: enzyme blank. For SPS Cel: only Celluclast 1.5L loaded, Cel+Nov: Celluclast supplemented with Novozym 188. For all other substrates, the two-enzyme system was applied.

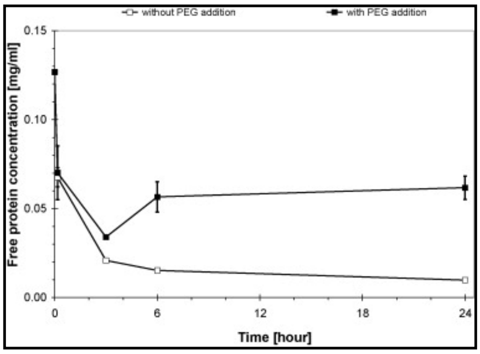

Figure 7.4- Free protein concentration in the supernatant during the first 24-h hydrolysis of SPS. Hydrolysis was performed at 2% DM, pH 4.8, and 50 °C using 20 FPU/g DM Celluclast 1.5 L, supplementd with Novozym 188 to 20 IU/g DM. The mean values of triplicates and standard deviations are presented.

7.2.4 Free FP activity in the hydrolysis supernatants

The free FP and β-glucosidase activities at the end of the 72 h hydrolysis have been measured (*Figure* 7.5A and 7.5B). In general free enzyme activities tend to decrease during enzymatic hydrolysis, due to both the productive binding of cellulases and occurs in the early stage of hydrolysis, when cellulose is still present,[28,29] and the unproductive binding of cellulases on the substrates, especially on lignin.[11,14,15] In our experiment in case of SPS (Cel+Nov) about 90% of the initial FPA has disappeared from the hydrolysis supernatant, while in case of other substrates, the decrease of enzyme activity was less pronounced, resulting in 50-60% decrease. Considering the SPS (Cel), the decrease of FPA in the hydrolysis supernatant was remarkably higher than for SPS (Cel+Nov). For all substrates, decrease of FPA in supernatant was reduced by PEG addition. The highest effect of PEG addition was in case of SPS (7.7-fold higher activity for SPS (Cel+Nov) and about 90-fold increase for SPS (Cel)), while no significant difference ($p=0.571$) due to PEG addition was observed on

SPSSB. For SPCS, SPH, SPW and SPWS, 53%, 64%, 68% and 51% increase in FPA was observed, and the difference was found to be significant (p<0.0015).

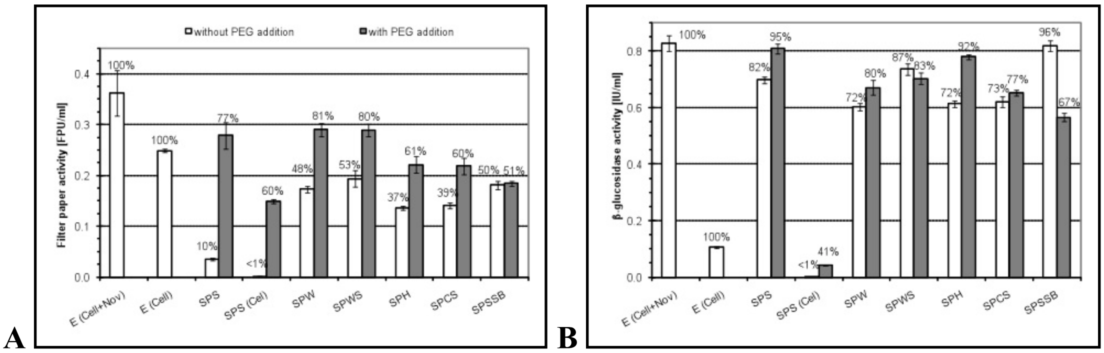

Figure 7.5- FP (A) and β-glucosidase (B) activities in the hydrolysis supernatants. The ratio compared to the originally loaded activity is indicated on the top of each column. E: enzyme blank. For SPS Cel: only Celluclast 1.5L loaded, Cel+Nov: Celluclast 1.5L supplementd with Novozym 188. For all other substrates, the two-enzyme system was applied.

The decrease in protein concentration can be regarded as adsorption, in contrast to the results achieved with free enzyme activity mesurements, where other factors, like enzyme inactivation or synergistic effects may also occur.The effect of PEG addition on free protein concentration and free FPA showed a significant correlation (r=0.868), suggesting that the most of the protein detected was involved in cellulose degradation.

During steam pretreatment, lignin undergoes several structural changes, like (1) melting and reprecipitating, resulting in dense lignin-agglomerates,[30] (2) degradation and repolymerisation,[31] and (3) other side-chain reactions.[31] As decrease of free FPA was found to be different using various steam pretreated substrates, and similar (acid catalysed steam pretreatment) treatment was applied for the substrates, there might be difference in the lignin structures of the substrates even though the same kind of pretreatment has been used. These lignin structures tend to (1) bind cellulases and (2) adsorb PEG to a different extent. The results show that the positive effect of PEG addition is not universal, since there were substrates, like SPSSB or SPCS, where no positive effect could be observed, while it was pronounced in case of SPS. This could be due to the typical softwood lignin structure of spruce that does not show the occurrence of p-hydroxyl and condensed phenolic OH groups, while hardwood lignins contain syringyl units and annual plant lignins contain also significative amounts of p-hydroxyphenyl units.

For β-glucosidase, the decrease of enzyme activity and the difference between the 'without PEG' and the 'with PEG' cases were less pronounced when both Celluclast 1.5L and Novozym 188 was used (*Figure* 7.5B). The positive effect of PEG addition on the free β-glucosidase activity was significant for SPS (Cel+Nov) (p=7·10-4), SPS (Cel) (p=10-5), SPW (p=0.017) and SPH (p=4·10-5), while the increase has not reached the level of significance with SPCS (p=0.063). Reverse effect of PEG addition on free β-glucosidase activity in case of SPSSB (p=4·10-5) and SPWS (p=0.117) could be noticed. The most pronounced difference was observed in hydrolysis of SPS with Celluclast 1.5L alone, where a 100-fold increase in free β-glucosidase activity was observed due to the addition of PEG.

There are contradictory results in the literature about β-glucosidase adsorption. Several papers reported significant β-glucosidase adsorption on lignocelluloses[2,32] while others observed less binding of β-glucosidases,[29,33,34] latter are more comparable with our previous result obtained with SPS (Cel+Nov). The main difference in the referred papers was the origin of the enzymes used in the hydrolysis/adsorption experiments. To clarify this problem, hydrolysis of SPS has also been performed with *Trichoderma* enzymes alone (SPS (Cel)), i.e. without *Aspergillu*s *niger* β-glucosidase supplementation. Surprisingly, when only *T. reesei* β-glucosidase (Celluclast 1.5L only) was present in the hydrolysis mixture almost no free β-glucosidase activity was observed after 72-hour hydrolysis; compared to the other case (SPS (Cel+Nov)), where *A. niger* β-glucosidase (Novozym 188) was also present. This drastical decrease in β-glucosidase activity has been reflected in the decrease of FPA as well. Addition of PEG 4000 has increased efficiently the free β-glucosidase activity in hydrolysis supernatant when only *T. reesei* enzyme was present, and had less impact on the β-glucosidase activity of the supernatant in case of the Celluclast 1.5L+Novozym 188 mixture. The difference in the adsorption behaviour of β-glucosidases from different enzyme sources may be due to their molecular properties. These enzymes lack carbohydrate binding modules, which were previously shown to be important in unproductive binding of cellulases.[15] Other surface properties affecting the enzyme adsorption on lignin, like exposed hydrophobic amino-acid side chains or different degree in protein glycosylation could also be the reason for this difference.

7.2.5 Pyrolysis-GC/MS analysis of steam pretreated substrates

The steam pretreated substrates were analysed for the characteristics of the lignin structure by Py-GC/MS. When the pyrolysis parameters are set carefully, mostly the ether bonds between the monomeric units are disrupted during pyrolysis and monomeric lignin products are released. However, other chemical bonds are also affected; mainly the aliphatic side chains are cleaved. The main groups of lignin pyrolysis products are visualised in *Figure* 7.6. SPS, the substrate with the highest affinity for adsorbing enzymes has differed regarding the ratio of pyrolysis products compared to the other substrates (*Table* 7.3). It released the highest yield of dihydroxybenzenes, although the original spruce lignin contains mostly 2-methoxyphenol segments (data not shown). This can be explained by the demethylation of the aromatic methoxyl-groups during the steam pretreatment which is in good accordance with previous reports.[35,36] Relatively high ratio of pyrolysis products with carbonyl groups on the alkyl side chain were also produced from SPS together with SPW and SPH. On the other hand, small amount of alkenyl phenols and H-lignins (no methoxyl substitutions) are formed from the wood samples (SPS and SPW) and these products are, as expected, more abundant from the other plant samples.

Table 7.3- Ratio of lignin derived pyrolysis products for various steam pretreated substrates. The phenolic compounds were measured by GC/MS.

	Carbonyl compounds	Dihydroxy-benzene compounds	Vinyl compounds	Alkenyl compounds	H-lignin
	Pyrolysis product/ total lignin products				
SPS	0.154	0.273	0.100	0.158	0.152
SPW	0.171	0.123	0.168	0.225	0.159
SPWS	0.124	0.110	0.225	0.283	0.200
SPH	0.173	0.030	0.228	0.430	0.151
SPCS	0.052	0.034	0.441	0.506	0.510
SPSSB	0.065	0.022	0.388	0.467	0.360

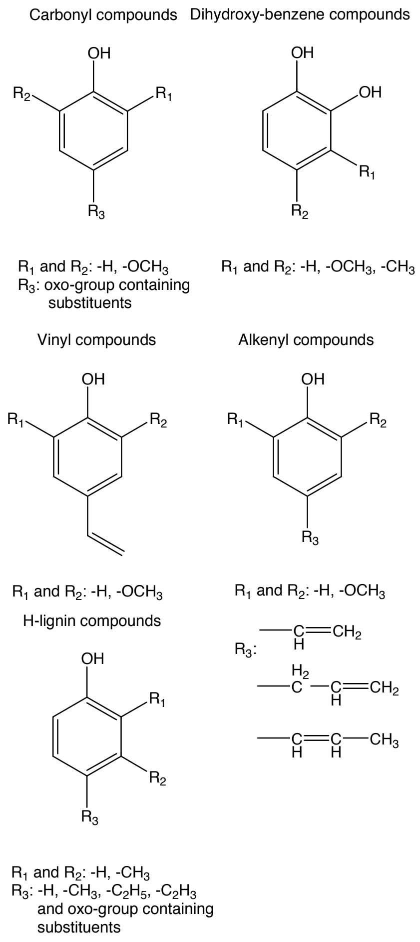

Figure 7.6- Groups of lignin pyrolysis products.

