BIOTECHNOLOGICAL EMPLOYMENT OF OLIVE OIL MILL WASTEWATERS FOR WATER REMEDIATION AND POTENTIAL BIODIESEL PRODUCTION

Veria Addorisio

Dottorato in Scienze Biotecnologiche – XXIII ciclo
Indirizzo Biotecnologie per le Produzioni Vegetali
Università di Napoli Federico II

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Dottorando: Veria Addorisio

Relatore: Dott.ssa Filomena Sannino
Dott. César Mateo Gonzalez

Coordinatore: Prof. Giovanni Sannia

“Nessun problema può essere risolto allo stesso livello di conoscenza che è stato generato"

Albert Einstein

Gli scienziati non si vergognano dei loro errori, né si arrendono davanti ai risultati infruttuosi e ai fallimenti, ma ne approfittano per trovare nuove opportunità e per andare avanti.

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RIASSUNTO

Le acque di vegetazione (AV) o reflui oleari costituiscono il principale sottoprodotto della lavorazione delle olive e sono prodotte in elevate quantità in tutti i Paesi del Bacino del Mediterraneo. Le AV causano gravi problemi ambientali per le notevoli proprietà inquinanti, attribuibili soprattutto alla presenza di Na^+ e di sostanze di natura organica quali i polifenoli.

Nell'ambito delle biotecnologie vegetali, l'utilizzo dei reflui oleari come biomasse vegetali ha dato origine a nuove ricerche volte alla loro utilizzazione e valorizzazione attraverso trattamenti biologici e chimici, al fine di ottenere prodotti ad elevato valore aggiunto e, allo stesso tempo, consentire l'abbattimento del loro potere inquinante.

Precedenti ricerche sulle AV hanno condotto al recupero e alla caratterizzazione da tali reflui della componente polimerica metallo-organica con proprietà umo-simili e di polielettrolita, denominata polimerina. La porzione organica di questa matrice è costituita da un polisaccaride libero e da un pigmento bruno polimerico formato, a sua volta, da una componente polisaccaridica, una catecolmelaninica ed una proteica, legate tra di loro con legami covalenti, ad idrogeno ed interazioni CH-π, formando una struttura supramolecolare. La frazione metallica, costituita da potassio, sodio, calcio, magnesio, ferro, zinco e rame, si lega a quella organica mediante legami ionici e di chelazione. Tale matrice, per la sua somiglianza alle sostanze umiche, per la presenze di macro e micronutrienti e per la sua scarsa fitotossicità, comparata alle AV tal quali, potrebbe essere impiegata in agricoltura come bio-ammendante e/o bio-integratore di metalli. Inoltre, per la presenza nella sua struttura chimica di gruppi -COOH carbossilici e di -OH fenolici e polisaccaridici, la polimerina potrebbe rappresentare un bio-materiale da utilizzare in processi di biotecnologia ambientale, in particolare per la decontaminazione di acque inquinate da composti tossici, mediante adsorbimento degli stessi.

Il primo obiettivo della presente tesi è stato l'uso della polimerina per la decontaminazione di acque inquinate da agrofarmaci. E' stata valutata la capacità di adsorbimento della polimerina nei confronti del cyhalofop acido (CyA), un erbicida arilossifenossipropionico di nuova generazione; è stato effettuato uno studio comparativo con una ferridrite sintetica e un complesso organo-minerale, ferridrite-polimerina, quest'ultimo ottenuto per immobilizzazione della polimerina stessa su ferridrite mediante coprecipitazione di una soluzione di nitrato ferrico in presenza della polimerina. Inoltre, è stato effettuato un altro studio comparativo sulla capacità di adsorbimento della polimerina e di due matrici minerali mesoporose, Al_2O_3 e Fe_2O_3, nei confronti dell'acido 4-cloro-2-metil fenossiacetico (MCPA), un erbicida fenossiacetico, e della 2-cloro-4,6-bis(etilammino)-1,3,5-triazina (simazina), uno clorotriazinico.

Il Chyalofop butile (CyB), acido 2-[4-(4-ciano-2-fluoro-fenossi)fenossi] propanoico, butyl estere (R), introdotto di recente dalla Dow AgroSciences, è usato per il controllo in fase di post-emergenza per il riso ed è specifico per il controllo dei giavoni (*Echinochloa spp*.). La forma commerciale è l'estere butilico del cyhalofop che agisce come pro-erbicida e facilita il suo assorbimento da parte della cuticola della pianta; poche ore dopo l'assorbimento avviene la trasformazione del cyalofop butile in cyhalofop acido, che rappresenta la forma biologicamente attiva e quella che si ritrova nelle acque inquinate.

Tra i diversi inquinanti comunemente presenti nei suoli e nelle acque, l'MCPA riveste un interesse particolare, si tratta di un erbicida fenossiacido usato in fase di

post-emergenza per il controllo annuale delle infestanti dei cereali, dei prati e degli alberi. E' molto solubile in acqua (273.9 mg/L), altamente mobile e sembra avere effetti mutageni e carcinogenici.

La simazina è un erbicida sintetico usato per il controllo in fase di pre-emergenza per il controllo delle infestanti a foglia larga e delle erbe annuali in agricoltura. La simazina è il secondo pesticida più frequentemente riscontrato nelle acque di falda e superficiali negli Stati Uniti, in Australia e in Europa. Persiste nell'ambiente anche dopo otto mesi dalla sua applicazione e non è facilmente degradabile dai microbi.

Gli studi di adsorbimento sono stati realizzati considerando diversi parametri, tra cui l'effetto del pH, del tempo di contatto e della quantità di matrice adsorbente. L'adsobente più efficiente per il cyhalofop è risultato essere la ferridrite, seguita dal complesso ferridrite-polimerina e dalla polimerina, mentre per l'MCPA e la simazina i dati sperimentali hanno evidenziato l'elevata capacità di adsorbimento dell'Al_2O_3 rispetto al Fe_2O_3 e alla polimerina.

In particolare il CyA si lega alla ferridrite mediante una combinazione di legami ionici e ione-dipolo, al complesso ferridrite-polimerina mediante legami ionici e alla polimerina mediante legami ad idrogeno.

La polimerina adsorbe l'MCPA mediante la formazione di legami ad idrogeno e la simazina mediante legami ad idrogeno e ionici. L'MCPA si lega ad Al_2O_3 e Fe_2O_3 mediante una combinazione di interazioni ioniche e ione-dipolo. La simazina è adsorbita su Fe_2O_3 mediante legami ad idrogeno e per l'elevata acidità dell'adsorbente anche mediante interazioni elettrostatiche; mentre, l'Al_2O_3 adsorbe la simazina solo mediante legami ad idrogeno. La più alta capacità di adsorbimento di Al_2O_3 rispetto a Fe_2O_3 nei confronti di MCPA e simazina è dovuta alla più elevata area superficiale di questa matrice, nonché alla presenza di micropori secondari che hanno un'influenza positiva nell'uptake di entrambi gli erbicidi.

Acque simulatamente contaminate con CyA sono state completamente purificate dopo due cicli di adsorbimento su ferridrite e cinque cicli sul complesso ferridrite-polimerina, mentre le stesse acque presentavano un residuo costante di erbicida dopo cinque cicli di adsorbimento su polimerina. Lo stesso esperimento condotto per MCPA e simazina ha mostrato che l'Al_2O_3 permetteva la totale rimozione di MCPA dopo quattro cicli di adsorbimento ed una rimozione dell'84% di simazina dopo solo 2 cicli, mentre l'Fe_2O_3 permetteva una rimozione del 92% di MCPA e del 69% di simazina dopo cinque cicli. Esperimenti di adsorbimento ciclico di MCPA e simazina su polimerina hanno indicato che non era possibile una totale rimozione di entrambi gli agrofarmaci dalle acque simulatamente inquinate.

Una matrice adsorbente ideale dovrebbe avere un'elevata area superficiale, pori uniformemente accessibili e una stabilità sia fisica che chimica. E' noto che la capacità adsorbente di una matrice è influenzata dalla sua area superficiale, che aumenta col diminuire delle dimensioni delle particelle, inoltre la distribuzione delle dimensione dei pori è un altro parametro importante per un ottimale processo di adsorbimento. Pertanto, con l'introduzione dei nano-ossidi l'efficienza della rimozione di inquinanti può essere notevolmente aumentata. Le matrici minerali possono essere usate come adsorbenti per una rimozione rapida ed efficace di pesticidi ionici e ionizzabili in aree soggette ad inquinamento puntiforme. Inoltre, Al_2O_3 essendo rigenerato mediante incenerimento può essere considerato per trattamenti su piccola scala e su scala industriale.

Un'ulteriore strategia innovativa volta alla valorizzazione delle AV riguarda il loro uso come mezzo di crescita di microrganismi oleaginosi con il duplice scopo della potenziale produzione di biodiesel e della riduzione dell'impatto ambientale negativo associato allo smaltimento di tale refluo. La richiesta di metil esteri di acidi grassi (GAMEs) come materiale di partenza per la produzione di biodiesel è notevolmente aumentata. I microrganismi oleaginosi sono in grado di produrre e accumulare elevate quantità di lipidi che, pertanto, possono essere usati per la potenziale produzione di biodiesel. Il secondo obiettivo di questa tesi è stato quello di valutare la capacità di *Lipomyces starkeyi*, un lievito oleaginoso, di sopravvivere e proliferare in presenza di AV come mezzo di crescita. *L. starkeyi* è in grado di accumulare un'elevata quantità di lipidi riutilizzandone solo una piccola parte. Tale lievito accresciuto in presenza di AV tal quali, senza supplemento esterno di carbonio, ha mostrato una significativa riduzione del carbonio organico totale (TOC) e del contenuto di fenoli del refluo. Test di fitotossicità condotti su semi di *Lactuca sativa* usando AV prima del trattamento microbiologico hanno dimostrato che è necessaria una diluizione del 25% delle acque per ottenere un lieve aumento dell'indice di germinazione, mentre per le acque trattate e diluite del 10% è stato osservato un significativo aumento della germinabilità. Inoltre, una componente delle AV che avrebbe potuto inibire la crescita microbica è l'olio di oliva residuo presente nel refluo, è stata misurata l'attività lipasica prima e dopo il test di fermentazione ed i dati sperimentali hanno mostrato che non solo l'olio di oliva residuo non inibisce la crescita del lievito, ma la sua attività lipasica extracellulare incrementava significativamente nel corso della fermentazione delle AV. La preliminare diluizione delle AV ha consentito una più efficiente rimozione dei componenti tossici, conducendo ad una riduzione quasi completa del TOC e del 50% dei fenoli. La biomassa oleaginosa di *Lipomyces* è stata successivamente recuperata per estrarne la frazione lipidica (superiore al 20%), ed infine l'analisi della distribuzione dei residui degli acidi grassi (netta prevalenza di acido oleico) ha dimostrato che il materiale lipidico ottenuto rappresenta una materia prima ideale per la sintesi di biodiesel di II generazione.

Infine, abbiamo studiato l'effetto dell'immobilizzazione di due laccasi dai funghi *Myceliophthora thermophila* e *Trametes versicolor* su differenti supporti. Questi enzimi sono in grado di degradare un'ampia varietà di substrati, abbondanti nelle AV, come fenoli, ammine aromatiche, idrossindoli, composti fenotiazinici e molecole lignino-simili. Il terzo scopo di questa tesi è stato quello di studiare l'aumento della stabilità operazionale e della durata degli enzimi dopo il processo di immobilizzazione. La laccasi è una ossidasi a più atomi di rame, capace di catalizzare l'ossidazione di una ampia gamma di substrati con la simultanea riduzione dell'ossigeno ad acqua. La bassa specificità di substrato delle laccasi e la loro capacità di ossidare inquinanti le ha rese interessanti per il loro uso nel trattamento di acque di scarto e in processi di bioremediation. Molte laccasi da differenti fonti potrebbero essere considerate per applicazioni biotecnologiche, tuttavia, le laccasi da piante superiori e da batteri sono meno capaci di degradare polimeri di origine fenolica, inclusa la lignina, rispetto alle laccasi da funghi. In particolare, è ben documentato che funghi termofili possono essere un'eccellente fonte di enzimi industriali termostabili e la tolleranza termica è una caratteristica interessante per molte applicazioni biotecnologiche degli enzimi. Nella presente tesi abbiamo usato una laccasi fungina (Novozym 51003) da un ascomicete termofilo, *Myceliophthora thermophila,* e una laccasi da un fungo basidiomicete, *Trametes versicolor.* Quest'ultimo risulta essere particolarmente interessante in quanto la sua

laccasi ha un elevato potenziale redox (785 mV). Tuttavia, gli enzimi in forma solubile hanno alcune caratteristiche svantaggiose come l'instabilità alle alte temperature, a valori estremi di pH e nei confronti di solventi organici, di conseguenza non possono essere usati per molti cicli di reazione.

L'immobilizzazione delle due laccasi usate nel presente lavoro di tesi ci ha permesso di ottenere catalizzatori eterogenei, caratterizzati da differente attività e stabilità, in vista di un futuro utilizzo a livello industriale, in processi di ossidazione di prodotti altamente tossici ed in grado di operare in diverse condizioni di pH, temperatura o presenza di agenti denaturanti. Entrambi gli enzimi sono stati completamente purificati in un sola fase di adsorbimento-desorbimento usando un supporto a scambio anionico.

L'immobilizzazione della laccasi da *Myceliopthora thermophila* è stata realizzata usando differenti supporti, ottenendo derivati con differente attività e stabilità. L'enzima non amminato è stato immobilizzato su supporti amminati e funzionalizzati con glutaraldeide, cioè contenenti un elevato numero di cariche positive ed in grado di immobilizzare il biocatalizzatore attraverso le aree di superficie ricche in cariche negative nette. La successiva amminazione della laccasi ha permesso di ottenere derivati in cui l'enzima fosse immobilizzato con un rendimento più elevato e, di conseguenza molto più stabili di quelli in cui l'enzima non è amminato. I derivati più stabili in tutte le condizioni di inattivazione saggiate sono risultati essere quelli in cui l'enzima amminato è stato immobilizzato su supporti eterofunzionali di nuova generazione attivati con acido imminodiacetico (IDA) e gruppi glyoxyl. L'immobilizzazione su questi suppporti si realizza in due steps: nel primo step l'enzima è adsorbito al supporto a pH neutro mediante l'interazione tra le cariche negative del supporto e le zone di superficie ricche in cariche positive nette, nel secondo step, alcalinizzando il pH, si realizza l'interazione tra i gruppi amminici nucleofili presenti nelle arre di superficie in cui l'enzima è stato previamente adsorbito, ed i gruppi aldeidici del glyoxyl agarosio, dando origine ad un'intensa interazione covalente multipuntuale. Tale derivati sono risultati essere 30 volte più stabili nelle diverse condizioni di pH e temperatura saggiate, e leggermente più stabili anche in presenza di solventi organici e di agenti denaturanti quali l'urea, rispetto al derivato in cui l'enzima è legato al supporto mediante un unico legame covalente, nelle stesse condizioni sperimentali. Per quanto riguarda la laccasi da *Trametes versicolor*, l'enzima è stato prima amminato e poi immobilizzato su supporti a base di glyoxyl agarosio. Si sono ottenuti derivati con differenti fattori di stabilizzazione, in base alle condizioni di incubazione scelte durante il processo di immobilizzazione. La migliore stabilizzazione è stata ottenuta dopo incubazione a pH 10, tali derivati sono 70 volte più stabili, nelle diverse condizioni di temperatura e pH valutate, se comparati con l'enzima solubile o con il corrispondente derivato immobilizzato mediante un unico legame covalente, inoltre si è osservato anche un fattore di stabilizzazione pari a 2, in seguito al trattamento con solventi organici.

In conclusione, nella presente tesi sono state proposte differenti strategie volte all'utilizzazione delle AV, mediante il recupero di una matrice polimerica (polimerina) usata come adsobente per decontaminare acque inquinate da pesticidi e, allo stesso tempo, volte alla valorizzare di questo refluo, usandolo come mezzo di crescita di microrganismi oleaginosi, per la potenziale produzione di biodiesel. Inoltre, fra le tecnologie sviluppate per smaltire le AV, l'uso di laccasi immobilizzate su una nuova generazione di supporti rappresenta una strategia innovativa e di grande importanza.

ABSTRACT

Olive oil mill wastewaters (OMW) are dark-colored wastes characterized by high values of COD (chemical oxygen demand) and BOD (biological oxygen demand). OMW contains high amounts of organic and inorganic compounds. The first category includes mostly sugars, polyphenols, organic acids, proteins, fatty substances, mixed phenol-polysaccharide polymers, polyalcohols, cellulose and hemicellulose, pectins and tannins. The inorganic substance, present in lower amount, includes mainly potassium and, to a lesser degree, sodium, calcium and magnesium as cations, and chlorides, phosphates and sulphates as anions. OMW, for their composition, are particularly dangerous for soil and waters if dispersed unprocessed because of phenolic compounds and long-chain fatty acids which have phytotoxic and antimicrobial properties. So, the aim of this thesis is to develop integrate strategies aimed at valorisation and/or disposing of OMW.

In order to obtain by-products to reuse in environmental technology processes the recovery of organic matter from this waste was performed. Polymerin, the high molecular organic fraction of OMW, is a polyelectrolyte with humic-like properties and can be used as a potential bio-filter for decontamination of polluted wastewaters because exhibited very interesting sorption capacities for cationic and anionic heavy metals, ionic or ionisable pesticides and hydrophobic organic compounds. The first aim of this thesis is the use of polymerin for the decontamination of wastewaters contamined by pesticides. A study was performed on the sorption capacity of polymerin towards the cyhalofop acid (CyA), a new generation aryloxyphenoxypropionic herbicide, and compare it with a mineral matrix, the ferrihydrite, and an organo-mineral one, the ferrihydrite-polymerin complex. Moreover, we performed another comparative study on sorption capacity of polymerin and two mesoporous mineral matrices, Al_2O_3 and Fe_2O_3 towards the 4-chloro-2-methylphenoxyacetic acid (MCPA), a phenoxyacetic herbicide, and 2-choloro-4,6-bis(ethylamino)-s-triazine (simazine), a chlorotriazinic one.

Cyhalofop-butyl (CyB), 2-[4-(4-cyano-2-fluoro-phenoxy)phenoxy]propanoic acid, butyl ester (R), recently introduced from Dow AgroSciences, is used for the post-emergence control of grasses in rice, mainly against barnyard grass (*Echinochloa* species) and silver top (*Lepthochloa fusca* species). The ester presents a low water solubility, but it hydrolyzes rapidly into its corresponding derivative 2-[4-(4-cyano-2-fluorophenoxy) phenoxy] propionic acid (CyA), which is much more soluble and presents the effective herbicide action. Moreover, CyA is the effective chemical compound occurring in water after its application on the crop because it originates by the rapid hydrolysis of its corresponding butyl ester form.

Among the different pollutants commonly found in soil and waters, MCPA deserves a particular interest. It is a post emergence phenoxy acid herbicide extensively used in agriculture to control annual and perennial weeds in cereals, grasslands, trees, and turf. It is very soluble (273.9 mg/L in water, at neutral pH), highly mobile, and can leach from soil and it is suspected for mutagen and carcinogen properties. This compound has been found in well water in some countries and is classified by the U.S. Environmental Protection Agency (EPA) as a potential groundwater contaminant.

Simazine is a synthetic *s*-triazine herbicide widely used for pre-emergence control of broad-leaf weeds and annual grasses in agricultural and non-crop fields. Simazine

is the second most commonly detected pesticide in surface and groundwaters in the United States, Australia and Europe. It is persistent in the environment up to eight months and not easily degraded by microbes. Due to the carcinogenic potential of *s*-triazines, simazine presence in water is of increasing concern.

Effects of pH, contact time, initial concentration and sorbent dosage on the sorption of the herbicides were investigated. The most efficient sorbent for cyhalofop acid showed to be ferrihydrite followed by ferrihydrite-polymerin complex and polymerin; while for MCPA and simazine the collected data evidenced the greater sorption efficiency of Al_2O_3 with respect to Fe_2O_3 and polymerin.

In particular, cyhalofop acid bonds to ferrihydrite by a combination of ionic and ion-dipole bonds, the ferrihydrite-polymerin complex by ionic bonds and polymerin by hydrogen ones. Sorption of MCPA on polymerin occurred by the formation of H-bonds while for simazine by hydrogen and ionic bonds. Moreover, MCPA is assumed to be bond to Al_2O_3 and Fe_2O_3 by a combination of ionic and ion-dipole interactions. Simazine is sorbed on Fe_2O_3 by hydrogen bonds and because of the high acidity of this matrix also by electrostatic interactions, while on Al_2O_3 sorption occurred only by hydrogen bonds. So, the highest superficial surface area of Al_2O_3 than Fe_2O_3 and the presence of secondary small pores at boundary of micropores region has a positive influence in the uptake of simazine and MCPA.

Simulated wastewaters contaminated with cyhalofop acid were completely purified by two sorption cycles on ferrihydrite and five cycles on the ferrihydrite-polymerin complex, whereas the same wastewaters maintained a constant residue even after five sorption cycles on polymerin. The same experiment carried out for MCPA and simazine indicated that Al_2O_3 allowed the total removal of MCPA by four sorption cycles and a removal of 84% of simazine after only two sorption cycles, whereas on Fe_2O_3 MCPA was removed for 92% after five cycles and simazine for 69% after five cycles. Cyclic sorption experiments on polymerin revealed that no total removal was possible for both pesticides.

An ideal sorbent should have a high surface area (i.e., high density of sorption sites), uniformly accessible pores and physical and/or chemical stability. It is believed that the sorption capacity of a sorbent is largely determined by the surface area available which increases with decreasing the particle size although the pores size distribution is also decisive for an optimal sorption process. Therefore, with the introduction of nanoscaled oxide materials, the pollutant removal efficiency can be increased dramatically.

So, mineral matrices can be used as sorbents for a fast and highly efficient removal of ionic and ionisable pesticides and as a suitable filters for the decontamination of point sources. Moreover, Al_2O_3 can be regenerated by incineration method and could be considered for small-scale treatment systems and industrial scale.

A further innovative strategy, aimed to enhance the valorisation of OMW, concerns their use as grow media for oleaginous microorganisms, for potential biodiesel production, so this use could be a convenient alternative to avoid the negative environmental impact associated with its discharge. The demand for fatty acid methyl esters (FAMEs) as diesel fuel (biodiesel) has increased significantly. Microorganisms can accumulate high levels of lipids and do not require arable land. Therefore, the microbial lipids can potentially be used as raw material for biodiesel production using

the common way to produce FAMEs in the biodiesel industry. The second aim of this thesis is to investigate the potential of *Lipomyces starkeyi*, an oleaginous yeast able to survive and proliferate in the presence of olive oil mill wastewaters, as a source of lipids. *L. starkeyi* proved to store large amounts of lipids, showing only a minimal reutilization of the stored ones. It was grown in the presence of undiluted OMW, without external organic supplements, producing a significant reduction of both the total organic carbon (TOC) and the total phenol content. The OMW treated by *L. starkeyi* showed a significant increase of the germination index. The preliminary dilution of OMW enhanced the reduction of polluting components of OMW, leading to a complete TOC removal, as well as to lower levels of residual phenols. The activities of extracellular lipases and esterases significantly increased in the course of the OMW fermentation. A significant increase in lipid yield was observed in *L. starkeyi* in the course of the OMW treatment, particularly enhanced when the feedstock was preliminarily diluted. The fatty acid distribution showed a prevalence of oleic acid, demonstrating the potential of *L. starkeyi* as a source of lipids to be used as a feedstock for the synthesis of II generation biodiesel.

Finally, we investigated the effect of immobilization of two laccases, from the fungi *Myceliophthora thermophila* and *Trametes versicolor*, on different supports. These enzymes are able to degrade a wide array of substrates, abundant in olive oil mill wastewaters, such as phenols, aromatic amines, benzenethiols, hydroxyindoles and phenothiazinic compounds. The third aim of this thesis is to evaluate the enhance of operational stability and durability of the enzymes after immobilization. Laccase is a multi-copper oxidase, able to catalyze the one-electron oxidation of a wide array of substrates with the simultaneous reduction of oxygen to water. The low substrate specificity exhibited by laccase and its ability to oxidize priority pollutants has attracted interest for its use in wastewaters treatment and bioremediation. Many laccases from different sources could be considered for biotechnological applications, but laccases from higher plants and bacterial strains are less capable to degrade polymers of phenolic origin, including lignin, than laccases from fungi. In particular, it is well documented that thermophilic fungi may comprise a rich source of thermostable industrial enzymes. Furthermore, the thermal tolerance is an attractive feature for many biotechnological applications of enzymes. In the present thesis we used a fungal laccase (Novozym 51003) from the thermophilic ascomycete *Myceliophthora thermophila* and a laccase from basidiomycete fungus *Trametes versicolor*. This latter was reported to have the highest redox potential among laccases; i.e., 785 mV versus the standard hydrogen electrode, which makes this enzyme particularly interesting since high redox potentials correlate with high activity. However, enzymes are soluble and they cannot easily be used for many reaction cycles, are quite unstable under conditions of high temperatures, extreme pHs or use of organic co-solvents or toxic products, because of that the enzymes have to be stabilized to convert the process in a profitable one. The immobilization of two laccases presented in this thesis allowed to obtain heterogeneous catalysts with good perspectives to be used at industrial level in different processes of oxidation of products highly toxic. The use of different techniques of immobilization allowed having catalysts with different activities and stabilities for their use in processes in which different conditions of pH, temperature or the presence of denaturant agents (unfolding agents or organic cosolvents) are required.

Both the enzymes were able to be purified almost completely in only one step of adsorption-desorption using anionic exchanger supports. The immobilization of

laccase from *Myceliopthora thermophila* was performed using different supports by which catalyst with different activities and stabilities were obtained. Non aminated enzyme only could be immobilized on supports capable to immobilize through the richest place in negative charges as aminated or glutaraldehyde supports. The amination of this laccase was performed in a very simple way, so the immobilization of the aminated enzyme allowed obtaining derivatives more stable than that obtained with the non aminated enzyme. The most stable derivatives in all assayed inactivation conditions were those where the aminated enzyme was immobilized on heterofunctional supports activated with iminodiacetic acid (IDA) and glyoxyl groups. These derivatives were immobilized through the richest place in positive charges and were more than 30 times more stable than soluble enzyme in some of the assayed conditions; in addition they resulted slightly more stable against the presence of organic cosolvent or in the presence of unfolding reagents (urea).

As regards *Trametes versicolor* laccase, the enzyme was first aminated and than immobilized on glyoxyl supports obtaining derivatives with different stabilization factors depending of the incubation conditions during the immobilization process. The best stabilizations were obtained after incubation at pH 10 where it was possible to obtain stabilization factors over 70 times if compared with the stability of the soluble enzyme or the correspondent one point immobilized derivative (cyanogen bromide support). In addition the best derivatives were at least twice more stable against organic solvents.

In conclusion, in the present thesis different strategies aimed at a biotechnological employment of olive oil mill wastewaters have been proposed, in order to remediate water polluted from pesticide and, at the same time, to valorize this waste using it for potential biodiesel production. Moreover, among the technologies developed to disposing of OMW, the use of laccases immobilized on new generation supports is an innovative and great important strategy.

1. GENERAL INTRODUCTION

1.1 Olive oil industry by-products: production, environmental problems and regulation

The olive oil production is a significant agricultural activity with a great economic importance particularly in Mediterranean countries, which produce more than 98% of the world's olive oil, estimated at over 2.5 million metric tons per year. About 75% is produced in the European Union (EU). Over the last decade, olive oil production has increased about 40% worldwide and Europe has obtained an increase of 45% in production. Spanish olive oil production, which represents about half of European production, has shown an increase of almost 80% during that time (FAOSTAT, 2009).

Two different extraction systems are used in the production of extra-virgin olive oil: the traditional olive oil mill, which implements a discontinuous pressing process, and the continuous three phase system, which works by centrifugation. In both processes, two by-products are obtained: a solid and liquid residue, this latter is named olive oil mill wastewaters (OMW). In the last years, a new centrifugation extraction system has been developed, so-called "two-phase", that generates a single phase made up of a mixture of the solid residue and OMW. In summary, for oil extraction 3-processes are applied (figure 1):

a. Pressing, traditional system

b. Continuous process with three-phase decanters

c. Continuous process with two-phase decanters

In press process, the traditional method, the olives are washed, crushed and kneaded with addition of water called "process water". The resulting paste is then pressed to separate the oil and the vegetation water which is the liquid phase of the process from the solid phase that is called olive cake. Finally, the oil is separated from the wastewater by vertical centrifugation or decanting (Demichelli&Bontoux, 1996). In centrifugation system, horizontal centrifuge (decanter) is used. It substitutes the press, allowing continuous operations. Depending on the decanter used during the production with centrifugation systems, two processes can be distinguished:

3-Phase System: It requires "process water" and produces: oil, wastewaters and olive cake. This process needs addition of significant quantities of water, therefore, it generates a considerable volume of olive oil mill wastewates (three times more than the traditional system).

2-Phase System: No "process water" is added and only two phases are produced : oil and olive cake. This system is ecologically attractive because the aqueous phase (olive oil mill wastewaters) is almost reduced to zero. The major part of vegetation water is retained by the solid phase. For the two systems, the obtained solid phase is rich in water (50-60% of water) and contains 2-3% of residual olive oil.

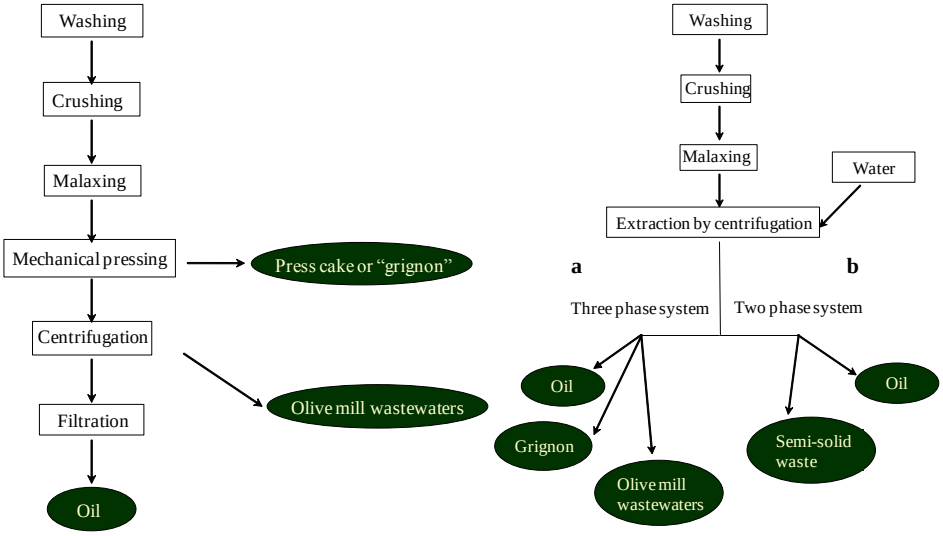

Figure 1. Pressing and continuous three phase (a) and two phase (b) systems for olive oil production.

Solid and liquid OMW are dark-colored wastes and contain high amounts of organic materials. They are composed of many complex substances that are not easily degradable. OMW generally contain, as dissolved or suspended substances, mostly sugars, polyphenol compounds, organic acids, proteins and related nitrogen compounds, fatty substances, mixed phenol-polysaccharide polymers, polyalcohols, cellulose and hemicellulose, pectins and tannins. The inorganic substances, present in lower amount, include mainly potassium and, to a lesser degree, sodium, calcium and magnesium as cations, and chlorides, phosphates and sulphates as anions. Phenolic compounds, which are usually present in OMW consist of monocyclic aromatic molecules, such as hydroxytyrosol, tyrosol, catechol, methylcatechol, caffeic acid, and higher molecules mass compounds obtained through their polymerisation. For this chemical composition OMW are characterized by high values of COD (chemical oxygen demand) and BOD (biological oxygen demand). Chemical composition of OMW is reported in table 1.

PARAMETER	OMW (Conventional Process)	OMW (Continuous Process)
pH	4.5-5.0	4.7-5.2
Total solids (%)	12	3
Total mineral SS (%)	1.5	0.4
Suspended solids (%)	0.1	0.9
COD (g/L)	120-130	40
BOD (g/L)	90-100	33
Sugar (%)	2.8	1.0
Total nitrogen (%)	5-2	0.28
Organic acids (%)	0.5-1.0	-
Polyalcohols (%)	1.0-1.5	1.0
Pektin, tannin etc. (%)	1	0.37
Polyphenols (%)	1.0-2.4	0.5
Oil (%)	0.03-10	0.5-2.3

Table 1. Chemical composition of OMW obtained from conventional and continuous processes.

Therefore, the olive oil mill effluent causes an important environmental problem. Deterioration of natural water bodies due to olive oil mill wastes is a serious problem as indicated by coloring, appearance of an oily shine, and increased oxygen demand, they also affect the soil quality. As already observed, one feature that makes olive oil mill wastewaters particularly harmful if dispersed unprocessed, such as by directly pouring it into soils as a fertilizer, is its content of polyphenol compounds. These latters, while in the olives are protective agents with antioxidant and bacteriostatic functions until the olives ripen, when are contained in the olive processing wastes may have a phytotoxic and antibacterial action (Capasso et al., 1992; Capasso et al., 1995; DellaGreca et al., 2004). Phenols in seeds have also been proposed as germination inhibitors (Khan and Ungar, 1986). The presence of phenols causes the inhibition of germination of *Atriplex Triangularis* and *Pinus laricio* seeds (Muscolo et al., 2001). Among phenolic compounds, gallic acid significantly reduces the larval growth of *Spodoptera frugiperda* neonates (Bulla et al., 2004). Consequently, if applied as received, olive oil mill wastewaters could have a negative environmental impact and the disposal of raw OMW by spreading over soils according to the laws of various producing countries may impact greatly on land and water environments for the broad spectrum toxicity. In this light should be necessary to reduce their negative impact before discharged. Pollution abatement for olive oil mill wastewaters is difficult for the high organic loading, seasonal operation, high territorial scattering and for the presence of organic compounds, which are hard to biodegrade such as phenolic compounds and long-chain fatty acids (Ergüder et al., 2000).

For this reason, several provisory legislation and ministerial decrees were promulgated and strategic plans were applied in some countries. One of them has foreseen spreading of wastewaters on land, such as in Italy. The Italian Government in 1996 promulgated a regulation about olive waste disposal and/or recycle, the law 574/96: “New regulation about the agronomical use of olive oil mill waste”. This law permits the agronomical utilization of olive oil mill wastes upon approval of the local Authority. Ten articles state the possibility of spreading the olive wastewaters as fertilizers on croplands. These articles regulate the maximum amount of waste, deriving from pressing or continuous processes, that can be spread, the distribution modality and explain which are the land that are excluded from spreading.

1.2 Technologies aimed at recycling and/or disposing of olive oil mill wastewaters

Various methods for the treatment of olive oil mill wastewaters and olive residues can be used. The great variety of compounds of the waste needs several technologies to remove the harmful compounds for the environment. A lot of factors must be considered to choose the most suitable strategies, e.g. waste state (liquid or solid), waste amount, costs and investment, required area, specific training of the workers, noise and odour emissions and seasonal trouble.