7.2.6 Lignin structure analysis

In order to clarify the chemical modifications occurred in lignins before and after the hydrolysis processes, we used an advanced heteronuclear magnetic resonance analysis, the ^{31}P NMR, after sample phosphitylation with 2-chloro-4,4’,5,5’-tetramethyl-1,3,2-dioxaphospholane. On *Figure* 7.7, spectrums of the steam pretreated materials are shown, together with the detailed signal assignment and in *Table* 7.4 the quantitative data, for the functional groups, before and after the hydrolysis, obtained by phosphorus NMR are reported.

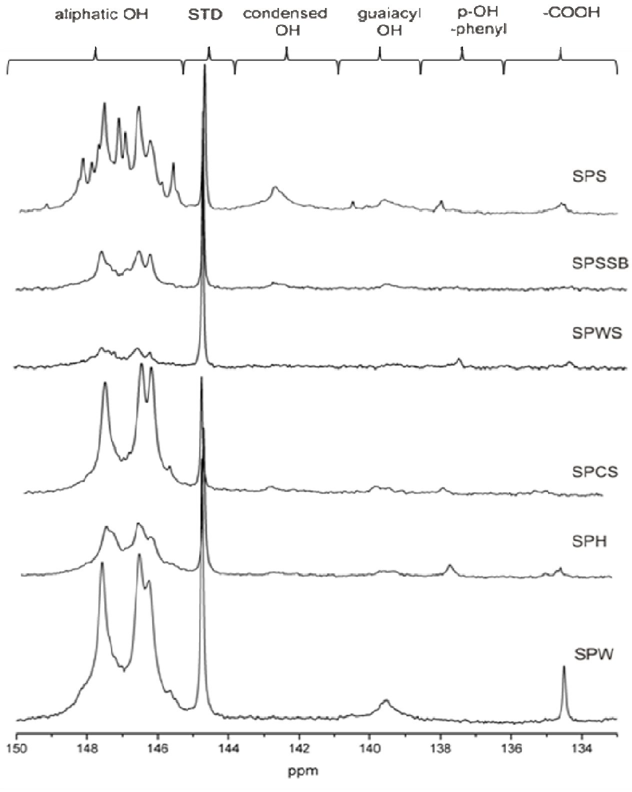

Figure 7.7- ^{31}P NMR spectra of steam pretreated substrates, with the chemical shifts related to the various structures.

As steam pretreated samples contained significant amount of cellulose and some hemicellulose besides lignin, a factor was calculated from the carbohydrate conversions for the hydrolysis residue samples. Multiplying the results obtained by NMR shows the quantity of various hydroxyl groups present in hydrolysis mixture. The amount of aliphatic hydroxyl groups showed large variations, while most of the substrates contained between 1 and 2 mmol/g SPWS and SPCS contained significantly higher and lower amount, 3.05 mmol/g and 0.52 mmol/g, respectively. This is probably connected with the pretreatment conditions; for most of the substrates sulphur-dioxide catalyst was used during pretreatment, where for wheat straw, sulphuric acid was supplied. In the corn stover pretreatment no catalyst was added. On the basis of guaiacylic OH groups content; the softwood steam pretreated samples could be divided in two different groups: SPW and SPWS with an average content of 0.15 mmol/g of this functional group, and SPH, SPCS, and SPSSB, with an average amount more than two times lower (0.06 mmol/g). In the case of condensed hydroxyl groups, SPW and SPWS contained significantly higher amount compared (average amount 0.42 mmol/g) to the other substrates (average amount 0.1 mmol/g).

Table 7.4- ^{31} P NMR analysis of the original steam pretreated substrates and that of the hydrolysis residue.*

Sample[1]	Relative quantity in hydrolysis[2]	Aliphatic OH	Condensed/ siryngyl OH	Guaiacyl OH	p-OH phenyl	COOH
SPS 1	1.000	1.760	0.086	0.116	0.003	0.079
SPS 2	0.482	2.173	0.296	0.258	0.072	0.195
SPS 3	0.453	2.584	0.462	0.417	0.143	0.173
SPW 1	1.000	1.957	0.428	0.158	0.079	0.194
SPW 2	0.371	2.925	0.493	0.162	0.068	0.133
SPW 3	0.341	3.258	0.731	0.257	0.127	0.180
SPWS 1	1.000	3.050	0.421	0.173	0.113	0.127
SPWS 2	0.371	3.931	0.473	0.274	0.187	0.234
SPWS 3	0.373	2.748	0.465	0.277	0.176	0.222
SPH 1	1.000	1.130	0.132	0.072	0.028	0.098
SPH 2	0.381	4.350	0.280	0.122	0.071	0.251
SPH 3	0.356	4.339	0.290	0.145	0.079	0.162
SPCS 1	1.000	0.516	0.078	0.042	0.055	0.083
SPCS 2	0.498	1.689	0.124	0.053	0.081	0.114
SPCS 3	0.518	2.949	0.384	0.198	0.172	0.329
SPSSB 1	1.000	1.264	0.103	0.066	0.058	0.117
SPSSB 2	0.471	2.626	0.274	0.149	0.184	0.243
SPSSB 3	0.454	2.753	0.218	0.107	0.134	0.219

* Amount (mmol/g) of aliphatic, phenolic and carboxylic OH groups present on pretreated material as evaluated by quantitative ^{31} P NMR analysis. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative 31P NMR acquisition. The maximum standard deviation of the reported data was 2 x 10^{-2} mmol/g, while the maximum standard error was 1 x 10^{-2} mmol/g. [1]Steam pretreated substrates: 1) steam pretreated material; 2) hydrolysis residue; 3) hydrolysis + PEG residue. [2]Calculated from the carbohydrate conversions.

After the hydrolysis, both with and without PEG addition, the residual lignins showed an increase of aliphatic, condensed and guaiacyl OH groups with respect to the preatreated material. As a general trend the increase in aliphatic OH groups could be explained as the occurrence of hydrolysis reactions of linkages in the lignin side chains, and alternatively to the hydrolysis of lignin-carbohydrate complexes. Moreover, the increase in the amount of guaiacyl OH groups usually suggests the occurrence of alkyl-phenyl ether bond cleavage reactions which finally result in lignin depolymerisation, while the increase of condensed phenolic OH groups is due to the occurrence of oxidative coupling processes on phenolic end groups. However, under the reaction conditions used no oxidation reactions are likely to occur unless the enzymes used were contaminated with traces of oxidative enzymes. Such modifications in lignin structure might be due to hydrolysis of ester bonds throughout the lignin backbone. This finding would explain the relative high amount of carbonyl groups detected by Py-GC-MS.

7.2.7 Mechanism of PEG effect o enzyme adsorption

Addition of PEG in enzymatic hydrolysis of steam pretreated substrates was shown to increase free cellulase activity in the hydrolysis supernatant. PEG has been previously found to adsorb on the substrate in enzymatic hydrolysis[14] and when incubated with SPS alone.[15] In addition, insignificant PEG adsorption was observed on microcrystalline cellulose,[15] suggesting that PEG adsorbs on the surface of the lignin. Our experiments have shown that the amount of dihydroxybenzene pyrolysis products correlated with the increase of free FPA due to PEG addition.

The interactions occurring in protein adsorption have been recognised as non-covalent interactions like (1) hydrogen bonding, (2) electrostatic or (3) hydrophobic interactions.[37] It has been shown, that surfaces with polyethylene oxide grafts show greatly reduced protein adsorption.[38] PEG can attach to the lignin (1) terminally, or (2) by interaction between the lignin and an internal atom of the polymer. The first interaction results in a 'fur-like' coat on the lignin surface with high volume excluded by PEG and require high PEG dosage to cover the whole surface, while the latter results in a thin PEG layer on the surface and require less PEG.

Our results suggest that the interaction is formed between PEG and the phenolic hydroxyl groups of lignin. The importance of phenolic hydroxyl groups in lignin and cellulase inactivation has already been hypothesized.[39] Lignin modified by hydroxypropylation (specific modification of phenolic hydroxyl group) was found to be less detrimental to cellulose hydrolysis, than unmodified lignin.[39] Phenolic hydroxyl groups exposed on the lignin surface can easily form hydrogen bonds with the internal oxygen atoms of the PEG polymer. This would mean that in this case PEG is accepting, and free phenolic hydroxyl group of lignin is donating the hydrogen bond. It has already been suggested that ethylene oxide based surfactants are hydrogen bond accepting ones.[15] However, the occurrence of hydrophobic interaction cannot be excluded with our experimental results.

7.3 Conclusion

Addition of PEG polymer to enzymatic hydrolysis of steam pretreated lignocelluloses was found to be beneficial by increasing the free cellulase activity in the hydrolysis supernatant. The increased concentration of active cellulases can result in a faster and more efficient cellulose conversion, a possible decrease in enzyme need or possibility for enzyme recycling.

The positive effect of PEG addition was found to be different using various pretreated substrates, with steam pretreated spruce showing the highest affinity for enzymes. The results suggest that the different degree of increased free cellulase activity obtained by PEG addition is based on the various structures of lignin in the substrates. It was concluded that phenolic hydroxyl groups exposed on the lignin surface interacts with PEG through hydrogen bonding, forming a layer of PEG on lignin surface, which prevents unproductive binding of cellulases on lignin.Decrease of free β-glucosidase activity was found to be dependent on the source of the enzyme. *Trichoderma reesei* β-glucosidase (in Celluclast 1.5L) tends to bind unproductively in higher degree than *Aspergillus niger* β-glucosidase (in Novozym 188). Our results suggests that increasing β-glucosidase secretion of T. reesei in the enzyme fermentation is not the key solution for the problem of β-glucosidase deficiency of the culture broths, but addition of β-glucosidase from other enzyme sources may be more beneficial.

7.4 Materials and methods

7.4.1 Substrates and enzymes

Six steam pretreated substrates were used in the hydrolysis experiments; one from softwood (spruce), one from hardwood (willow), and four perennial crop residues (wheat straw, hemp, sweet sorghum bagasse and corn stover). Preatreatment conditions are summarised in *Table* 7.5. The substrates were rinsed with triple amount of warm (~60 °C) distilled water to remove much of the soluble compounds.

Celluclast 1.5L had an overall cellulase activity of 88.8 FPU/ml, and β-glucosidase activity of 38.6 IU/ml. Novozym 188 had 408 IU/ml β-glucosidase activity.

7.4.2 Enzymatic hydrolysis

Enzymatic hydrolysis was performed at 50 °C, pH 4.8 (in 0.05 M sodium acetate buffer) in 100 ml screw-capped bottles at 300 rpm agitation speed using magnetic stirrers. Experiments were carried out in 50 g batches with a substrate concentration of 2% DM. The low substrate concentration was applied to eliminate the mass transfer problems. Enzyme loading of Celluclast 1.5L was 20 FPU/g dry substrate, and 40 IU/g DM Novozym 188. Hydrolysis of SPS was also performed without β-glucosidase supplementation, named SPS (Cel), while as SPS (Cel+Nov) when both Celluclast and Novozym were used). Hydrolysis was performed in triplicate with and without addition of 2.5 g/l PEG with an average molecular mass of 4000 g/mol (Merck, Hochenbrunn, Germany).

Table 7.5- Steam pretreatment conditions of the six samples.

Raw material	Reactor type	Impregnation	Temperature	Residence Time
spruce (SPS)	batch	2.5% SO_2	210°C	5 min
willow (SPW)	batch	2% SO_2	190°C	12 min
wheat straw (SPWS)	batch	0.2% H_2SO_4	190°C	10 min
hemp (SPH)	batch	2% SO_2	205°C	5 min
sweet sorghum bagasse (SPSSB)	batch	2% SO_2	190°C	10 min
corn stover (SPCS)	flow-through	-	210°C	15 min

7.4.3 Preparation of supernatant

Before measuring the residual enzyme activities and protein content in the hydrolysis supernatant, 15 ml of each sample was concentrated ten times, using an ultrafiltration device (Vivaspin 4 centrifugal filter unit with 10-kDa polysulfone membrane, Sartorius, Germany) and “washed” with buffer (5 ml, 0.05 M Na-acetate, pH 4.8) to remove sugars and other small molar mass compounds that interfere with enzyme activity measurements.

7.4.4 Enzyme activities and protein content

Filter paper activity (FPA) measurements were carried out according to Mandels *et al.*,[40] with the modification, that an enzyme dilution releasing 1 mg glucose was used instead of 2 mg. To keep the pH at 4.8, sodium acetate buffer (0.05 M) was used. β-glucosidase activity was measured according to Berghem and Pettersson[41] with minor modifications. The substrate was 5mM 4-nitrophenyl-β-D-glucopyranoside (PNPG). A volume of 1 ml preincubated substrate was mixed with 0.1 ml diluted enzyme solution and incubated for 10 min at 50 ◦ C. The reaction was terminated by addition of 2 ml of 1 M Na_2CO_3 solution and then diluted with 10 ml distilled water. The amount of the liberated 4-nitrophenol was measured at 400 nm against a blank.

Protein content in the hydrolysis supernatant was measured according to Bradford[42] using Coomassie Brilliant Blue dye with BSA as a standard.

7.4.5 Determination of protein adsorption and free residual enzyme activities

Total protein concentration and residual enzyme activities (FP and β-glucosidase) were measured in the hydrolysis supernatants in order to quantify the protein adsorption and the free residual enzyme activities. Prior to analysis, 15 ml of each sample was concentrated, using an ultrafiltration device (Vivaspin 4 centrifugal filter unit with 10 kDa polysulfone membrane, Sartorius, Germany) and “washed” with buffer (5 ml, 0.05 M Na-acetate, pH 4.8) to remove sugars and other small molar mass compounds that interfere with enzyme activity measurements.

7.4.6 Analysis

Lignin and carbohydrate content of raw and pretreated materials were analysed using the NREL protocol[43] with minor modifications. The amount of oven dried (105 °C) sample was 0.5 g and was hydrolysed with 2.5 ml 72% sulphuric acid at room temperature for 2 h. After the reaction time was reached, this mixture was diluted with 72.5 ml distilled water and the hydrolysis was continued at 121 °C for 60 min. The samples were filtered and washed with distilled water through G4 glass filter crucibles. The remaining lignin on the filter was dried at 105 °C, weighed and placed in a furnace at 550 °C for 6 h. The Klason lignin content was taken as the ash free residue after acid hydrolysis.

7.4.7 Pyrolysis gas chromatography/mass spectrometry (Py-GC/MS)

Approximately 0.6 g samples were pyrolyzed at 600°C for 20 s in helium atmosphere using a Pyroprobe 2000 pyrolyzer interfaced to an Agilent 6890A/5973 gas chromatograph/mass spectrometer. The pyrolysis products were separated on an HP-5MS capillary column (30 m x 0.25 mm, 0.25 μm film thicknesses). The GC oven was programmed to hold at 40°C for 2 min and then increase the temperature to 300°C (hold 5 min) at a rate of 6°C/min. The mass spectrometer was operated in the electron impact mode at 70 eV electron energy. The mass range *m/z* 14-500 was scanned by the mass spectrometer.

7.4.8 ^{31}P NMR lignin analysis

Steam pretreated samples were prepared as previously described by Crestini and Argyropoulos.[43] Thirty milligrams of dry lignin was accurately weighted and was dissolved in a solvent mixture composed of pyridine and deuterated chloroform, 1.6:1 (v/v) ratio (0.4 ml). The derivatisation agent, 2-chloro-4,4’,5,5’-tetramethyl-1,3,2-dioxaphospholane (0.1ml), the internal standard (N-hydroxynaphthalimide) and the relaxation reagent (chromium(III) acetylacetonate) solution (0.1 ml), were added. Quantitative ^{31}P NMR spectra were obtained on a Bruker 300 NMR spectrometer by using previously described methods.[44,45] The ^{31}P NMR data reported are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2 x 10^{-2} mmol/g, while the maximum standard error was 1 x 10^{-2} mmol/g.

7.4.9 HPLC

Liquid samples from raw material analysis and enzymatic hydrolysis upon their monomer sugar concentrations were determined with a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 65°C. The eluent, 5 mM H_2SO_4 was used at a flow rate of 0.5 ml/min. Concentration of carbohydrates was detected upon their refractive index. All samples were filtered through a 0.2 μm pore size filter before analysis to remove solid particles.

7.4.10 Statistical analysis

Statistical evaluation of the results obtained was performed using Statistica 9.0 software (Statsoft Inc., OK, USA).

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8: UNDERSTANDING THE MELANIN CHEMICAL STRUCTURE AND THE REACTION WITH LACCASE

8.1 Introduction

Melanins are a broad array of pigments widely distributed in the animal kingdom that are involved in human pigmentation and related pathological conditions.[1] Their distinguishing features include a high molecular weight, lack of a well defined physical and chemical characteristics. In fact, the main difficulties in the study of this polymer lie in the high insolubility and in the lack of a well defined spectral behaviour.

According to an early view, eumelanins are complex polymers generated from random coupling of indole monomeric units. In 1992 a titrimetric method for the quantification of moderately acidic and weakly acidic groups in sepia melanin was reported, although it was suitable for the determination of functional groups present on the melanin surface only.[2] However, these techniques do not allow a rapid and quantitative identification and evaluation of melanin substructures. The determination of the empirical formula from elemental analysis data,[3] showed that the oxygen content of melanins does not match with the previously hypothesized polymeric or oligomeric simple dihydroxyindolil substructure.

Direct chemical characterisation of melanins has been attempted by FT-IR[4] and CPMAS C-NMR.[5] Melanin oligomers were also characterised by MALDI Mass Spectrometry.[6] The structures of synthetic and natural melanins were examined by solid-state NMR using cross polarization magic angle spinning and high –power proton decoupling. Resonances consistent with known pyrrolic and indolic structures and signals due to aliphatic residues were detected in all the samples examined.[7]

The early primary objective of this part of my PhD thesis was to develop a simple and reliable quantitative technique of melanin analysis which would provide information on the fundamental environments present in melanins; namely the phenolics, the carboxylic, the aliphatic hydroxyl and the indolic and amino substructures. There are many examples where magnetic resonance techniques when applied to polyphenolic polymers have proven to be excellent analytical tools for the structural elucidation of these complex biopolymers. Part of my Ph.D. project has been focused on the development of solution ^{31}P based novel magnetic resonance methods aimed to expand the frontiers of application of NMR to melanin analysis. I report here the development of a new ^{31}P-heteronuclear correlated NMR spectroscopic technique applied at the elucidation of eumelanin structure. This solution NMR technique is capable of detecting and quantitatively evaluate all functional groups in melanins possessing reactive hydroxyl groups, *i.e.* phenolic OH, carboxylic acids and the different amino groups in aminoacids, indole and pyrrole substructures after selective and quantitative functionalisation with a suitable phosphitylating reagent.

The study of different melanins substructures by this new technique allowed for the first time to clarify the basic overall melanin chemical structure, since the samples were all well solubilised. Considerations concerning melanin structure as unlocked by quantitative solution ^{31}P-NMR of suitably phosphitylated melanin samples are also reported. Moreover it is here proposed a new approach, based on melanin degradation, that could be useful to study in detail not only the melanogenesis but also many aspects correlated with pigmentation disorders. In particular melanin degradation has been obtained by use of natural oxidative enzymes with the LMS. This approach could be innovative and can lead to the development of skin agents more effective but expecially safer with respect to the systems based on melanogenesis inhibition.