The more conventional methodologies used to dispose of OMW are listed below, with their advantages and disadvantages:

1. Evaporation lagoons

One of the treatment methods is to collect wastewater in the lagoons and let them evaporate. Seasonally production of wastewaters and this easy way, led to be preferred this method for long time. Disadvantages of the method are necessity of large fields and the distance between manufacturing plant and the field and also wastewater leakage into the groundwaters.

2. Membrane Process

Membrane filtration such as reverse osmosis or ultrafiltration is applied on olive oil mill wastewaters after some pre-treatment steps. In this case, wastewater is separated into two phases: treated wastewater (permeate), and concentrate (retentate). The concentrate can be sent to incineration or landfilled for final disposal.

3. Chemical Oxidation

Chemical oxidation using ozone or advanced oxidation technologies based on the generation of hydroxyl radical is a possible way to reduce COD and polyphenol content.

4. Irrigation

In this method, olive oil mill wastewaters are before concentrated in lagoons, and after pH correction, they can be used for irrigation. This is a rather cheap process, it can fertilize the soils, however, the controlled discharge is very important due to high amount of phenolic substances.

5. Membrane process

Membrane filtration such as reverse osmosis or ultrafiltration is applied on olive oil mill wastewaters after some pretreatment steps. In this case, wastewaters is separated into two phases: treated wastewaters (permeate), and concentrate (retentate). The concentrate can be sent to incineration or landfilled for final disposal.

6. Composting

Olive oil mill residues composting, which permits the degradation of toxic compounds, is suggested by different researchers who also propose to reuse the cured compost in agriculture as eco-compatible and good quality organic amendments and fertilizers (Tomati et al., 1996; Cegarra et al., 1996; Ranalli et al., 2002). In addition, composts from different vegetal origin, due to their beneficial effects, are also suggested to be used as alternative means to control plant pathogens (Lazarovits, 2001; El-Masry et al., 2002; Raviv, 2005).

7. Aerobic Biological Treatment of Olive Oil Mill Wastewaters

Olive oil mill wastewaters are rich in phenols, inhibiting biodegradation and showing some degree of ecotoxicity. Therefore, in order to achieve the aerobic biological treatment of OMW, the key is the elimination of the phenols. The major point is the decolorization, biodegradation and biotransformation of the phenolic compounds, abundant in the olive oil mill wastewaters, by different kinds of microorganisms. The process needs to be effective for degradation of both simple phenols and high molecular weight polyphenols. Either free or immobilized microorganisms can be used in continuous or batch processes. The pre-treatment of OMW with the white rot fungi can decrease the COD concentration. The presence of phenolic inhibitors requires the use of microorganisms able to utilize aromatic compounds.

Nowadays, researchers propose numerous methods for the treatment of olive oil mill wastewaters and olive residues developing new technologies aimed at increasing the value of OMW or making the old methodologies cheaper and more effective. However, the most of the methods above reported have been tested in laboratory scale and only some of them used in industrial scale.

1.3 Application of OMW in environmental technologies

1.3.1 Water pollution from pesticides

Environmental pollution is contamination of air, water and land from man-made wastes. Pollution is the introduction of contaminants into the environment that causing harm or discomfort to humans and other living organisms. Pollutants can be naturally occurring as substances or energies, but are considered contaminants when in excess of natural levels. Some pollutants are biodegradable and therefore will not persist in the environment in the long term. However, the degradation products of some pollutants are themselves polluting.

The quality of soil and surface waters deserves particular attention because of its importance in the ecosystem survival as well as in the supply of water for drinking and amenity use. Thus, their protection and, eventually, their remediation is a necessity of primary importance.

Water pollution is any chemical, physical or biological change in the quality of water that has a harmful effect on any living organisms that drinks or uses or lives it. There are several classes of water pollutants. The first are disease-causing agents. These are bacteria, viruses, protozoa and parasitic worms that enter into sewage systems and untreated waste. A second category of water pollutants is oxygen-demanding wastes; wastes that can be decomposed by oxygen-requiring bacteria.

When large populations of decomposing bacteria are converting these wastes, it can deplete oxygen levels in the water. This causes the death of other organisms in the water, such as fish. A third class of water pollutants is water-soluble inorganic pollutants, such as acids, salts and toxic metals.

Another class of water pollutants are nutrients; they are water-soluble nitrates and phosphates that cause an excessive growth of algae and other water plants, which deplete the water's oxygen supply. Water can also be polluted by a number of organic compounds such as oil, plastics and pesticides, which are harmful to humans and all plants and animals in the water. A very dangerous category is suspended sediments, because they cause depletion in the water's light absorption and the particles spread dangerous compounds such as pesticides through the water. Finally, water-soluble radioactive compounds can cause cancer, birth defects and genetic damage and are thus very dangerous water pollutants.

Water pollution is usually caused by human activities. Pollution is often classed as point sources or nonpoint sources pollution. Point sources pollution is contamination that enters the environment through any discernible, confined, and discrete conveyance, such as a smokestack, pipe, ditch, tunnel, or conduit; they remain a major cause of pollution to both air and water and are differentiated from non-point sources, which are those that spread out over a large area. Examples of nonpoint sources are: acid deposition from the air, traffic, pollutants that are spread through rivers and pollutants that enter the water through groundwater. Nonpoint pollution is hard to control because the perpetrators cannot be traced. Point sources of water pollution include municipal sewage treatment, plant discharges and industrial plant discharges. Examples of point source water pollution include agricultural and urban runoff, and runoff from mining, and construction sites. Industrial point sources can contribute pollution in the form of toxic chemicals and heavy metals and are the result of industrial using waters used in production processes.

So, the ability for pollutants to reach surface water or groundwater is enhanced by the amount of precipitation (rain) or irrigation. The release of pollutants into the environment can kill organisms outright, change the biogeochemical conditions and processes occurring within a system and result in systemic changes that degrade habitats and make disorders in ecological processes. In rural areas, irrigation run-off from farming activities may sometimes contains insecticides, fertilizers and herbicides that have been applied to crops. This runoff may affect aquatic and marine organisms living in the catchments and their associated estuaries and in-shore marine ecosystems. In the first part of this thesis we focus the attention on water decontamination from an agricultural point source pollution, as pesticides.

Food and Agriculture Organization (FAO) has defined the term of *pesticide* as: *"any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies".*

Pests are those organisms like weeds, insects, bacteria, fungi, viruses and animals which can i) reduce the quality and quantity of food produced by lowering production and destroying stored produce; ii) harm our animals (like fleas, worms and diseases) and iii) compete with humans for food and affect the health, welfare and way of life of people.

In many countries, pesticides are considered an essential part of agriculture. It is believed that if the pesticides are not used to protect plants from insects and other pests, the crop may have negative economic effects. But excessive use of pesticides may have serious environmental consequences. There are many examples of entire communities suffering from chronic pesticide poisoning. Even when used properly, some pesticide chemicals remain in the environment for years, evaporating into the atmosphere and polluting the entire planet. FAO is constantly researching and testing ways of reducing farmers' dependency on pesticides. Less pesticide means fewer toxic chemicals in the environment, fewer accidental poisonings and lower expenses for the farmer.

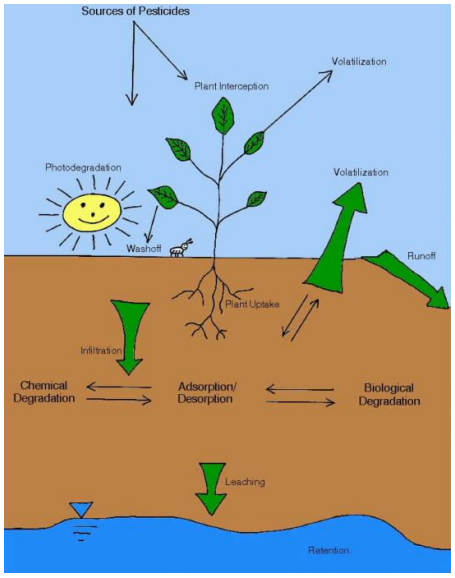

Figure 2. Pesticide movement in the environment.

In figure 2 is simplified the complex movement of pesticides in the environment after their application on crops. Only a part of the applied amount of a pesticide is bioactive, the rest is distributed in the environmental. In fact, the agrochemicals can move from the application point to different environmental compartments, such as air, soil and water. They can be degraded from sunlight, and this process usually leads to the formation of less harmful breakdown products but in some instances can produce more toxic products or they can be volatilized, polluting the atmosphere. In other cases, pesticides may be washed off the soil into nearby bodies of surface water. In the soil, agrochemicals, if not adsorbed, may undergo processes of chemical or biological degradation, and may percolate through the soil to lower soil layers and groundwaters. All these processes are significantly influenced by chemical-physical properties of pesticides, such as water solubility, hydrophobic/hydrophilic characteristics, vapour pressure, acidity and basicity and so on. Production and uses of pesticides are still increasing but must to be strongly controlled to minimize the health and environmental problems. It is more economical to reduce the applied amounts than to decompose the non-bioactive amounts, if ever possible (Fait et al., 2007). An important way to achieve this aim is the design of formulation which combine an optimum of bioactivity with a minimum amount of pesticides.

1.3.2 Recovery of polymerin, an agricultural by-product, as new biosorbent matrix

The protection of surface and groundwaters from pollutants of different chemical nature as heavy metals, herbicides and polycyclic aromatic hydrocarbons (PAHs), derived from agricultural and industrial activities, is a very important actual environmental problem, considering also the viable importance of the water, especially in the poor countries in the world. Contamination arising from sources such as non approved use, poor practice, illegal operations, accidental releases from the farmyard, and inputs of washings is increasingly recognized as contributing to water contamination and is defined as point source (Spanoghe et al., 2004; Guzzella et al., 2006; Muller et al., 2002). The application of treatment systems at the farm level could allow an improved protection of the aquatic ecosystems and a reduction of water treatment costs. In this light, the possibility of using vegetable biomass wastes as a biofilter for the remediation of wastewaters generated at point sources has been widely described (Hesketh et al., 1996; Akhter et al., 2007; Rodriguez-Cruz et al., 2007). In the past 10 years, biological systems, known originally as biobeds (Castillo et al., 2008), were developed to remediate surface water point-source pollution from pesticides. The use of free-cost organic biomass from agricultural and industrial wastes could represent a cheap and effective method for the protection of waters. For this reason, many studies have been carried out in the past 20 years by the international scientific community on water protection and remediation by using sorption processes. In particular, several studies have dealt with sorbents of a vegetable nature derived from industrial and agricultural waste, with the double objective of recycling natural free-cost material and ecologically disposing or reusing the exhausted sorbents.

During the olive oil production process, olive oil mill wastewaters (OMW) are obtained. These waste waters have significant polluting properties due to its high levels of chemical oxygen demand (COD), biological oxygen demand (BOD) and phenols (Demicheli and Bontoux, 1997; Arienzo and Capasso, 2000). Polluting properties are mainly due to their polyphenol content and its synergy with other

naturally occurring compounds (Capasso et al., 2002b) makes OMW unsuitable for discharge to soil and waters.

Among several natural organic sorbents investigated in the last fifty years, polymerin, a humic acid-like polymer, represents the characteristic dark polymeric pigment of OMW. This polymeric organic fraction was recovered from OMW by a three-step purification procedure (figure 3). In the first step 1L of OMW was centrifuged, filtered and concentrated to 0.5 L. In the second step, the concentrated sample was precipitated with cold methanol and centrifuged. In the third step, the dark precipitate was re-dissolved in ultra pure water, dialyzed and the non-permeated fraction was freeze-dried, giving a brown residue, named *polymerin* (Capasso et al., 2002a).

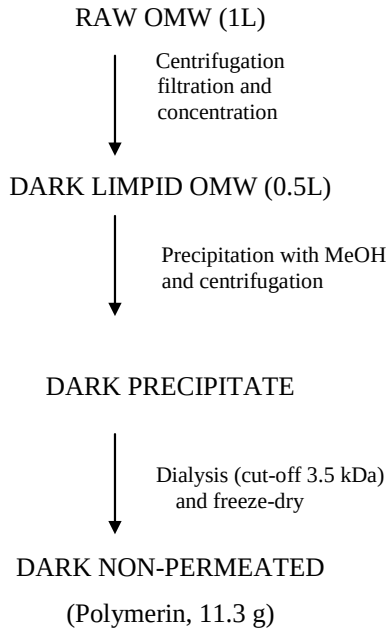

Figure 3. Recovery of polymerin from olive oil mill wastewaters.

As reported by Capasso et al., 2002a the chemical analysis show that polymerin is composed of carbohydrates, melanin and proteins (table 2). In particular, the monosaccharides and amino acids content is reported in tables 3 and 4. Moreover, it contains also metals, mainly K and, to a lesser extent, Na, Ca, Mg, Zn, Fe and Cu.

ORGANIC COMPOSITION	mg/100 mg
Polysaccharides	47.4 (± 0.007)
Proteins	20.4 (± 0.034)
Melanin	30.2 (± 0.01)
Metals	2
TOTAL	**100**
C	46.0 (± 0.205)
N	2.4 (± 0.007)
O + H	49.6

Table 2. Chemical composition of polymerin as reported by Capasso et al., 2002a.

	% of sugar present in polymerin	% of monosaccharides in the polysaccharide component
Arabinose	20.9	38
Galactose	10.45	19
Glucose	9.9	18
Ramnose	5.5	10
Glucuronic acid	4.4	8
Galacturonic acid	3.85	7
Total	**55**	**100**

Table 3. Monosaccharide composition of polymerin determined by HPAEC-PAD expressed in weight % as reported by Capasso et al., 2002a.

Amino acid	% of amino acids present in polymerin	Number of amino acids in the protein component
Asp + Asn	1.98	15
Glu + Gln	2.02	14
Cys	0.1	1
Thr	0.87	5
Ser	1.37	13
Pro	1.1	11
Gly	2.29	31
Ala	1.36	15
Val	1.35	12
Met	0.37	2
Ile	0.6	5
Leu	1.15	9
Tyr	0.55	3
Phe	0.54	3
Lys	0.054	1
His	0.088	1
Arg	0.64	4
Total	**16.6**	**143**

Table 4. Amino acidic composition of polymerin determined by automated Amino Acid Analyzer expressed in weight (%) as reported by Capasso et al., 2002a.

Studies carried out by gel filtration chromatography on polymerin show that polymerin is composed of a free polysaccharide and a supramolecular aggregate of protein, melanin and polysaccharide, strongly attached to each other by a combination of covalent, hydrogen bonds and CH/π interactions.

Both the supramolecular aggregated portion and the free polysaccharide are bound to metals and this interaction occurs through carboxylate and other nucleophile functional groups, also in chelating form, identified in the acidic amino acids and uronic acids and in the melanin system (Capasso et al., 2002a).

As reported by Capasso et al., 2002b polymerin derivatives, obtained by saturation of this matrix with micronutrients (Cu, Zn, Mn and Fe) and tested on tomato plants showed that only Mn derivative of polymerin caused witling of the plants, whereas all the other metal derivatives were non toxic. This suggests that these derivatives may be used as bio-amendments and/or metal-biointegrator; in addition, the melanins are humic acid-type polymers which are involved in soil fertility (Paim et al., 1990; Biliska, 1996).

Polymerin shows much lower COD and BOD values in comparison with those of raw OMW (Arienzo and Capasso, 2000). Finally, the polyelectrolyte characteristics of polymerin and its relatively high content of the easily removable K suggest that this biomaterial may be used as a potential bio-filter to clean up polluted waters.

1.3.3 Removal of pesticides by sorption on polymerin

The removal of organic pollutants from waters is performed using specific treatments and/or processes as membrane filtration, ozonization, oxidation and phytoremediation, but one of the most important is adsorption.

However, the methods currently used for the removal of pollutants are often found to be expensive and ineffective. Hence, an urgent need exists to develop less expensive, simpler and more efficient methods for contaminant removal from water. Adsorption is one of the most relevant methods that has been successfully applied for heavy metals and organic chemicals removal from hazardous wastes due to low maintenance costs, high efficiency and ease of operation. Sorption reactions generally occur over a short period of time, however if the adsorbed contaminant begins to be incorporated into the structure of the sorbent, a slow occurring reaction, known as absorption, begins to take place. The difference between adsorption and absorption is that adsorption is the attraction between the outer surface of a solid particle and a contaminant, whereas absorption is the uptake of the contaminant into the physical structure of the solid.

The use of vegetable biomasses for decontamination of wastewaters by adsorption of pollutants is of great importance and it is an environmentally clean process.

Pesticides, as other organic pollutants, when in contact with organic materials, tend to be adsorbed. For example, sorption of paraquat on ayous (*Triplochiton schleroxylon*) sawdust was 36.83 $mmolKg^{-1}$ (Nanseu-Njiki et al., 2001). Spent diatomaceous earth from wine processing and its activated product had a larger sorption capacity of paraquat corresponding to 19 and 95 $mmolKg^{-1}$ (Tsai et al., 2005). Sorption of 2,4-D on activated carbon derived from date stones was 1076 $mmolKg^{-1}$ (Hameed et al., 2009).

In particular, the utilization of polymerin for remediation of contaminated waters by pesticides and other chemicals could represent a convenient method of bioremediation, because this material derives from a cost-free agricultural waste and possesses humic acid-like characteristics. Polymerin and its derivatives have shown very high and versatile sorption capacities for toxic inorganic or organic (Sannino et

al., 2008: De Martino et al., 2009; Sannino et al., 2009a, 2009b; Iorio et al., 2008) compounds.

In precedent studies the use of polymerin has been proposed, in particular, polymerin is able to sorb paraquat and 2,4-D. Decontamination of simulated wastewaters by cyclic sorption indicated a total removal of paraquat after three cycles and a partial one of 2,4-D after two cycles (Sannino et al., 2008).

Studies reported by Iorio et al. 2008, indicated that polymerin sorbed also nonpolar compounds as phenanthrene (phen). The results showed that polymerin and the dried complex Al_2O_3-polymerin sorbed an high amount of phen than Al_2O_3 nanoparticles. This latter sorbent has a positively charged surface, and due to the hydration layer, it is not an effective sorbent for phen. By coating the oxides with negatively charged organic matter, it is possible to change the hydrophilic surfaces into hydrophobic ones, which have greater affinity for phen. The results obtained on cyclic sorption indicated that only two cycles were necessary for the total remediation of water using either polymerin or polymerin-Al_2O_3, whereas too many cycles were required using Al_2O_3 nanoparticles alone. In these studies polymerin and its derivatives proved also to be the most efficient sorbent for the removal from waters of organic pollutants as paraquat, and phen while for 2,4-D a residual amount remained in solution in the cyclic sorption experiments.

Another study conducted on sorption of heavy metals on polymerin indicated that the individual sorbing capacities of Zn, Cu(II), Cr(III) on polymerin was considerable and followed the order Cr(III)>Cu(II)>Zn. The high sorption of the selected heavy metals on polymerin was consistent for the presence of negative sites on the surface of the organic matter. A study on the sorption mechanism of the selected heavy metals on polymerin, by using a combination of Diffuse Refractance Infrared Fourier Transformed (DRIFT) and Atomic Absorption Spectrometry (AAS) analysis, indicated that these heavy metals were substantially sorbed on polymerin by ion exchange with Ca and K, naturally occurring in polymerin (Capasso et al., 2004) and a concomitant complexation with the OH groups of its polysaccharide component. Polymerin has been shown to be very suitable for the removal of heavy metal cations from simulated polluted waters by cyclic sorption, with the additional advantage to release in water useful metals such as K, Mg, and Ca, natively occurring in the sorbent. More specifically, it has been shown to remove very efficiently Cr(III) and Cu(II), but to lesser extent Zn. Sorption of Zn, Cu, and Cr(III) on polymerin is higher than sorption on other bio-sorbents described in the literature (Volesky and Holan, 1995; Aderhold et al., 1996; Gardea-Torresday et al., 2001; Schneegurt et al., 2001; Wilson et al., 2003). Sorption studies were also conducted on the Cr(VI), the toxic chromium form. The maximum amount sorbed of Cr(VI) was 195 $mmolkg^{-1}$. X-ray photoelectron spectroscopy (XPS) and DRIFTS investigations showed that on polymerin Cr(III), mainly (75%), and Cr(V) (25%) were the effective sorbed chromium forms, which are formed by the redox reaction at pH 4.0 of Cr(VI) with the CH_2OH groups of the polysaccharide moiety of this sorbent (Sannino et al., 2009a). A comparative studies carried out on a complex ferrihydrite-polymerin and only ferrihydrite showed that the maximum sorption of Cr(VI) was 206 and 255 $mmolkg^{-1}$, respectively. Finally, polymerin sorbed also As(V) and the amount was 384 $mmolkg^{-1}$. The same study conducted on ferrihydrite-polymerin and ferrihydrite showed a maximum sorbed amount of 880 and 743 $mmolkg^{-1}$ respectively. Experiments of cyclic sorption carried out on polymerin indicated that the total removal of Cu(II) and Zn was performed after three cycles while four and five sorption cycles were necessary for Cr(VI) and As(V).

The reduction from Cr(VI) to Cr(III) indicate that it is possible to recover a useful metal of considerable industrial interest. In this way polymerin may be used in environmental technology processes to generate Cr(III) from Cr(VI) formed in the oxidation process of some tannery plants, before these wastes are discarded. Finally, ferrihydrite-polymerin could represent an efficient sorbent for the removal of metalloid oxyanions from contaminated waters. These data promote the use of this matrix for sorption studies of pesticides actually employed.

1.3.4 Removal of pesticides by sorption on nanoparticles

Nanotechnology, which is defined as “understanding and control of matter at dimensions of roughly 1-100 nm, where unique phenomena enable novel applications,” is making a significant impact on our everyday lives. Although nanotechnology has been used in fields such as medicine, biotechnology and electronics, its beneficial application to drinking water treatment has begun only recently (Simon, 2005; Glenn, 2006). Because of their size, nanomaterials can exhibit an array of novel properties that can be used to develop new technologies and improve existing ones. Characteristics such as large surface area, potential for self assembly, high specificity, high reactivity, and catalytic potential make nanoparticles excellent candidates for water treatment applications. In particular, nanomaterials, as adsorbent matrices, could remove contaminants from drinking water during municipal treatment. The performance of adsorbent media in a fixed bed column depends mainly from two factors: the adsorption capacity of the media and its mass transport kinetics. Since pore and surface diffusion generally control the internal mass transport, it is expected that usage of materials and shapes which facilitate these types of internal transport, to beneficially affect the mass transport kinetics. External mass transport depends on particle size of the media, which can be controlled during the fabrication process. Nanoparticles have been proven to possess great potential for the removal of many kinds of pollutants.

For example, carbon nanotubes (CNTs) and fullerenes, recently discovered allotropes of carbon, have been proposed for the adsorption of polycyclic aromatic hydrocarbon (PAHs) (Kun Yang et al., 2006), chlorobenzenes (Xianjia Peng et al., 2003), herbicides (Qingxiang Zhou et al., 2006) as well as cadmium (Yan-Hui Li et al., 2003a), zinc, copper (Yan-Hui Li et al., 2003b), and chromium (Ze-Chao Di et al., 2006) from water. Metal nanoparticles have also been proposed as adsorbents: silica-magnetite for phenols, zero valent iron for cobalt, aluminum oxides for PAHs (Iorio et al., 2008) and sulphur mustard, Fe_2O_3 for copper, titanium silicate for cadmium. Moreover, natural and synthetic clay mineral nanoparticles, such as montmorillonite and layered double hydroxides (LDHs), have been studied for their potential use in water decontamination. Montmorillonite nano-composites have been proposed for the removal of radiostrontium in complex ionic environments, Mg/Al-LDH holds promise for the removal of hexavalent chromium. Several studies have reported that nanoparticles possess higher adsorption capacity than larger particles of the same nature. For example, a study carried out by Iorio et al. 2008 demonstrated that aluminum oxides nanoparticles (60 nm) sorbed an amount of phenanthrene 70 times higher than microparticles (100 μm). Inorganic metal oxides, Fe_2O_3, are known to be capable of binding to metal ions in solutions and in the nanoscale are potentially highly efficient, their use is thus becoming very attractive in a new area of adsorption or recovery of metal ions from industrial wastes or natural water streams.

Therefore, in this thesis we investigate the sorption capacity of new mesoporous metal oxides, Al_2O_3 and Fe_2O_3, for the removal of current used pesticide from polluted waters, like simazine and MCPA.

1.4 Microbial conversion of olive oil mill wastewaters

The research on OMW valorisation and bioremediation concerns anaerobic treatment for biogas production and aerobic treatment (e.g. for single cell protein production using yeasts or fungi) (Fiestas Ros De Ursinos and Borja-Padilla, 1992, 1996; Ramos-Cormenzana et al., 1996).

A further innovative strategy, aimed to enhance the valorisation of OMW, concerns their use as grow media for oleaginous microorganisms, as sources of lipids for biodiesel production. Demand for fatty acid methyl esters (FAMEs) as diesel fuel (biodiesel) has increased significantly due to the instability of petroleum prices and the development of government measures in many countries around the world that establish a minimum proportion of biofuel for all petrol and diesel used in transport. For instance, the European Union establishes a minimum content of 5.75% of biofuel by 2010 (European Union Directive 2003/30/EC) and the United States plans to increase the amount of bioethanol and biodiesel to 12.95 and 36 billion gallons by 2010 and 2022, respectively (Energy Independence and Security Act of 2007). Biodiesel constitutes a renewable fuel that is compatible with current commercial diesel engines and has clear benefits relative to diesel fuel including enhanced biodegradation, reduced toxicity and a lower emission profile. However, biodiesel presents some disadvantages. One of its drawbacks is the high manufacturing cost, which is mainly due to the high cost of the vegetable oil. Actually, 70-90% of the biodiesel production cost corresponds to raw vegetable oil. In addition, the biodiesel industry competes with the food industry for oil crops. In fact, it has been calculated that a very large percentage of the current available arable land is required to achieve the current biofuel objectives using crops such as rapeseed or sunflower.

Therefore, it is necessary to explore new raw materials that reduce the biodiesel price without competing with food production. In this context, oils from microorganisms (also called single-cell oils) constitute a promising alternative for producing biodiesel since they present many advantages over vegetable oils from oleaginous plants. Microorganisms can accumulate high level of lipids and do not require arable land. In addition, the production of these microorganisms does not compete with food production since biomass residuals can be used as carbon source. Microorganisms which accumulated more than 20-25% lipids are usually referred to as oleaginous species (Ratledge, 1991). In most cases, the oil from these microorganisms is in the form of triglycerides, which are also the main component in vegetable oils and animal fats. Therefore, the microbial lipids can potentially be used as raw material for biodiesel production using the common way to produce FAMEs in the biodiesel industry. The principal oleaginous microbial species are microalgae, bacteria, fungi and yeasts. The use of microorganisms as a source of lipids has been extensively investigated for their application as food additives, pharmaceuticals and feed ingredients for aquaculture (Ratledge, 1991; Becker et al., 1994; Ratledge, 2002; Alvarez and Steinbuchel, 2002). Microorganisms are sources of edible oils because they have the ability to produce oils rich in polyunsaturated fatty acids, which are in demand as dietary supplements and for infant nutrition. More recently, some works have dealt with the use of oleaginous microorganisms for biodiesel production.

Particularly, microalgae, which capture carbon dioxide by transformation into lipids using sunlight, have attracted recent attention and investment for biofuel production because of their higher oil productivity and faster growth compared to conventional energy crops (Chisti, 2007). However, these photosynthetic microorganisms have problems associated with their growth in bioreactor systems due to the necessity of light supply and large acreages. In addition, the economics of producing biodiesel from microalgae need to improve to make it competitive with diesel (Chisti, 2007). Through changing culture conditions or using genetic engineering modifications, some autotrophic microalgae can be converted to heterotrophic microalgae and such heterotrophic microalgae can also accumulate oils using organic carbon as the carbon source instead of CO_2 (Li and al., 2008). Conversely, little information has been reported so far on the use of lipids from yeast, fungi and bacteria for biodiesel production. Oleaginous microorganisms are defined as microbial with the content of microbial lipid excess of 20%. Biodiesel production using microbial lipids, which is named as single cell oils (SCO), has attracted great attention in the whole world. The first commercial production of an SCO did not begin until 1995 and this only lasted for 6 years before it was closed down as no longer being cost effective. In recent years, a large number of hydrophobic lipid accumulating microorganisms have been studied as substrates for SCO production, especially used in the production of biodiesel. In microorganisms, the extent of lipid accumulation is determined by the genetic constitution, as maximum attainable lipid contents can vary enormously among species and even among individual strains. According to different microorganisms and different culture conditions (such as temperature, pH, culture time, etc.), oil content and composition are different (Alvarez et al., 2002; Papanikolaou et al., 2004; Papanikolaou et al., 2002). In comparison to the microalgae, the growth of these microorganisms can be carried out in conventional microbial bioreactors, which will improve the biomass yield and will reduce the cost of biomass and oil productions. Some yeast strains, such as *Rhodosporidium* sp., *Rhodotorula* sp. and *Lipomyces* sp. can accumulate intracellular lipids as high as 70% of their biomass dry weight. Oleaginous yeasts and molds accumulate triacylglycerols rich in polyunsaturated fatty acids or having specific structure, somewhat limited: oleic (18:1) and linoleic (18:2) acids together with palmitic (16:0) or palmitoleic acids (C16:1) are the most frequently found fatty acids, and nearly all of them are unsaturated fatty acid. Based on these data, oleaginous yeasts and fungi are potential alternative oil resources for biodiesel production. In our case, we investigate the potential of *Lipomyces starkeyi*, an oleaginous yeast able to survive and proliferate in the presence of olive oil mill wastewaters, as a source of lipids. Moreover, the use of this waste product as a free grow media could be a convenient strategy for biodiesel production and at the same time could avoid the negative environmental impact associated with its discharge.

1.5 Biotechnological employment of laccases for potential treatment of olive oil mill wastewaters

Enzymes are biological catalysts that promote the transformation of chemical species in living systems. These molecules, consisting of thousands of atoms in precise arrangements, are able to catalyze the multitude of different chemical reactions occurring in biological cells. Their role in biological processes and in health and disease has been extensively investigated. They have also been a key component in many ancient human activities, especially food processing, well before their nature or function was known. Because of their excellent functional properties (activity, selectivity, and specificity), enzymes are able to catalyze the most complex

chemical processes under the benign environmental conditions (Wong and Whitesides, 1994). Enzymes are able to catalyze, under very mild conditions, very fast modifications of a unique functional group (between several similar groups) existing in only one substrate in the presence of other very similar molecules. Therefore, they may be excellent industrial catalysts in a number of areas of chemical industry such as fine chemistry, food chemistry, and analysis.

Enzymes, in addition to their excellent catalytic properties, also have some characteristics that are not very suitable for industrial and biotechnological applications, in fact they are soluble catalysts, usually very unstable, they may be strongly inhibited by substrates and products; for these reasons they only work well on natural substrates and under physiological conditions (figure 4). In most cases, enzymes have to be greatly improved before their use in industrial and biotechnological processes. The engineering of enzymes, from biological to chemical industries, is one of the most exciting, complex, and interdisciplinary goals of biotechnology.

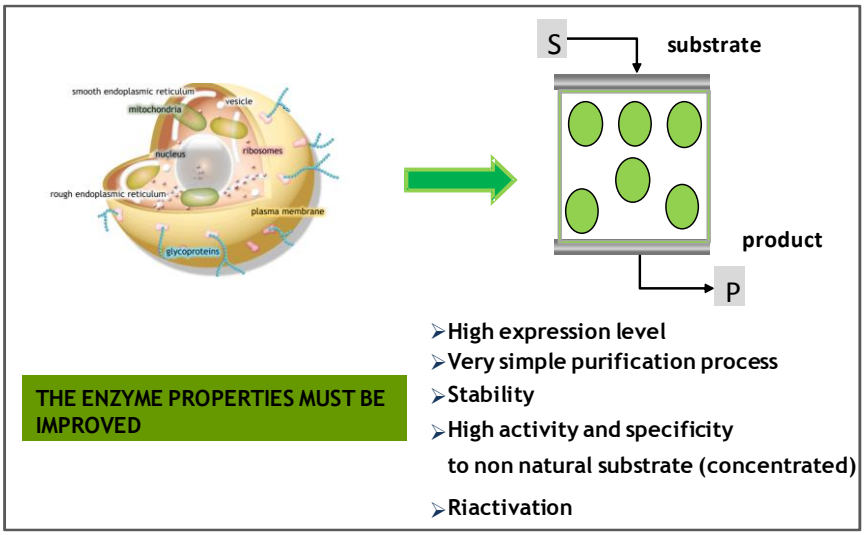

Figure 4. Enzymes: from living creatures to biotechnological applications.

Both phytotoxicity (Martìn et al., 2002; Casa et al., 2003; Sampedro et al., 2005; Bonanomi et al., 2006) and antimicrobial properties (Moreno et al., 1987; Kotsou et al., 2004) of olive oil mill wastewaters have been ascribed to the phenolic fraction. In addition to phenols, the high contents in lignin of the waste (Madejòn et al., 1998; Felizòn et al., 2000) might negatively affect the performances of a composting process. (Tuomela et al., 2000). Therefore, biodegradation of both lignin and phenols are key processes in OMW upgrading. White-rot fungi (WRF) are widely recognized as the most effective lignin degraders in nature, in fact, these fungi can be considered suitable candidates in OMW upgrading. The lignin degrading ability of white-rot fungi seems to be associated with the release of extracellular enzymes, which mainly include laccase (EC 1.10.3.2), manganese peroxidase (MnP, EC 1.11.1.13), and lignin peroxidase (LiP, EC 1.11.1.14) and these enzymes could participate both in the removal of monomeric phenols and in the decolorization of olive residues. In recent years, laccase has become of interest due to its potential biotechnological applications and pollution abatement effect.

This multi-copper oxidase, widely distributed in higher plants and in some bacterial strains of *Azospirillum lipoferum* and *Alteromonas* sp. too, is able to catalyze the one-electron oxidation of a wide array of substrates, such as phenols, aromatic amines,

benzenethiols, hydroxyindoles and phenothiazinic compounds, with the simultaneous reduction of oxygen to water (Thurston, 1994). The low substrate specificity exhibited by laccase and its ability to oxidize priority pollutants has attracted interest for its use in wastewaters treatment and bioremediation. The use of peroxidases and tyrosinase for the removal of aromatic pollutants from process streams has been proposed also (Nicell et al., 1993) but laccase presents the distinct advantage that it does not require the addition of hydrogen peroxide like peroxidases, and it generally exhibits a broader substrate specificity than tyrosinase

1.5.1 Immobilization of laccases

The immobilization of enzymes is a usual requirement to use them at industrial scale. Under this perspective, the immobilization of enzymes must be able to allow the reuse or continuous use of the biocatalyst. From this industrial point of view, simplicity and cost-effectiveness are the key properties of immobilization techniques. In addition, a long term industrial reuse of immobilized preparations also requires the preparation of very stable derivatives that also have suitable functional properties for a given reaction (e.g. activity and selectivity). The practical development of protocols for immobilization of enzymes is intimately related to simplicity, cost effectiveness and stability of the enzyme.