8.2 Results and discussion

8.2.1 Melanin Uv-Vis characterisation

In this study melanin from different origin were considered: the natural pigment from *Sepia officinalis;* melanins obtained by chemical or enzymatic reaction, starting by different precursors. In particular the enzymatic reactions were performed using the enzyme tyrosinase and the precursors used in both the enzymatic and the chemical processes were L-DOPA, dopamine, tyrosine and 5,6-dihydroxyindole-2-carboxylic acid (DHICA).

The insolubility of melanin in almost all the organic solvents constitutes a problem for the characterisation of the chemical properties of this polymer. For the spectrophotometric analysis, first Oikawa and Nakayasu[8] reported a spectrophotometric assay of melanins based on solubilising the deproteinised melanin in Soluene-100, a toluene solution containing 0.5 M of dimethyl-n-dodecyl-n-undecylammonium hydroxide. Further investigation demonstrated that eumelanins can be completely dispersed in Soluene-100 giving a solution that shows no light scattering.[9] Later to this solvent the Soluene-350was preferred, a stronger organic base containing toluene (60-80% w/v), dimethyl-n-dodecyl-n-undecylammonium hydroxide (20-40 % w/v) and methanol (2.5-10% w/v), that is able to dissolve all the constituents of cells, including the melanin pigment.[10] In fact Soluene-350 is a tissue solubiliser widely used in liquid scintillation counting, has a higher capacity to dissolve tissued constituents and to retain water than Soluene-100.

After the complete solubilisation in Soluene-350, melanin samples were characterised by their absorption in UV-Vis (290 nm<λ< 600nm). In *Figure* 8.1 is shown a typical spectrum obtained by the analysis of dopamine melanin, obtained by the enzymatic process.

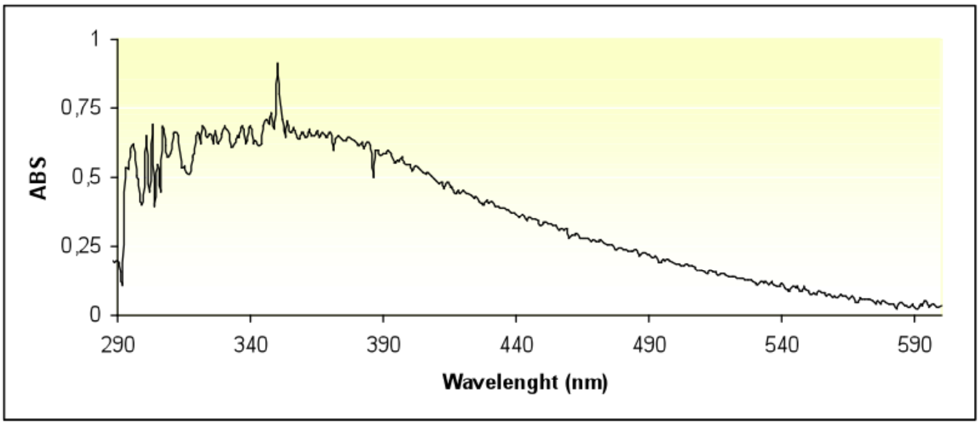


Figure 8.1- Uv-Vis analysis at 290 nm <λ< 600 nm of dopamine melanin (10 μg/ml) obtained by the enzymatic oxidation of dopamine.

For all the melanins considered in this study, a typical UV-Vis spectrum was obtained, and in all the cases a maximum absorbance at 350 nm was recorded, as previously reported.[11]

8.2.2 Phosphitylation reactions and quantitative ^{31}P-NMR of melanin model compounds

Melanin structural characterization is a challenging research topic since to date, due to low solubilità, a detailed structural characterization of this elusive biopolymer has not been accomplished by solution methods. The only available data reported are relative to solid state ^{13}C NMR spectra. In an attempt at developing an innovative analytical technique for melanin structural characterization the attention was focused on the possible application of heteronuclear ^{31}P NMR. The rationale behind this approach consists in the selective phosphytylation of phenolic indolic, amino and carboxylic groups present in melanins. The labeling of such moieties with phosphorous atoms would allow an easy identification by ^{31}P-NMR of the suitably phosphytilated polymer due to the extreme sensitivity of ^{31}P-chemical shift upon the phosphorous atom chemical environment.

As such, a number of melanin model compounds were selected in order to be phosphytilated and submitted to solution ^{31}P NMR analysis. *Figure* 8.2 shows the melanin model compounds selected. In order to develop a solution analytical protocol the correct choice of a suitable solvent system is of pivotal relevance.

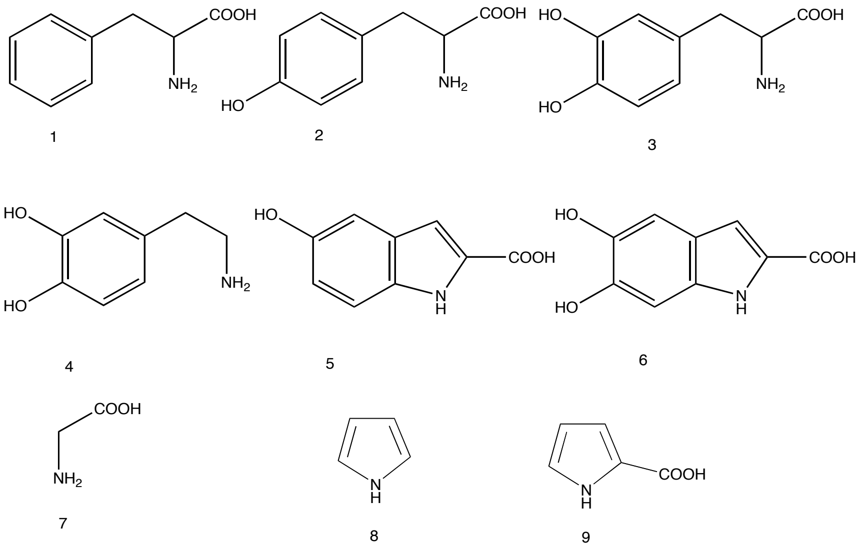


Figure 8.4- Melanin model compounds 1) phenylalanine; 2) tyrosine; 3) L-DOPA; 4) dopamine; 5) 5-hydroxyindol-2-carboxylic acid; 6) 5,6-dihydroxyindol-2-carboxylic acid; 7) glycine; 8) pyrrole; 9) 2-pyrrolcarboxylic acid.

Recently it has been reported[12,13] that some ionic liquids can effectively dissolve biopolymes, also in the cases were a good solubilisation it's a really difficult task to achieve. Ionic liquids are non-aqueous solvents composed of an organic cation (most often an alkyl-substituted imidazolium or pyridinium cation) and an inorganic anion. Similar to polar solvents such as dimethyl formamide and dimethyl sulfoxide, ionic liquids can dissolve a wide range of organic compounds.[14]

Due to the fact that they are not flammable and they do not have a detectable vapor pressure, they are established as convenient green solvents, capable of replacing organic solvents as a reaction medium in a number of organic synthetic and biocatalytic reactions.[15] Among the ionic liquids, I chose to use 1-butyl-3-methylimidazolium chloride ([bmim]Cl) because of its high capacity to dissolve another biopolymer with similar solubility problems with respect to melanin, the wood.[16,17] The structure of bmimCl is shown in *Figure* 8.3.

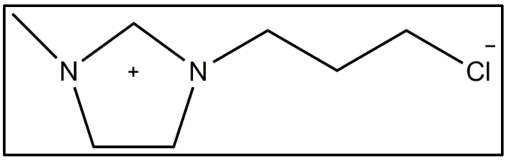


Figure 8.3- The chemical structure of the ionic liquid 1-butyl-3-methylimidazolium chloride ([bmim]Cl).

After the dissolution in the ionic liquid (IL), the melanin model samples were phosphitylated by adding a mixture of Py_{d5} and 2-chloro-4,4',5,5'-1,3,2-dioxaphospholane (Cl-TMDP). Cl-TMDP has been reported to react quantitatively with aliphatic alcohols, phenols and carboxylic acids and polyphenols in pyridine and triethylamine. Its reaction with labile protons proceeds in accordance with that depicted in *Figure* 8.4 and described in *Chapter* 2. The fact that three oxygen atoms surround the phosphorus atom in the phosphite esters formed ensures that the ^{31}P NMR signals of such derivatives will be singlets containing no coupling information.

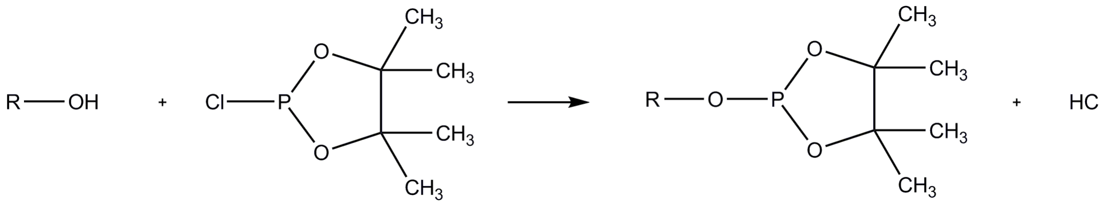
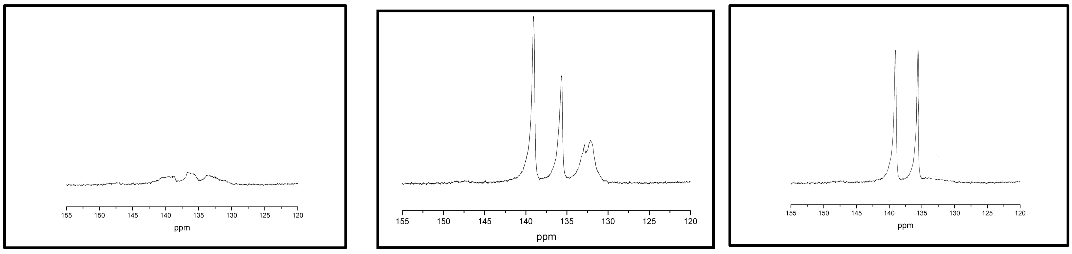


Figure 8.4- Phosphitylation of melanin with 4,4',5,5'-tetramethyl-chloro-1,3,2-dioxaphospholane (TMDP)with R=CH_3, and chloro-1,3,2-dioxaphospholane (DP) with R=H.

The extent of functionalization in ionic liquid of the different melanin functional groups was evaluated by reaction of TMDP with several melanin model compounds and aminoacids (*Figure* 8.2).

In order to quantitatively evaluate the amount of different functional groups a suitable internal standard is needed. In this respect the choice was on N-hydroxyphtalimmide. With the aim to find the best solubilisation conditions and the reactivity maximum in IL, the experiments were carried out at different temperatures (RT, 30 °C, 40 °C, 60 °C and 80 °C) and for different reaction times (1 h, 2 h, 3 h, 8 h, 12 h). The maximum reactivity rate, together with the best solubilisation, was observed performing the reaction at 60°C for 3 h. At higher temperatures the reactivity of model compound decreased, probably due to irreversible degradation processes. Nevertheless, leaving the sample in IL at 60 °C for 3 h with Cl-TMDP, there was a complete quantitative reactivity for COOH and NH groups, while it was not possible to detect signals for the phenolic OHs. In particular, the phenolic OHs signals were detected at RT, even if the reaction for these groups were non quantitative; increasing the temperatures the signal for the phenolic OH groups completely disappeared. *Figure* 8.5 three ^{31}P NMR spectra of 5-hydroxyindole-2-carboxylic acid **5**, in different experimental conditions are shown.



A B C

Figure 8.5- 31 P NMR spectra of HICA in different phosphytilation condition: A. 120°C for 1 min; B. RT; C. 60 °C.

Successively, under the same experimental conditions, all the remaining model compounds were analyzed. *Table* 8.1 reports the chemical shifts of different functional groups in melanin model compounds completely solubilised in IL and derivatised with TMDP and submitted to ^{31}P-NMR. The derivatization extents were evaluated in the presence of a suitable amount of internal standard by integration and comparison of the areas of the NMR peaks due to the different functional groups.

The use of IL leads to a quantitative reactivity of COOH and NH groups, but it was not possible to detect the phenolic OH groups, probably due to the higher temperatures used in the experimental conditions. Since the use of IL did not allow the analysis of phenolic OH groups in melanins, due to incomplete phosphytilation, it was necessary to change both the solvent system and the phosphytilating agent in order to improve the reactivity of the system.

To increase the reactivity, expecially of the phenolic groups, another phosphytilating agent, chloro-1,3,2-dioxaphospholane (Cl-DP), was choosen. The phosphytilation reaction on melanin model compounds reported in *Figure* 8.2 was carried out in pyridine and chloroform 1,6:1 (*solvent system I*), a solvent system previously applied in the quantitative dissolution and phosphytilation of lignins.[18,19] The choice of a basic solvent is due to the necessity to neutralize the hydrochloric acid that arises from the derivatization reaction as shown in *Figure* 8.1.When this solvent system was used for Cl-DP phosphytylation and ^{31}P-NMR only oxygen containing moieties in melanin model compounds were found quantitatively functionalized.

On the contrary, amino and indolyl moieties were found only partially phosphitylated and did not allow their quantitative evaluation (*Table* 8.2).

Table 8.1- ^{31}P NMR chemical shifts of melanin model compounds solubilised in IL and derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP).

Model compound number	Solvent system	NH_2	Phenolic OH	COOH	NH
Phenylalanine **1**	**IL**	140,6	-	134,7	-
Tyrosine **2**	**IL**	140,5	N.D.*	134,9	-
L-DOPA **3**	**IL**	140,8	N.D.*	134,8	-
Dopamine **4**	**IL**	141,8	N.D.*	-	-
5-hydroxyindol-2-carboxylic acid **5**	**IL**	-	N.D.*	134,9	140,6
5,6-Dihydroxyindol-2-carboxylic acid **6**	**IL**	-	N.D.*	134,7	140,5
Glycine **7**	**IL**	140,6	-	-	140,4
Pyrrole **8**	**IL**	-	-	-	140,5
2-pyrrolcarboxylic acid **9**	**IL**	-	-	134,7	140,6

*N.D.= not detected or present in traces.

In particular, using the solvent system I and Cl-DP for the melanin model compounds analysis, the COOH and OH groups were found to be quantitative, while the amino and indolyl moieties showed a reactivity between the 20% and the 50%. Furthermore the signal of phosphitylated pyrrolic moiety was overlapped with that of carboxylic acids. On the basis of these results, with the aim to obtain a quantitative analysis of all the functional groups in melanin and no signal overlapping, another solvent system was identified and tested. Verkade[20] reported the derivatization of amines with Cl-TMDP and Cl-DP in triethylamine, but no data were available in the literature about the reaction yields. On the basis of these data, a mixture of $Et_3N:CDCl_3=1.6:1$ was used (*solvent system II*). The reaction was carried out at 20°C during 90 min and 15 min for Cl-DP respectively. In *Table* 8.2 reports the chemical shifts of different functional groups in melanin model compounds derivatised with Cl-DP and dissolved in Et_3N. Five resonance regions could be identified from the analysis of melanin models NMR spectra. More specifically carboxylic acids ranged from 134 to 135,2 ppm while phenolic OH groups were found at 137,8 to 139,0 ppm. The NH_2 resonances of aromatic aminoacids ranged from 149,9 to 151,8 ppm, the indolic NH from 143,4 to 144,1 ppm and the pyrrole NH from 134,1 to 134,7 ppm. In this case the resonance due to the phosphitylated pyrrolic moiety was no longer overlapped with the carboxylic acids one (*Table* 8.2 entries 8 and 9). The phosphitylating system consisting in Cl-DP in triethylamine-$CDCl_3$ showed a high efficiency both in the resolution of all the different resonances of melanin model compounds and in their quantitative derivatization as shown by the integrals values of the spectra carried out in the presence of a suitable internal standard.

Table 8.2- ^{31}P NMR chemical shifts of melanin model compounds derivatized with 2-chloro-1,3,2-dioxaphospholane (DP).*

Model compound number	**Solvent system**	**NH_2**	**Phenolic OH**	**COOH**	**NH**
Phenylalanine **1**	**I** **II**	N.D.* 143,9	- -	N.D.* 127,1	- -
Tyrosine **2**	**I** **II**	N.D.* 144,0	128,6 128,6	N.D.* 126,9	- -
L-DOPA **3**	**I** **II**	N.D.* 144,0	129,1-129,5 129,1-129,5	N.D.* 127,0	- -
Dopamine **4**	**I** **II**	N.D.* 144,4	129,6-129,8 129,6-129,8	- -	- -
5-hydroxyindol-2-carboxylic acid **5**	**I** **II**	N.D.* 150,1	127,9 128,0	128,0 127,0	N.D.* 132,85
5,6-Dihydroxyindol-2-carboxylic acid **6**	**I** **II**	- -	129,2-129,3 129,2-129,3	127,2 127,2	N.D.* 133,0
Glycine **7**	**I** **II**	N.D.* 141,6	- -	N.D.* 126,8	- -
Pyrrole **8**	**I** **II**	- -	- -	- -	123,5 123,5
2-pyrrolcarboxylic acid **9**	**I** **II**	- -	- -	127,2 127,3	123,6 123,6

*N.D.= not detected or present in traces.

In an effort to examine the stability of melanin models phosphitylated with Cl-DP, solutions of the models 1-9 were quantitatively analyzed after 30, 60, 90, 120, 150 min from the onset of the phosphitylation reactions. The reaction showed to be quantitative after 10 min and remained thereafter constant up to 150 min. It is possible to conclude from data obtained from melanin models that it is in principle possible to distinguish and quantitatively evaluate all the phenolic OH groups, pyrrole and carboxylic acids. In tyrosine, dopamine and L-DOPA, the NH, besides the phenolic hydroxy and carboxylic groups can be quantified and identified.

In order to obtain a better separation in the chemical shift of COOH and OH groups and a quantitative analysis of NH_2 groups, the melanin model compounds analysis was also performed with Cl-TMDP, a phosphytilating agent that present a higher stability than DP due to the presence of four methyl groups. Then, all the melanin model compounds analised with DP in presence of the two different solvent systems, were also analised after the phosphitylation with Cl-TMDP, again in presence of the two different solvent systems. *Table* 8.3 reports the chemical shifts of the different functional groups of melanin model compounds.

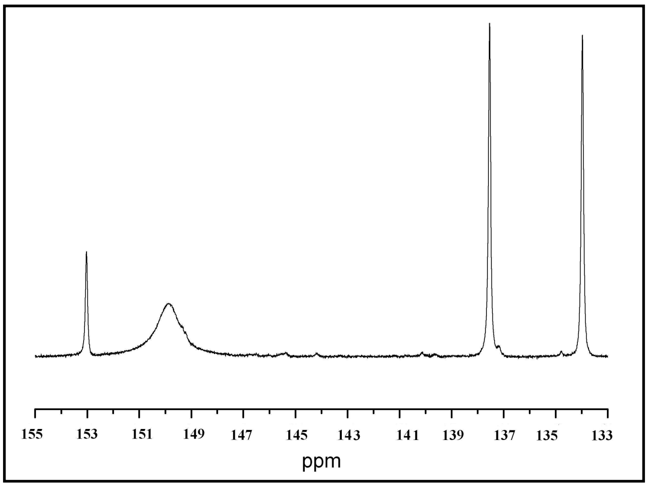
When solvent system I was used, all the phenolic groups were found quantitatively derivatised and gave well resolved spectra. Their chemical shift ranged from 146 to 140 ppm upfield from phosphoric acid. These data are in accord with other previous reported on phenolic derivatives phosphitylated under analogous reaction conditions.[21] In all cases phenols were quantitatively reacting with Cl-TMDP. Carboxylic acids were found also fully derivatised with a chemical shift range from 135,0 and 134,2 ppm. Also in this case data were in full accord with previous literature reports. ^{31}P NMR experiments showed that in pyridine:$CDCl_3$ aminoacids **1,2,3,7** are not reactive with Cl-TMDP at 20°C during 12 h. In the case of tyrosine **2** or *l*-dopa **3** only the peaks due to phenolic OH groups were detected. It is interesting to note that when dopamine **4**, phenylalanine **1** or glycine **7** were analysed both the amino and carboxylic groups were found completely unreactive. On the contrary, when 5-hydroxyindole-2-carboxylic acid **5** or 5,6-dihydroxyindole-2-carboxylic acid **6** were analysed the carboxylic moieties were visible in a range from 135,0 to 134,2 ppm and were quantitatively derivatised. The lower basicity of indole derivatives with respect to pyridine, in fact, allows the carboxylate groups to efficiently react with Cl-TMDP.