Many protocols for enzyme immobilization are reported in the literature and they should be reassessed according to a number of criteria:

1. No need to use toxic or hazardous reagents during and after the immobilization process.

2. The use of very stable previously activated supports is a clear advantage for the biotechnological industries.

3. The possibility of associating the immobilization process with the improvement of functional properties (activity, stability and selectivity).

4. The preparation of immobilized derivatives useful in different reactions, reaction media or reactors.

5. The preparation of derivatives that can be used in different applications such as fine chemistry, biosensors, environmental processes and so on.

Covalent immobilization or strong physical adsorptions of enzymes, fully dispersed on the internal surface of porous supports, may promote very interesting stabilizing effects such as the immobilized enzyme is not able to undergo any intramolecular process (autolysis, proteolysis, aggregation) and the immobilized enzyme is not able to perform undesirable interactions with large hydrophobic interfaces (air/oxygen bubbles or immiscible organic solvents). Under certain experimental conditions, these random immobilization protocols may promote very important stabilizations of immobilized enzymes with regard to soluble enzymes, as soluble enzymes are able to do aggregations and interactions with hydrophobic interfaces.

There are many methods that allow the immobilization of enzymes for their industrial and biotechnological applications and in this thesis we use some of them with the aim of increasing the operational stability and durability of *Myceliophthora thermophila* and *Trametes versicolor* laccases after immobilization.

1.6 Aim of the thesis

The main objective of this thesis is to develop innovative strategies aimed at valorisation and/or disposing of olive oil mill wastewaters. This waste product, as

previously reported, is a vegetable biomass waste produced in a very limited period of the year, presenting disposal problems for its polluting properties, which are documented by high chemical oxygen demand (COD) and biological oxygen demand (BOD). Polluting properties are mainly due to their polyphenol content which makes OMW unsuitable for discharge to soil and waters. The organic matter, recovered from this waste and named polymerin, has a humic acid-like nature, a good sorbing capacity of anionic and cationic heavy metals, as previously mentioned, and may be employed as biofilter for the removal of ionic and ionizable pesticides from surface waters. The use of polymerin for remediation of pesticide contaminated waters may represent a convenient method for bioremediation, because this material derives from a free cost agricultural waste, it has a low phytotoxicity and very low BOD and COD values, compared to raw OMW. Therefore, its use may contribute to the resolution of OMW disposal problem. In fact, the employment of polymerin in wastewaters remediation processes may also enrich them in biologically useful nutrients as Ca, Mg and K, which are released from polymerin during the clean-up process. The application of polymerin to a larger range of pollutants, makes this bio-sorbent useful and convenient. In particular, we want to study the sorption capacity of polymerin toward different herbicides, the cyhalofop acid, an aryloxyphenoxypropionic herbicide, and compare it with a mineral matrix, the ferrihydrite, and an organo-mineral one, the ferrihydrite-polymerin complex. Moreover, we perform a comparative sorption study on polymerin sorption of simazine, a chemical from chlorotriazine, and MCPA, a phenoxyacetic herbicide, between polymerin and a new generation of mesoporous mineral matrices, Al_2O_3 and Fe_2O_3.

To bring down the negative impact of olive oil mill wastewaters, we also use this waste as grow media for an oleaginous yeast, *Lypomyces starkeyi*, with the aim of obtain lipids suitable for biodiesel production.

Finally, we investigate the effect of immobilization on different supports of two laccase, from the fungi *Myceliophthora thermophila* and *Trametes versicolor*, able to degrade a wide array of substrates, abundant in olive oil mill wastewaters, such as phenols, aromatic amines, benzenethiols, hydroxyindoles and phenothiazinic compounds, to evaluate the increase of operational stability and durability of the enzymes after immobilization.

In conclusion, the different objectives that we aim to achieve in this thesis are dealing with the following topics:

- Comparative study on the sorption capacity of cyhalofop acid, a new generation and currently used herbicide, on polymerin, ferrihydrite and on a ferrihydrite-polymerin complex (chapter 2);
- Sorption capacity of simazine and MCPA on polymerin, Al_2O_3 and Fe_2O_3 (chapter 3);
- Microbial conversion of olive oil mill wastewaters (chapter 4);
- Immobilization and stabilization of Myceliophthora thermophila and Trametes versicolor laccases (chapter 5).

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2. COMPARATIVE STUDY ON THE SORPTION CAPACITY OF CYHALOFOP ACID ON POLYMERIN, FERRIHYDRITE AND ON A FERRIHYDRITE-POLYMERIN COMPLEX

2.1 INTRODUCTION

Agriculture has become extremely intensive and productive by using a large variety of pesticides. But this benefit is associated with the risk of environmental pollution, and the soil and surface waters are mostly exposed. The quality of soil and water bodies deserves particular attention because of its importance in the survival of the ecosystem as well as in the water supply for human consumption; therefore, their protection and the development of new remediation technologies are a necessity of primary importance. Surface and groundwaters have been shown to be contaminated with a range of pesticides. Research over recent years has focused on the nonpoint sources of pesticide contamination resulting from application to agricultural land (Carter, 2000; Spanoghe et al., 2004; Guzzella et al., 2006). However, point sources such as nonapproved use, poor practice, illegal operations, accidental release from farmyards, and inputs of washings are increasingly recognized as contributing to water contamination (Mason et al., 1999; Carter, 1999; Kruger and Nilsson, 2001; Yoder et al., 2001).

Several workers have indicated that point sources (i.e. the spills and washings from the farmyard) can contribute between 18% and 84% of the pesticide load measured in individual catchments (Higginbotham et al., 1999; Muller et al., 2002). Better training of sprayer operators and good machinery maintenance can reduce the number of spills; releases to the farmyard due to spray tank washings could be minimized (Rose et al., 2004). The application of treatment systems at the farm level could allow an improved protection of the aquatic ecosystem and a reduction of water treatment cost (Mason et al., 1999).

The use of vegetable biomass with high sorption capacity for remediation of waters contaminated by pesticides or metals has been widely described in the literature over the last ten years (Schneegurt et al., 2001; Mameli et al., 2004). In this light, the utilization of the polymeric organic fraction of olive mill wastewaters (OMW), named polymerin (Capasso et al., 2002), for remediation of contaminated waters with pesticides could represent a convenient method of bioremediation. This material derives from a free cost agricultural waste and possesses humic acid-like characteristics. Previously, is demonstrated that polymerin exhibits very interesting sorption capacities for cationic and anionic heavy metals (Capasso et al., 2004; Sannino et al., 2006) or ionic and ionizable pesticides, such as paraquat and 2,4-D, used as models (Sannino et al., 2008).

Thus, in this chapter we study the sorption of cyhalofop acid[1] (CyA) by polymerin. Cyhalofop-butyl (CyB), 2-[4-(4-cyano-2-fluoro-phenoxy)phenoxy]propanoic acid, butyl ester (R), recently introduced from Dow AgroSciences, is an aryloxyphenoxypropionate herbicide for the post-emergence control of grasses in rice, mainly against barnyard grass (*Echinochloa* species) and silver top (*Lepthochloa fusca* species). The ester of the acid acts as a pro-herbicide. The formulation as ester facilitates uptake through the plant cuticle, and once it has

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entered into the plant, it is transformed within a few hours into 2-[4-(4-cyano-2-fluorophenoxy) phenoxy] propionic acid (CyA), which is the effective active form. Moreover, CyA is the effective form occurring in water after its application on the crop because it originates by the rapid hydrolysis of its corresponding butyl ester form.

In particular, we want to study the sorption capacity of polymerin toward the cyhalofop acid and compare it with a mineral matrix, the ferrihydrite, and an organo-mineral one, the ferrihydrite-polymerin complex.

A ferrihydrite was selected as a cationic mineral in the present study, and the main objective of this chapter is to find, by a comparative study on the sorption of CyA on polymerin, ferrihydrite, and a ferrihydrite-polymerin complex, the most efficient and convenient sorbent among the three selected matrices, to be used as a potential filter for the decontamination of wastewaters in point sources. Comparative studies were carried out on (i) the sorption capacity of CyA on polymerin, ferrihydrite, and the ferrihydrite-polymerin complex and (ii) the removal of CyA from simulated wastewaters by cyclic sorption of the herbicide on the selected sorbents renewed at each cycle. With the aim to elucidate the different sorbing capacities of CyA on the selected matrices, the interaction mechanism was also studied, mainly by Diffuse Refractance Infrared Fourier Transformed (DRIFT) analysis.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Provider	Reagents
Dow Agrosciences B.V. Rotterdam (The Netherland)	• Cyhalofop-butyl, (R)-2-[4-(4-cyano-2-fluorophenoxy) phenoxy] propionate (CyB), 99.0% purity
Sigma-Aldrich (St. Louis, MO, EEUU)	• Hydrochloridric acid (HCl) • Sodium hydroxide (NaOH) • Iron (III) chloride ($FeCl_3$) • Sodium chloride (NaCl) • Potassium chloride (KCl)
BDH Reagents (Poole, U.K.)	• Stock standard solutions of metal cation (1g/L) for AAS
Aldrich (Chemical, Co., Milwaukee, WI, USA)	• Potassium bromide (KBr)
Spectrum Labs.com	• Cellulose dialysis membranes (cut-off 3,500 Da)

All solvents are of HPLC grade (Carlo Erba, Milan, Italy) and are used without further purification. All other chemicals are obtained from Sigma-Aldrich unless otherwise specified.

2.2.2 Methods

2.2.2.1 Pesticide preparation

Cyhalofop-butyl, (R)-2-[4-(4-cyano-2-fluorophenoxy) phenoxy] propionate (CyB), (figure 1a) was supplied by Dow Agrosciences. Its corresponding acid, (R)-2-[4-(4-cyano-2-fluorophenoxy)phenoxy]propionic acid (CyA) (figure 1b), was prepared by alkaline hydrolysis of CyB. In particular, an aqueous solution of 0.05N sodium hydroxide (NaOH) was added to 100 mL of CyB suspension (1 g) in CH_3CN/H_2O 50/50 v/v, up to pH 9.0 under stirring at room temperature. The pH of the mixture was checked throughout and brought to a value of 9.0 by NaOH addition until the suspension turned into solution. CH_3CN was evaporated, and diluted HCl was added to the solution. A white precipitate was obtained, which was filtered and repeatedly washed with H_2O to give the pure compound CyA (Pinna et al. 2008).

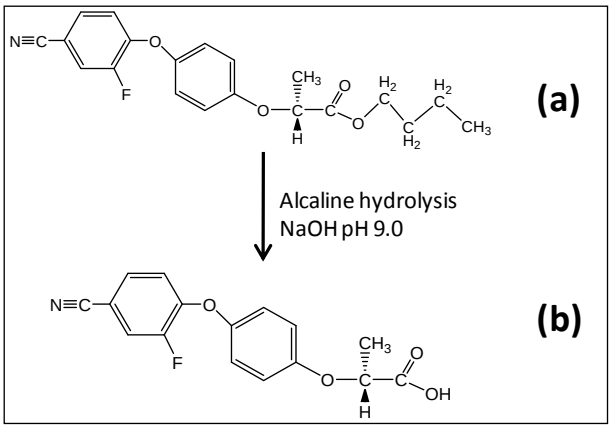


Figure 1. Structural formulas (a) Cyhalofop-butyl, (R)-2-[4-(4-cyano-2-fluoro-phenoxy)phenoxy]propionate (CyB), and **(b)** Cyhalofop-acid, (R)-2-[4-(4-cyano-2-fluorophenoxy)phenoxy]propionic acid (CyA).

2.2.2.2 Preparation of sorbents

Polymerin

OMW were obtained from a pressure processing system located in Monopoli (Puglia, Italy). Polymerin was prepared according to the procedure reported by Capasso et al. 2002 (figure 2).

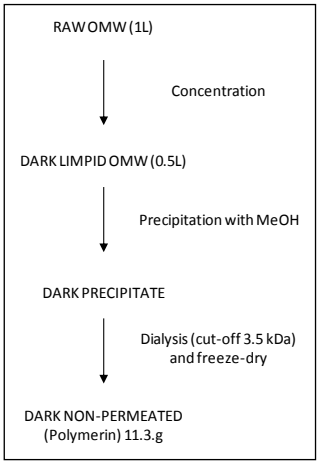


Figure 2. Recovery of polymerin from olive mill waste waters.

In the first step, a dark, muddy sample of OMW was centrifuged and filtered, obtaining a dark, clear solution, which was concentrated to half of initial volume. In the second step, the concentrated sample was precipitated with cold methanol and centrifuged. In the third step, the dark precipitate was re-dissolved in ultrapure water,

dialyzed and the non permeated fraction was lyophilized, leaving a brown residue, named polymerin.

Ferrihydrite and Ferrihydrite-Polymerin complex

Ferrihydrite was prepared according to the following procedure. A stock solution (1 L) of 0.1M $FeCl_3$ was potentiometrically titrated (TIM 856 Titration Manager automatic titrator, Analytical De Mori, Milano, Italy) to pH 6.0 by adding 1M NaOH at a feed rate of 0.5 mL min^{-1}. The precipitate was kept in a propylene container and aged at 20°C for 24 h. After aging, the sample was centrifuged, washed, and dialyzed in deionized water and freeze-dried, obtaining an iron oxy-hydroxide mineral [$(Fe(OH)_x$] known as a ferrihydrite (Violante et al. 2003).

The ferrihydrite-polymerin complex was obtained by adding 1.0 g of polymerin to a 500 mL 0.1M $FeCl_3$ solution and by titrating the solution with 1M NaOH to reach pH 6.0 using an automatic titrator (TIM 856 Titration Manager). The precipitate obtained was washed, dialyzed against deionized water to reach a conductivity of 1.0μS, and then lyophilized.

2.2.2.3 Physical-chemical characterization of the sorbents

Diffuse Reflectance Infrared Fourier Transform Spectroscopy (DRIFT) Analysis

Sample preparation for DRIFTS determinations was performed as follows: a quantity of herbicide corresponding to the maximum sorption value obtained on the isotherm of CyA was added to each matrix at the optimal sorption pH (pH 4.5 for polymerin and pH 3.5 for ferrihydrite and ferrihydrite-polymerin complex). After incubation, the samples were centrifuged, and the precipitates were washed twice with 0.03M KCl to remove the excess of herbicide; then the samples so obtained were lyophilized. Finally, 0.20 mg of each sample was mixed with 200 mg of KBr (FTIR grade). The mixture was finely ground in an agate mortar and transferred to a sample holder. Its surface was smoothed with a microscope glass slide, and DRIFT spectra were recorded. The DRIFT spectra were obtained using a Perkin-Elmer Spectrum One FT-IR spectrometer (U.S.A). The instrument had a special resolution of 1 cm^{-1}, which was used in all spectral determinations.

Point of zero charge

The point of zero charge (PZC) of raw polymerin was determined according to the method reported by Tsai at al., 2003 using the potentiometric titration curve as a function of pH at different ionic strengths. In particular, dry sample of polymerin (250 mg) were dissolved in 100 mL of electrolyte solution (0.1, 0.01 and 0.001M NaCl), brought to pH 9.0 with 2M NaOH and successively brought to pH 2.0 with 0.05M HCl by an automatic titrator (VIT 909 Videotritator, Copenhagen) under nitrogen stream. As regards ferrihydrite and the ferrihydrite-polymerin complex, the determination of PZC was performed according to the method described by Mustafa et al. (2004). Dry samples (100 mg) were dissolved in 40 mL of 0.001M KCl solution as a background electrolyte and equilibrated for 30 min by means of a magnetic stirrer. Successively, the initial pH of the solution was adjusted to pH 4.0 by the addition of either 0.1M HCl or 0.1M KOH. The suspension was equilibrated for another 10 min, and the pH was then measured. The pH suspension was recorded every 2 min as a function of volume of titrant added, until pH 10 was reached. The PZC of two sorbents was determined from the variation of surface charge density as a pH function.

Specific surface area

Specific surface area (SSA) was determined from the nitrogen adsorption isotherm using the BET method with a Gemini 2370 instrument (Micrometrics, U.S.A.).

Carbon elemental and metals analysis

Carbon elemental analysis was performed using a Fisons EA 1108 elemental analyzer (Fisons Instruments S.p.a., Milano, Italy) for hydrogen, carbon, nitrogen, and sulfur (HCNS).

The metals were determined by a Perkin-Elmer Instruments (Analyst 700 U.S.A.) atomic adsorption spectrometer equipped with deuterium arc background correction (Capasso et al., 2004). Air-acetylene flame was used as the atomization source. The average and standard deviation of three absorption measurements were recorded for each sample.

2.2.2.4 Sorption methodology

Stock solution of pesticide was prepared by dissolving 125 mg of CyA in 500 mL of 0.03M KCl and alkalizing the solution at pH 10.0 with KOH (final concentration 0.830mmol/L). This solution was then kept refrigerated. The sorption experiments were conducted at a solid/liquid ratio of 0.5 obtained by adding 10 mg of sorbent dried at 100°C for 1 h, to a final volume of 20 mL, at 25°C. The pH was controlled by the addition of 0.01mM HCl or KOH to the suspension. After incubation, the samples containing polymerin were centrifuged at 4,500 rpm for 30 min using Centrifugal Filter Devices equipped with Amicon Ultra membrane with a cut off of 3,000 Da, while those containing ferrihydrite or ferrihydrite-polymerin were only centrifuged at 7,000 rpm for 30 min. The amount of herbicide sorbed on each matrix was calculated as the difference between the initial quantity of pesticide added and that present in the equilibrium solution. Blanks of pesticide in the same experimental conditions were analyzed in order to check pesticide stability and sorption to vials.

Analytical determination of CyA

CyA was analyzed with an Agilent 1200 Series HPLC apparatus (Wilmington U.S.A.), equipped with a DAD array and a Chem Station Agilent Software. A Macharey-Nagel Nucleosil 100-5 C18 column (stainless steel 250 x 4 mm) was utilized. An isocratic elution was performed at a flow rate of 1 mL min^{-1} with acetonitrile (50%) and phosphate buffer (50%) (0.04M, pH 2.95). The detector was set at 250 nm. A sample volume of 20 µL was used for injection. The quantitative determination of CyA was performed elaborating the corresponding calibration curve (0.016-0.75mM).

Effect of pH

Experiments were carried out by adding a fixed pesticide concentration, 0.17mM, and varying the pH, from 3.0 to 11.0. The suspensions were shaken for 24 h. The herbicide solution was separated from the sorbent and analyzed as described above.

Effect of Time

Experiments were performed using 0.17mM of CyA at pH 4.5 for polymerin and pH 3.5 for ferrihydrite and ferrihydrite-polymerin complex, respectively. The suspensions were stirred for 0.25, 0.5, 1, 2, 4, 6, 8, 24, 30, 48, and 72 h.

Sorption Isotherms

Different volumes of a stock solution of herbicide (0.83mM) were added to each sorbent to give an initial concentration ranging from 0.030 to 0.60mM of CyA. The pH of each suspension was kept constant at pH 4.5 for polymerin and pH 3.5 for ferrihydrite and ferrihydrite-polymerin, respectively, by the addition of 0.10 or 0.01M HCl or KOH. The samples containing ferrihydrite were shaken for 4 h and those with polymerin or ferrihydrite-polymerin complex for 24 h; then after centrifugation, the supernatants were analyzed as described above.

2.2.2.5 Cyclic sorption on Polymerin, Ferrihydrite and Ferrihydrite-Polymerin complex

Cyclic experiments for the sorption of CyA were carried out at pH 4.5 for polymerin and pH 3.5 for ferrihydrite and ferrihydrite-polymerin complex. In the first cycle, a predetermined quantity of herbicide stock solution was pipetted into the flasks to give 0.575mM concentration. The samples were treated according to the procedure previously described. After the contact time, the sorbent was separated from the suspension by centrifugation, and the supernatant was analyzed to determine the amount of sorbed pesticide. For the second cycle, 20 mL of supernatant was added to the fresh sorbent (10 mg). The experiments were repeated until the equilibrium concentrations remained constant.

2.2.2.6 Data analysis

All experiments were performed in triplicate, and the relative standard deviation was lower than 4%. The kinetic data were analyzed using the Lagergren equation (Namasivayama and Yamuna, 1995):

$$\log(q_e - q)=\log q_e - K_1 t/2.303$$

where q_e and q are the amount of herbicide sorbed ($mmolkg^{-1}$) at equilibrium and at time t, respectively, K_1 is the rate constant of sorption (min^{-1}), and t is the time (min).

The sorption data were analyzed according to the Langmuir and Freundlich's equations. The first equation can be written as follows:

$$C_s=X_m KC/1+ KC$$

where C_s ($mmolkg^{-1}$) is the amount of CyA sorbed, K ($Lmmol^{-1}$) is the Langmuir constant related to the binding energy, X_m ($mmolkg^{-1}$) is the maximum amount of CyA sorbed and C is the equilibrium concentration of CyA ($mmolL^{-1}$) in according to Giles et al. 1974.

The Freundlich equation is:

$$C_s=K_F C^{1/N}$$

where C_s and C are defined in the equation reported above, $K_F[(mmolkg^{1})/(mmolL^{-1})^{1/N}]$ and N (dimensionless) are constants that give an estimate of the sorption capacity and intensity, respectively. The distribution coefficient K_d, defined as the ratio of the concentration of pesticide sorbed per unit weight of sorbent to its equilibrium concentration (Lkg^{1}), at saturation was also determined.

2.3 RESULTS AND DISCUSSION

2.3.1 Characterization of sorbents

Polymerin was recovered from OMW and characterized chemically and spectroscopically according to the procedure described by Capasso et al. 2002. This

polymeric mixture was confirmed to be a humic-like polyelectrolyte consisting of carbohydrates, melanin, and proteins. It also contains metals such as Ca, Mg, K, Na, and Fe that are naturally bound or chelated through carboxylate anions and other characteristic nucleophilic functional groups present in polymerin. Analysis of relative molecular weight showed that polymerin consists of two component: a free polysaccharide fraction of 45,000 Da and a supra-molecular aggregate of all organic and inorganic components of about 3,500-10,000 Da. Polymerin was co-precipitated with Fe to obtain an insoluble organo-mineral complex called ferrihydrite-polymerin, in order to study the influence of ferrihydrite, an iron oxy-hydroxide, on the sorption capacity of organic component. The organo-mineral complex shows a surface area (380.7 m^2g^{-1}) lower than that of ferrihydrite (484.2 m^2g^{-1}) because of the presence of polymerin (312 m^2g^{-1}), which partially covers the ferrihydrite surface. The chemical and physicochemical properties of the selected sorbents are reported in table 1.

Polymerin	
Polysaccharide (%, w/w)	43.07
Protein (%, w/w)	22.40
Metals (%, w/w)	4.77
Carbon (%, w/w)	41.49
Point of zero charge (PZC)	2.2
Surface Area (m^2/g)	312
Ferrihydrite	
Point of zero charge (PZC)	9.4
Surface Area (m^2/g)	484.2
Ferrihydrite-Polymerin Complex	
Point of zero charge (PZC)	7.7
Surface Area (m^2/g)	380.7
Carbon (%, w/w)	4.35

Table 1. Characterization of sorbents.

2.3.2 Sorption studies

The results obtained by studies of CyA sorption on the selected sorbents as a function of pH show the greatest sorption capacity of CyA on polymerin at pH 4.5 and on both ferrihydrite-polymerin and ferrihydrite at pH 3.5, whereas a significant decrease of the herbicide on all matrices is shown up to pH 6.0 (figure 3).

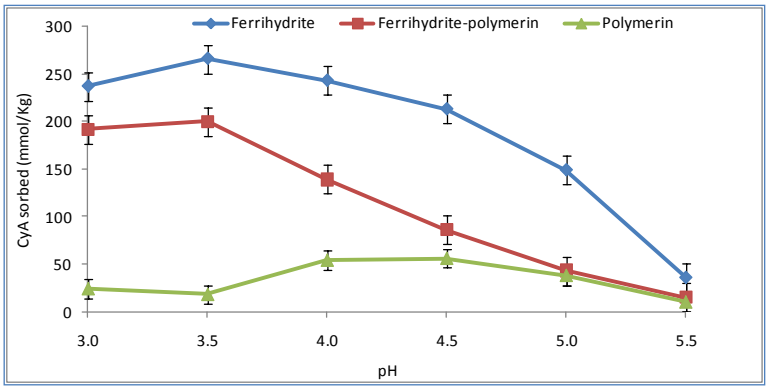


Figure 3. Effect of pH on the sorption of CyA by polymerin, ferrihydrite and ferrihydrite-polymerin complex.

Polymerin could be considered as a weak acid polyelectrolyte because of its carboxylic and phenol groups, and it is characterized by a pk_a of 4.5 (Capasso et al., 2002) and a PZC of 2.2 (Sannino et al., 2008). The point of zero charge (PZC) indicates the pH value at which the surface charge of a molecule is neutral (the negative charge number is the same of the positive one). When pH>PZC the polymerin has a surface negative charged, on the contrary, when pH<PZC the

sorbent is positive charged. Sorption process could be very probably attributed to the prevalent formation of hydrogen bonding between the OH alcoholic groups of the polysaccharide component and the nitrile group, the ethereal groups, and the undissociated carboxylic group of CyA (pK_a=3.8) (European Commission Directive, 2002). Therefore, at pH 4.5 the effect of hydrogen bonding is higher than the repulsion between the ionized carboxylic groups of polymerin and the herbicide. On the contrary, a significant reduction of sorbed CyA is detected at pH > 4.5 because the herbicide and polymerin carboxylic groups are mainly in dissociated form, with a consequent dominant effect of the repulsion. This behaviour is confirmed in previous studies conducted on the sorption of the phenoxy acid 2,4-D on polymerin as a function of pH (Sannino et al., 2008). The results reported in figure 3 show a greatest sorption at pH 3.5 on ferrihydrite and ferrihydrite-polymerin complex. At this pH, both the sorbents have all surface positive charged by OH^{2+} groups because of the PZC value, 9.4 for ferrihydrite and 7.7 for ferryhydrite-polymerin (table 1), whereas the proportions of un-ionized and ionized herbicide carboxylic groups are roughly estimated as 50/50%. This means that ionized carboxylic groups ($-COO^-$) of CyA form ionic bonds with the positive surface of both sorbents. The hydrogen bond to the protonated hydroxyl group presents a very high mobility due to the positive charge on the oxygen atom. In these conditions and considering the aqueous medium where the adsorption occurs, it can be supposed that the formation of hydrogen bonds contributes to the stabilization of the interaction between the anionic form of CyA and the positively charged surface of the matrices, very likely attenuating the ionic character of the bond. A moderate decrease of sorbed herbicide is observed on the ferrihydrite-polymerin complex due very probably to both the lower PZC and surface area with respect to ferrihydrite, which was attributable very likely to the presence on the mineral component of a small amount of the sorbed polymerin. An additional ion-dipole bond is hypothesized in the system CyA-ferrihydrite between the undissociated carboxylic group of the acid and the positive surface of ferrihydrite. All previous hypotheses are confirmed by the DRIFTS analyses reported later. Previous studies confirm that the iron oxides exhibit a strong sorption of ionic and ionizable pesticides at low pH and a decreasing sorption as pH increases (Clausen and Fabricious, 2001).

Kinetic studies (data not shown) on the sorption of CyA on polymerin are carried out at pH 4.5 while on ferrihydrite-polymerin complex and ferrihydrite at pH 3.5. The results obtained by the Lagergren equation show that the sorption of CyA on each sorbent fits a first-order mechanism (r^2>0.90). However, this process is very fast on ferrihydrite, reaching the sorption equilibrium within 1-2 h, but is much slower on ferrihydrite-polymerin complex and polymerin, it being observed within 24 h. In correspondence, the rate constants determine from the slopes of the plots of $\log(q_e-q)$ versus t are 0.43 for ferrihydrite and 0.23 and 0.11 min^{-1} for ferrihydrite-polymerin and polymerin, respectively. Therefore, an incubation period of 4 h for ferrihydrite and 24 h for polymerin and ferrihydrite-polymerin complex is utilized.

The sorption isotherms of CyA (figure 4) on polymerin, ferrihydrite, and ferrihydrite-polymerin complex fit the Langmuir equation (table 2).

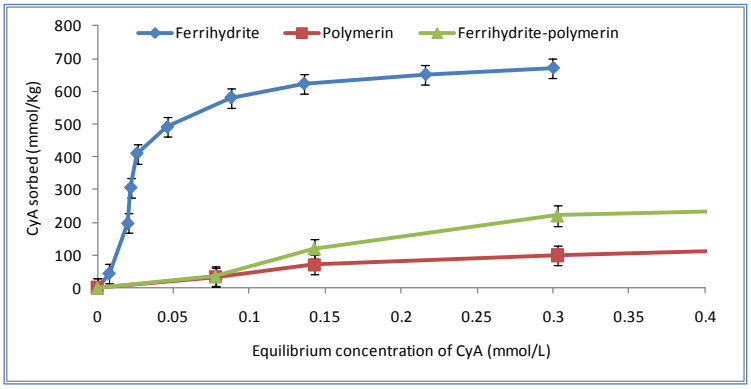


Figure 4. Sorption isotherm of CyA. The experiment was conducted at pH 4.5 on polymerin and at pH 3.5 on ferrihydrite and ferrihydrite-polymerin complex.

The shape of the isotherms is different and is mainly distinguished by the initial slope. In fact, the sorbed amount of herbicide on polymerin increases slowly when the CyA equilibrium concentration increases, whereas an opposite behaviour is detected with the other two sorbents, which show a higher affinity. Actually, at a 0.20 $mmolL^{-1}$ equilibrium concentration of CyA, the amount of sorbed herbicide significantly increases from 75 on polymerin to 300 and 600 $mmolkg^{-1}$ on ferrihydrite-polymerin and ferrihydrite, respectively. Therefore, the amount of CyA sorbed on all selected sorbents follows the order ferrihydrite>ferrihydrite-polymerin>polymerin. This trend is confirmed analyzing both the Langmuir (x_m and k) and Freundlich constants (K and N), and also the K_d values (table 2), showing that ferrihydrite sorbs CyA with both the greatest binding energy and sorptive intensity.

Langmuir parameters				
	X_m ($mmolkg^{-1}$)	K ($L\ mmol^{-1}$)	r^{2a}	
Polymerin	194.97	3.45	0.99	
Ferrihydrite	796.98	27.12	0.98	
Ferrihydrite-Polimerin complex	492.01	10.05	0.99	
Freundlich parameters				
	K ($mmol\ Kg^{-1}$)/($mmol\ L^{-1}$) $^{1/N}$	N	r^{2a}	$K_d{}^b$ ($L\ Kg^{-1}$)
Polymerin	229.40	1.33	0.98	194.77
Ferrihydrite	951.48	4.52	0.98	3080.90
Ferrihydrite-Polimerin complex	551.188	2.89	0.99	1128.70

Table 2. Langmuir and Freundlich parameters for the sorption of CyA on polymerin, ferrihydrite and ferrihydrite-polymerin complex. [a] Determination coefficient. [b]Distribution coefficient.

The sorption capacity of CyA on polymerin is similar to that of the phenoxy acid herbicide 2,4-D (Sannino et al., 2008). In particular, the maximum individual sorbed amount (x_m) of 2,4-D and CyA on the previous bio-organic sorbent is 183.15 and 194.97 $mMkg^{-1}$, respectively. On the contrary, their corresponding constant related to the binding energy (k) shows opposite values (8.47 and 3.45 for 2,4-D and CyA, respectively) suggesting a weaker bonding affinity between the functional groups of CyA and the polymerin surface, compared to that of 2,4-D. The weaker interaction of CyA than 2,4-D could be very probably due to a higher steric hindrance of the larger molecule of CyA, which is, however, balanced by the hydrogen bond between its several functional groups and polymerin.

2.3.3 Sorption mechanism

The sorption mechanism of CyA on the selected sorbents was also investigated, by DRIFTS analysis, with the aim to elucidate their different sorption capacities. To

this end, the preliminary DRIFTS characterization of the selected three matrices was performed.

The DRIFT spectrum of polymerin (figure 5a)

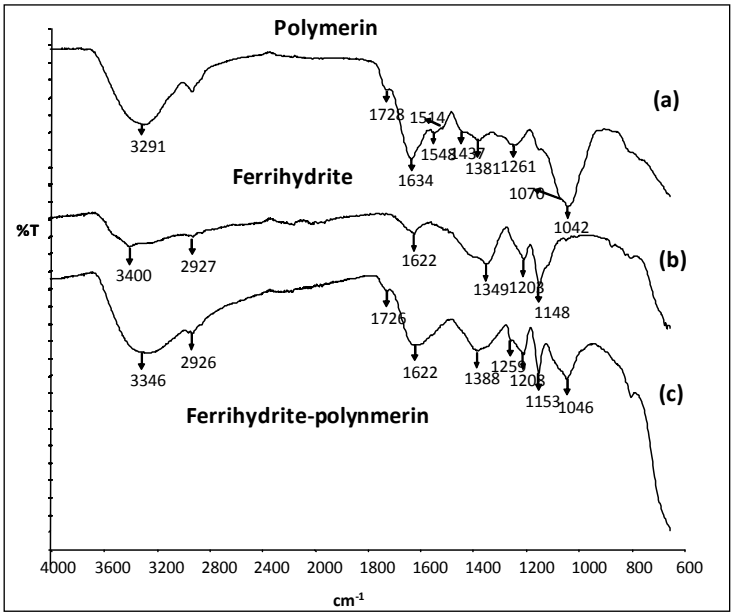


Figure 5. DRIFT spectra: (a) polymerin**, (b)** ferrihydrite**, (c)** ferrihydrite-polymerin complex.

shows a very strong and broad band at 3291 cm^{-1} (OH groups), four weak, albeit correlated, bands at 1728 and 1261 cm^{-1}, and 1437 and 1381 cm^{-1} (stretching of methylester groups, and symmetric and asymmetric bending of the corresponding CH_3 groups, respectively), and absorption at 1634 cm^{-1} (aromatic ring and CO peptide groups stretching). The absorptions at 1548 and 1514 cm^{-1} are also assignable to aromatic ring stretching. Moreover, the absorption at 1634 cm^{-1} included the symmetric stretching of COO^- groups, to which the asymmetric stretching of the same group included in the band at 1437 cm^{-1} is correlated. A very strong absorption at 1070 and 1042 cm^{-1} (stretching of the C-OH bond of the polysaccharide component of polymerin) is finally observed. In conclusion, polymerin is assessed to be a humic acid like polymer very similar to humic acids as shown in table 3.

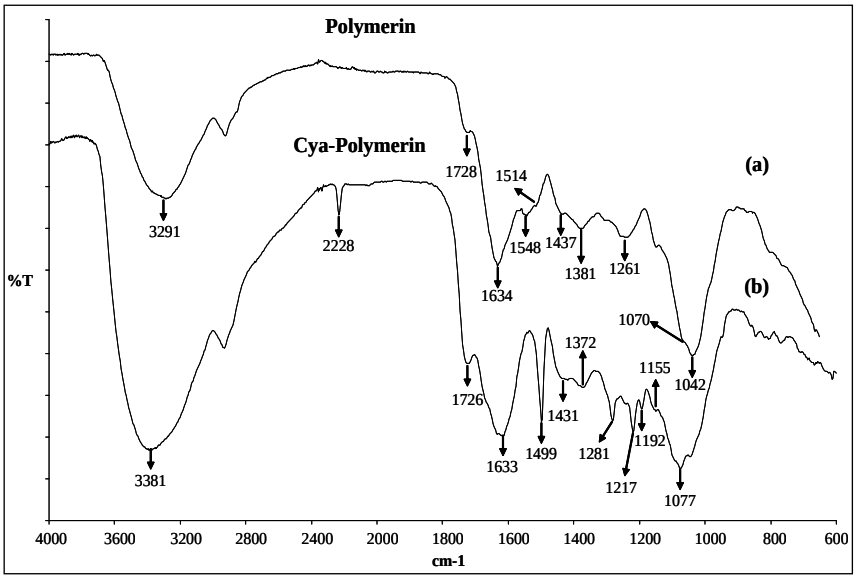
Characteristic band (nm) of chemical group	Polymerin	Humic acid
OH of phenol, alcohol and carboxylic acid	3291	3400-2900
C-H of ester (O-CH_3)	2924	
Carbonyl ester (CO-OCH_3) + COOH	1728	1725-1720
Aromatic nucleus + peptide C=O + symmetric COO^-	1634 and 1548-1514	1590-1517
Asymmetric COO^-, symmetric and asymmetric ester OCH_3	1437 and 1381	1400-1390
Ester OC-OCH_3	1070-1042	1280-1200
Alcoholic C-OH	1072	1170-950
Alcoholic OH and Ester OC-OCH3	1042	

Table 3. DRIFT data of polymerin compared with humic acids.

The DRIFT spectrum of ferrihydrite (figure 5b) shows a characteristic and very intense band at 3400 cm^{-1} (stretching of the OH groups present in high amount in the mineral) and two other strong and intense and correlated bands at 1203 and 1148 cm^{-1} (bending of the OH groups).

The DRIFT spectrum of the ferrihydrite-polymerin complex (figure 5c) shows a strong and broad band at 3346 cm^{-1} (OH stretching), deriving from the overlap and the shift of the broad bands observed at 3400 and 3291 cm^{-1} in the spectrum of ferrihydrite and polymerin, respectively, and very likely attributable to the ion-dipole bonding between the ionic OH^{2+} groups of the former matrix and the dipole -OH groups of the latter matrix.

In fact, the PZC of ferrihydrite is 9.4, whereas that one of polymerin is 2.2; therefore, the -OH groups of the former matrix are totally positively charged, and the alcoholic -OH groups of the latter are not charged at all but are in dipole form. In other terms, the formation of a H-bond in this conditions is excluded. In addition, the spectrum of ferrihydrite-polymerin evidences two weak and correlated bands at 1726 and 1259 cm^{-1} (stretching of methyl ester groups of polymerin), and two intense and broad bands at 1622 and 1388 cm^{-1} corresponding to respective symmetric and asymmetric stretching of the polymerin $-COO^{-}$ groups bound by ionic linkage to OH^{2+} groups of ferrihydrite, which originate from the shift of the respective bands at 1634 and 1381 cm^{-1} observed in the polymerin spectrum. Finally, the DRIFT spectrum of the ferrihydrite-polymerin complex shows that a medium band appeared centred at 1046 cm^{-1}, which derives from an intense and split band at 1070 and 1042 cm^{-1} (C-OH stretching of polymerin polysaccharide component), respectively, originally present in the polymerin spectrum and attributable to the ion dipole bond as previously described. In summary, all previous data clearly indicate that polymerin is fixed on ferrihydrite by a combination of the dipole-ion bonding between the OH groups of the former organic matrix and the OH^{2+} groups of the latter mineral matrix, and ionic bonding between the $-COO^{-}$ groups of the organic sorbent and OH^{2+} groups of the mineral sorbent. The sorption mechanism of CyA on the selected matrices is then assessed by the DRIFTS analysis of CyA-polymerin, CyA-ferrihydrite, and CyA-ferrihydrite-polymerin systems. In particular, the DRIFT spectrum of the CyA-polymerin system, registered at pH 4.5 (figure 6b), compared to that of polymerin (figure 6a), shows the appearance of a new band at 1499 cm^{-1} due to the stretching of CyA benzene rings, which is slightly shifted with respect to the band at 1506 cm^{-1} observed in the spectrum of the herbicide (figure 6g). In addition (figure 6b), two new bands at 1281 and 1217 cm^{-1}, evidence a shift and reduction with respect to those observed at 1292 and 1227 cm^{-1} in the spectrum of CyA (figure 6g).



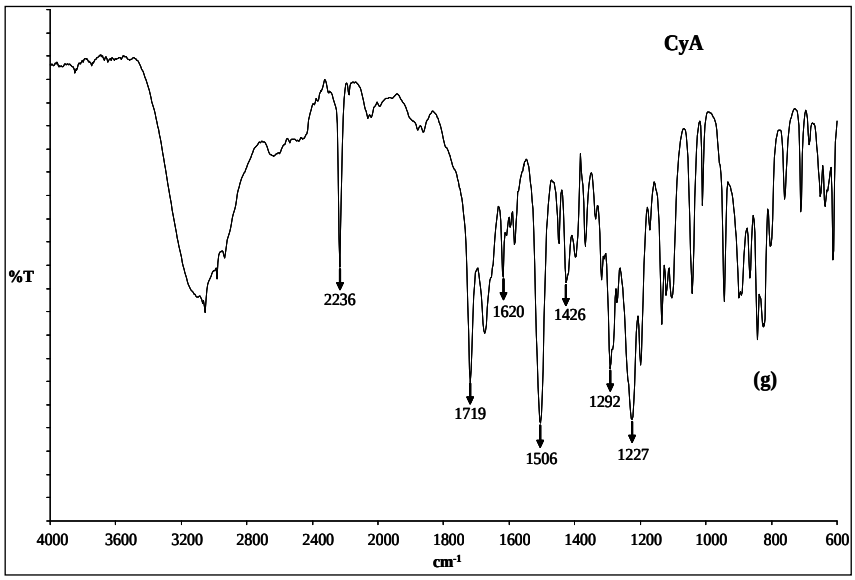


Figure 6. DRIFT spectra: (a) polymerin**, (b)** CyA-polymerin, **(g)** CyA.

The considered absorptions are assignable very probably to the stretching modifications deriving from hydrogen bonding between each ethereal oxygen of the herbicide (figure 1b) and the OH alcoholic groups of polymerin. Still, the DRIFTS spectrum of CyA-polymerin (figure 6b) shows a band at 2228 cm^{-1} reduced and shifted as compared to the characteristic sharp and intense band that appears at 2236 cm^{-1} in the CyA spectrum (figure 6g), attributable to the stretching of CyA nitrile group as well as the weak band, which appears at 1077 cm^{-1} (figure 4b), originating from the modification and reduction of the signal at 1070 and 1042 cm^{-1} present in the spectrum of polymerin (figure 6a) and due to the stretching of the OH groups of the polysaccharide component of polymerin. All last modifications concerning the spectrum of figure 6b are attributable to the formation of hydrogen bonds between the OH groups of the polymerin polysaccharide component and the nitrile group, and the ethereal groups and the undissociated carboxylic group of CyA. The DRIFT spectrum of CyA-ferrihydrite (figure 6d) shows a new band at 1734 cm^{-1} by comparison with the spectrum of ferrihydrite (figure 6c).

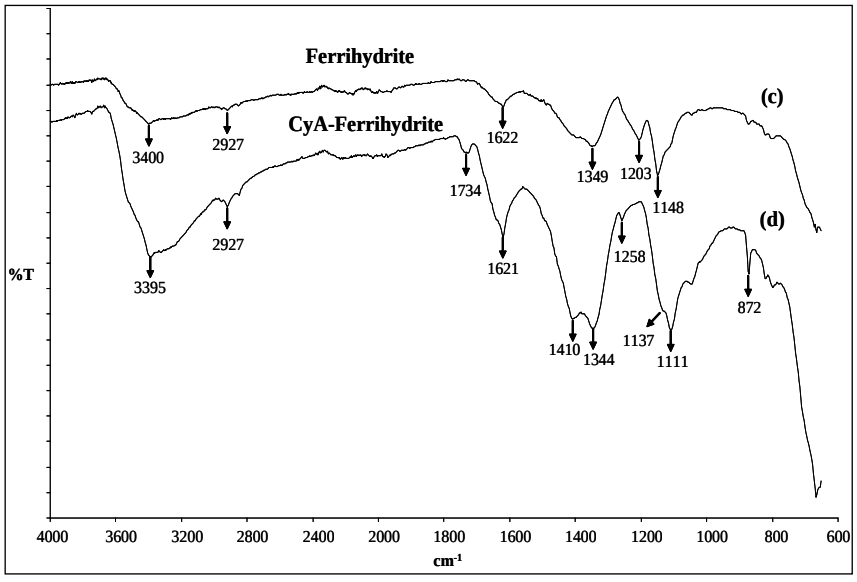


Figure 6. DRIFT spectra: (c) ferrihydrite, **(d)** CyA-ferrihydrite.

It is the shifted band with respect to that observed at 1719 cm^{-1} in the CyA spectrum (figure 6g), due very likely to the stretching variation of the CyA undissociated carboxylic group, as a consequence of dipole-ion bonding between this latter group and the OH^{2+} groups of ferrihydrite. In addition, the bands observe at about 1621 cm^{-1} and 1410 cm^{-1} are very likely originated from the modification of those appearing in the spectrum of CyA at 1620 cm^{-1} and 1426 cm^{-1} (figure 6g), respectively. These latter are very probably attributable to the asymmetric and symmetric stretching of CyA COO^- groups, which interact by ionic bonding with the positive OH^{2+} groups of ferrihydrite. In addition, the band at 1621 cm^{-1} in the spectrum of figure 6d includes the band at 1618 cm^{-1} observed in the spectrum of ferrihydrite (figure 6c). Furthermore, the strong bands at 1203 and 1148 cm^{-1} observed in the ferrihydrite spectrum, due to the OH bending, appear strongly modified in the spectrum of CyA-ferrihydrite (figure 6d), where the first disappears, and the second is reduced and shifted to 1111. Such a modification could be also attributed to an ionic bonding between the OH^{2+} groups of ferrihydrite and COO^- groups of CyA. Finally, the DRIFT spectrum of CyA-ferrihydrite-polymerin, registered at pH 3.5 (figure 6f), shows, as a main modification with respect to the spectrum of ferrihydrite-polymerin complex (figure 6e), the disappearance of the band at 1208 cm^{-1} and the strong reduction of the band at 1153 cm^{-1} (both corresponding to the OH bending of ferrihydrite), due very probably to the ionic bonding between the COO^- groups of CyA and the OH^{2+} groups of ferrihydrite. The broadband at 1621 cm^{-1} and the broad and slightly split band at 1380 cm^{-1} observed in the spectrum of figure 6f appear, even weakly, modified with respect to the corresponding bands observed in the spectrum of figure 6e at 1622 and 1388 cm^{-1} (symmetric and asymmetric stretching of polymerin COO^- groups bound by ionic linkage to OH^{2+} groups of ferrihydrite) because of the contribution of the bands previously identified at 1621 cm^{-1} and 1410 cm^{-1} in the spectrum of CyA-ferrihydrite (see figure 6d). In conclusion, CyA is bound to polymerin by hydrogen bonding, to the ferrihydrite-polymerin complex by ionic bonding and to ferrihydrite by a combination of dipole-ion and ionic bonding.

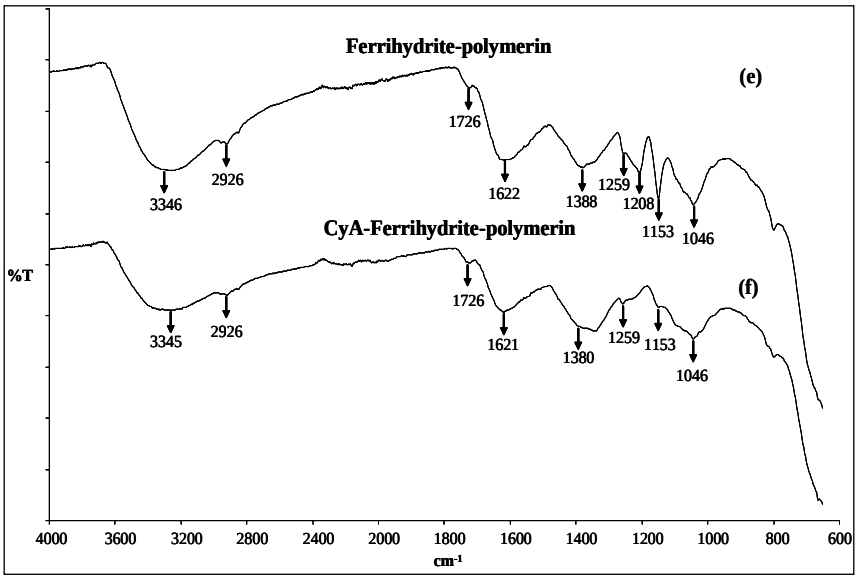


Figure 6. DRIFT spectra: (e) ferrihydrite-polymerin complex, **(f)** CyA-ferrihydrite-polymerin complex.

These results could confirm the following sorption capacity order, ferrihydrite > ferrihydrite-polymerin > polymerin for CyA, already observed in the corresponding sorption isotherms (figure 4 and table 2).

2.3.4 Cyclic removal of CyA by polymerin, ferrihydrite, and ferrihydrite-polymerin from simulated wastewaters

Experiments on cyclic sorption investigate the potential to remove CyA from simulated wastewaters. Figure 7 shows that almost a total removal of CyA on ferrihydrite and the ferrihydrite-polymerin complex is performed after two and five sorption cycles, respectively, whereas decontamination is still incomplete on polymerin even after five cycles, leaving a constant residue of 0.11 $mmolL^{-1}$.

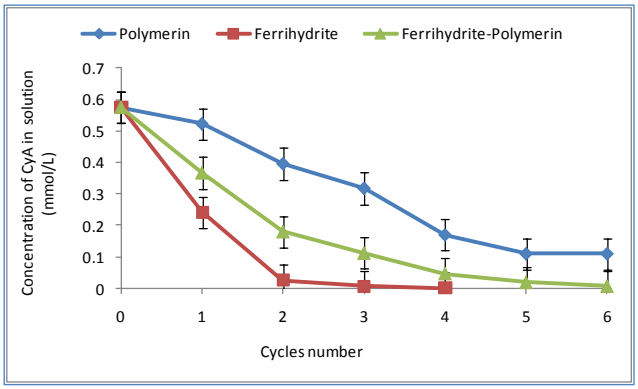


Figure 7. Cyclic removal of CyA by polymerin, ferrihydrite and ferrihydrite-polymerin from simulated wastewater. (The sorbent was renewed in each cycle).

A similar behaviour is observed in a previous study on the cyclic sorption of 2,4-D on polymerin (Sannino et al., 2008), also confirming for the CyA-polymerin system that the acid-base equilibrium of CyA is strongly in competition with the sorption equilibrium between the undissociated form of herbicide and polymerin. Therefore, ferrihydrite shows to be the most efficient sorbent for the removal of CyA from contaminated waters. In conclusion, among the selected investigated sorbents, ferrihydrite proves to be the most convenient for possible use as a filter for the control of wastewater contamination with CyA in point sources. In fact, this matrix allows the almost total removal of the herbicide from contaminated waters by only two sorption cycles, using the usual procedure of centrifugation for the separation of purified waters; in addition, it can be prepared more easily and at lower cost than the other two sorbents.

2.4 CONCLUSIONS

The use of a mineral (ferrihydrite) and an organomineral complex (ferrihydrite-polymerin) as filter for the remediation of waters contaminated with CyA from point sources is proposed here for the first time. Moreover, ferrihydrite is much more advantageous than polymerin and ferrihydrite-polymerin complex. In fact, simulated wastewaters contaminated with cyhalofop acid are completely purified by two sorption cycles on ferrihydrite and five cycles on the ferrihydrite-polymerin complex, whereas the same wastewaters maintain a constant residue even after five sorption cycles on polymerin.

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3. COMPARATIVE STUDY ON THE SORPTION CAPACITY OF SIMAZINE AND MCPA ON POLYMERIN, Al_2O_3 AND Fe_2O_3

3.1 INTRODUCTION

The extended contamination of soils and water bodies from the widespread use of pesticides in modern agriculture is constantly increasing and consequently is necessary to develop new remediation technologies for the safeguard of the environment. Accidental releases, illegal operations, or not-appropriate disposal of wastes from farms are the main point sources of soil and water pollution by pesticides. Traditional treatment processes have been employed for the remediation of waters polluted by pesticides including membrane technology (ultrafiltration, nanofiltration, and reverse osmosis), solid phase extraction, ozone and chemical oxidation, Fenton degradation, photocatalysis and adsorption on various supports. In particular, the adsorption for its low maintenance costs, high efficiency, and ease of operation, is proved to be one of the most attractive and effective techniques successfully used for heavy metals and organic chemicals removal from hazardous wastes and has a great environmental significance (Sannino et al., 2008 and 2009). Among the available adsorbents, activated carbon is the preferred solid because of its high effectiveness. The adsorption properties result from its high surface area, meso- and micropore structure, and broad range of surface functional groups. However, there are many disadvantages like high energy demands and consequently high costs of regeneration or most times renewal of adsorbent. Also clays are widely used as adsorbents due to their high specific surface area. Nevertheless, the anionic character of clay minerals limits their usefulness as adsorbents of acid herbicides when these latter are present in their anionic form. Some studies have proposed the use of natural organic materials or wastes as biosorbent (Rodriguez-Cruz et al., 2007; Wang et al., 2007) due to high sorption capacity of organic matter (Gunasekara and Xing, 2003; Kang and Xing 2005). In particular, polymerin, the polymeric organic fraction recovered from olive oil mill wastewater (OMW), exhibits very interesting sorption capacities for cationic and anionic heavy metals (Capasso et al., 2004; Sannino et al., 2009), ionic or ionisable pesticides (Sannino et al., 2008), and hydrophobic organic compounds (Iorio et al., 2008). The development of new sorbents for application in water decontamination is of great interest for the removal of organic pollutants. An ideal sorbent should have a high surface area (i.e., high density of sorption sites), uniformly accessible pores and physical and/or chemical stability (Yoshitake et al., 2002). It is believed that the sorption capacity of a sorbent is largely determined by the surface area available which increases with decreasing the particle size although the pores size distribution is also decisive for an optimal sorption process. Therefore, with the introduction of nanoscaled oxide materials, the pollutant removal efficiency can be increased dramatically. Mesoporous materials, a class of nanoporous materials, have attracted a lot of attention in both scientific and industrial communities since the introduction of well-ordered mesoporous silicas which possess large surface areas and uniform and tunable pore sizes (2-50 nm) (Lee, 2003; Kim et al., 2004). The great interest of these materials as adsorbents for environmental remediation is linked not only to their high surface area but also to their fast contaminant sorption kinetics. Recent works have shown that mesoporous materials can have large adsorption capacity, good selectivity and improved recoverability for the removal of toxic compounds from aqueous solutions. Nanometer-sized pores within mineral particles are thought to contribute to retention of organic pollutants in soils and sediments (Goyne et al., 2004). Studies of hydrophobic organic contaminants (HOCs) indicate that the interaction between

compounds and mineral micropores is the key of high sorption process. Although hydrophobic micropores are likely important for HOC adsorption (Werth and Reinhard, 1997; Li and Werth, 2001), hydrophilic porous surfaces may also be significant, since steric effects, slow diffusion, and tortuosity may inhibit the desorption process. Some studies support the likelihood that mineral mesopores may enhance organic compound sorption processes. For example, the structure of water in close proximity to mineral surfaces differs from that in the bulk (Pignatello, 1999). This may create favourable conditions for contaminant sorption within mesopores, even though the extent of structured water may not permeate throughout a mesopore as it apparently does within a micropore. Previous studies (Mastral et al., 2001) report that the increasing of benzene rings number in polynuclear aromatic compounds results in more favourable sorbate/sorbate interactions within mesopores, while Zimmerman et al. (2004) observe that nitrogenous organic compounds smaller than one-half mesopore diameter exhibite a greater adsorption on mesoporous alumina and silica, relative to non porous analogues. Therefore, several works have investigated the sorption of HOCs and heavy metals on mesoporous materials (Farrell and Reinhard 1994; Wang and Lo 2009), but few studies (Goyne et al., 2004) have been carried out on the sorption of ionic organic pollutants.

Among the different pollutants commonly found in soil and waters, 4-chloro-2-methylphenoxyacetic acid[2,3] (MCPA) deserves a particular interest. MCPA is a post emergence phenoxy acid herbicide extensively used in agriculture to control annual and perennial weeds in cereals, grasslands, trees, and turf. It is very soluble (273.9 mg/L in water, at neutral pH), highly mobile, and can leach from soil and it is suspected for mutagen and carcinogen properties. This compound has been found in well water in some countries and is classified by the U.S. Environmental Protection Agency (EPA) as a potential groundwater contaminant (Walker and Lawrence, 1992).

Simazine[1,3], (2-chloro-4,6-bis(ethylamino)-s-triazine) is a synthetic *s*-triazine herbicide widely used for pre-emergence control of broad-leaf weeds and annual grasses in agricultural and non-crop fields (Gunasekara et al., 2003). Simazine is the second most commonly detected pesticide in surface and groundwater in the United States, Australia and Europe (Troiano et al, 2001), it is persistent in the environment up to eight months and not easily degraded by microbes. Due to the carcinogenic potential of *s*-triazines, simazine presence in water is of increasing concern (Hayes et al., 2006).

Therefore, the objective of this chapter is to evaluate (i) the sorption capacity of a bio-sorbent (polymerin) with two commercial nanosized metal oxides (Al_2O_3 and Fe_2O_3), for simazine and MCPA removal from polluted waters, (ii) the removal of both

A version of this chapter has been published:

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[2]**Addorisio, V.**, Pirozzi, D., Esposito, S., Sannino, F., (2010). Remediation of waters polluted with simazine by sorption on mesoporous metal oxides. Submitted to *Environmental Science and Technology*.

[3]De Martino, A., **Addorisio, V.**, Sannino, F., Iorio, M., Capasso, R., (2009). Potential remediation of waters contaminated with pesticides by sorption on recycled organic matter. *Progress in Environmental Science and Technology*, Vol II, part B, published by Science Press ISBN 978-7-03-024459-8.

pesticides from simulated wastewaters by cyclic sorption of the herbicide on the selected sorbents renewed at each cycle and (iii) the regeneration of metal oxides.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Provider	Reagents
Sigma-Aldrich *(St. Louis, MO, EEUU)*	• 4-Chloro-2-methylphenoxyacetic acid (MCPA), 99% purity • 2-chloro-4,6-bis(ethylamino)-1,3,5-triazine (simazine), 99.0% purità • Hydrochloridric acid (HCl) • Hydroxy potassium (KOH) • Potassium Chloride (KCl)
IoliTec Nanomaterials *(Denzlingen, Germany)*	• γ-Aluminium (Al_2O_3) nanosized oxides, 99.9% purity • Iron(III) (Fe_2O_3) nanosized oxides, 99.5% purity
Spectrum Labs.com	• Cellulose dialysis membranes (cut-off 3,500 Da)

All solvents are of HPLC grade (Carlo Erba, Milan, Italy) and are used without further purification. All other chemicals are obtained from Sigma-Aldrich unless otherwise specified.

3.2.2 Methods

3.2.2.1 Physical characterization of the sorbents

Polymerin was prepared and characterized in according to the procedure reported in the chapter 2 of this thesis.

Specific surface area

The specific surface area (SSA) of Al_2O_3 and Fe_2O_3 was calculated by the Brunauer-Emmett-Teller (BET) method (Rouquerol et al., 1999). N_2 adsorption-desorption isotherms at 77 K were obtained by a Micromeritics Gemini II 2370 apparatus. Before each measurement the sample was degassed at 250°C for 2 h under N_2 flow. Pore volumes were determined from the amount of adsorbed N_2 at P/P°= 0.98 (desorption curve), assuming the presence of liquid N_2 (density=0.807 g cm^{-3}) in the pores under these conditions. The average values of pore diameters (d_p) were calculated from the relation $d_p=4V/A_{BET}$, where V is total pore volume. The Barrett-Joyner-Halenda (BJH) approach (Rouquerol et al., 1999) was used to calculate pore size distribution of the sample using the desorption data.

Point of zero charge (PZC)

The point of zero charge (PZC) of Al_2O_3 and Fe_2O_3 was performed according to the method described by Mustafa et al. (2004). Dry samples (100 mg) were dissolved in 40 mL of 0.001M KCl solution as a background electrolyte and equilibrated for 30 minutes by means of a magnetic stirrer. Successively, the initial pH of the solution was adjusted to pH 4.0 by the addition of either 0.1M HCl or 0.1M KOH. The

suspension was equilibrated for another 10 minutes, and the pH was then measured. The suspension pH was recorded every 2 min as a function of volume of titrant added until the pH 10 was reached. The PZC of two sorbents was determined from the variation of surface charge density as a function of pH.

3.2.2.2 Sorption methodology

Stock solutions were prepared by dissolving 2 mg of simazine in 500 mL of 0.03M KCl (final concentration 4 mgL^{-1}), and 100 mg of MCPA in 500 mL of 0.03M KCl (final concentration 200 mgL^{-1}). These solutions were then kept refrigerated. Preliminary sorption experiments were conducted at solid/liquid ratio of 0.5 obtained by adding 10 mg of support to a final volume of 20 mL, using a fixed pesticide concentration of 10 $\mu molL^{-1}$, varying the pH from 3.0 to 7.0 and for an incubation time of 24 h. The pH was controlled by addition of 0.01mML^{-1} HCl or KOH to the solution. After incubation, the polymerin was separated from the solution by centrifugation at 5,500 rpm for 20 min, using an Amicon Ultra system (Millipore) having two transversal membranes with cut-off of 5,000 Da, the Al_2O_3 and Fe_2O_3 were separated by centrifugation at 7,000 rpm for 20 min. The amount of simazine and MCPA sorbed was calculated as the difference between the initial quantity of pesticide added and that present in the equilibrium solution. Blanks with the same amount of sorbents in 0.03M KCl were subjected to the same experimental procedure. Blanks of pesticides in 0.03M KCl were analyzed in order to check pesticide stability and adsorption to vials and Amicon Ultra system.

Analytical determination of pesticides

Simazine and MCPA were determined using an Agilent 1200 Series HPLC equipped with a DAD array and a ChemStation Agilent Software. A Macharey-Nägel Nucleosil 100-5 C18 column (stainless steel 250×4 mm) was utilized. The mobile phase, comprising a binary system of 65:35 acetonitrile: water and 50:50 acetonitrile: phosphate buffer (0.1%, pH 2.0) for simazine and MCPA respectively, was pumped at 1 $mLmin^{-1}$ flow, in an isocratic mode. The detector was set at 220 nm for simazine and at 225 nm for MCPA. Injection volume was 20 µL. The quantitative determination of simazine and MCPA was performed elaborating their corresponding calibration curves between 0.05-4 mgL^{-1} for simazine and 0.05-200 mgL^{-1} for MCPA.

Effect of solid/liquid ratio

Precedent studies (Sannino et al., 2008) showed that the optimal solid/liquid ratio for the sorption experiments with polymerin was 0.5 and all experiments were conducted in this condition, at pH 3.5 and 4.5 for simazine and MCPA, respectively. For Al_2O_3 and Fe_2O_3, ratios of 0.1, 0.5, 1.0, and 2.0 were obtained by adding 2.0, 10, 20, and 40 mg of mesoporous oxides to a final volume of 20 mL. The samples with MCPA were incubated at pH value of 4.0 and 3.5 for Al_2O_3 and Fe_2O_3, respectively, the ones with simazine were incubated at pH values of 6.5 and 3.5 for Al_2O_3 and Fe_2O_3, respectively. All experiments were carried out for 24 h by adding a fixed pesticide concentration, 10 $\mu molL^{-1}$ at 20°C.

Effect of Time

Kinetic studies were performed at optimal pH values, adding 10 $\mu molL^{-1}$ of simazine and MCPA to polymerin, Al_2O_3 and Fe_2O_3. The suspensions were stirred for 0.5, 1, 1.5, 2, 4, 5, 8, 16, 24, 30, 48 and 72 h.

Sorption Isotherms

Different volumes of stock solutions of herbicides were added to each sorbent to give an initial concentration ranging from 0.05 to 500 $\mu molL^{-1}$ for MCPA and 0.5 to 10.69 $\mu molL^{-1}$ for simazine. The samples were incubated for 20, 180 and 1440 min for Al_2O_3, Fe_2O_3 and polymerin, respectively, at the optimum pH value for each sorbent.

3.2.2.3 Cyclic sorption on polymerin, Al_2O_3 and Fe_2O_3

Cyclic sorption experiments were carried out at optimum pH of sorbents, as previously determined, and using in the first sorption cycle 10.69 and 500 μML^{-1} for polymerin and 10 and 100 μML^{-1} for Al_2O_3 and Fe_2O_3 of simazine and MCPA, respectively. All samples were treated according to the procedure previously described. After the contact time, the sorbent was separated from the suspension by centrifugation and the supernatant was analyzed to determine the amount of sorbed pesticide. For the second cycle, the supernatants were added to fresh sorbent maintaining constant the solid/liquid ratio. The experiments were repeated until the equilibrium concentrations remained constant.

3.2.2.4 Data analysis

All experiments were performed in triplicate, and the relative standard deviation was lower than 4%. The kinetic data were analyzed using the Lagergren's first order (Namasivayama and Yamuna, 1995) and Ho's second order (Ho and McKay, 1999) kinetic equations.

$$\text{Lagergren's equation: } \log(q_e - q_t) = \log q_e - K_1 t/2.303$$

where q_e and q_t are the amount of herbicide sorbed ($\mu molkg^{-1}$) at equilibrium and at time t, respectively, K_1 is the rate constant of sorption (min^{-1}), and t is the time (min).

$$\text{Ho's equation: } t/q_t = 1/k_2 q_e^2 + (1/q_e)t$$

where, q_e and q_t are described above and k_2 is pseudo-second order rate constant of the reaction ($Kg\ \mu mol^{-1}\ min^{-1}$).

The half-life time $t_{1/2}$, that is, the time required for the sorbent to uptake half of sorbate amount that will be retained at the equilibrium is determined by following equation:

$$t_{1/2} = 1/k_2 q_e$$

The sorption data were analyzed according to the Freundlich's equation:

$$C_s = K_F C^{1/N}$$

where C_s is defined as the amount of pesticide sorbed ($\mu molkg^{1}$), C is the equilibrium concentration of pesticide ($\mu molL^{-1}$), $K_F[(\mu molkg^{1})/(\mu molL^{-1})^{1/N}]$ and N (dimensionless) are constants that give estimates of the sorption capacity and intensity, respectively, according to Giles et al., (1974).

After log linearization: $\log C_s = \log K_F + 1/N \log C$

Freundlich parameters K_F and N values were determined from the curve obtained after linear regression of $\log C_s$ versus logC (intersection with Y-axis and slope, respectively).

The distribution coefficient K_d (Lkg^{-1}) was determined as follows:

$$K_d = C_s / C$$

Equilibrium concentration (q_e) was calculated considering as reference concentration 0.1 ($K_{d0.1}$) and 0.01 ($K_{d0.01}$) of the solubility concentration of pesticides.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of sorbents

Polymerin was recovered from OMW and characterized chemically and spectroscopically according to the procedure reported in chapter 2 of this thesis. The porosity of the Al_2O_3 and Fe_2O_3 was analyzed to get insight into their adsorption capability. N_2 adsorption-desorption isotherms for Al_2O_3 and Fe_2O_3 are reported in figure 1. The isotherms are of type IV for Al_2O_3 and of type IIb for Fe_2O_3 according to the IUPAC classification (IUPAC recommendation, 1985) with large adsorption volumes at P/P0 > 0.4 and a desorption hysteresis. Although both the isotherms show a desorption hysteresis, characteristic of mesoporous materials, the type of hysteresis is different for the two samples, indicating a dissimilar mesoporous structure.

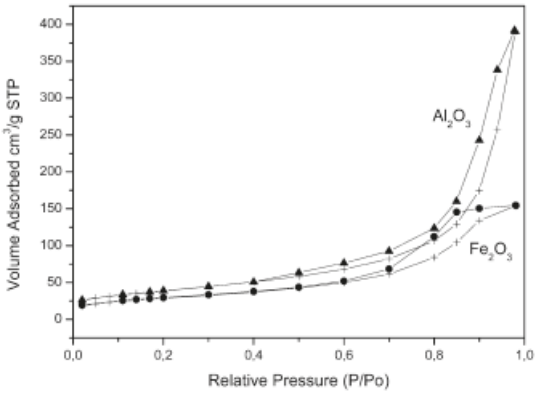


Figure 1. N_2 adsorption-desorption isotherm of mesoporous metal oxides, Al_2O_3 and Fe_2O_3.

The isotherm of Al_2O_3 does not exhibit any limiting adsorption at high P/P0, revealing a type H3 hysteresis according to the IUPAC classification (IUPAC recommendation, 1985). This behaviour is observed with not rigid aggregates of plate like particles giving rise to slit-shaped pores. Because of the delayed capillary condensation, multilayer adsorption is able to proceed on the particle surface until a high value of P/P0 is reached. The isotherm of Fe_2O_3 shows a plateau at a high value of P/P0, and a type H2 loop can be detected. This situation is often associated with disordered materials where the distribution of pore size and shape is not well-defined. The pore structures in these materials are complex, and often they are made up of interconnected networks of pores of different size and shape. The adsorption isotherms were elaborated using the BET method, and the corresponding surface areas are reported in table 1, together with the total pore volume and the estimated average pore diameter.

Sample	Pore volume (cm^3g^{-1})	Particle size (nm)[a]	Average pore diameter, d_p (nm)	Surface area (m^2g^{-1})	Point of zero charge (PZC)
Al_2O_3	0.723	20	14.8	195	9.1
Fe_2O_3	0.239	10-20	9.2	106	10.1
Polymerin	-	-	-	312	2.2

Table 1. Physical and chemical properties of Al_2O_3, Fe_2O_3 and polymerin.

[a] Provided by the supplier

It can be observed that the surface area of aluminium oxide (195 m^2g^{-1}) is much greater than that of iron oxide (106 m^2g^{-1}), meanwhile the aluminum oxide possesses a porous structure with a total pore volume much higher than that of iron oxide. The average values of pore diameter, in both selected supports, are further evidence that most of the porosity is due to quite large cavities, with size far from that of MCPA (~7-9 Å) (Gimeno et al., 2003) and simazine (~7-8 Å). It is worth underlining the greater porosity of the aluminum oxide with respect to the iron oxide, as indicated by the total pore volume together with the higher average values of pore diameter (table 1). To obtain the pore size distribution of these materials, the desorption data were elaborated by the BJH method and reported in figure 2.

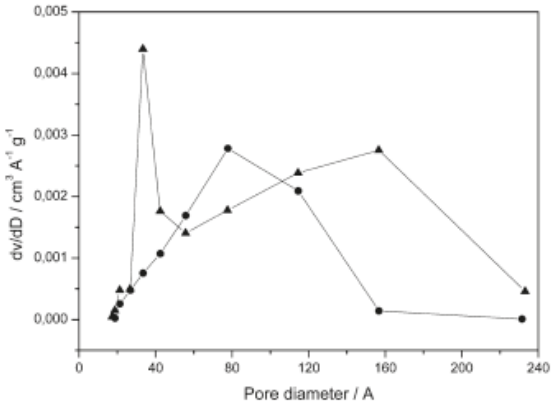


Figure 2. Pore size distribution of mesoporous metal oxides: Al_2O_3 (♦) and Fe_2O_3 (●).