Table 8.3- ^{31}P NMR chemical shifts of melanin model compounds derivatized with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP).

Model compound number	**Solvent system**	**NH_2**	**Phenolic OH**	**COOH**	**NH**
Phenylalanine **1**	**I** **II**	N.D.* 150,1	- -	N.D.* 135,0	- -
Tyrosine **2**	**I** **II**	N.D.* 150,11	137,8 137,8	N.D.* 134,5	- -
L-DOPA **3**	**I** **II**	N.D.* 149,9	137,8-138,5 138,9	N.D.* 134,2	- -
Dopamine **4**	**I** **II**	N.D.* 151,8	138,8-139,0 138,8-139,0	- -	- -
5-hydroxyindol-2-carboxylic acid **5**	**I** **II**	- -	137,9 138,0	135,0 134,7	N.D.* 143,4
5,6-Dihydroxyindol-2-carboxylic acid **6**	**I** **II**	- -	138,3-138,0 138,3-138,0	135,2 134,5	N.D.* 144,1
Glycine **7**	**I** **II**	N.D.* 147,1	- -	N.D.* 134,0	- -
Pyrrole **8**	**I** **II**	- -	- -	- -	143,5 144,2
2-Pyrrolcarboxylic acid **9**	**I** **II**	- -	- -	135,0 134,6	143,7 144,2

*N.D.= not detected or present in traces.

Thus solvent system I is suitable for direct quantitative evaluation of aliphatic and phenolic hydroxyl groups and COOH units from indolecarboxylic derivatives in melanins, but it is inable to detect all the functional groups present in the amino acids. In an effort to tune the reactivity of melanin model compounds in order to efficiently derivatize amino acid moieties, the solvent system was modified by introduction of triethylamine instead of pyridine. Solvent system II showed to increase significantly the reactivity of melanin model systems. The NH and COOH groups of all the model compounds tested were found quantitatively reactive at 20°C. It is interesting to note that the chemical shift of the NH_2 group in tyrosine, dopa, dopamine, phenylalanine and glycine was found at 150 ppm and was not overlapped to other resonances although very broad due to nitrogen relaxation. In *Figure* 8.6 is show a typical ^{31}P NMR spectrum of tyrosine and HICA.



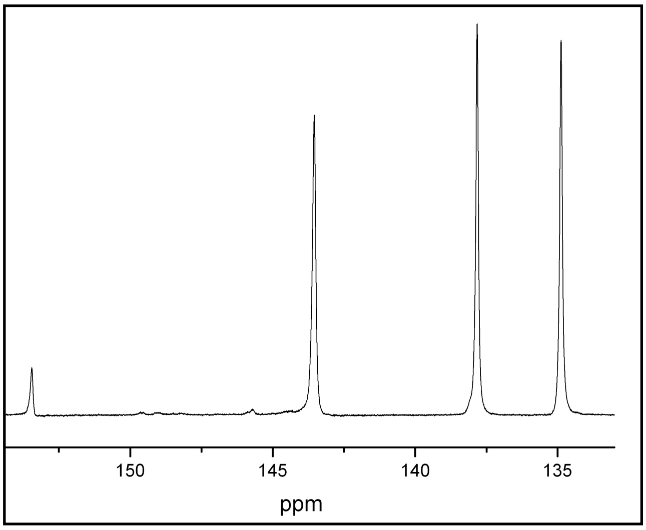


Figure 8.6-^{31} P NMR spectrum of tyrosine (above) and HICA (down) solubilised with Et_3N and derivatized with Cl-DP showing the COOH signal at 134.5 ppm, the phenolic OHs at 137.8 ppm and the NH_2 at 150.1 ppm. The signal at 153.8 ppm is related to the internal standard.

Table 8.3 shows the chemical shifts of the different melanin model compounds studied. Solvent system II is thus extremely helpful for the quantitative selective evaluation of all phenolic groups, carboxylic acids in indolyl and aromatic or aliphatic structures, without any possible interference of residual aminoacidic groups from dopa or tyrosine that showed a marked different chemical shift. From the comparison of the phosphytilating agents and solvent systems used it resulted that the combination of Et_3N and Cl-TMDP can be considered the better system for the qualitative analysis of all the functional groups present in melanin model compounds, due to the high resolution of all the chemical shift without signal overlapping, and also for the quantification of all the groups.

8.2.3 Quantitative solution ^{31}P-NMR of melanins

Among the two different solvent systems used for melanin model compounds analysis, Et_3N was the solvent that gave the best performances, both in qualitative and in quantitative analysis. On the basis of these data, Et_3N was chosen for melanin samples analysis. Moreover, with the aim to completely clarify the melanin chemical structure and to obtain a more informative analysis, melanins was subjected to ^{31}P NMR analysis, after the dissolution in Et_3N, in presence of the two phosphitylating agents, Cl-DP and Cl-TMDP.

The variability of melanin samples was considered by the study of three different kind of samples. A first sample was obtained chemically starting from tyrosine. A second set of melanin samples was obtained by the polymerisation of tyrosine, L-DOPA, and dopamine in the presence of tyrosinase. These melanin models have been widely studied in the literature in order to elucidate the early stages of melanin biosynthesis and its hypothetical structure. In addition, melanin extracted from *Sepia officinalis* was chosen as a natural melanin sample. In *Figures* 8.6 and 8.7 are shown ^{31}P NMR spectra of different melanins in presence of DP and TMDP.

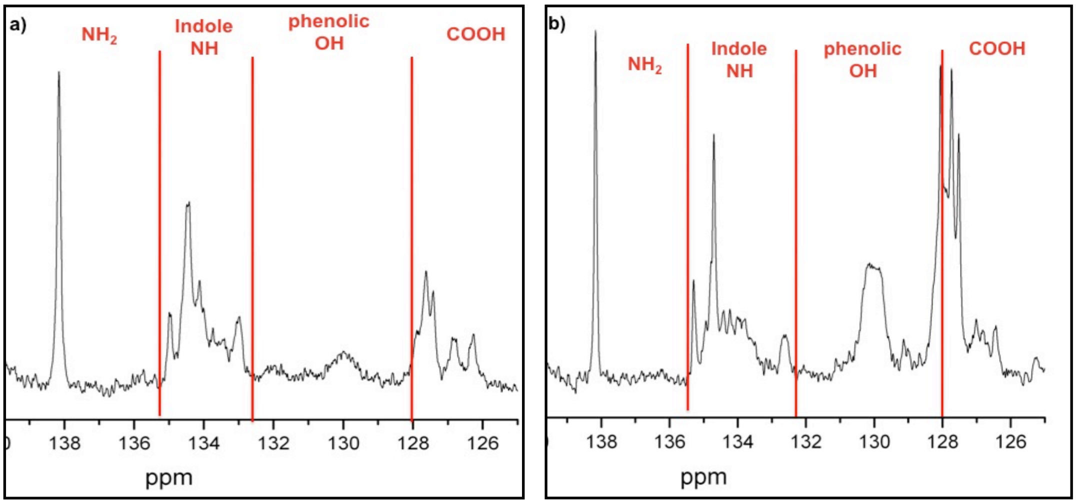


Figure 8.6- ^{31}P NMR spectra of melanin samples solubilised in Et_3N and derivatised with DP with the identified spectral regions of :a) enzymatic melanin from Dopa; b) melanin from *Sepia officinalis*.

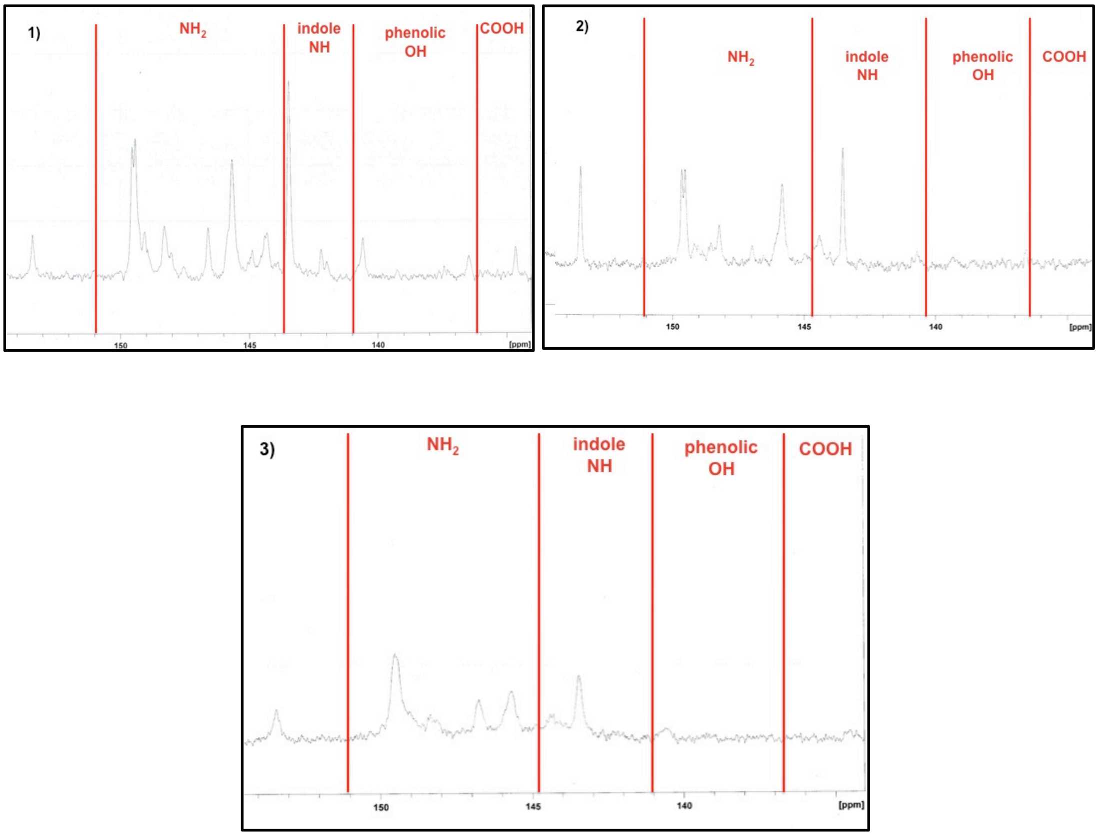


Figure 8.7- ^{31}P NMR spectra of melanin samples solubilised in Et_3N and derivatised with TMPDP with the identified spectral regions of :1) melanin from *Sepia officinalis;* 2)enzymatic melanin from Dopamine; 3) synthetic melanin from chemical oxidation of Dopa.