The iron oxide shows an unimodal distribution, and most of the N_2 volume is adsorbed in the pore range of 6-10 nm. For Al_2O_3 the distribution appears to be bimodal characterized by two maxima, the former at about 3 nm and the latter at about 15 nm. The peak in the range 20-40 Å is a tricky point because it could be caused by the forced closure of the H3 hysteresis loop.

3.3.2 Sorption studies

3.3.2.1 Effect of pH

The effect of pH on simazine sorption is shown in figure 3.

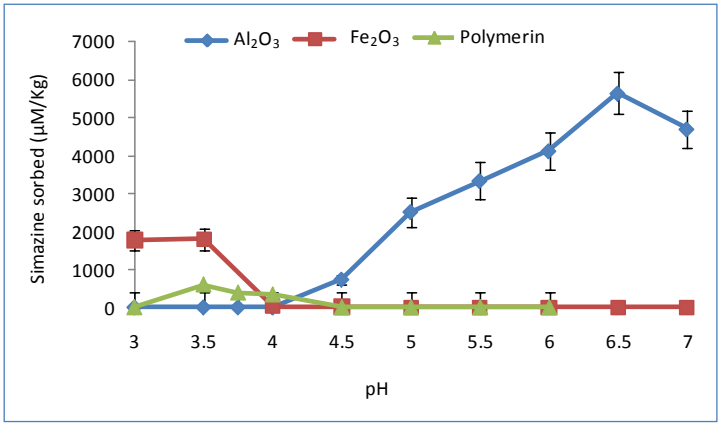
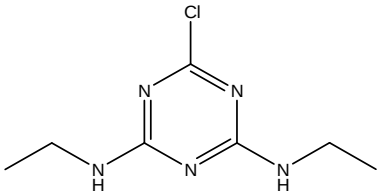


Figure 3. Effect of pH on simazine sorption on polymerin, Al_2O_3 and Fe_2O_3.

In particular, optimum pH sorption for simazine on polymerin is 3.5. The amount of sorbed pesticide decreases by increasing the pH up to 4.5 and is void above this value. Sorption of pesticide is function of polymerin PZC and pK_a. This latter is considered a weak acid polyelectrolyte with a PZC of 2.2 and a pK_a of 4.5 due to its carboxylic groups (Capasso et al., 2002). These values suggest that when pH>PZC

polymerin possesses a surface with negative charges. This hypothesis is supported by carboxylic acidity value of 9.7 meqg^{-1}. Simazine, a ionisable molecule with a pK_a of 1.70 (figure 4), at pH 3.5 shows a weak positive charged while the acidic groups of organic matter are for the 75% in neutral form, as R-COOH, and 25% in anionic forms, as R-COO$^-$, approximately. Then, at pH 3.5 sorption occurs probably by H-bonding between nitrogen of triazinic ring and secondary amino groups of lateral chain of simazine (figure 4) with carboxylic, phenolic and alcoholic functional groups of polymerin. Moreover, at this pH simazine can be probably sorbed through ionic bonding occurring between the little amount of its protonated secondary amino groups and carboxylate anions of polymerin.



Herbicide	Molecular weight (gmol^{-1})	Solubility in water (mgL^{-1})	pKa
Simazine	201.66	5.0[b]	1.70[b]

Figure 4. Structure formula and chemical properties of simazine.

Source [b] The Pesticide Manual. Charles R. Worthing and Raymond J Hance, The British Crop Protection Council, 9th edition.

Some authors report that interactions of simazine with humic substances occur through H-bonding, involving carbonyl groups of humic acids and secondary amine groups of simazine and proton transfer processes (Celis et al., 1998; Nearpass, 1995). Regarding to the sorption on polymerin under pH 3.5 and up to pH 4.5, the possibility of ionic and H-bonding is drastically reduced. In fact, at low pH, as humic acids, structure of polymerin collapses for the formation of intra- and intrermolecular H-bonding and hydrophobic interactions (Schimpf and Wahlund, 1997). This behaviour diminishes its flexibility and capacity to participate in external H-bonding (Alvarez-Puebla and Garrido, 2005). Increasing the pH up to 3.5 simazine tends to acquire neutral and successively anionic character while polymerin increases the negative surface charges. Consequently, no interaction are possible because of electrostatic repulsion between sorbent and sorbate prevents the formation of H-bonding. This finding suggests that sorption of simazine occurs prevalently a pH 3.5 for the instauration of an equilibrium between protonated and deprotonated amino groups of pesticide with functional groups of polymerin that allows the formation of ionic and H-bonding.

The results obtained from the sorption studies on the oxides show the greatest sorbed amount of simazine at pH of 6.5 and 3.5 on Al_2O_3 and Fe_2O_3, respectively. This behaviour could be explained by considering the simazine pK_a value (1.70) of and the PZC of two oxides, 9.1 and 10.1 for Al_2O_3 and Fe_2O_3, respectively. When the pH suspension is greater than the pK_a of the herbicide, simazine is prevalently deprotonated and is adsorbed by hydrogen bounds between the secondary amino groups of its lateral chain and the OH- groups of the Al_2O_3 and Fe_2O_3. Moreover, the intense “surface acidity” of Fe_2O_3 promotes the protonation of weak bases at pH 3.5. In fact , simazine molecules arrive at Fe_2O_3 interface mostly as molecular species, then because of the high hydrolysis constant of $10^{-2.19}$ of the Fe^{3+} ion, the complex-

bound water of hexaaqua ion $Fe(H_2O)_6)^{3+}$ is hydrolytically dissociated, especially at pH values greater than 3 (Cornell and Schwertmann, 1996). The hydrogen ions released during hydrolysis, however, are also capable of protonating the triazine molecules. As a consequence of protonation, the molecules are at least partially in the cationic form (secondary amino groups) and thus can be sorbed through electrostatic interactions with –OH groups of Fe_2O_3. This behaviour is completely opposite to that reported in literature; in fact, triazine molecules such as atrazine or simazine are scarcely sorbed on iron and aluminium oxides (Clausen and Fabricius, 2001). In our study, the high acidity of Fe_2O_3 and the presence of a porous structure allow the sorption of this herbicide. On the contrary, the highest sorption capacity of simazine on Al_2O_3 is detected at pH 6.5. In this case the "surface acidity" is less than Fe_2O_3 and it is not able to protonate a sufficient number of molecules, resulting in low sorption. On the other hand, by increasing pH solution, Al_2O_3 exhibits a greater number of –OH groups as undissociate form and simazine is present as molecular form. In these conditions, simazine can be probably sorbed by H-bonding between nitrogen of triazinic ring and secondary amino groups of lateral chain with –OH groups of Al_2O_3. Moreover, the highest superficial surface area of this oxide (195 m^2g^{-1}) than Fe_2O_3 (106 m^2g^{-1}), and the presence of secondary small pores at boundary of micropores region in Al_2O_3 could have a positive influence in the uptake of small organic molecules such as simazine (0.784 nm). In fact, for Al_2O_3 the pore size distribution appears to be bimodal, characterized by two maxima, the former at about 3 nm and the latter at about 15 nm. The peak in the range 20-40 Å is a tricky point because it could be caused by the forced closure of the H3 hysteresis loop. On the contrary, Fe_2O_3 shows an unimodal distribution and most of the N_2 volume is adsorbed in pore range of 6-10 nm. It is likely that the sorption energy increases in those pores whose dimensions approach to the herbicide dimensions (0.7-0.9 nm).

The effect of pH on MCPA sorption is shown in figure 5.

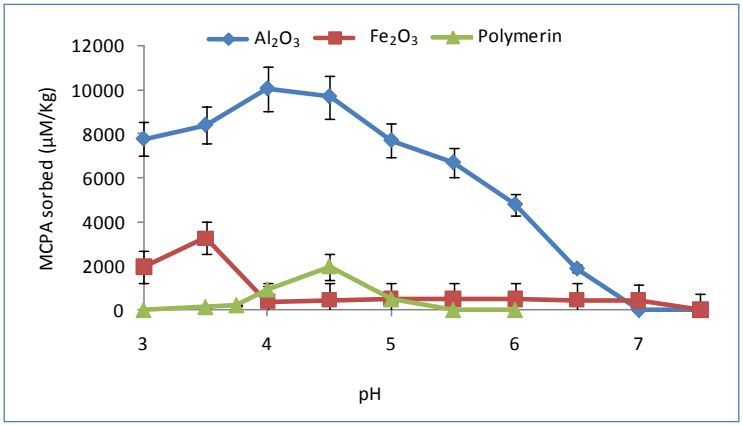
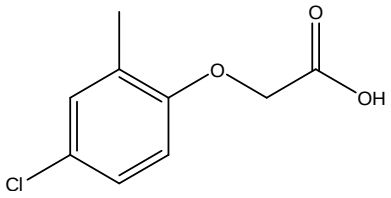


Figure 5. Effect of pH on MCPA sorption on polymerin, Al_2O_3 and Fe_2O_3.

In particular, optimum pH sorption of MCPA on polymerin is 4.5. MCPA, an anionic pesticides with a pK_a of 3.07 (figure 6), at pH 4.5 is almost completely in ionized form. So, sorption on polymerin occurs by the formation of H-bonding between the remaining indissociated form of pesticide and the carboxyl and -OH groups of organic matter. Up to pH 4.5 electrostatic repulsion between the anionic form of pesticide and negative charged surface of polymerin don't allow the formation of H-bonding. The behaviour is confirmed in previous studies conducted on the sorption of the phenoxy acid 2,4-D on polymerin as a function of pH (Sannino at al., 2008).



Herbicide	Molecular weight ($gmol^{-1}$)	Solubility in water (mgL^{-1})	pKa
MCPA	200.6	273.9[a]	3.07[b]

Figure 6. Structure formula and chemical properties of MCPA.

Source [a] MUCCINELLI M. Manual of pesticides, 2006, 11th ed., Edagricole, Italy;

Source [b] The Pesticide Manual. Charles R Worthing and Raymond J Hance, The British Crop Protection Council, 9th edition.

Sorption on Al_2O_3 and Fe_2O_3 shows the greatest sorbed amount of MCPA at acidic pH, with an optimum pH of 4.0 and 3.5 for Al_2O_3 and Fe_2O_3, respectively. This behaviour could be explained considering the pK_a value (3.07) of MCPA and the PZC of two oxides, 9.1 and 10.1 for Al_2O_3 and Fe_2O_3, respectively (table 1). Indeed, at sorption optimum pH (4.0 and 3.5 for Al_2O_3 and Fe_2O_3) both the oxides have the completely positive surface by OH^{2+} groups because of PZC value, whereas the proportions of un-ionized and ionized herbicide carboxylic group are roughly estimated as 40:60% and 50:50% for Al_2O_3 and Fe_2O_3, respectively. This means that the ionized carboxylic group ($-COO^-$) of MCPA forms ionic bonding with the positive surface of both the sorbents. The hydrogen bonded to the protonated hydroxyl group presents a very high mobility due to positive charge on oxygen atom. In these conditions and considering the aqueous medium where the adsorption occurs, it can be supposed that the formation of hydrogen bonds contributes to stabilize the interaction between the anionic form of MCPA and the positively charged surface of the matrices, attenuating very likely the ionic character of the bond. The amount of sorbed MCPA on Fe_2O_3 was much lower than that detected on Al_2O_3 due very probably to (i) the lower proportion of ionized herbicide thus available for ionic bonding with the matrix and (ii) the lower surface area and the lack of secondary small pores with respect to Al_2O_3. Previous studies confirm that metal oxides exhibit a strong sorption of ionic and ionizable pesticides at low pH and a decreasing sorption as pH increased (Clausen and Fabricius, 2001). Cho et al. (2006) investigated the sorption ability of MCPA on activated carbon and demonstrated a higher sorption of the herbicide at pH 3.5 and a decrease with increasing the pH of the solution.

3.3.2.2 Effect of solid/liquid ratio

In a previous study (Sannino et al., 2008) was reported that among 2.5, 1.25, and 0.5 solid/liquid ratio, the best condition for adsorption of 2,4-D and paraquat on polymerin was 0.5. For this reason we utilized 10 mg of polymerin in 20 mL of final solution in all experiments, at pH 3.5 and 4.5 for simazine and MCPA, respectively.

Sorption studies of simazine were carried out at pH 6.5 and pH 3.5, the ones of MCPA at pH 4.0 and 3.5 on Al_2O_3 and Fe_2O_3, respectively, and varying the amount of sorbent, (ratios of 0.1, 0.5 1.0 and 2.0).

Both the oxides show a higher sorption capacity at a solid/liquid ratio corresponding to 0.1 for MCPA and 0.5 for simazine (figure 7a and 7b).

Regarding the simazine the amount sorbed on Al_2O_3 is significant at the solid/liquid ratio of 0.1 and drastically increases by increasing the amount of oxide. However, at 1.0 and 2.0 solid/liquid ratio for both oxides, no sorption is observed. For Fe_2O_3 a considerable amount of simazine sorbed is detected only at solid/liquid ratio 0.5.

As regards the MCPA, the amount sorbed on Al_2O_3 significantly decreases by increasing the amount of oxide. For Fe_2O_3 a drastic reduction is observed at a solid/liquid ratio of 0.5 while no sorption is observed at solid/liquid ratio of 1.0 and 2.0

This behaviour could be explained by considering the mass transfer resistance involved in the sorption process. The sorption on a solid surface takes place in several steps, such as external diffusion, internal diffusion, and actual sorption. Intra particle diffusion has been generally considered as the rate controlling step in liquid-phase sorption.

Evidently, the amount of oxide and the resistance to the diffusion inside the particle are directly proportioned , and the result is a lower sorption of the herbicide. This would explain no sorption of the herbicides at solid/liquid ratio 1.0 and 2.0. On the contrary, at lower solid/liquid ratio, although the low resistance to the diffusion inside the particle, the higher amount sorbed on Al_2O_3 than Fe_2O_3 can be attributable to higher volume and diameter of pore as well as the surface area (table 1).

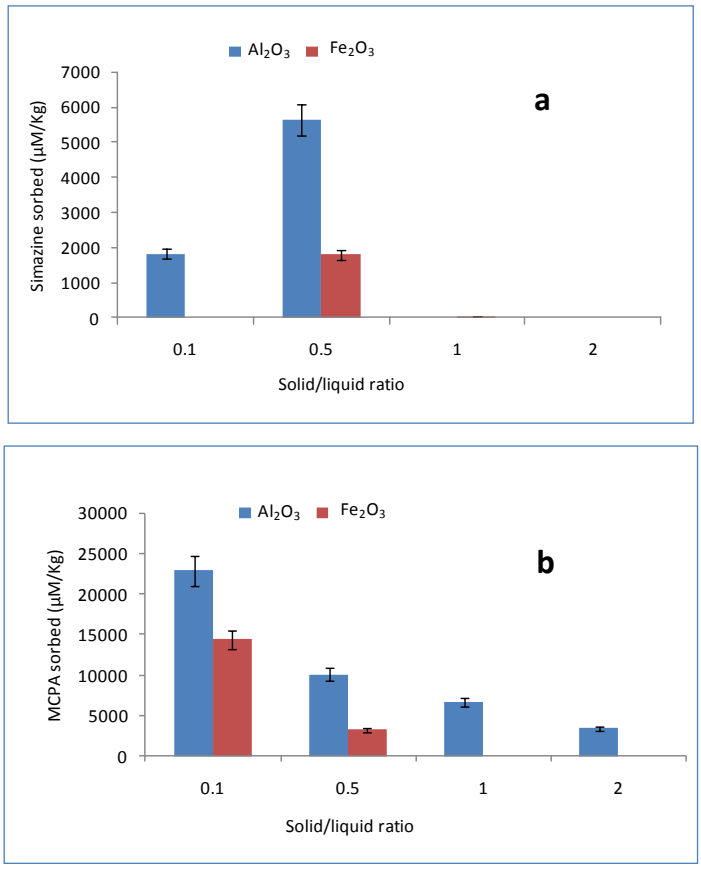


Figure 7. Effect of solid/liquid ratio on the sorption of simazine (a) and MCPA (b) on Al_2O_3 and Fe_2O_3.

3.3.2.3 Effect of time

The effect of time on simazine and MCPA sorption is shown in figure 8a and 8b.

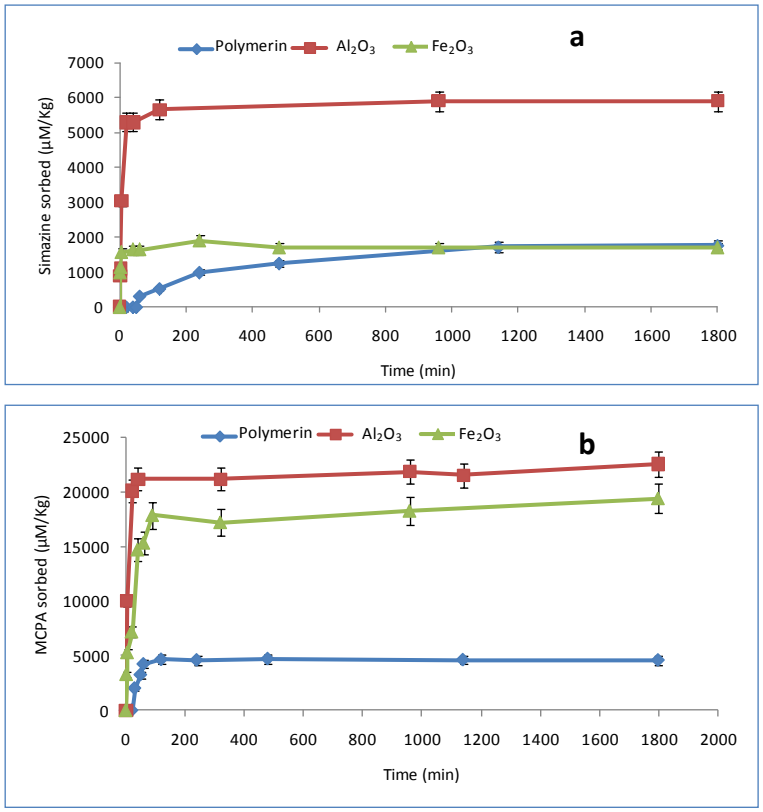


Figure 8. Effect of the time on simazine (a) and MCPA (b) sorption by polymerin, Al_2O_3 and Fe_2O_3.

Kinetic studies on polymerin, carried out at pH 3.5 for simazine and pH 4.5 for MCPA, show that MCPA is rapidly sorbed, reaching the sorption equilibrium within 4-8 h; no appreciable changes in the sorbed amount are observed after this time. As regards simazine, the data show an increase in sorption up to 24 h and no variation after this time. Huang et al. (1996) report that the sorption of organic compounds onto non-porous mineral surface is relatively fast. Similarly, sorption of simazine and MCPA onto polymerin consists of an initial fast sorption to the surface of organic matter and a final slow sorption to the internal surface. Kinetic models, using Lagergren and Ho's equations, are applied to the experimental data and the parameters are presented in table 2. Sorption of the two pesticides fits a pseudo-second order equation. In fact, R^2 of pseudo-second order equation, 0.993 and 0.998 for simazine and MCPA, respectively, is better than the one of the first-order equation (0.7244 and 0.2362, for simazine and MCPA, respectively). Higher value of k_2 for MCPA indicates a higher amount of polymerin necessary to sorb the pesticide respect to simazine. But only apparently polymerin has a best sorption performance for simazine. In fact considering half life time, $t_{1/2}$, polymerin needs two times longer to sorb at equilibrium half simazine respect to MCPA.

	Pseudo-second order			Pseudo-first order	
	K_2	R^2	$t_{1/2}$	K_1	R^2
Simazine	0.49	0.993	12	1	0.7244
MCPA	3.26	0.998	6	1	0.2366

Table 2. Kinetics parameters of simazine and MCPA sorption on polymerin.

Regarding the two mesoporous oxides, sorption process of simazine is very fast on Fe_2O_3, reaching the sorption equilibrium after 5 min, but it is much slower on Al_2O_3, it being observed within 120 min. Therefore, an incubation period of 20 min for a Fe_2O_3 and 180 min for Al_2O_3 is utilized.

As regards MCPA, sorption process is very fast on Al_2O_3, reaching the sorption equilibrium after 5 min, but it is much slower on Fe_2O_3, it being observed within 90 min. So, an incubation time of 20 min for Al_2O_3 and 180 min for Fe_2O_3 is utilized. Previous studies (Goyne et al., 2004) show similar results in the sorption process of 2,4-D on three Al_2O_3 adsorbents with varying degrees of mesoporosity (pore diameter 2-50 nm).

The rapid sorption of both pesticides on Al_2O_3 and Fe_2O_3 further suggests that the sorption mechanism is mainly due to electrostatic attraction, and it also implies that the mesopores are not a limiting factor for the herbicide diffusion into the interior of the mesoporous oxides.

The parameters obtained by the Lagergren equation show that the sorption of MCPA and simazine on each mesoporous oxide fits a first order mechanism (R^2> 0.90; table 3). In correspondence, the rate constants determined from the slopes of the plots of log(q_e-q) versus t are 0.830 and 0.510 min^{-1} for simazine and 0.230 and 0.908 min^{-1} for MCPA on Fe_2O_3 and Al_2O_3, respectively.

	Simazine		MCPA	
Pseudo-first order	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3
K_1	0.510	0.830	0.908	0.230
R^2	0.9	0.9	0.9	0.9

Table 3. Kinetics parameters of simazine and MCPA sorption on Al_2O_3 and Fe_2O_3.

3.3.2.4 Sorption isotherms

Sorption isotherms of both pesticides on polymerin, Al_2O_3 and Fe_2O are well-fitted by the linearized form of Freundlich equation (r^2=0.99), (figures 9 and 10). Freundlich parameters for MCPA sorption studies are listed in table 4. In according to the classification of Giles et al., (1974) values of 1/N<1 indicate that the experimental sorption isotherms are L-type for all sorbents. As showed by $Kd_{0.1}$ and $kd_{0.01}$ values (table 9) the MCPA sorption on three sorbents decreases with the increasing in herbicide equilibrium concentration. In particular, at low concentration, ($Kd_{0.01}$) the amount sorbed on Fe_2O_3 is more higher than the others two sorbents but at high concentration ($Kd_{0.1}$) the amount sorbed on Al_2O_3 is slightly higher than Fe_2O_3 (848.41 against 762.47 Lkg^{-1}) but always more higher than polymerin (262.33 Lkg^{-1}). This data are in according with the values of K_f reported in table 9. So, the sorption order of MCPA on the three sorbents is Al_2O_3> Fe_2O_3>polymerin.

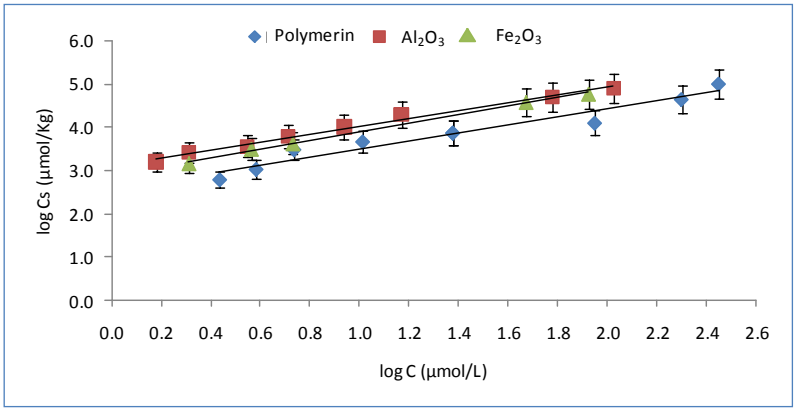


Figure 9. Sorption isotherm of MCPA on polymerin, Al_2O_3 and Fe_2O_3.

Freundlich parameters of MCPA sorption isotherm				K_d[b] (L Kg^{-1})	
	Log K_F (mmol Kg^{-1})/(mmol L^{-1}) $^{1/N}$	1/N	r^{2a}	q_e=0.1q_s	q_e=0.01q_s
Polymerin	2.5822	0.9235	0.99	262.33	312.86
Al_2O_3	3.1197	0.9105	0.99	848.41	1042.57
Fe_2O_3	2.9055	0.9891	0.99	762.47	4719.23

Table 4. Freundlich parameters for MCPA sorption on polymerin, Al_2O_3 and Fe_2O_3.

[a] Correlation coefficient. [b] Distribution coefficient.

Non linear sorption isotherms for MCPA and similar acidic pesticides are frequently reported (Thorstensen et al., 2001; Socias-Viciana et al., 1999). This might be explained by an increased difficulty to access the active sorption sites when pesticide concentrations in solution are elevated. Moreover, non linear isotherms for organic compound sorption to natural organic matters, like polymerin, are due to sorbent heterogeneity and limited sorption site abundance (Schwarzenbach et al., 2003).The textural analysis data for mesoporous sorbents would suggest that the interconnected networks of pores of different size and shape are accountable for two different sorption processes. The highest sorption observed with Al_2O_3 can be explainable probably by considering the presence of secondary small pores at boundary of micropore region in Al_2O_3 (figures 1 and 2) that have a positive influence in the uptake of small organic molecules (i.e., organic molecules having sizes in the range of 6-8 Å). It is likely that the sorption energy increases in those pores whose dimensions approach to the herbicide dimensions (0.7-0.9 nm).

Freundlich parameters for simazine sorption studies are listed in table 5.

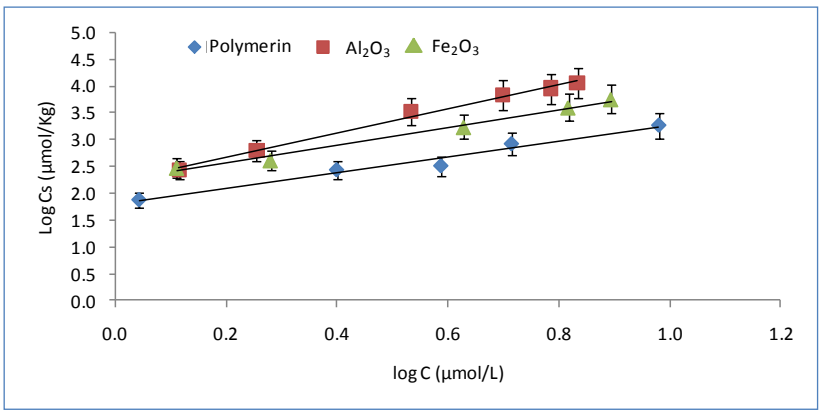


Figure 10. Sorption isotherm of simazine on polymerin, Al_2O_3 and Fe_2O_3.

Freundlich parameters of simazine sorption isotherm				K_d[b] (L Kg^{-1})	
	Log K_F (mmol Kg^{-1})/(mmol L^{-1}) $^{1/N}$	1/N	r^{2a}	q_e=0.1q_s	q_e=0.01q_s
Polymerin	1.9245	1.2857	0.99	109.192	56.557
Al_2O_3	2.2556	2.2521	0.99	567.363	31.751
Fe_2O_3	2.2209	1.6769	0.99	309.219	65.067

Table 5. Freundlich parameters for simazine sorption on polymerin, Al_2O_3 and Fe_2O_3.

[a] Correlation coefficient. [b] Distribution coefficient.

In this case, the 1/N values analysis show that the experimental sorption isotherms are S-type for all three sorbents. The S-type curve observed indicates that the presence of already sorbed molecules favours the sorption process which is called “cooperative”, macroscopically the effect is similar to an increase in available sites over time. This behaviour can be explained by assuming that the molecules already sorbed on the surface modify the affinity towards the others present in solution, favouring the sorption. K_d values (table 5) are calculated when C was 0.1 and 0.01 of C_s ($K_{d0.1}$ and $K_{d0.01}$) and indicate that simazine is sorbed more effectively at high concentration.

The Freundlich constants, K_f and 1/N, (table 5) showing that Al_2O_3 sorbs the herbicide with a higher sorptive intensity and binding energy than Fe_2O_3 and polymerin.

3.3.3 Cyclic removal of simazine and MCPA by polymerin, Al_2O_3 and Fe_2O_3

Experiments of cyclic sorption with simulated wastewaters were conducted for the total removal of pesticides from polluted waters, (figures 11, 12, 13).

No total removal is possible for simazine sorbed on polymerin. Starting from an initial concentration of 10.5 $\mu molL^{-1}$; a removal of 81% is achieved after 6 cycles of sorption, these data indicate that exists a threshold concentration value of 2.1 $\mu molL^{-1}$, at which no sorption occur, and this value is independent of sorbent amount and of cycles number. Moreover, a removal of 84% of simazine on Al_2O_3 is performed after only two sorption cycles, whereas a decontamination of 69% is observed on Fe_2O_3 even after five cycles and a constant amount of 3.07 $\mu molL^{-1}$ is detected. The behaviour of two oxides towards simazine shows the different sorption capacities, as reported previously, and confirm the bigger sorption capacity of Al_2O_3 respect to Fe_2O_3.

As regards MCPA, starting from an initial pesticide concentration corresponding a 450 $\mu molL^{-1}$, a removal of 83% is achieved after 4 cycles of sorption on polymerin. Also in this case there is a threshold concentration value of 79.50 $\mu molL^{-1}$, at which no sorption occurs. Results obtained for MCPA are in accordance with those reported by Sannino et al. (2008) for 2,4-D. On the other hand, a total removal of MCPA on Al_2O_3 is performed after four sorption cycles, whereas decontamination is still incomplete on Fe_2O_3 even after five cycles, leaving a constant residue of 7.5 $\mu molL^{1}$. The behaviour of two oxides toward MCPA shows, also in this case, their different sorption capacity, with an herbicide removal of 100% and 92% for Al_2O_3 and Fe_2O_3. The threshold concentration existing for MCPA is due to the acid-base equilibrium which is strongly in competition with the sorption equilibrium between the undissociated form of the same pesticide and the sorbents. For simazine the equilibrium existing between the protonated and the neutral form generates the same

competition. Therefore, Al_2O_3 is shown to be the most efficient sorbent for the removal of MCPA and simazine from contaminated waters.

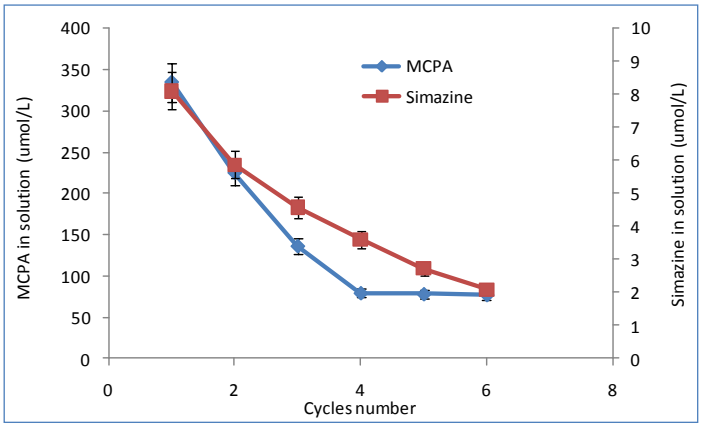


Figure 11. Cyclic removal of simazine and MCPA on polymerin.

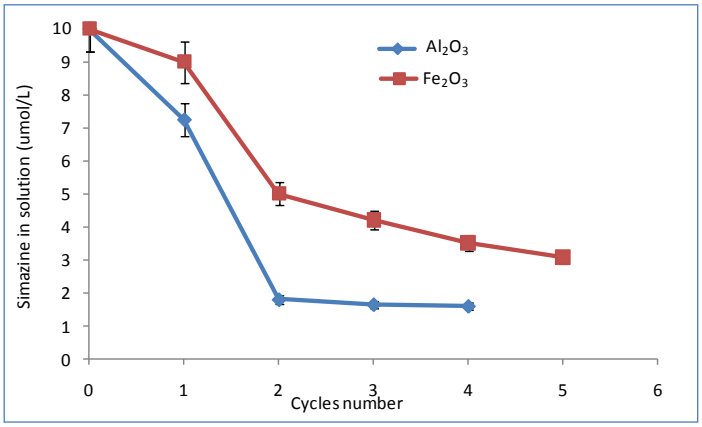


Figure 12. Cyclic removal of simazine on Al_2O_3 and Fe_2O_3.

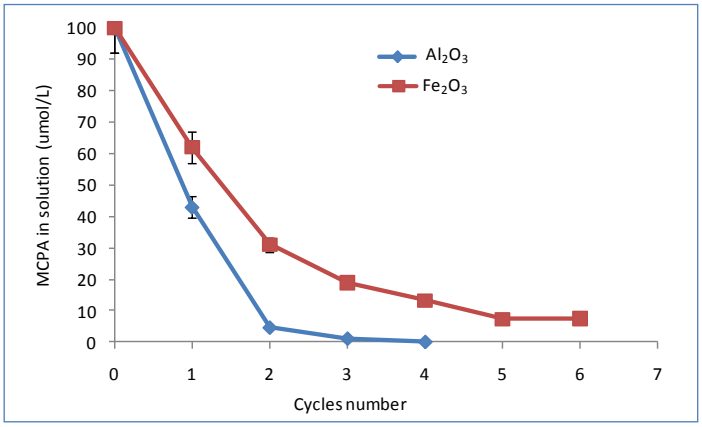


Figure 13. Cyclic removal of MCPA on Al_2O_3 and Fe_2O_3.

3.3.4 Regeneration of Al_2O_3 and Fe_2O_3

In a wastewater treatment process that uses a sorption metodology, the regeneration of the sorbent is crucially important. Nowadays, in many applications, reuse of the sorbent through regeneration of its sorption properties is an economic necessity. Desorption agents (e.g. sodium hydroxide solution) are commonly used to recover sorbents such as Fe- and Al- based supports (Kym and Lee, 2004). However, the utilization of a desorption agent has some disadvantages because it increases the operating cost and waste solution containing NaOH discarded from the regeneration of the sorbent causes environmental pollution. The incineration method

could be considered as alternative way for the regeneration of the sorbents, thus avoiding the use of hazardous desorption agents. To assess the feasibility of this choice, Al_2O_3 and Fe_2O_3 are annealed at 500°C for 1 h; subsequently, to ascertain if the textural properties are retained, a physical analysis on heat treated oxides is performed through the analysis of the relative N_2 adsorption-desorption isotherms. The porosity of Al_2O_3 and Fe_2O_3 is fully analysed and the physical properties of the heat treated samples are compared. The notation used for the samples is referred to the chemical formula followed by a number indicating the temperature of the heat treatment, Al_2O_3500 and Fe_2O_3500; the samples before the heat treatment are simply denoted with the chemical formula, as in the text. The isotherm of Al_2O_3500 perfectly resembles that of Al_2O_3, indicating that the mesoporous structure is not damaged by the annealing. The adsorption isotherms are elaborated using the BET method, and the corresponding surface areas are reported in table 6 , with the total pore volume and the estimated average pore diameter, all textural properties of Al_2O_3 are well preserved after the heat treatment. The present observation drives us to consider the incineration method for the regeneration of aluminum oxide.

Sample	Pore volume (cm^3g^{-1})	Average pore diameter, d_p (nm)	A_{BET} (m^2g^{-1})
Al_2O_3	0.723	14.8	195
Al_2O_3 500	0.770	14.7	200
Fe_2O_3	0.239	9.2	106
Fe_2O_3 500	0.0650	7.9	33

Table 6. Comparison of surface area, pore volume and average pore diameter of Al_2O_3 and Fe_2O_3.

Furthermore, at the same time, the thermal stability of Fe_2O_3 is not comparable to the one of Al_2O_3 from the pore structure point of view. The heat treatment strongly alters the textural properties of the sample and a drastic collapse of the surface area is observed (table 6). The desorption data are elaborated by the BJH method and we observe that the pore size distribution of iron oxide has been modified by the annealing procedure. The comparison between the pore size distribution of Fe_2O_3 and Fe_2O_3500 samples (figure 14) indicates that the mesoporous structure has been completely destroyed by the heat treatment at 500°C, making the iron oxide not recoverable by incineration.

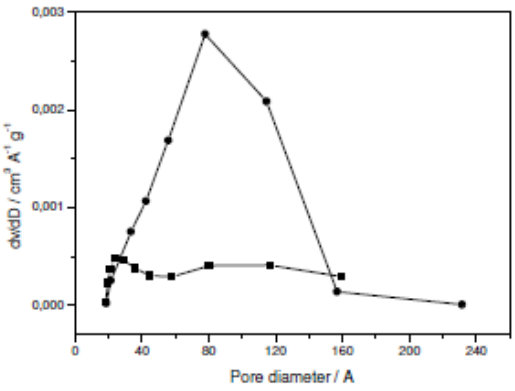


Figure 14. Pore size distribution of mesoporus Fe_2O_3 (●) and Fe_2O_3 500 (■).

To investigate the reason of the observed thermal behaviour of porous structure, X-ray diffraction are performed on iron oxide before and after the heat treatments at 500°C. The XRD patterns, reported in figure 15, clearly show that the annealing of Fe_2O_3 promotes a phenomenon of crystallization probably referable to the phase transition γ-Fe → α-Fe (Wang and Lo, 2009; Grabis et al., 2008).

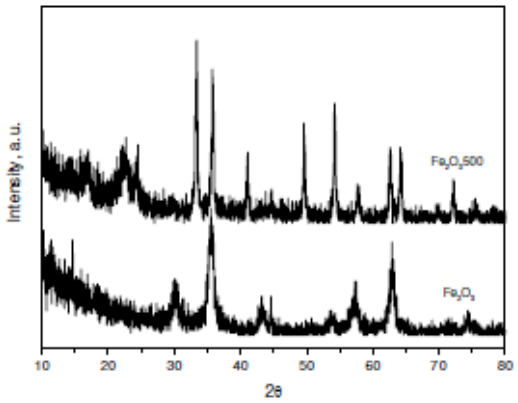


Figure 15. XRD patterns of Fe_2O_3 and Fe_2O_3 500.

Finally, polymerin is a cheap sorbent, recovered from olive mill waste water, and its recycling is not convenient. Moreover, the thermal treatment reported above for Al_2O_3 cannot be performed on polymerin because at high temperature it loses its chemical nature.

3.4 CONCLUSIONS

In conclusion, in this chapter we highlight the capability of the selected oxides compared with polymerin to work as sorbents for a fast and highly efficient removal of simazine and MCPA from contaminated waters. Moreover, we evidence the influence of both chemical composition and mesoporous structure on the sorption capability and the possibility of use these sorbents as suitable filters for the decontamination of polluted point sources. Al_2O_3 is resulted a highly efficient sorbent respect to Fe_2O_3 and polymerin to remediate wastewaters contaminated by MCPA and simazine. In particular, Al_2O_3 turns out to allow the total removal of MCPA from contaminated waters after four sorption cycles, by using the usual procedure of centrifugation for the separation of purified waters. Moreover, Al_2O_3, being regenerated by incineration, could be considered for small-scale treatment systems and industrial scale.

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4. MICROBIAL CONVERSION OF OLIVE OIL MILL WASTEWATERS INTO LIPIDS SUITABLE FOR BIODIESEL PRODUCTION

4.1 INTRODUCTION

In recent years, biodiesel is attracting increasing interest as a substitute for petroleum-based diesel, due to the negative environmental effects of fossil fuels combustion and the concerns about petroleum supplies. As a matter of fact, biodiesel is a biodegradable, nontoxic, and clean biofuel that can be obtained from renewable sources. Unfortunately, the starting materials traditionally used for biodiesel synthesis, namely, vegetable oils, animal fats, and (more recently) waste cooking oils, cannot realistically satisfy the demand for biodiesel at the current rate of consumption (Christi, 2007; You et al,., 2008). In addition, the cost of the biodiesel, which is mainly due to (70-85%) the vegetable oils used as feed stocks, still exceeds that of the mineral diesel. Alternative sources of triacylglycerols (TAGs) are also necessary to reduce the social cost of biodiesel production, as the increase of the latter is leading to significant increases in the price of vegetable oils, a basic food in many underdeveloped countries, as well as to the deforestation of large areas. Thus, new cheaper sources of lipids are needed for biodiesel to be a competitive and sustainable fuel, and the development of non traditional processes for the production of TAGs, to be used as feedstock for biodiesel production, is presently targeted by a growing number of research work (Christi, 2007; Srivastava and Prasad, 2000). Oleaginous microorganisms, which have the ability to produce more than 20% of their weight in the form of lipids, are attracting increasing interest as a potential source of TAGs (Papanikolaou et al., 2007; Angerbauer et al., 2008). The basic physiology of lipid accumulation in such microorganisms has been well-studied (Holdsworth and Ratledge, 1988a; Naganuma et al., 1985b). It is known that lipid production requires nitrogen-limiting conditions. An important advantage offered by the application of the oleaginous microorganisms stems from their ability to produce aerobically lipids from residual organic matters. Consequently, to optimize the cost of the process, as well as to increase its environmental benefit, residual materials have been tested as possible nutrients for the oleaginous microorganisms, such as sewage sludge (Angerbauer et al., 2008), lignocellulosic materials (Dai et al., 2007), and hydrolyzed tomato waste (Fakas et al., 2008).

Olive oil production is a significant agricultural activity with great economic importance, particularly in Mediterranean countries. However, it generates high amounts of waste waters derived from the olive mill process (OMW). This waste causes disposal problems because of its highly polluting properties, which are documented by high chemical oxygen demand (COD) and biological oxygen demand (BOD) values (Arienzo and Capasso, 2000). Different studies have been so far devoted to the application of biological treatments for the reduction of the high organic carbon contents of the OMW (Diamadopoulos and Paraskeva, 2006). Most of these studies describe treatments based on the use of yeasts (Lanciotti et al., 2005; Papanikolaou et al., 2008) or white rot fungi (D'Annibale et al., 2004; Laconi et al., 2007). It has been shown (Field and Lettinga, 1989) that the phenolic components of OMW may inhibit the growth of microorganisms, limiting the efficiency of the digestion processes. As far as we know, no many investigations have focused on the biological synthesis of lipids starting from OMW-based media. The natural habitat of *Lipomyces starkey*i are soil and ensilage (Lodder, 1970), where the microorganisms degrade carbohydrates using extracellular carbohydrolases and contribute to the biodegradation of herbicides (Nishimura et al., 2002). *L. starkeyi* has been proved to

store large amounts of lipids (Holdswoth et al., 1988b), showing only a minimal reutilization of the stored lipids (Holdswoth and Ratledge, 1988a). Lipid accumulation is affected by the concentration of some ions like Zn^{2+} and Mn^{2+} (Naganuma et al., 1985a; Naganuma et al., 1985b). So far, sewage sludge has been the only residual material tested as a culture medium for *L. starkeyi* growth (Angerbauer et al., 2008).

In this chapter[4] we have studied the capability of an oleaginous yeast, *Lipomyces starkeyi,* to grown in the presence of olive oil mill wastewaters (OMW), and we investigated the conversion of OMW into microbial lipids as an alternative feedstock for the synthesis of biodiesel.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Provider	Yeasts
Department of vegetal biology (Perugia, Italy)	• *Lipomyces starkeyi* • *Cryptococcus curvatus* • *Rhodosporidium toruliodes* • *Rhodotorula glutinis*
Provider	**Reagents**
Sigma-Aldrich (St. Louis, MO, EEUU)	• Potato dextrose agar

All solvents are of HPLC grade (Carlo Erba, Milan, Italy) and are used without further purification. All other chemicals are obtained from Sigma-Aldrich unless otherwise specified.

4.2.2 Methods

4.2.2.1 Microorganisms and culture medium

The microorganisms were kept on potato dextrose agar (Sigma) at T= 5±1°C and cultivated in a synthetic N-limiting medium, containing (g/L): KH_2PO_4, 1.0; $MgSO_4$ $7H_2O$ (BDH), 0.5; $(NH_4)_2SO_4$, 2.0; yeast extract, 0.5; and glucose, 70.0. The growth was carried out under aerobic conditions at 30°C on a rotary shaker at 160 rpm (Minitron, Infors HT, Switzerland).

4.2.2.2 OMW

OMW was obtained from the Casa Olearia Italiana (Monopoli, Italy). Samples were immediately frozen at -20°C until further use. Before each experimental test, OMW samples were defrozen, and the solids were removed by centrifugation (4,000 rpm, 30 min, 20°C) in a thermostatic centrifuge (Rotanta 460R, Hettich, United States). The pH of OMW after centrifugation was 4.68. The composition of the OMW is given in table 1.

[4] A version of this chapter has been published.
Yousuf, A., Sannino, F., **Addorisio, V.**, Pirozzi, D. (2010).
Microbial conversion of olive mill wastewaters into lipids suitable for biodiesel production.
Journal of Agricultural and Food Chemistry, 58, 8630-8635.

COMPONENT	CONCENTRATION (g/L)
Water	965.40
Non aqueous components	4.70
Sugars	12.79
Phenols	9.14
Proteins	3.41
Lipids	0.72
Dry weight (105°C)	47.20
Mineral residue (550°C)	11.00
Fe	0.01
Mg	0.19
Ca	0.26
K	2.85
Na	0.65
Cu	0.002
Zn	0.003

Table 1. Chemical composition of OMW.

4.2.2.3 Fermentation in OMW

The fermentation tests were carried out using a fixed volume (150 mL) of OMW (both raw or diluted), without external organic supplement, in a 500 mL conical flask. A preliminary centrifugation of OMW (2,000 rpm, 10 min) was carried out before each test. The liquid medium was inoculated with 2 mL of microorganism suspension, obtained by dissolving 5 loops of solid culture in 8 mL of physiological solution. The flasks were incubated in a rotary shaker at an agitation rate of 160±5 rpm and an incubation temperature of T = 30±1°C.

4.2.2.4 Lipid extraction and measurement

Methanol (5.0 mL) and chloroform (2.5 mL) were added to 200 mg of dry biomass and vortexed for 5 s. Subsequently, the cells were disrupted for 12 min in an Ultrasonic Homogenizer (Omni Ruptor 250, United States) at 50% power and 90% pulser. The cells were then filtered off with Whatman no. 1 filter paper, and the solvent-lipid mixture was placed in a 50 mL tube fitting with centrifuge racks. The layers were separated by centrifugation for 10 min at 2,000 rpm in a thermostatic centrifuge (Rotanta 460R, Hettich) at 20°C. The lower layer was then transferred to a pear-shaped flask with a Pasteur pipet. Again, 10 mL of 10% (v/v) methanol in chloroform was added to the residue, a new centrifugation was carried out, and the lower phase was added to that from the first extraction. The solvent in the pearshaped flask was evaporated to dryness (BUCHI Rotavapor R-200, Switzerland), and the extracted weight was finally recorded after drying at 105°C for 1 h.

4.2.2.5 Biomass analysis

The biomass concentration in the synthetic medium was measured by optical density (OD) determination at 600 nm. When microorganisms were cultured in the OMW, the OD measurement could not be carried out due to the darkness of the medium. Consequently, the total count of microorganisms was carried out by sequential dilution and insemination in plate count agar medium (Difco Laboratories, Detroit, MI). The colonies were counted after 24 h of culture on agar medium. After each fermentation test, the biomass was recovered by centrifugation (3,500 rpm for

10 min) and lyophilized (LYOBETA- 50, Spain), to enable the determination of the dry biomass and the lipid concentration measurement.

4.2.2.6 Fatty acids composition

The fatty acids composition was determined by GC analysis on a Shimadzu GC 17/3 gas chromatograph equipped with a flame ionization detector, following the method suggested by Li and co-workers (Li et al., 2007). Briefly, wet cell pellets from 1 mL of culture were treated with 0.5 mL of a 5% KOH-methanol solution at 65°C for 50 min. After the addition of 0.2 mL of BF_3 diethyl etherate and 0.5 mL of methanol, the mixture was refluxed for 10 min, cooled, diluted with distilled water, and extracted with petroleum ether. The organic layer was collected and washed with distilled water. The GC analysis of the fatty acid methyl esters was carried out using N_2 as the carrier gas (40 mL/min), an injection temperature of 230°C, an oven temperature of 190°C, and a detector temperature of 230°C.

4.2.2.7 Chemical analysis

The chemical analyses of OMW samples before and after treatment with microorganisms were performed according to the Rodier methods (Rodier, 1984), and each test was performed in triplicate. In particular, the cations (Fe, Mg, Ca, K, Na, Cu, and Zn) were determined by atomic absorption spectrometry (Perkin-Elmer Analyst 700). The total sugar and protein contents were measured according to anthrone (Fong et al., 1953) and Bradford (Bradford, 1976) methods, respectively, by using in the first analysis an equimolar standard solution of galactose and mannose (50%w/v) and, for the second, a bovine sieroalbumine solution as the standard. The total phenolic content was estimated according to the Folin method (Box, 1983), using gallic acid as the standard. The total organic carbon (TOC) measurements were carried out with a TOC-$V_{CSH-CSN}$ (Shimadzu, Japan), upon suitable dilution of a culture medium sample. The TOC values were obtained by subtracting the IC (inorganic carbon) value from the TC (total carbon) value.

4.2.2.8 Extracellular lipase and esterase activity

The activity of lipases and esterases in the culture medium was measured after biomass removal by centrifugation, using p-nitrophenyl-butyrate (pNPB) in 10 mM sodium phosphate buffer, pH 7.0, at 37°C. One unit of activity was defined as the amount of enzyme that releases 1 µmol of p-nitrophenol per minute.

4.2.2.9 Phytotoxicity test

The OMW phytotoxicity was assessed on the seeds of *Lactuca sativa* species purchased by “La Semiorto Sementi” located in Sarno, Italy. The bioassays were carried out according to U.S. EPA procedures (U.S. EPA, 1996). A suitable volume (5 mL) of OMW, both before or after treatment, with *L. starkeyi*, diluted (1:10 and 1:25) with deionized water, was added to 100 mm diameter Petri dishes containing a filter paper disk (Whatman no. 1, Ø 90 mm). Ten seeds were placed on each paper disk, and for each sample tested, three replicates were prepared. Controls with deionized water were also run. The plates were incubated in a growth chamber (Angelantoni HCT 120) in the dark at 23°C, and after 72 h, the germinated seeds were counted, and the rootlet of each germinated seeds was measured with a ruler. Each experiment was repeated three times. A primary root >2 mm was considered as the end germination point. Seed germination and root elongation at the end of the bioassays were measured, and the relative index of germination (GI) was calculated according to the following formula:

$$\%GI = (Gs/Gc) \times (Ls/Lc) \times 100$$

where Gs and Gc are the number of germinated seeds in the sample and in the control, respectively, and Ls and Lc are the average root length of seedlings for the samples and for the control, respectively.

4.2.2.10 Statistical analysis

All experiments have been carried out adopting a sample size of at least n=3. The hypothesis tests for the GI data were carried out by a one-sided *t* tests (Himmelblau, 1970), with significance levels of α= 0.01%.

4.3 RESULTS AND DISCUSSION

4.3.1 Fermentation in synthetic medium

Oleaginous yeasts accumulate lipids as storage materials only under N-limiting conditions. Consequently, different oleaginous microorganisms (*L. starkeyi, C. curvatus, R. glutinis, and R. toruloides*) were preliminary cultured in a synthetic medium (described in the Materials and Methods) with a C/N ratio=58. The growth profiles reported in the figure 1a demonstrate that the growth kinetics are substantially similar. The lipid yield obtained with *L. starkeyi* after 120 h of growth (12.4%) was higher as compared to these pertaining to the other microorganisms. In addition, *L. starkeyi* has been proved to store large amounts of lipids, showing only a minimal reutilization of the stored lipids (Holdsworth et al., 1988b). For these reasons, *L. starkeyi* was selected for the subsequent tests.

Figure 1b describes the growth profile of *L. starkeyi* under multiple additions of fixed amounts (3 g each) of the nitrogen source $(NH_4)_2SO_4$ after a stationary phase was established. The experimental data show that, after each addition of $(NH_4)_2SO_4$, a new exponential phase starts, leading to an increase in the biomass concentration obtained under N-limiting conditions, although the increases in the biomass concentration are progressively reduced. The lipid yields obtained after the first and the second additions of $(NH_4)_2SO_4$ (see figure 1b) were 14.1 and 15.5, respectively, showing that operation under N-limiting conditions also allows an increase in the lipid fraction of the biomass.

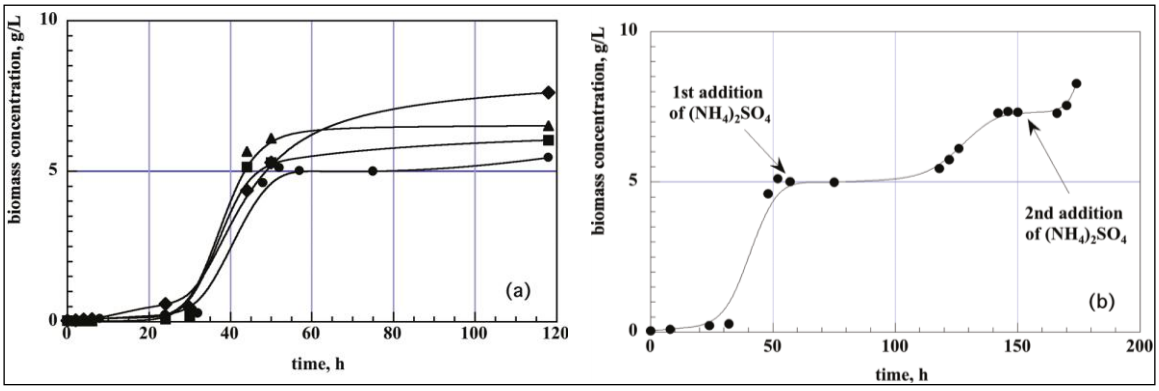

Figure 1. (a) Growth kinetics of four oleaginous yeasts using a N-limiting synthetic medium in batch cultures: *C. curvatus* (▲), *R. glutinis* (■), *R. toruloides* (♦), and *L. starkeyi* (●). Operating conditions: T = 30°C and 160 rpm. The medium composition is as in the Materials and Methods. **(b) Growth of *L. starkeyi* using a N-limiting synthetic medium in batch cultures, under multiple additions of the nitrogen source.** Operating conditions: T=30°C and 160 rpm. The medium composition is as in the Materials and Methods.

4.3.2 Fermentation in the presence of OMW

L. starkeyi is cultured in the presence of the OMW, without external organic supplement. Experimental tests are carried out in the presence of raw OMW (after a preliminary centrifugation), as well as in water mixtures containing 50 and 25% OMW, respectively. The biomass concentration is evaluated in terms of colony-

forming units (CFU), as OD measurements could not be carried out due to the darkness of the medium. Typical growth profiles are reported in figure 2a. The results show an initial increase of CFU limited to the first 3 days. The biomass growth is slightly slower when the yeasts are cultured in the presence of diluted OMW. Subsequently, *Lipomyces* cultured in 25% OMW are for about 170 h in a stationary phase, followed by a progressive reduction of CFU. *Lipomyces* cultured in the presence of 100 and 50% OMW survives at a constant biomass concentration for an even longer period. The OMW are a very complex medium (see table 1). Consequently, the biomass growth in OMW could be affected by different C sources (sugars, lipids, and phenols). Although it is likely that the microorganisms degrades preferentially the C sources that are more easy to metabolize (sugars), we first measure the degrading activity of the yeasts in terms of TOC levels, to evaluate subsequently the variation of the different classes of components. The TOC levels registered in the course of the same tests are reported in figure 2b. A complete removal of the organic carbon is carried out only in the presence of 25% OMW. However, in all tests carried out, the OMW reduction is mostly achieved in the first 3 days, that is, in the period of the CFU growth, showing the use of the organic carbon as a primary carbon source (Yesilada et al., 1998).

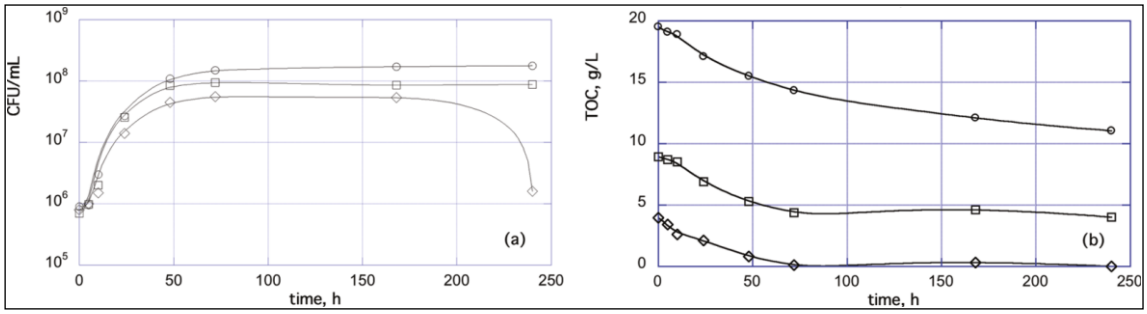

Figure 2. (a) Growth of *L. starkeyi* in batch cultures, in the presence of raw OMW (O), or in water mixtures containing 50% OMW (□) and 25% OMW (✧). Operating conditions: T=30°C and 160 rpm. The OMW composition is as in the Materials and Methods. **(b) TOC measurements during the culture of *L. starkeyi* in batch cultures, in the presence of raw OMW (O), or in water mixtures containing 50% OMW (□) and 25% OMW (✧).** Operating conditions: T= 30°C and 160 rpm. The OMW composition is as in the Materials and Methods.

4.3.3 Logistic model for the biomass growth

To model the biomass production and the TOC abatement, a numerical model is built based on the experimental data obtained under different experimental conditions. The biomass production rate is obtained by a biomass balance:

$$dX/dt = \mu \qquad (1)$$

The specific growth rate is defined adopting the logistic model:

$$\mu = \mu max\ (1- X/Xmax) \qquad (2)$$

The TOC profiles are described adopting the hypothesis of proportionality between TOC reduction rate and biomass growth rate:

$$d(TOC)/dt = \mu X\ (1/ Y_{X/TOC}) \qquad (3)$$

where $Y_{X/TOC}$ is the ratio of the amount of biomass produced to the amount of TOC consumed (g biomass/g TOC). Equations 1-3 were integrated using a fourth-order Runge-Kutta integration method. The least-squares method is used to obtain the parameter estimates. The model fitted the experimental data (see the results in table 2) with R^2 values higher than 0.95. Figure 3 shows a graphic comparison between the model predictions (dashed line) and the experimental results of a typical test. Standard errors, reported in figure 3, never exceed 14%.

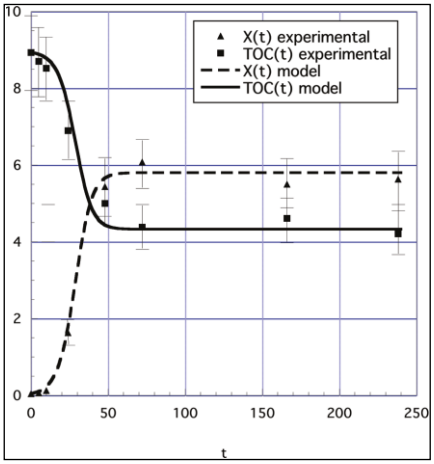

Figure 3. Comparison of experimental measurements of biomass concentration (X, g/L) and TOC (g/L) with the theoretical data obtained with the logistic model, with reference to the culture of L. *starkeyi* in batch cultures, in the presence of raw OMW. Operating conditions: T = 30°C and 160 rpm. The OMW composition is as in the Materials and Methods. Standard error bars are reported.

4.3.4 Growth parameters of *L. starkeyi* in the presence of OMW

A detailed comparison between the experimental and the theoretical data is given in table 2. As higher OMW concentration values are adopted (experiments E1, E2, and E3 in table 2), the specific growth rate (μmax) slightly reduces.

Experiment	OMW Fraction (%)	Dilution factor	pH control	Addition of glucose	T (°C)	g/L		1/h		g/L		g/g	
						X_o	$(TOC)_0$	μmax (exp)[a]	μ_{max} (pred)	X_{max} (exp)[a]	X_{max} (pred)	$Y_{X/TOC}$ (exp)[b]	$Y_{X/TOC}$ (pred)
E1	25	4	No con.	No add.	30	0.0512	3.95	0.181	0.190	3.20	2.81	1.32	1.30
E2	50	2	No con.	No add.	30	0.0488	8.93	0.171	0.175	5.90	5.81	1.29	1.25
E3	100	1	No con.	No add.	30	0.0576	19.05	0.128	0.132	10.4	10.0	1.20	1.28
E4	100	1	No con.	C_{glu}=70 g/L	30	0.0539	20.1	0.127	0.136	11.1	10.8	1.31	1.22
E5	100	1	pH 5.5	No add.	30	0.0510	20.1	0.130	0.135	11.0	10.7	1.24	1.30
E6	100	1	pH 6.5	No add.	30	0.0491	20.0	0.127	0.134	10.6	10.5	1.30	1.27
E7	100	1	No con.	No add.	25	0.0512	19.9	0.071	0.078	6.12	5.86	1.29	1.20
E8	100	1	No con.	No add.	25	0.0522	20.1	0.099	0.097	11.0	10.3	1.34	1.31

Table 2. Comparison of experimental values of the growth parameters with the theoretical data obtained with the logistic model, with reference to the culture of *L. starkeyi* in batch reactors, under different experimental conditions. [a]The experimental value of μ_{max} was calculated from the equation $\mu_{max} = \ln (X_2/X_1)/(t_2 - t_1)$, assuming a constant growth rate in the early exponential phase. [b]The experimental value of $Y_{X/TOC}$ was calculated from the slope of the curve of the biomass concentration (X) as a function of the TOC.

This result agrees with previously reported data, concerning the bacterial growth in the presence of OMW (Yesilada et al., 1998), and is due to the higher concentration of the phenolic components of OMW. In principle, another component of OMW that may inhibit microbial growth is the olive oil. However, it is ascertained (table 3) that the lipase activity of *L. starkeyi* in presence of OMW increases significantly. Consequently, the yeasts should be able to metabolize olive oil residuals. The maximum values of biomass concentration (Xmax), reported in table 2, increase with the initial OMW concentration, with the maximum value of Xmax obtained when using raw OMW (10.4 g/L). The biomass yield based on TOC consumption ($Y_{X/TOC}$) appears to be substantially constant, suggesting that changes in the OMW concentration do not cause a significant increase in the maintenance requirements.

Dilution factor	OMW fraction (%)	U/mL	
		Lipase activity before test	Lipase activity after test
1	100	145	1040
2	50	134	827
4	25	122	710

Table 3. Extracellular lipase activity before and after *L. starkeyi* culture in the presence of OMW.

Table 4 describes the reduction of sugars, proteins, and phenols concentration obtained during the *L. starkeyi* cultures at different concentrations of OMW (experiments E1, E2, and E3 in table 2). The experimental data demonstrate that efficiency of the yeasts in metabolizing the phenols is higher as the preliminary dilution of OMW increases, yielding phenol removal efficiencies of 43, 47, and 53% in the presence of OMW volumetric fractions of 100, 50, and 25%, respectively.

Dilution factor	%			
	OMW fraction	Removal of sugars content	Removal of proteins content	Removal of phenols content
1	100	48	82	43
2	50	54	87	47
4	25	86	98	53

Table 4. Sugars, proteins and phenols content removal (%) obtained during the *L. starkeyi* culture in the presence of undiluited and diluited OMW as compared with untreated OMW.

These results indicate the critical concentration of phenols in OMW, preventing the growth of *Lipomyces*, to be higher than 9 g/L. The higher concentrations of OMW also reduce the removal of sugars and proteins (table 4). Again, this detrimental effect is attributed to the higher initial concentration of phenols. The fraction of protein removed is in any case higher than 80%, reaching 98% in the presence of OMW volumetric fraction of 25%. The highest efficiency of sugars removal is obtained with the most diluted OMW sample. In any case, the sugars removal is never complete, demonstrating that the break in the biomass growth was not due to the exhaustion of sugars. This conclusion is confirmed by a further test: a glucose amount corresponding to a concentration of 70 g/L is added to raw OMW before the test beginning. In this case, the growth curves do not change significantly (experiment E4 in table 2). To check whether the growth of L*. starkeyi* in the presence of OMW is carried out under N-limiting conditions, supplementary additions (3 g) of the nitrogen source $(NH_4)_2SO_4$ are tried once a stationary phase is established during the culture in the presence of OMW (data not shown). In the latter tests, no further increases are observed in the biomass concentration, neither reductions in the TOC levels. The initial pH of OMW (both raw or diluted) used in the experimental tests is between 4.7 and 5.0. A slow pH increase is observed in the course of the OMW fermentations, although the pH change in a single test is never higher than 1.2 pH units. To ascertain the actual effect of the pH, specific tests (experiments E5 and E6 in Table 2) are carried out, making every day pH adjustments to constant pH values (pH 5.5 and pH 6.5). The results reported in table 2 show that the pH control does not affect appreciably the growth kinetics (i.e., μmax) and stoichiometry ($Y_{X/TOC}$ and Xmax). Experimental tests (experiments E7 and E8 in table 2) are carried out to evaluate the effect of the temperature. When the OMW fermentation is carried out at 35°C, both μmax and Xmax are lower as compared to the corresponding values observed at

30°C. The data obtained at a temperature of 25°C show a significantly lower value of μmax, although the Xmax value is close to that obtained at 30°C. In the course of OMW fermentation, the extracellular activity of lipases and esterases increases significantly (table 3), in agreement with the literature data (Goncalves et al., 2009; Eroglu et al., 2004). Consequently, a further potential benefit of the OMW treatment with *L. starkeyi* is related to the production of these enzymes. The lipase and esterase activities are higher as the OMW concentration increases, probably due to the induction produced by the TAGs (olive oil) contained in the OMW.

4.3.5 Phytotoxicity

The results on GI of *L. sativa* seeds of untreated and treated samples of raw OMW are reported in the table 5. When no sample dilutions are carried out before the GI test, both the untreated OMW and the *Lipomyces*-treated OMW were completely phytotoxic. Consequently, further GI tests are carried out upon dilution of samples. When testing diluted samples (1:10 v/v), the untreated OMW remain completely phytotoxic, whereas the *Lipomyces*-treated OMW show a significantly high GI (125.5). To achieve a significant reduction of the phytotoxic activity of the untreated OMW, a higher dilution ratio (1:25) is required.

Dilution ratio	GI	
	Untreated OMW	*Lypomyces*-treated OMW
1	0	0
10	0	125.5
25	105.0	121.0

Table 5. GI of *L. sativa* seeds on untreated and treated samples of raw OMW.

The hypothesis that the GI of the OMW is actually increased in the course of the treatment is confirmed by a one-sided t test (Himmelblau, 1970), with a significance level of α = 0.01%. As widely reported in the literature (Iamarino et al., 2009; Jaouani et al., 2003), the phenols are considered the compounds mainly responsible for the OMW phytotoxicity. Consequently, the increase of GI likely originated from the reduction of phenols (see **table 5**).

4.3.6 Lipid yield and composition

The amounts of lipids extracted from *L. starkeyi* cultured in OMW are reported in table 6 in terms of lipid yield. The experimental data show that a 50% dilution of OMW results in a significant increase in the concentration of lipids (28.6 against 22.4%), although a further increase in wastewater dilution produces only a minimum improvement.

Sample	Lipid yield (%)
L. Starkey grown in synthetic medium	12.1
L. Starkey grown in undiluted OMW	22.4
L. Starkey grown in 50% OMW	28.6
L. Starkey grown in 25% OMW	29.5

Table 6. Lipid concentration in *L. starkeyi* after 10 days of culture in OMW.

The fatty acids distribution in the lipids accumulated in the *L. starkeyi* grown in OMW is described in the table 7. The composition indicates a clear prevalence of

oleic acid, in agreement with results obtained using different oleaginous microorganisms (Li et al., 2007; Papanikolaou et al, 2008; Papanikolaou and Aggelis, 2007). The total content of saturated fatty acids is low enough to allow an excellent cold behaviour of biodiesel, reducing its tendency of crystallization or gelling at low temperatures.

Sample	%
Myristic acid C 14:0	<1
Palmitic acid C 16:0	19.1
Palmitoleic acid C 16:1	0.5
Stearic acid C 18:0	8.5
Oleic acid C 18:2	49.1
Linoleic acid C 18:2	18.8
Linolenic acid C 18:3	3.5
Arachidonic acid C 20:0	0.3
Free fatty acids	14.5

Table 7. Distribution of fatty acids in the lipids accumulated in the *L. starkeyi* grown on OMW.

4.4 CONCLUSIONS

Negative environmental consequences of fossil fuels and concerns about petroleum supplies have spurred the search for renewable transportation biofuels. Among current shortage of petrodiesel oil and highest production cost of biodiesel using plant oil or animal fat, the major challenge mankind confronts in this century is developing oleaginous microorganisms to obtain large amount of standard biodiesel for industry. At present, however, neither bioethanol nor biodiesel is competitive with conventional fuels in the whole world. Only by improving the quality and reducing the cost of biodiesel, it can compete with conventional fuels. Producing low cost microbial diesel primarily requires the development of high lipid content microorganisms that would be becoming a potential and promising way in the future.

In particular, the accumulation of lipids by *L. starkeyi* may occur in the presence of OMW with no preliminary treatments and without external organic supplement. The growth of the yeasts is associated with a significant reduction of both the TOC and the total phenols content. The increase of the GI of the OMW after the biological treatment demonstrates the environmental benefits that can be achieved by this process. The use of diluted wastewaters increases the fraction of the organic compounds (TOC, total phenols, and proteins) removed, although the kinetics of the biomass growth is slower. The lipid concentration (between 20 and 30%) in the microorganisms increases in the course of the OMW treatment, particularly in the presence of the preliminary diluted feedstock. The fatty acids distribution demonstrates the usability of the lipids accumulated in the *L. starkeyi* as feedstock for biodiesel synthesis, so the use of this waste product as a free grow media could be a convenient strategy for biodiesel production and at the same time could avoid the negative environmental impact associated with its discharge.