Table 9.4 shows the chemical shift range of the different functional groups in melanins. It is possible to identify main chemical shift regions. The aminoacidic NH_2 were found at 137-141 ppm with Cl-DP and at 145-151 ppm with Cl-TMDP. The regions between 132 and 135 ppm and 142-144 ppm has been unambiguously attributed to indolyl NH groups in Cl-DP and Cl-TMDP, respectively. Phenolic OH groups showed a chemical shift of 128-131 ppm with DP and 137,8-139,3 ppm with TMP. Finally the carboxylic acids lie in the region between 127 and 128 ppm with Cl-DP and 134-135 ppm with Cl-TMDP.

Table 8.3- Chemical shift range of the different functional groups in melanins derivatized with DP and TMDP in Et_3N.

Functional group	Chemical shift with DP	Chemical shift with TMDP
COOH	127-128	134-135
phenolic OH	128-131	137,5-139,3
Indole NH	132-135	142-144
NH_2 aminoacids	137-141	145-151

8.2.4 On the melanins molecular structure as revealed from quantitative ^{31}P-NMR

The quantitative evaluation of the different functional groups in different melanins is reported in *Table* 8.4. Data are reported in mmol/g. It has been reported that the empirical formula from elemental analysis of different melanins ranges from $C_{7,67}H_{5,51}NO_{3,68}$ (MW:170) in sepia melanin to $C_{7,88}H_{4,65}NO_{3,78}$ (MW:173) and $C_{8,79}H_{5,76}NO_{4,28}$ (MW:193) for tyrosine enzymatic and chemical melanins respectively. We would therefore expect about two phenolic hydroxyl groups per melanin subunit, that means about 11,8 and 10,4 mmol/g of phenolic hydroxyl groups in sepia and enzymatic melanins and chemical melanin, repectively. That means that in all the melanin samples examined there is less than one phenolic hydroxyl group per five indole units. It follows that the molecular structure of melanin is more complex than hypothesized before with the phenolic hydroxyl groups either extensively oxidised to quinone or involved in the interunit bondings as aryl ether groups or both.

Table 8.4- ^{31}P NMR analysis of sepia melanin, enzymatic polymerisation melanin and chemical melanin phosphitylated with DP.*

Functional group (mmol/g)	Sepia melanin	Enzymatic polymerisation melanin	Chemical polymerisation melanin
Indole NH	0,24	0,04	0,01
Phenolic OH	0,06	0,09	0,03
COOH	0,05	0,11	0,01
Pyrrole NH	1,33	0,61	0,91

*Amount (mmol/g) of indole NH, phenolic OH, pyrrole NH and carboxylic OH groups present on different type of melanin, as evaluated by quantitative ^{31}P-NMR analysis. The ^{31}P-NMR data reported in this regard are averages of three phosphytylation experiments followed by quantitative ^{31}P-NMR acquisition. The maximum standard deviation of the reported data was $2x10^{-2}$ mmol/g, while the maximum standard error was $1x10^{-2}$ mmol/g.

The presence of carboxylic acid units in melanin enzymatically synthesized from dopamine **4** (*Figure* 9.5), a monomer lacking of the COOH moiety, clearly suggests that the origin of COOH could be, besides the indolecarboxylic acid monomeric subunit, in the further oxidation and cleavage of the indolyl ring. The amount of COOH units identified in the different melanin samples ranged from 1 to 10% of the theorethical indolil units as calculated on the basis of the empirical formula, being 2,5% in enzymatic dopamine melanin.

8.2.5 Melanin oxidation with laccase

After the spectrophotometric characterisation of melanin samples of different origin, the results obtained were used for a comparison of the spectral behaviour of melanins before and after enzymatic treatments. In particular, the chemical and enzymatic melanins and also melanin from *Sepia* were subjected to the oxidation both with laccase and LMS. Although melanins are considered very stable compounds, under special conditions, chemical or photochemical degradation is possible[22] as well as biodegradation by fungi.[23] In nature, the fungal laccase catalyses the formation of melanin by oxidizing L-DOPA, initiating a series of presumably spontaneous reactions that ultimately leads to the polymerization of the pigment in the cell wall.[24] By the other side, it has been also reported that some fungi are able to degrade specific melanins of different origins.[26-27] Recently, Rätto *et al*.[28] showed that white–rot basidiomycetes outperformed fungi from other taxa to decolorize *Aureobasidium pullulans* melanin in vitro. The white-rot fungi secrete a mixture of oxidative enzymes that act directly or indirectly on lignin; among them the most relevant ones are laccase, manganese peroxidase and lignin peroxidase. The performances of this enzymes, in particular of laccase, can be increased using compounds that act as oxidation mediators.

With the aim to induce the enzymatic degradation, melanin samples have been treated with laccase from the white rot fungus *Trametes versicolor* with or without the presence of two different mediators, HBT or VA. Due to the recalcitrant properties of the polymer, the enzymatic treatments were carried out for 12 h at 40 °C. After the treatments, the samples were acidified, washed many times and freeze dried. Finally the reaction products were subjected to spectrophotometric analysis. The results of UV-Vis measurements at 350 nm are shown in *Figure* 8.8.

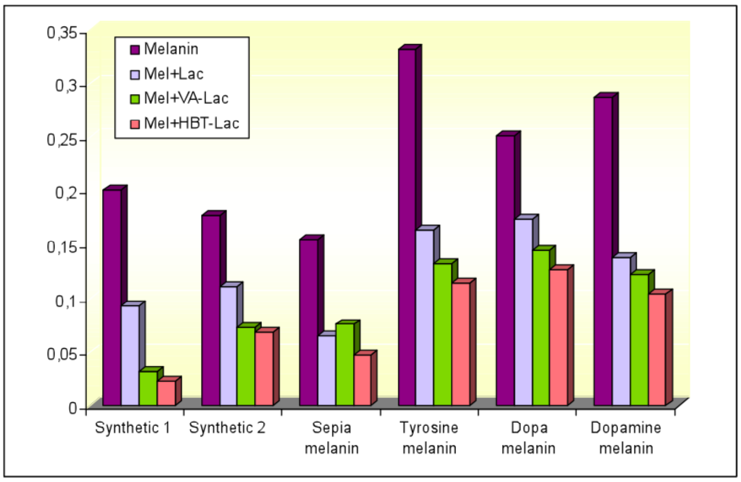


Figura 8.8- Absorbance values (A_{350}) after laccase treatment.

In all the samples, the melanins oxidized with laccase showed a decrease in absorbance values, respect to the native ones. In particular, after the treatment with laccase the absorbance values showed a decrease of about 50% of the values observed for the untreated samples. This spectral behaviour indicates that the oxidation leads to changes in the polymer that gave as results its decolorization. In presence of mediators, HBT or VA, the decrease in the absorbance values was more relevant; using the LMS, a stronger polymer oxidation occurs, with respect to the use of the only laccase. Among VA and HBT, this latter can be considered a more effective mediator in melanin oxidation. The effect of HBT was more evident on synthetic and sepia melanin; enzymatic melanins showed no significative differences when treated with VA or HBT.

The occurrence of decolorization processes after the treatment with laccase and LMS demonstrated that natural enzymes can successfully act as whitening agents on melanin. The use of oxidative enzymes on melanin constitutes a new approach for detailed studies on pigmentation disorders. Moreover the use of laccase and LMS appears to be promising and important because an enzyme of biological origin could be more acceptable and safer for cosmetic purposes than chemical formulations.

8.3 Conclusion

For the first time, in this study, an advance heteronuclear spectroscopic technique was used for the characterisation and elucidation of the chemical structure of melanin, an highly complex biopolymer formed by the enzymatic oxidation of tyrosine. The most relevant problem in melanin study is its insolubility; due to this, to date, only a partial characterisations of the chemical structure in a solid state is reported in literature. In this study, all the solvent systems used gave an high solubility level that allows the first solution NMR analysis. The spectroscopic analysis of melanin model compounds and of melanins from different origin, demonstrated that the ^{31}P NMR constitutes a rapid reliable and quantitative technique for the elucidation of chemical structure of the polymer. This constitutes an important tool to shed light on melanin structure and biosynthesis process. From the study of different melanins by ^{31}P NMR it appears clearly that the chemical structure of melanin accepted to date should be revised on the basis of new acquired structural data. More specifically, the amount of phenolic hydroxyl groups is actually much lower than expected. This is in accord with the results reported about the melanins elemental analyses that show also a higher oxygen content than expected for simple dihydroxyindole subunits. Moreover, a low amount of phenolic hydroxyl groups is also supported by the Raper-Mason theory on melanogenesis (*Figure* 8.9).[30] The major steps in eumelanin formation is considered the cyclization of dopaquinone to leucodopachrome, which is immediately oxidized to form dopachrome. The dopachrome is tautomerically rearranged to form 5,6-dihydroxyndole-2-carboxylic acid. But, acc ording to the study of Mason,[30] the dopachrome may also spontaneously decarboxylate to 5,6-dihydroxyindole (DHI) which will rapidly oxidize to form indole 5,6-quinone. Finally, the polimerization of the quinone to eumelanins occurs.