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5. IMMOBILIZATION AND STABILIZATION of *Myceliophthora thermophila* and *Trametes versicolor* LACCASES

5.1 INTRODUCTION

Several types of industrial and agricultural wastes contain phenol compounds. For example, chlorophenols, largely used as wide-spectrum biocides, and nitrophenols, widely used in the chemical industry, accumulate in soils, sediments, surface waters, and animals because of their continuous usage and recalcitrant nature (Gianfreda et al., 2002a). Moreover, a lot of phenol compounds are recognized as endocrine disruptors since they possess estrogenic or antiestrogenic activities so interfering with the endocrine system. These recalcitrant compounds produced in industries as paper, oil or different ink industries are in general not able to be degraded by the active lodes of the treatment factories. Because of that the general trend is treating these compounds with different enzymes in order to transform into a degradable compounds or into other not toxic ones (Georgieva et al., 2008). The use of enzymes in these processes has a lot of advantages because of their high activity in environmental conditions such as room temperature, neutral pH or atmospheric pressure, their high selectivity and specificity. One of the enzymes are being used extensively is laccase.

Laccases are produced from different sources (Duran et al., 2002). This enzyme is able to oxidate different aromatic compounds such as phenol or different dye compounds into other compounds easily to be degraded by the active lodes. (Duran, 2002). Laccases (EC 1.10.3.2, *p*-diphenol: dioxygen oxidoreductase) belong to the so-called blue-copper family of oxidases. Laccases catalyze the oxidation of a variety of organic compounds including methoxyphenols, phenol, *o*- and *p*-diphenols, aminophenols, polyphenols, polyamines, and lignin-related molecules. Laccase can catalyze the oxidation of *o*-, *m*-, and *p*-benzenediols and phenol to *o*-, *m*-, *p*-quinones or radical species (Freire, 2001) and does not require hydrogen peroxide as co-substrate or any cofactors for the catalytic reaction. Other important biotechnological applications of laccases include: lignin modification (Camarero et al., 2002), paper strengthening (Liu et al., 2009; Chandra et al., 2004), juice manufacture (Berka et al., 1998) and hair colouring (Aaslyng et al., 1999). Figure 1 illustrates the catalytic cycle of laccase and the proposed mechanisms for the reduction and reoxidation of the copper sites. (Duran et al., 2002).

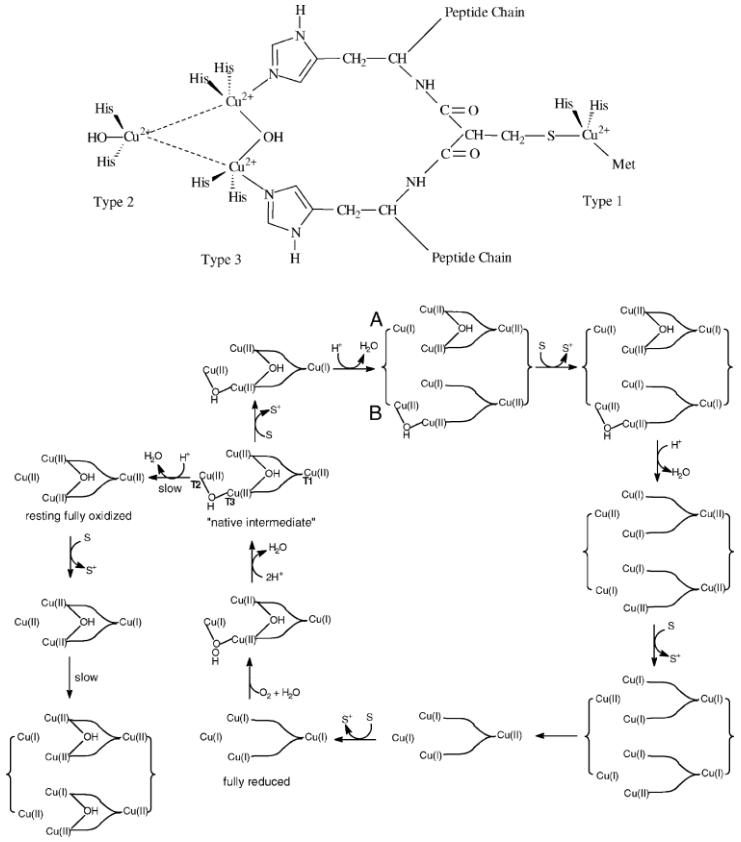


Figure 1. Catalytic cycle of laccase (modified from Solomon et al., 1996)

Starting from the “native intermediate,” the substrate reduces the Type 1 site, which in turn transfers the electron to the trinuclear cluster. Two possible mechanisms for the reduction of the trinuclear cluster are shown: (A) the Type 1 and Type 2 sites together reduce the Type 3 pair and (B) each copper in the trinuclear cluster is sequentially reduced by electron transfer from Type 1 site, in which case the Type 3 no longer acts as a two-electron acceptor. Slow (left) decay of the “native intermediate” leads to the resting fully oxidized form. In this form, the Type 1 site can still be reduced by substrate, but electron transfer to the trinuclear site is too slow to be catalytically relevant (Solomon et al., 1996).

However, the use of enzymes is not always easy on an industrial scale because these have a number of limitations due to their biological origin. So enzymes are soluble and they cannot easily be used for many reaction cycles, are quite unstable under conditions of high temperatures, extreme pHs or use of organic co-solvents or toxic products, are inhibited by different reagents and products and catalyze reactions under physiological conditions; because of that enzymes have to be stabilized to convert the process in a profitable one.

Within the present thesis is proposed the use of immobilized enzymes to have them as heterogeneous catalyst so that they can be used for many reaction cycles or continuous reactors. Although in the literature different methods to immobilize enzymes are described, most of these methods do not improve or even worse sometimes the properties of the soluble enzymes. A key parameter in these processes of immobilization is to obtain more stable catalysts that the soluble enzyme so that it can thereby increase their productivity improving the profits of the process. (Forde et al., 2010).

5.1.1 *Myceliophthora thermophila* and *Trametes versicolor* laccases

Many laccases from different sources could be considered for various industrial and biotechnological applications, but laccases from higher plants and bacterial strains are less capable to degrade polymers of phenolic origin, including lignin, than laccases from fungi. In particular, it is well documented that thermophilic fungi may comprise a rich source of thermostable industrial enzymes. Furthermore, thermal tolerance is an attractive feature for many biotechnological applications of enzymes.

The thermophilic fungus *Myceliophthora thermophila* was described previously as a producer of cellulase and xylanase enzymes with pronounced thermal resistance (Berka et al., 1998) and in the present theis we use a fungal laccase (Novozym 51003) from this ascomycete. Moreover, laccase from basidiomycete fungus *Trametes versicolor* was reported to have the highest redox potential among laccases; i.e., 785 mV versus the standard hydrogen electrode (Nakamura, 1958; Yaropolov et al., 1994), which makes this laccase particularly interesting since high redox potentials correlate with high laccase activity (Li et al., 1999; Xu, 1996). In addition, the ability of this laccase to exert its catalytic activity on many types of aromatic compounds has been demonstrated (Cantarella et al., 2003; Johannes and Majcherczyk, 2000; Kang et al., 2002; Keum and Li, 2004; Kulys et al., 2003; Roy-Arcand and Archibald, 1991; Tanaka et al., 2001; Tsutsumi et al., 2001; Ullah et al., 2000). However, the stability of this laccase and its ability to catalyze the transformation of substrates under various reaction conditions has not been well characterized.

5.1.1.1 Modelling of *Myceliophthora thermophila* and *Trametes versicolor* structure

To design a rational immobilization protocol is important to know the 3D structure, the catalytic and the inactivation mechanism of the target enzyme. Like *Trametes versicolor*, *Myceliophthora thermophila* laccase is a single polypeptide with three internal disulfide bonds and four copper atoms. Both the nucleotide sequence of the gene and the amino acid sequence have been determinate. This last sequence (Svendsen, 2009, patent US 7622287) contain 573 amino acids and was overlapped, using the EXPASY server (*modelling* application and *alignment* modality) to all sequences of proteins that have been crystallized. The selected sequence is belonging to *Melanocarpus albomyces* laccase (PDB code: 1-GWO). The alignment of these two sequences (method compositional matrix adjust) showed an amino acid identity percentage of 74% (415aa/559aa), an omology of 84% (474aa/559aa) and a gaps of 0% (0aa/559 aa), (figure 2).

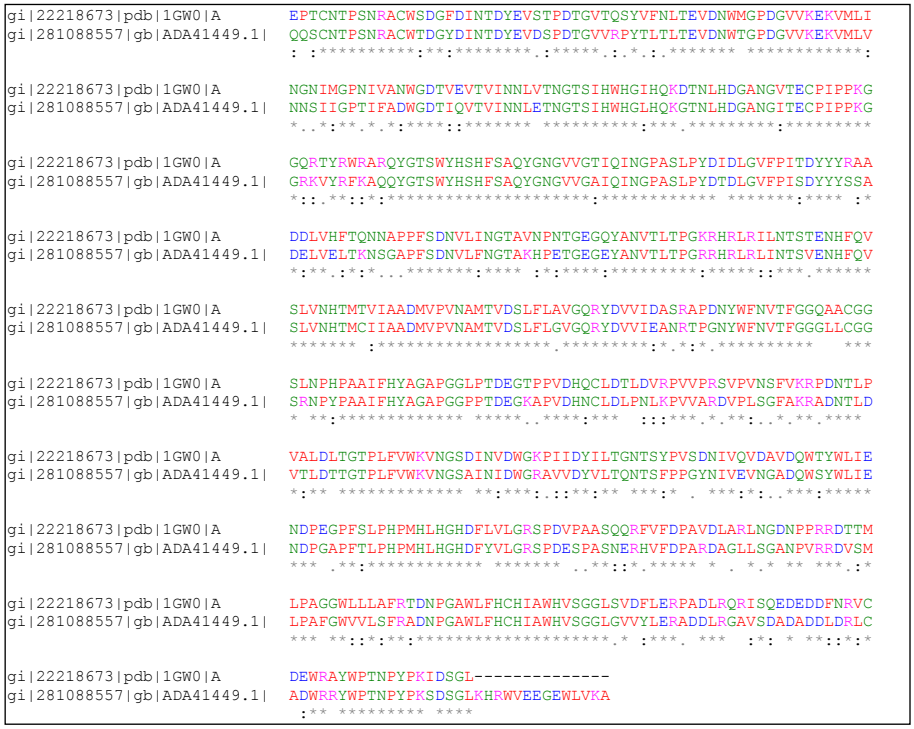


Figure 2. Structure-based sequence alignment. ★identity ● homology ▬ difference

All images of the resulting model were produced with the molecular visualization system: Pymol 099. *Myceliophthora thermophila* laccase has one mononuclear copper site containing one type-1 Cu (blue Cu), and a trinuclear copper site containing one type-2 Cu (normal Cu) and two type-3 Cu (coupled binuclear Cu). Substrates are oxidized near the mononuclear site, and the electrons are transferred to the trinuclear site, where the molecular oxygen is reduced. Neither the electron transfer mechanism nor the oxygen reduction to water is fully understood (Hakulinen et al., 2002). The model elaboration show that, like *M. albomyces* laccase (MaL), *Myceliophthora thermophila* laccase is a monomer consisting of three cupredoxin-like domains, see figure 3.

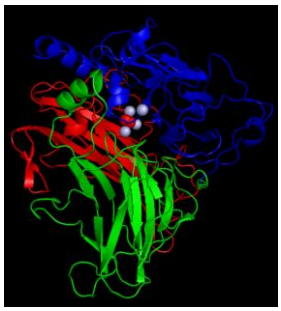


Figure 3. Three dimensional structure of *Myceliophthora thermophila* laccase. Domain red includes residues that participate in the binding the coppers at the trinuclear site. Domain green contains residues that take part in the substrate binding. Domain blue contains residues that participate in the binding of coppers at the mononuclear and trinuclear site, as well as in substrate binding.

According to SDS-PAGE, the enzyme estimated mass is ≈83 KDa and its isoelectric point is 4.2 (Claus et al., 2002). The structure is stabilized by three disulfide bridges, the first located in domain red, the second between domains red and blue and the third located in domain green, near the substrate-binding site. The figure 4 show the three-dimensional model of the mononuclear and trinuclear cluster, the oxygen molecule is located in the middle of the two type-3 coppers.

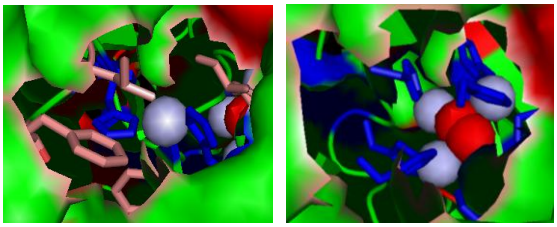


Figure 4. Copper sites. (A): mononuclear and **(B)** trinuclear site: Oxygen atoms are represented by red balls, copper atoms are represented by grey balls.

The model study shows that the secondary structure is rich in β-strands and poor in helix content, see figure 5,

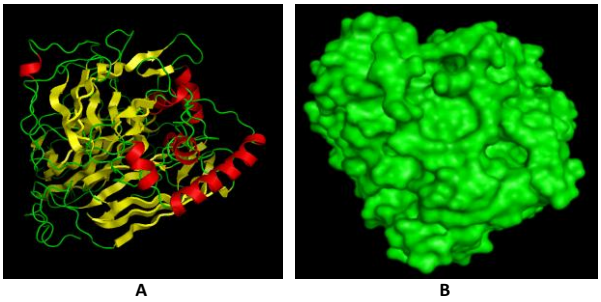


Figure 5. (A): Secondary structure, the β-strands are represented in yellow, the helixes in red and the loops in green. (**B):** Molecular surface.

and that a narrow tunnel, leading to the type-2 copper (Cu 4) may provide access for the solvent molecules, but the C-terminal residues pack against the tunnel (figure 6). The function of the tunnel is uncertain; it could form an access route for the oxygen molecule to enter the trinuclear copper site. The entrance of an oxygen molecule and exit of a water molecule through this tunnel would require a conformational change in the C-terminus, to open the tunnel and this suggests that C-terminal amino acids might have a role in the function of all fungal laccases.

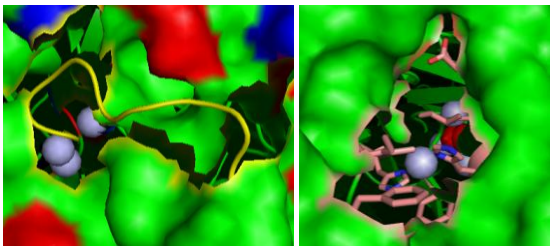


Figure 6. Molecular surface with tunnel: the loop is represented in yellow and the copper atoms are represented by grey balls.

Recently, the structure of laccase from *Trametes versicolor* was determined in its glycosylated, fully functional form at 1.9 Å resolution. *T. versicolor* laccase is a globular protein of about 500 amino acids and contains three cupredoxin-like β-sandwich domains, similar to those found in ascorbate oxidase and in ceruloplasmin.

Like *Myceliophthora thermophila* laccase, the structure of *Trametes versicolor* is a monomer, organized in three sequentially arranged domains, and has dimensions of about 65 X 55 X 45 Å, with a molecular mass of about 70 KDa. Each of the three domains is of similar β-barrel type architecture, related to the small blue copper proteins such as azurin or plastocyanin. The tri-nuclear copper cluster (T2/T3) is embedded between domains 1 and 3 with both domains providing residues for the coordination of the coppers. Finally, at the C-terminal end of domain 3, three

sequentially arranged α-helices complete the fold. A 13-aminoacid-long α-helix at the C-terminal portion is stabilized by a disulfide bridge to domain 1 (Cys-85–Cys-488), and a second disulfide bridge (Cys-117–Cys-205) connects domains 1 and 2. Both N-terminal and C-terminal amino acids benefit from hydrogen bonding networks to the rest of the protein, providing sufficient rigidity.

The electrostatic surface potential distribution of *Trametes versicolor* laccase reveals a dominance of negative charges, which is in accordante with the acidic isoelectic point of about 3.5. From the crystal structure of an enzyme/substrate complex, we know that the substrate binds in a small negatively charged cavity near the copper T1 site. The negative charges located at this site may have functional significance since they could stabilize the radical cation products that are formed during the catalytic cycle. The oxygen-reducing site at the T2/T3 cluster has access to solvent through two channels, which lead to the type-3 copper sites and to the type-2 copper site, respectively. The latter site is more exposed and more labile as compared with the other two at the T3 site. It appears that the solvent channels of the blue copper oxidases are well suited to allow fast access of dioxygen molecules to the trinuclear cluster and subsequently easy release of water.

5.1.2 Immobilization of laccases

There are many methods that allow the immobilization of enzymes for their industrial and biotechnological use; they generally can be divided into reversible and irreversible ones.

5.1.2.1 Reversible methods for enzyme immobilization

The principal characteristic of these derivatives is the possibility of detach the enzyme from the support under gentle conditions. The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays, the support can be regenerated and re-loaded with fresh enzyme (figure 7). The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems (Gupta et al., 1992).

Adsorption

The simplest immobilization method is the adsorption. Among these methods there are different kinds of adsorptions:

1- Non specific adsorption: it is the simplest immobilization method and is mainly based on physical adsorption or ionic binding (Messing, 1976; Woodward, 1985). In physical adsorption the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. The nature of the forces involved in noncovalent immobilization results in a process that can be reversed by changing the conditions that influence the strength of the interaction. Immobilization by adsorption is a mild, easy to perform method and usually preserves the catalytic activity of the enzyme. However these methods are economically attractive, but may suffer from problems such as enzyme leakage from the support and the interactions are relatively weak.

2- Ionic binding: an obvious approach to the reversible immobilization of enzymes is to base the protein-ligand interactions on principles used in chromatography. For example, one of the first applications of chromatographic principles in the reversible immobilization of enzymes was the use of ion-exchangers

(Tosa et al., 1967; Sharp et al., 1969). The method is simple and reversible but, in general, it is difficult to find conditions under which the enzyme remains both strongly bound and fully active. More recently, the use of immobilized polymeric-ionic ligands has allowed for modulation of protein-matrix interactions and has thus optimized the properties of the derivative. However, problems may arise from the use of a highly charged support when the substrates or products themselves are charged; the kinetics are distorted as a result of partition or diffusion phenomena. Therefore, enzyme properties, such as pH optimum or pH stability, may change (Goldstein, 1972; Goldman et al., 1968). Although this could pose a problem it could also be useful to shift the optimal conditions of a certain enzyme towards more alkaline or acidic conditions, depending on the application (Guisan et al., 1994).

3- Hydrophobic adsorption: another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. Hydrophobic adsorption has been used as a chromatographic principle for more than three decades. It relies on well-known experimental variables such as pH, salt concentration, and temperature (Porath, 1987). The strength of interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule.

4- Affinity binding: the principle of affinity between complementary biomolecules has been applied to enzyme immobilization. The remarkable selectivity of the interaction is a major benefit of the method. However, the procedure often requires the covalent binding of a costly affinity ligand (e.s., antibody, or lectin) to the matrix (Solomon et al., 1987).

Chelation of metal binding

Transition metal salts or hydroxides deposited on the surface of organic carriers become bound by coordination with nucleophilic groups on the matrix. Mainly titanium and zirconium salts have been used and the method is known as “metal link immobilization” (Cabral and Kennedy, 1991; Cabral et al., 1986; Kennedy and Cabral, 1985). The metal salt or hydroxide is precipitated onto the support (e.g., cellulose, chitin, alginic acid, and silica-based carriers) by heating or neutralization.

Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal; therefore some of the positions remain free to coordinate with groups from the enzymes. The method is quite simple and the immobilized specific activities obtained with enzymes in this way have been relatively high (30-80%) However, the operational stabilities achieved are highly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably related to the existence of non-uniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be immobilized on the solid supports by means of stable covalent bonds. The metal ions are then bound by coordination and the stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a strong chelator such as ethylene diamine tetraacetic acid (EDTA) when desired. These metal chelated supports were named Immobilized Metal-Ion Affinity (IMA) adsorbents and have been used extensively in protein chromatography (Porath, 1992; Kagedal, 1998). The

approach of using different IMA-gels as supports for enzyme immobilization has been studied using Eschericia coli β-galactosidase as a model (Brena et al., 1994).

Formation of disulfide bonds

These methods are unique because, even though a stable covalent bond is formed between matrix and enzyme, it can be broken by reaction with a suitable agent such as dithiothreitol (DTT) under mild conditions. Additionally, because the reactivity of the thiol groups can be modulated via pH alteration, the activity yield of the methods involving disulfide bond formation is usually high provided that an appropriate thiol-reactive adsorbent with high specificity is used (Carlsson et al., 1998).

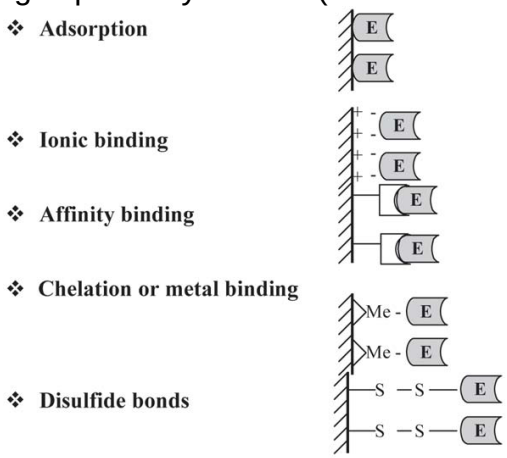


Fig. 7. Approaches to enzyme immobilization, reversible methods.

5.1.2.2 Irreversible methods for enzyme immobilization

The concept of irreversible immobilization means that, once the biocatalyst is attached to the support, it cannot be detached without destroying either the biological activity of the enzyme or the support. The most common procedures of irreversible enzyme immobilization are covalent coupling, entrapment or micro-encapsulation, and cross-linking (*see* figure 8).

Formation of Covalent Bonds

Immobilization of proteins by methods based on the formation of covalent bonds is among the most widely used. An advantage of these methods is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. However, in order to achieve high levels of bound activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage to the support; this may prove a difficult requirement to fulfill in some cases. A simple procedure that sometimes improves the activity yield is to carry out the coupling reaction in the presence of substrate analogs (Mattiasson and Kaul, 1991). Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

A wide variety of reactions have been developed depending on the functional groups available on the matrix (Scouten, 1987). Coupling methods in general can be divided in two main classes: (1) activation of the matrix by addition of a reactive function to a polymer and (2) modification of the polymer backbone to produce an activated group (table 1). The activation processes are generally designed to generate electrophilic groups on the support which, in the coupling step, react with the strong nucleophiles on the proteins. The basic principles controlling the course of covalent coupling to the matrices are analogous to those used for the chemical modification of proteins. The most frequently used reactions involve the following

side chains of the amino acids: lysine (ε-amino group), cysteine (thiol group), and aspartic and glutamic acids (carboxylic group).

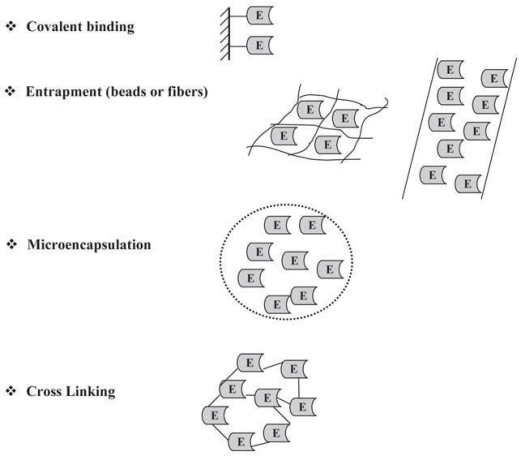


Fig. 8. Approaches to enzyme immobilization, irreversible methods.

Activation method	Group that reacts
Tresyl chloride	Thiol, amines
Cyanogen bromide	Amine
Bisoxiranes (epoxides)	Thiol, amine
Epichlorohydrin	Thiol, amine
Glutaraaldehyde	Amine
N-Hydroxy-succinimidyl	Amine

Table 1. Covalent Coupling Methods of Enzymes: activation of matrix.

There are many commercially available supports for immobilization; the best choice in each case requires the consideration of some relevant properties of the catalyst and the intended use. However, it is usually necessary to try more than one approach and then adapt a method to the specific circumstances (White and Kennedy, 1980; Taylor, 1991). The covalent reactions commonly employed give rise to enzymes linked to the support through either amide, ether, thio-ether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and, in many cases, it is also stabilized, which will be discussed later in results and discussion. However, because of the covalent nature of the bond, the matrix has to be discarded together with the enzyme once the enzymatic activity decays. The benefit of obtaining a leak-proof binding between enzyme and matrix resulting from these reactions is partially offset by the cost, in terms of generally low yield of immobilized activity and by the nonreversible character of this binding.

Entrapment

The entrapment method is based on the occlusion of an enzyme within a polymeric network that allows the substrate and products to pass through but retains the enzyme (O'Driscoll, 1976). This method differs from the coupling methods described above, in that the enzyme is not bound to the matrix or membrane. There are different approaches to entrapping enzymes such as gel (Bernfeld and Wan, 1963) or fiber entrapping (Dinelli et al., 1976) and micro-encapsulation (Wadiack and Carbonell, 1975). The practical use of these methods is limited by mass transfer limitations through membranes or gels.

Although as described above, there are numerous methods of immobilization, in this thesis are used some new immobilization methods developed in the enzyme engineering laboratory in the “Instituto de Catalisis” in Madrid. The use of some of these immobilization methods allows the immobilization involving different surface regions and through different amount of reactive groups. The stabilization of enzymes using immobilization methods is based in the fact that multipoint covalent attachment of enzymes on highly activated pre-existing supports, via short spacer arms and through a number of residues on the enzyme surface, can promote dramatic stabilization of the three-dimensional structure of the immobilized enzyme.

In this case, the relative distances among the residues involved in multipoint immobilization ought to be maintained unaltered during any conformational change induced by a distorting agent such as heat, organic cosolvents or extreme pH (Klibanov, 1979; Gianfreda et al., 2002b). The intensity of conformational changes involved in enzyme inactivation may be strongly reduced and the immobilized enzyme may become strongly stabilized. In fact, a number of enzymes have been dramatically stabilized by multipoint covalent immobilization as compared with one-point immobilized counterparts where the stability is in general similar to the soluble enzyme (Bolivar et al., 2006a; Bolivar et al., 2006b) (figure 9).

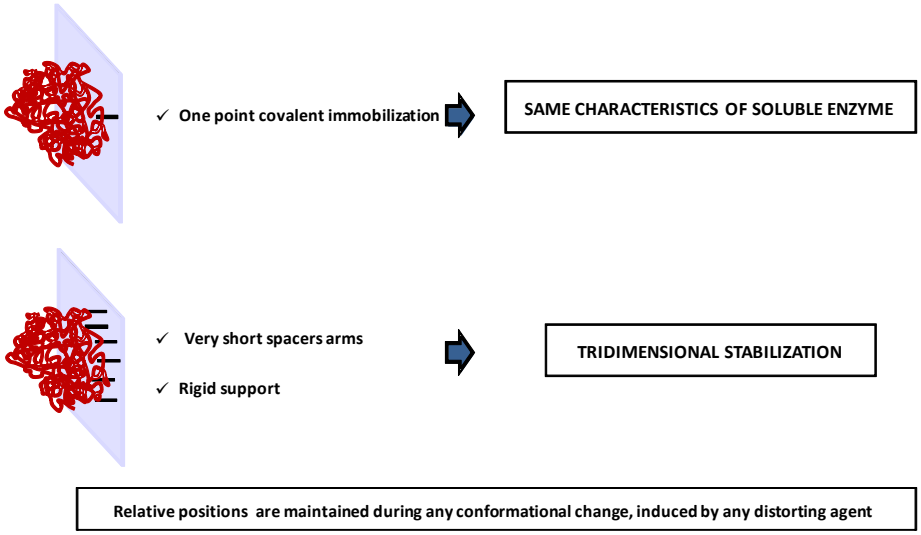


Fig. 9. One and multipoint covalent immobilization.

5.1.2.3 Immobilization on anionic exchanger supports

This is probably the oldest and simplest immobilization method. The immobilization of proteins via their carboxylic groups may be an excellent method for preparing industrial derivatives (e.g., industrial enzymes, immobilized antibodies, and so on). In fact, the sum of Asp and Glu residues usually constitutes the major fraction of surface groups in proteins. In this case, the support used is a new amine-agarose gel containing primary amino groups with a very low pK value and secondary amino groups with a relatively high pK. These groups exhibit a monoaminoethyl-N-aminoethyl structures and the support is called (MANAE-agarose). This gel was prepared by reaction of aldehyde-agarose gel (glyoxyl agarose) with ethylenediamine and further reduction with sodium borohydride (Fernandez-Lafuente et al., 1993) (figure 10). The use of low-ionic strength media allows the enzyme to be ionically adsorbed on the support (mainly through carboxylic-amine ionic interactions) and the process is very fast.

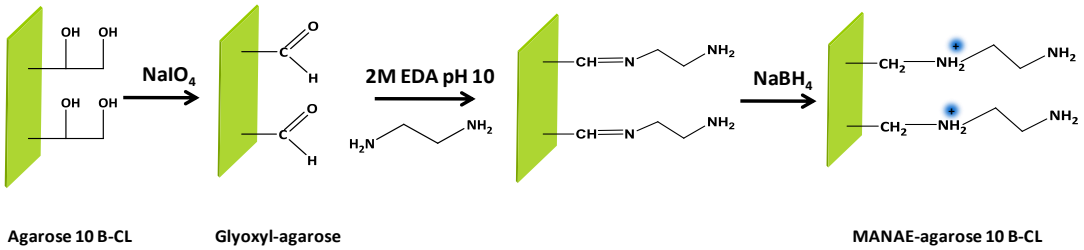


Figure 10. Schematic representation of (Monoamino-N-aminoethyl)-(MANAE-agarose) support structure. The support has two ionizable groups with pK values of 6.8 and 10.0, related to the primary and secondary amine groups respectively.

However the use of these supports allows immobilizations that in general are quickly and mild, this method has an advantage that is the fact that desorption of the enzyme can be promoted. This desorption to the reaction medium is a problem because the loss of the productivity of the immobilized catalyst and in addition the reaction product become contaminated by the enzyme. Furthermore, it is reported that most of the times no significant stabilizations are produced after immobilization on these supports. Because of that in the present thesis other covalent techniques are going to be used.

5.1.2.4 Immobilization on glutaraldehyde supports

Immobilization on aminated supports previously activated with glutaraldehyde is the most popular techniques to immobilize enzymes (Burteau et al., 1989; Van Aken et al., 2000; Dos Reis et al., 2003; Magnan et al., 2004; Seyhan et al., 2004). The methodology is quite simple and efficient and, in some instances, it even allows to improve enzyme stability by multipoint or multisubunit immobilization (Lamas et al., 2001; Barros et al., 2003). Moreover, glutaraldehyde has also been very used to introduce intermolecular crosslinking in proteins (Fernandez-Lafuente et al., 1995b; Visuri et al., 1999; Schoevaart et al., 2004) or to modify adsorbed proteins on aminated supports (Hwang et al., 2004).

The exact structure of glutaraldehyde on the support is still under discussion (Migneault et al., 2004), but given the high stability of the amino-glutaraldehyde bond, the formation of some kind of cycle seems to be a likely possibility. It has been shown by Monsan (1978) that it is relatively simple to activate aminated supports with glutaraldehyde, having between one or two molecules of glutaraldehyde per primary amino groups. His results pointed out that the use of more drastic conditions promoted a remarkable increase in the amount of glutaraldehyde introduced in the support, suggesting an uncontrolled glutaraldehyde polymerization. However, by controlling the activation conditions, it may be possible to activate all amino groups in a support with only one glutaraldehyde molecule per amino group or with two glutaraldehyde molecules, instead of using random activations that could produce a mixture of monomers and dimers. A second aspect to be considered is that glutaraldehyde is used to activate aminated supports. This means that below each glutaraldehyde molecule, there could be one or two amino groups (e.g. in the case of epoxy or aldehyde supports activated with ethylenediamine) (Fernandez-Lafuente et al., 1993) that can confer some ionic exchanger features to the support.

Hence, the supports could be considered as heterofunctional matrices and a physical adsorption could be possible before the covalent reaction. In our case (figure 11) the support is activated with two glutaraldehyde molecules and it could ionically adsorb the enzyme through the amino groups present under the glutaraldehyde molecules. The monomer and the dimmer of glutaraldehyde have very different reactivity, while the dimmer is able to rapidly immobilize proteins via a

direct covalent attachment; the monomer glutaraldehyde yields a very low immobilization rate. However, both kinds of groups are able to rapidly react with the nucleophiles of the protein surface after the protein ionic exchanger adsorption.

Secondly, the existence of one or two ionic groups (amino groups) under the glutaraldehyde must be considered, since it provides a certain anionic exchanger nature to these supports. This fact explains the importance of controlling the ionic strength using this support. Thus, immobilization at low ionic strength seems to proceed via a first ionic interchange of the protein on the amino groups of the support, followed by a very rapid "intramolecular" reaction between nucleophiles of the protein and very near glutaraldehyde groups in the support. If high ionic strength is used, immobilization will proceed slower, but the enzyme will be immobilized directly via a covalent attachment. As usually a neutral pH value is used to prevent support inactivation, this reaction should involve the most reactive amino groups in the protein. This means that, just by altering the ionic strength during the immobilization, we cannot only modify the immobilization rate, but also the region of the protein that is implied in the interaction with the support. Thus at low ionic strength the protein would be immobilized through the most negatively charged area of the surface. In contrast, at high ionic strength immobilization would occur through the region with the most reactive amino/s group/s. Thus, the activity/stability properties of enzymes immobilized on glutaraldehyde activated supports depend on the exact immobilization protocol employed. For example, the different groups may yield some differences in the stability, the shorter spacer arm (monomer) could give a higher rigidity, and conversely the longer spacer arm (dimmer) may allow the reaction with more groups and finally yield the best results (Betancor et al., 2006).

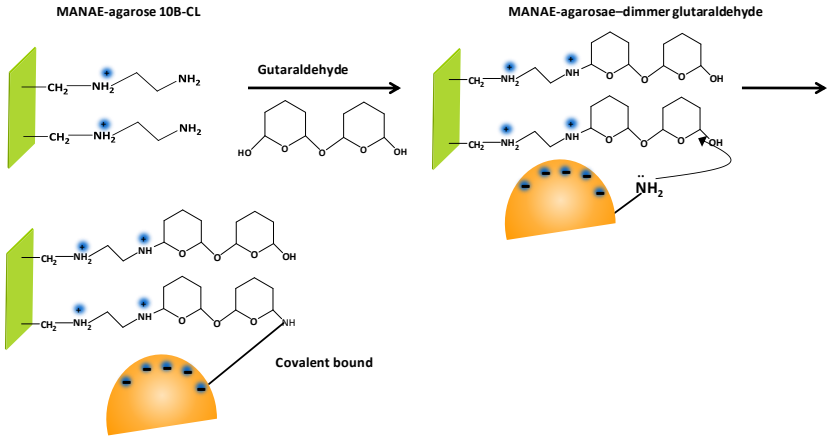


Figure 11. Schematic representation of the laccase immobilization on Monoamino-N-aminoethyl-(MANAE)-agarose dimmer glutaraldehyde support. The immobilization was conducted at pH 7.0 (phosphate buffer 5mM).