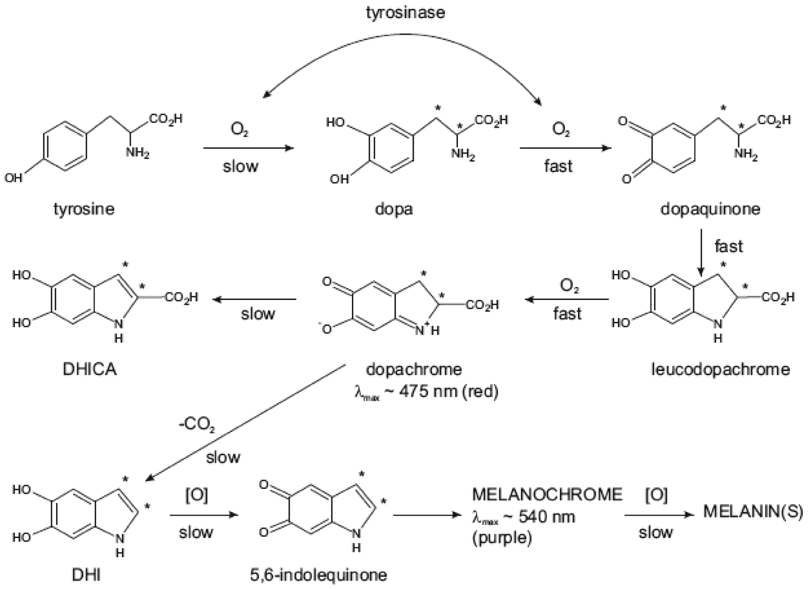


Figure 8.9- The Raper-Mason scheme of melanogenesis (from Mason *et al.*, 1948).

Considering this view, the quinones polimerization leads to the formation of a polymer with a low phenolic content and an increase indolic content with respect to a polimerization from DHICA. The ^{31}P NMR analysis seems to confirm that, for the major part, the eumelanins origin from the polimerization of quinone and only in a smaller part from DHICA polimerization.

The rest of this study aims to the UV-Vis characterisation of melanins from different origins and to the comparison of the spectral behaviour before and after the enzymatic treatment with laccase and LMS. Even if melanin is considered a polymer that is recalitrant to oxidation, prolonged treatments with laccase showed a positive effect on melanin degradation. Moreover, the melanin treatements with the LMS increase the oxidation, expecially in presence of HBT. The extention of the degradation was followed by spectrophotometric measurements, at the maximum absorbance value, that resulted in decolorization processes associated with the depolimerization. The use of natural enzymes and also of the LMS for melanin degradation costitutes a new approach that, with further investigations, could be lead to the development of skin agents more effective and safer than the ones actually used, that are based on melanogenesis inhibition.

Further studies are in course in order to further clarify melanin molecular structure and to understand the role of laccase in its decolorization.

8.4 Experimental

8.4.1 Melanin spectrophotometric assay

Melanin samples were solubilized as previously described.[11] In brief, sample aliquotes were dissolved in Soluene-350, stirred and heated at 50° C for 3h and analyzed for absorbances beetween 290 and 600 nm.

8.4.2 Synthesis of 5,6-dihydroxyindole-2-carboxylic acid (DHICA)

DHICA was prepared by a multistep synthesis as previously described.[31] $KFe(CN)_6$ (20,0 mmol) dissolved in 50 ml of water was added during 7 min to L-DOPA (5,0 mmol) in water (500 ml). During the addition pH was kept constant between 6,0 and 5,5 by addition of NaOH 1M. At the pH was then increased to 12,6 by addition of NaOH 1M and the reaction mixture was kept under stirring at 20°C during 15 min. The reaction was acidified to pH 1,2 with HCl 6M. The reaction mixture was entracted in ethyl acetate, washed with water, dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. 5,6-dihydroxyindol-2-carboxylic acid was purified by crystallization from acetone/hexane in a 60 % final yield.

8.4.3 Preparation of enzymatic melanins

Melanins were prepared by enzymatic oxidation of L-DOPA, dopamine, tyrosine and DHICA, respectively. Enzymatic oxidations were performed in a thermostatic bath at 25°C. Oxydations by tyrosinase/O_2 system were carried out according to Ito with minor changes.[11] The substrate (1mmol) in 80 ml of 0,1M phosphate buffer, pH 6,8 was incubated during 4 h with tyrosinase (16000 units) under oxygen flux. The reaction mixture was acidified to pH 3, centrifuged, washed repeatedly with water and freeze dried.

8.4.4 Preparation of chemical melanins

To a solution of tyrosine (1mmol) in 80 ml of 0,1M phosphate buffer, pH 6,8 $K_3Fe(CN)_6$ (1 mmol predissolved in buffer) was added under stirring at room temperature. After 2h the reaction mixture was acidified to pH 3, centrifuged, washed repeatedly with water, deionized by repeated washings with EDTA solutions freeze dried.[32]

8.4.5 Synthesis of ionic liquid

[Bmim]Cl (IL) was synthesized according to the general procedure provided by Zhang *et al.*,[33] with slight modification; both allyl chloride and 3-methylimidazolium were distilled prior use. [Bmim]Cl was further purified, to remove trace color, by dissolving the crude [bmim]Cl mixture in water and refluxing with activated charcoal (18 h). The solution was filtered through a silica plug, and the water was removed by distillation and dried for 2 days in vacuo to yield [bmim]Cl as a pale yellow cristalline solid, with a melting point of 52 °C.

8.4.6 NMR solution preparation

For the solubilisation in IL, this latter was charged into a 50 ml dried flask equipped with a mechanical stirrer, under an inert atmosphere of argon. The temperature of the dissolution process was controlled by an oil bath. The melanin sample was then added into the ionic liquid quickly and left to react for 2 hours. Then a solution of pyridine and phosphytilating agent was added and left to react another 2 hours.

A solvent mixture of pyridine and $CDCl_3$ (*solvent system* I) or alternatively $Et_3N/CDCl_3$ 1.6/1 v/v (*solvent system* II) was prepared under anhydrous conditions. The internal standard solution was prepared at the following concentrations: N-hydroxyphtalimmide, the internal standard, 21,32 mg/ml in the above described solvent solutions. The solutions were protected from moisture by the use of molecular sieves and were stored under nitrogen.

8.4.7 Model compounds phosphitylation

15 mg of melanin model compound were accurately weighed in a volumetric flask and suspended in 0,300 ml of the solvent solution. The internal standard solution was then added (0,100 ml) followed by the DP or TMDP (0,100 ml). The flask was tightly closed and put under stirring at 20°C during 90 min.

8.4.8 Melanin compounds phosphitylation

7-10 mg of melanin were accurately weighed in a volumetric flask and suspended in 0,300 ml of the solvent solution. The internal standard solution was then added (0,100 ml) followed by the chloro-1,3,2-dioxaphospholane (Cl-DP) or 2-chloro-4,4',5,5'-1,3,2-dioxaphospholane (Cl-TMDP) (0,100ml). The flask was tightly closed and put under stirring at 20°C during 90 min. The mixture become homogeneous upon reaction with the phosphytilating agent. When a higher concentration of melanin was used, the system was no longer homogeneous due to the scarce solubility of the melanins. Thus a final concentration of 1-2% w/w was used throughout all the experiments.

8.4.9 NMR spectroscopy

The NMR spectra were recorded on a Bruker 300 NMR spectrometer operating at 121.5 MHz for the phosphorus-31 nucleus. The probe temperature was 20°C. To eliminate NOE effects, the inverse gated decoupling technique was used. Typical spectral parameters for quantitative studies were as follows: 90° pulse width, sweep width of 6600 Hz. The spectra were accumulated with a delay of 15 s between successive pulses. Line broadening of 4 Hz was applied, and a drift correction was performed prior to Fourier transform. Chemical shifts were expressed in parts per million from 85% H_3PO_4 as an external standard. All chemical shifts reported are relative to the reaction product of water with Cl-DP or CL-TMDP, which has been observed to give a sharp signal in pyridine/$CDCl_3$ and in triethylamine/$CDCl_3$ at 132.2 and 121,1 ppm respectively.

The spin lattice relaxation profiles of the phosphorous atoms attached on melanin, ranged between 2 and 0,5 s. The spin lattice relaxation time of N-hydroxynapthalimmide was found 1.5s As such the experimental protocol for spectral acquisition of melanins was developed with a pulse delay of 15 s. The ^{31}P NMR data reported in this regard are averages of three phosphitylation experiments followed by quantitative ^{31}P NMR acquisition. The maximum standard deviation of the reported data was 2 x 10^{-2}mmol/g, while the maximum standard error was 1 x 10^{-2}mmol/g.

8.4.10 Enzyme activity assay

Free laccase activity was determined spectrophotometrically using ABTS as the substrate. The assay mixture contained 0.5 mM ABTS, 0.1 M sodium acetate pH 5 and an amount of enzyme; the substrate oxidation was followed by an absorbance increase at 415 nm for 1 min for the free enzyme and for 3 min for the immobilised enzyme. One activity unit was defined as the amount of enzyme that oxidised 1 mmol ABTS/min.[34] The immobilisation yield was calculated as the difference between the activity present in the immobilisation solution and that remaining in the supernatant at the end of the adsorption procedure.

8.4.11 Enzymatic treatments

The treatments were carried out in both the presence and absence of a mediator, namely HBT or VA. Melanin (80 mg) was suspended in acetate buffer pH 6 (40 ml) with or without the mediator (1 mM) and treated at 40 °C with the enzyme (60 U) under vigorous stirring to optimise the contact of the solution with air. After 24 h, the mixture was cooled, acidified at pH 3 and centrifuged. The residue was washed with water three times to eliminate solubilised lignin oligomers and then freeze-dried. The residual lignin structure after oxidation was analysed by UV-Vis spectrophotomer.

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APPENDIX