5.1.2.5 Immobilization on glyoxyl supports

As it was commented above when it is treated to greatly improve the stability of the enzymes, multipoint covalent attachment have to be promoted in order to rigidify the 3D structure of the enzyme. Probably, the best way to achieve this kind of immobilization consists in immobilizing through the amine groups of the protein. These groups are abundant on the protein surface and are quite reactive at moderate alkaline pHs because the lysines have a pK around 10.5 depending of the microenvironment.

Glyoxyl-supports have been described as a very adequate immobilization system to yield immobilized-stabilized proteins via multipoint covalent attachment. Many enzymes have been stabilized using this technique, e.g., penicillin G acylase from *E.*

coli (Alvaro et al., 1990) and *K. citrophila* (Guisàn et al., 1993), trypsin (Blanco and Guisàn, 1988), chymotrypsin (Guisàn et al., 1991), alcalase (Tardioli et al., 2003b), carboxypeptidase A (Tardioli et al., 2003a; Pedroche et al., 2002), FNR NADP-reductase, esterase (Bes et al., 1995; Fernandez-Lafuente et al., 1995a), thermolysin (Guisàn et al., 1997), DAAO (Betancor et al., 2003) catalases (Hidalgo et al., 2003) and lipases from different sources (Otero et al., 1991; Palomo et al., 2002). In fact, other research groups have also proven the feasibility of this immobilization technique to stabilize urokinase (Suh et al., 2003), l-aminoacylase (Toogood et al., 2002), chitosinase (Ickawa et al, 2002; Kuroiwa et al., 2002; Kuroiwa et al., 2003), enteroquinase (Suh et al, 2005). These good results should be the consequence of some peculiarity in the immobilization of proteins in this support when compared to other supports. Glyoxyl groups are linear aldehydes that form Schiff's bases with amino groups (Blanco and Guisàn, 1988). These supports are only capable to immobilize proteins if simultaneously are able to form several Schiff´s bases among several lisines and glyoxyl groups of the support. This special mechanism makes that the immobilization is performed through the richest region in lysine moieties of the protein surface.

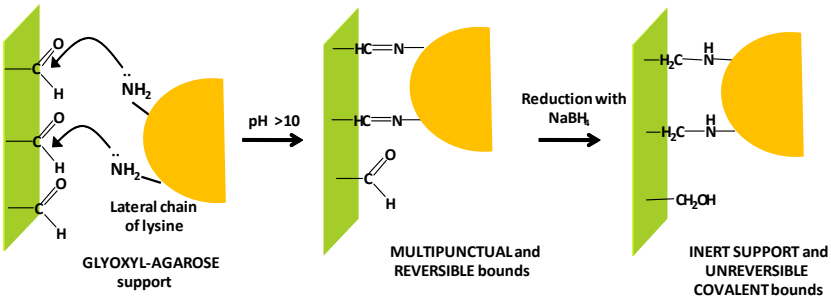


Figure 12. Schematic representation of the laccase immobilization on glyoxyl agarose 10 B-CL support. The immobilization was conducted at pH 10.05 (bicarbonate buffer 100mM), in these conditions the ε-NH_2 of lysine and NH_2-terminal can react with the aldehyde groups of the support; the last reduction step follow a more stable imino covalent bonds and reduce the remaining support aldehyde groups into inert hydroxyl groups.

5.1.2.6 Immobilization on heterofunctional glyoxyl supports

Although the immobilization on glyoxyl supports can be in most cases the most stabilizing because is directed by the richest region in lysines, and it is capable to maximize the amount of covalent linkages, in some cases there are other regions on the enzyme surface that are more sensitive to denaturing agents. For example, these regions could encompass unstable loops, domains close to the active center and hydrophobic pockets. Thus, their rigidification could result in a more stable attachment even though the number of covalent links is lower that obtained through linkage with the lysine-rich regions. In addition to stabilization, rigidification of the enzyme through these key regions could also promote a higher recovery of activity after immobilization.

The new supports contain a high concentration of non-reactive groups that are able to adsorb proteins at neutral pH as well as a high concentration of glyoxyl groups that are unable to covalently immobilize the enzyme at neutral pH. By using these supports, a two-step immobilization protocol is performed. In the first step, enzymes are adsorbed at neutral pH through surface regions that are complementary to the adsorbing groups on the supports, and in the second step, the adsorbed derivatives are incubated under alkaline conditions to promote an intramolecular multipoint covalent attachment between the glyoxyl groups of the support and the primary amino groups close to the adsorbed region of the enzyme (figure 13).

Novel heterofunctional glyoxyl supports are easy to prepare. Agarose gels can be carefully activated with epiclorhydrin. The activated supports have 40% epoxy groups, which are able to react with high concentrations of many small ligands bearing nucleophilic groups, such as amino groups and thiols. The activated supports also have 60% glyceryl groups, which can be converted into glyoxyl groups via periodate oxidation (Guisàn, 1988). At first glance, it appears that epiclorhydrin could produce 100% epoxy groups through reaction with all of the hydroxyl groups on the support, but the strong alkaline conditions required for activation promote the hydrolysis of a large number of epoxy groups into glyceryl groups instead. The quantification of the reactive groups show that the adsorbent groups are 70±3 µmol/mL and the glyoxyl groups are 105±5 µmol/mL. In this way, the supports would contain a large amount of glyoxyl groups that are capable of establishing intense multipoint covalent linkages, and therefore, a stable immobilized preparation could be obtained.

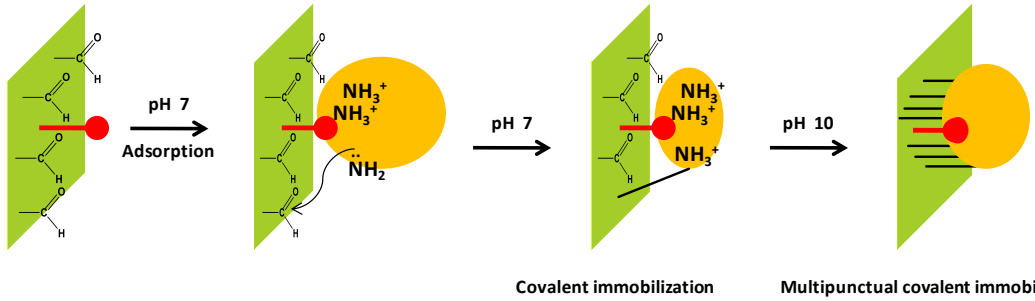


Figure 13: Mechanism of enzyme immobilization-stabilization on new heterofunctional supports. These novel heterofunctional supports allow the design of novel two-step immobilization.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Provider	Enzyme
Sigma-Aldrich (St. Louis, MO, EEUU)	• Bovine serum albumin (BSA) • *Trametes versicolor* laccase
Novozymes A/S (Bagvaerd, Denmark)	• *Myceliophtora thermophila* laccase
Provider	**Supports**
GE Healthcare (Little Chalfont, Buckinghamshire, UK)	• Ten percent beads crosslinked (10 B-CL) agarose • Carboxy methyl (CM)-Sepharose Fast Flow • DEAE-Sepharose Fast Flow • Cyanogen bromide (CNBr) activated Sepharose 4 B
Provider	**Substrate**
Sigma-Aldrich (St. Louis, MO, EEUU)	• 2,2'-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid): ABTS
Provider	**Reagents**

Sigma-Aldrich (St. Louis, MO, EEUU)	• Sodium borohydride ($NaBH_4$) • Ethylene diamine (EDA) • 1-ethyl-3-(3 dimethylaminopropyl)carbodiimide (EDAC) • Epichlorohydrin • β- mercaptoethanol • Triethylamine • Iminodiacetic acid (IDA) • Ethanolamine • Sodium metaperiodate ($NaIO_4$) • Sodium acetate trihydrate ($C_2H_3NaO_2$ $3H_2O$) • Sodium dihydrogen phosphate (NaH_2PO_4) • Sodium acetate ($C_2H_3NaO_2$) • Sodium hydroxide (NaOH) • Glycidol • Potassium iodide (KI) • 2,4,6-trinitrobenzenesulfonic acid (TNBS) • Borate • Picrylsulfonic acid • Sulphuric acid (H_2SO_4) • Chloridric acid (HCl)
Provider	**Reagent**
Fluka (Buchs, Suiza)	• Copper sulphate ($CuSO_4$)
Pierce (Rockford, IL, EEUU)	• Bradford reagent
Bio-Rad (Hercules, CA, EEUU)	• 30% acrylamide/bisacrylamide solution • 2% bisacrylamide solution
GE Healthcare (Little Chalfont, Buckinghamshire, UK)	• Low-molecular weight marker (LMW) (97,000-14,000 Da)
Spectrum Labs.com	• Cellulose dialysis membranes (cut-off 12,000-14,000 Da)

All other chemicals are obtained from Sigma-Aldrich unless otherwise specified.

5.2.2 Methods

5.2.2.1 Enzyme assay

Standard laccase activity was determined by oxidation of ABTS at room temperature. The reaction solution was composed of 3mM ABTS in 10mM sodium acetate buffer (pH 4.5). A suitable amount of enzyme was added and the oxidation of ABTS was followed by measuring the absorbance increase at 418 nm in a 1 mm path length spectrophotometric cell ($\varepsilon_{ABTS^{\cdot+}}$= 36000 M^{-1} cm^{-1}). One unit of laccase activity corresponds to the oxidation of 1 µmol ABTS per minute under these conditions. For the determination of immobilized enzyme activity, at fixed time intervals, an amount of biocatalyst preparation was incubated in 2 mL of 3mM ABTS in 10mM sodium acetate buffer (pH 4.5) solution with stirring and the absorbance at 418 nm was measured (Adinarayana Kunamneni et al., 2008).

5.2.2.2 Protein determination

The amount of protein of soluble enzymatic preparation was determinate by the method developed by Bradford (Bio-Rad protein assay), (Bradford, 1976). The bovine serum albumin (BSA) was used like a standard to make a calibration curve (0-1.5 mg/mL).

5.2.2.3 Enzyme purification

Laccase was purified by anionic adsorption on DEAE Sepharose Fast Flow support (table 2). The ion exchange group is a diethylaminoethyl group and the enzyme was adsorbed on DEAE Sepharose beads under continuous stirring in 25mM sodium phosphate buffer at pH 7.0. The activity of suspension and supernatant was periodically measured by using the ABTS assay. After enzyme adsorption, the laccase preparation was vacuum filtered using a sintered glass funnel and abundantly washed with 5mM sodium phosphate buffer at pH 7.0. Laccase was desorbed from DEAE Sepharose by suspending the immobilized enzyme in a 1/10 (w/v) ratio in 25mM sodium phosphate, 250mM NaCl buffer at pH 7.0, under continuous stirring at room temperature (figure 14). The activity of suspension and supernatant was periodically measured by using the ABTS assay until the supernatant activity was the 80% of the suspension activity. Only one protein band was detected by SDS-PAGE.

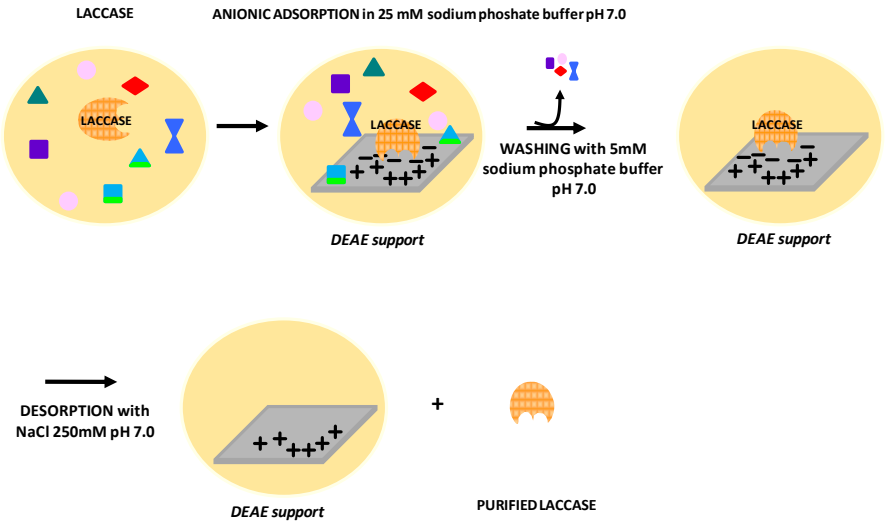


Figure 14. Schematic representation of the laccase purification strategy.

Property	Description
Ion exchange type	Weak anion
Total ionic capacity	0.11-0.16 mmol /mL media
Exclusion limit	4 x 0.16 (globular proteins)
Matrix	Cross-linked agarose, 6%
Bead form	Spherical, 45-165 µm

Table 2. Characteristics of DEAE Sepharose Fast Flow.

5.2.2.4 SDS-PAGE analysis of laccase

SDS-PAGE experiments were performed as described by Laemmli (Laemmli, 1970) in a SE 250-Mighty small II electrophoretic unit (Hoefer Co.) using 9 cm x 6 cm separation gel of 12% polyacrylamide. The enzyme, before and after purification, was boiled in Laemmli's disruption buffer that contains mercaptoethanol and SDS. Gel was stained with Coomasie brilliant blue® (R250) (Swank and Munkres, 1971). Low-molecular weight marker kit from GE Heallthcare was used (MW = 14,000-94,000 Da); a voltage of 150 V was applied for the separation.

5.2.2.5 Chemical amination of purified laccase

1 mL of purified laccase was added to 5 mL of 1M ethylene diamine (EDA) at pH 4.75. Different amounts of solid 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were added to the solution to a final concentration of 10 and 50mM. After 90 min of gentle stirring at 4°C, the solution was dialyzed 5 times with cellulose dialysis membranes (cut-off 12,000-14,000 Da) against 50 volumes of distilled water and stored at 4°C (Lopez-Gallego et al., 2005). It has been described that the use of 1M EDA at pH 4.75 and 10mM EDAC allows the full modification of the carboxylic groups of the protein surface, while using in 1M EDA at pH 4.75 and 1mM EDAC, the modification degree is only around 40–50% (Fernandez-Lafuente et al., 1992). In our case we aminated the soluble laccase using 10 and 50mM EDAC. During the amination processes, samples were withdrawn and the enzyme activity was determined as described above. The aminated enzymes are named as: laccase-NH_2 10mM (laccase modified with 10mM EDAC) and laccase-NH_2 50mM (laccase modified with 50mM EDAC).

5.2.2.6 Characterization of free laccase

pH stability

The effect of the pH on the purified and not purified enzyme stability was studied by incubating enzymes, at room temperature, in 25mM sodium acetate, phosphate and bicarbonate buffers at pH 5.0, 7.0 and 10.0, respectively. At different times the enzymes activity was assayed in standard conditions with ABTS, at room temperature.

5.2.2.7 Preparation of monofunctional supports

Cyanogen bromide (CNBr) activated sepharose 4 B support

5 mL of HCl solution in distilled water at pH 2.9 were added to 0.5 g of the freeze dried support for 30 minutes. The hydrate and active support has a mass of 1.55 g (when the dry support was completely wet and active, the weight of the support increased 3 times).

Monoamino-N-aminoethyl-agarose (MANAE-agarose) support

10 g of glyoxyl-agarose 10 B-CL were suspended in 2M ethylenediamine at pH 10.0 and the suspension was gently stirred for 2 h, to obtain a "quantitative aminations" (around 99% of aldehyde groups in the support converted into MANAE groups). Then, 1 g of solid $NaBH_4$ was added and the suspension was reduced for another 2 h; this step allows us: (i) a complete conversion of Schiff's bases into stable secondary amine bonds, (ii) a complete reduction of the almost negligible amount of remaining aldehyde groups into inert hydroxylic ones, and (iii) a complete reduction of a small percentage of immobilized nitro compounds formed as a consequence of the oxidation of ethylenediamine at pH 10.0. The reduced gel, monoaminoethyl-N-aminoethyl agarose (MANAE-agarose), was filtered and washed with 100 mL of 0.1M acetate, 1M NaCI at pH 5.0, with 100 mL of 0.1M bicarbonate, 1.0M NaCI at pH 10.0, and finally with 500 mL of deionized water (Fernandez-Lafuente et al., 1993). The concentration of amine groups in the support was first tested by titration of the activated gel with NaOH and further confirmed by colorimetric titration with picrylsulfonic acid. Titration of MANAE-agarose gels reveals the existence of two ionizable groups with pK values of 6.8 and 10.0 and with identical concentration, these two pK values are related to the primary and secondary amine groups respectively.

Dimmer glutaraldehyde Monoamino-N-aminoethyl-agarose (MANAE-agarose) support

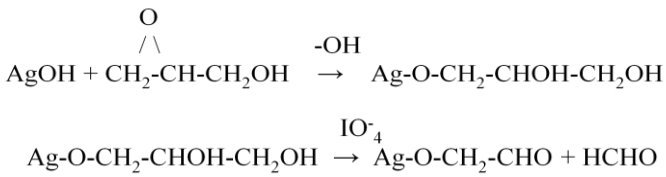
Glutaraldehyde agarose was prepared suspending 10 mL of MANAE agarose in 20 mL of 15% (v/v) glutaraldehyde in 200mM phosphate buffer pH 7.0. The suspension was kept under mild stirring at 25°C for 15 h. After that, the support was filtered and washed exhaustively with 25mM sodium phosphate buffer and then with distilled water (Betancor et al., 2006). The level of amine modification by glutaraldehyde was quantified by titration of the remaining primary amino groups with 2,4,6-trinitrobenzenesulfonic acid (TNBS). 2 mL of gels (MANAE or reduced glutaraldehyde activated agarose) were suspended in 8 mL of 0.1M borate pH 9. Then, 2 mL of 0.2M TNBS (aqueous solution) was added and the titration was performed for 1 h. The colored gels were extensively washed with 0.1M borate pH 9 and allowed to settle in a 1 mm path length spectrophotometric cell. After packing, the visible spectra of the colored gels were recorded using untitrated gels as blank.

Ten percent beads crosslinked agarose (10 B-CL) support

Activation of agarose gels

The activation of agarose gels was done according to the procedure previously described by Guisàn (Guisàn, 1988) with slight modifications. 30 mL of 10 B-CL agarose gel (0.7 g of swelling agarose is roughly equivalent to 1 mL) was suspended in a reaction mixture containing 6 mL of distilled water and 10 mL of 1.7M NaOH with 284 mg sodium borohydride ($NaBH_4$). These reducing conditions are necessary to avoid the oxidation of the gel. Glycidol (7.2 mL) was added dropwise to this suspension kept in an ice-bath in order to reach a 2M final concentration. The whole suspension was gently stirred overnight at room temperature. The activated gel was washed with abundant water (pH 7) and 300 mL water containing 300 µmoles $NaIO_4$ mL^{-1} gel to achieve multipoint attachment. This oxidative reaction was allowed to proceed for 2-3 h with stirring at room temperature. The glyceryl groups obtained in the etherification reaction by glycidol were oxidized specifically by periodate, mole to mole. This reaction is very important because we can know the number of aldehyde

groups that have been produced measuring the $NaIO_4$ that was not consumed in the reaction by titration with IK (Nevell, 1963).



5.2.2.8 Immobilization on monofunctional support

An enzyme solution at the indicated pH and conditions was mixed with the specified amount of different supports. At different times, samples of the supernatant, the support-enzyme suspension, and the initial enzyme solution without the support, were taken, and the activity was assayed. Immobilization yield refers to the percentage of enzyme that is immobilized on the support. Expressed activity is calculated as the ratio between the activity of the immobilized enzyme and that of the soluble purified enzyme before the immobilization. All experiments were performed using a little quantity of biocatalyst in order to avoid diffusion problems that could alter the apparent enzyme stability. All supports and the immobilized preparations were stored at 4°C.

Cyanogen bromide (CNBr) activated sepharose 4 B support, (Myceliophthora thermophila and Trametes versicolor laccases)

The immobilization was carried out adding to 1.55 g of support 10 mL of *Myceliophthora thermophila* soluble laccase (15 IU) prepared as previously described, and to 1 g of support 10 mL of of *Trametes versicolor* laccase (9 IU) in 25mM phosphate buffer at pH 7. The suspensions were left under mild stirring during 15 minutes at 4°C. After this time the supports were filtered and washed with 1M ethanolamine solution at pH 8 and incubated with the same solution in a 1/10 (w/v) ratio for 1.5 h to block the remained CNBr groups. Finally, the immobilized preparations were washed with distilled water, 25mM phosphate buffer at pH 7 and their activity were assayed as described above. The amination of *Trametes versicolor* CnBr derivative was performed with the procedure described above.

Monoamino-N-aminoethyl-agarose (MANAE-agarose) support, (Myceliophthora thermophila laccase)

1 g of support (MANAE-agarose) was suspended in 3.5 mL of 25mM potassium phosphate at pH 7 containing 15 IU of laccase. The suspension was gently stirred at room temperature for 2 h. Periodically, samples of the suspension and the supernatant were withdrawn and enzyme activity was analyzed as described above. When the immobilization was complete, the suspension was filtered, washed with distilled water, 25mM phosphate buffer at pH 7.

Dimmer glutaraldehyde Monoamino-N-aminoethyl-agarose (MANAE-agarose) support, (Myceliophthora thermophila laccase)

1 g of support (dimer glutaraldehyde MANAE agarose) was suspended in 6 mL of 25mM potassium phosphate at pH 7 containing 15 IU of laccase. The suspension was gently stirred at room temperature for 2 h. Periodically, samples of the suspension and the supernatant were withdrawn and enzyme activity was analyzed as described above. When the immobilization was complete, the suspension was filtered, washed with distilled water, 25mM phosphate buffer at pH 7 and its activity was assayed as described above.

***Ten percent beads crosslinked agarose (10 B-CL) support,* (*Myceliophthora thermophila* and *Trametes versicolor* laccases)**

Immobilization at pH 10

The immobilization was carried out by adding 1 g of support to 2 mL of 100mM sodium bicarbonate pH 10.05 and 1 mL of *Myceliophthora thermophila* purified enzyme (15 IU) to prevent diffusion limitations, and 1 g of support to 10 mL of the same buffer whit 10 IU of *Trametes versicolor* laccase. The suspensions were gently stirred at room temperature for 24 h. Finally, 0.1 mg/mL sodium borohydride ($NaBH_4$) was added under gentle stirring at 4°C to reduce the imino and aldehyde groups (Mateo et al., 2005). After 30 min, the immobilized enzymes were washed with an excess of distilled water and their activity was assayed as described above.

Immobilization at pH 9

The immobilization was carried out by adding 1 g of support to 5 mL of 100mM sodium bicarbonate pH 9.0 and 20 mL of *Myceliophthora thermophila* purified enzyme (15 IU) to prevent diffusion limitations, and 1 g of support to 10 mL of the same buffer whit 8 IU of *Trametes versicolor* laccase. The suspensions were gently stirred at room temperature for 8 and 2 h, respectively. After the enzyme immobilization at pH 9.0, the pH was adjusted at 10.05 and the suspensions of *Myceliophthora thermophila* and *Trametes versicolor* were kept, under gentle stirring at room temperature, for 18 and 2 h, respectively. Finally, 0.1 mg/mL sodium borohydride was added under gentle stirring at 4°C to reduce the imino and aldehyde groups (Mateo et al., 2005). After 30 min, the immobilized enzymes were washed with an excess of distilled water and their activity was assayed as described above.

5.2.2.9 Preparation of heterofunctional supports

Activation of agarose with epoxy groups

10 g of agarose 10 B-CL was suspended in 44 mL water, 16 mL acetone, 3.28 g NaOH, 0.2 g $NaBH_4$ and 11 mL epichlorhydrine. The suspension was stirred mildly for 16 hours and washed with an excess of water. For quantification of the activated epoxy groups, 1 g of the support was treated with 10 mL 0.5M H_2SO_4 for 2 hours to hydrolyze the epoxy groups. Then, this hydrolyzed support was oxidized with $NaIO_4$, as previously described (Guisán, 1988). The number of epoxy groups was calculated by the difference in periodate consumption between the hydrolyzed support and the initial epoxy support. Periodate consumption was quantified using potassium iodide as previously described (Nevell et al., 1963).

Modification of agarose supports with different reactive groups

Agarose epoxy-supports were modified with different moieties (figure 15). In all cases, the ratio of the modifying reactive solution to the support was 1/10 (v/w).

Cationic support: the epoxy-agarose support was modified with 1M triethylamine in 50% water/acetone at pH 12 for 24 hours at 25°C.

Anionic support: the epoxy-agarose support was treated with 0.5M iminodiacetic acid at pH 11 for 24 hours at 25°C.

Metal chelate support: the anionic support was modified with a 30 mg/mL solution of metallic salt ($CuSO_4$) at pH 7.0 for 1 hour at 25 °C.

Finally the supports were oxidized with sodium periodate, as previously described (Guisán, 1988).

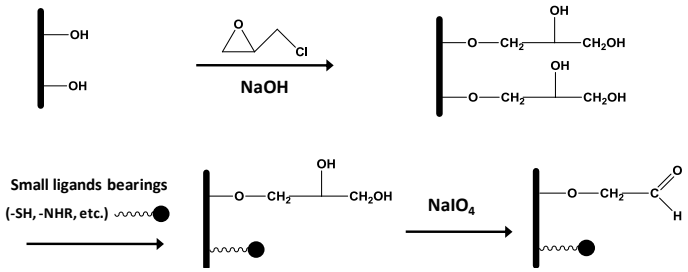


Figure 15. Preparation of different heterofunctional glyoxyl supports.

5.2.2.10 Immobilization on heterofunctional supports (*Myceliophthora thermophila* laccase)

Immobilization on amine-glyoxyl support (TEA)

The enzyme was added to a 5mM sodium phosphate buffer at pH 7 and 25^{o}C. Then, 1 g of support was suspended in 10 mL of the solution, with a maximum enzyme activity of 15 IU. Samples of the supernatants and suspensions were withdrawn, and periodically the enzyme activity was measured. The immobilization was complete in 1 h, the preparation was washed with the 5mM phosphate buffer pH 7, dried under vacuum and resuspended in 10 mL 5mM sodium hydrogen carbonate at pH 10.5 for 1h. Finally, the preparation was reduced by addition of 10 mg sodium borohydride. This suspension was stirred mildly for 30 minutes and then washed with distilled water, 5mM phosphate buffer at pH 7 and its activity assayed as described above.

Immobilization on carboxylic-glyoxyl support (IDA)

The laccase-NH_2 50mM was added to a 5mM sodium phosphate buffer at pH 8 and at 25^{o}C. Then, 1 g of support was suspended in 4 mL of the solution, with a maximum enzyme activity of 15 IU. Samples of the supernatants and suspensions were withdrawn, and periodically the enzyme activity was measured. The immobilization was complete in 1 h, the preparation was washed with the 5mM phosphate buffer pH 8, dried under vacuum and resuspended in 4 mL 5mM sodium hydrogen carbonate at pH 9 and 10.5 for 1 hour respectively. Finally, the preparation was reduced by addition of 4 mg sodium borohydride. This suspension was stirred mildly for 30 minutes and then washed with distilled water, 5mM phosphate buffer at pH 7 and its activity was assayed as described above.

Immobilization on chelate-glyoxyl support (IDA-Cu)

The laccase-NH_2 50mM was added to a 300mM sodium phosphate buffer and at pH 8 and 25^{o}C. Then, 1 g of support was suspended in 4 mL of the solution, with a maximum enzyme activity of 15 IU. Samples of the supernatants and suspensions were withdrawn, and periodically the enzyme activity was measured. After the immobilization was complete (1 h), the preparations was washed with the phosphate buffer, dried under vacuum and resuspended in 4 mL of 300mM sodium hydrogen carbonate at pH 9 and 10.5 for 1 hour respectively. Finally, the preparations were reduced by addition of 4 mg sodium borohydride. The metal chelate support was washed with 5 volumes of 50mM EDTA at pH 7 before the reduction step. The suspension with 4 mg sodium borohydride was stirred mildly for 30 minutes and then washed with distilled water, 5mM phosphate buffer at pH 7 and its activity was assayed as described above.

5.2.2.11 Inactivation of enzyme preparations

The different preparations of laccase were incubated at different temperature and pH values, in presence of denaturant agent as UREA, and organic solvents (dioxane). The derivatives were withdrawn and, at different times, samples of suspension were taken and the activity was assayed as previously described. In particular, laccase preparations (CNBr, carboxy and chelate-glyoxyl derivatives) were incubated in 4M UREA, pH 7 and 70% (v/v) dioxane in 10mM Tris pH 7, 4°C. All experiments were performed in triplicate, and the relative standard deviation was lower than 4%.

5.3 RESULTS AND DISCUSSION (*Myceliopthora thermophila laccase*)

5.3.1 Stability of soluble enzyme, effect of random immobilization

Although in the starting commercial extract the enzyme has a high purity (figure 16), when the commercial enzyme has to be immobilized is generally advisable to treat them through a semipurification process in order to avoid some compounds that are added as preservatives. Thus on one hand it is possible to study the real characteristics of the soluble enzyme that the presence of additives could mask and on the other hand some compounds that may interfere with the immobilization processes are eliminated. These compounds that are in general great size aminated compounds and they are not eliminable after a dialyzing can react with most of reactive groups of the supports. For this, a fairly effective strategy consists in adsorbing the enzyme on supports activated with ionic exchangers such as DEAE for one adsorbed the enzyme remove the not desired compounds by washing and subsequent desorption of the enzyme. Although when it comes to immobilize these compounds are undesirable, it has to take into account that they are added for increasing the stability of the enzyme preparations.

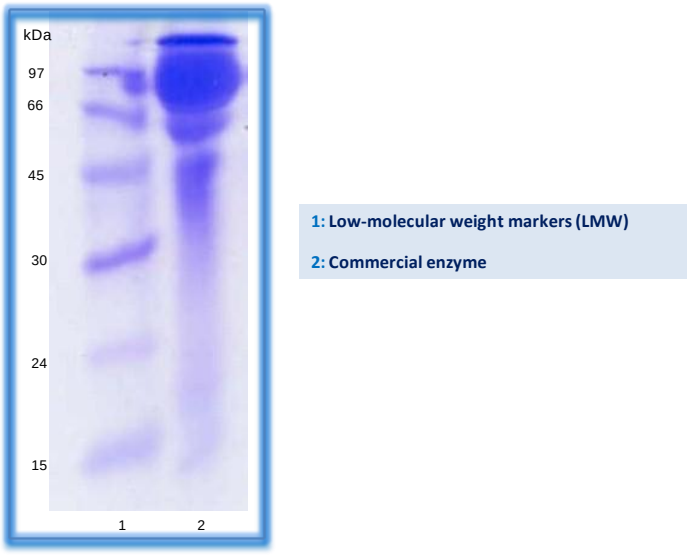


Figure 16. Electrophoretic profile of the not purified soluble enzyme.

Because of that some experiments to evaluate the real stability of the enzyme with and without the presence of those additives are proposed. The stability of the different preparations was studied at different pHs (figure 17). The wild enzyme was completely stable after incubation at acid pH as well as in neutral and basic pHs. Contrarily, when additives were eliminated the stability of the enzyme greatly decreased and was more stable at acid pH than at neutral and basic pH respectively.

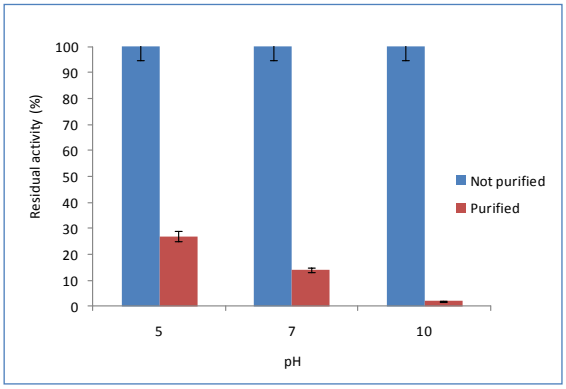


Figure 17. Stability/pH profile of the purified and not purified soluble enzyme. The experiment was conduct incubating the samples in 25mM sodium acetate, phosphate and bicarbonate buffers at pH 5.0, 7.0 and 10.0, respectively.

Besides this, generally soluble enzymes may suffer different undesirable processes such as aggregation, proteolysis or autolysis, so they do not show their real properties. So it is desirable to be immobilized on solid supports and through a single point of covalent attachment to the support. Under these conditions is described that the obtained derivatives have a very similar properties that soluble enzyme but avoiding all problems before mentioned. For this, it is proposed the immobilization on commercial supports activated with CNBr reactive groups highly reactive at neutral pH (figure 18). This support immobilize basically through the terminal amine yielding derivatives that normally have similar properties to the soluble enzyme. This derivative has a slightly higher stability than the corresponding soluble enzyme. The stabilizing factor was around 1.3 after incubation at 60ºC, pH 7.0 (data not show). Taking into account that the properties of the enzyme immobilized on CNBr activated supports are very similar to the soluble enzyme properties from now this derivatives will be used as reference for comparison of the different derivatives with the soluble enzyme.

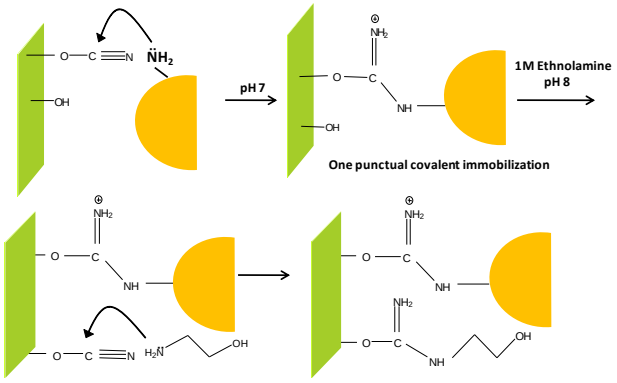


Figure 18. Schematic representation of the laccase immobilization on CNBr activated sepharose 4 B support. The immobilization was conducted at pH 7, in these conditions the most common primary amino group having a low pK value might be the terminal amino group of the protein. After one point immobilization, the support was incubated in 1M ethanolamine solution at pH 8 and to block the remained CNBr groups.

5.3.2 Immobilization on anionic exchanger support: Monoamino-N-aminoethyl-(MANAE)-agarose

Laccase was immobilized on supports activated with anionic exchangers as MANAE-agarose supports. At neutral pH, in general most of the proteins are able to be immobilized on these supports because most of the proteins have an isoelectric point between 4 and 5 and at neutral pH they are negatively charged. Thus at low ionic strength proteins are immobilized by an ionic exchange through the region of the enzyme surface richest in negative charges. When laccase was immobilized

under low ionic strength 100% of the enzymatic activity was adsorbed in less than 1 hour. The recovered activity for these derivatives was around 27% compared with the soluble enzyme that is a moderate recover taking into account that this method is very mild and little distorting. However, these supports are reversible so in some cases the enzyme can leak from the support to the reaction medium loosing the catalytic activity. Thus it would be very interesting its immobilization on solid supports but using covalent immobilization protocols.

5.3.3 Immobilization on dimmer glutaraldehyde (MANAE)-agarose support

In the context of the present thesis MANAE-agarose supports were activated with glutaraldehyde at neutral pH. Under the used conditions it is reported that supports activated with two glutaraldehyde molecules per amine group of the support are obtained (Betancor et al., 2006). Glutaraldehyde molecules are capable to covalently immobilize enzymes and under these molecules there are secondary amine groups with high capacity as anionic exchangers. Glutaraldehyde supports under low ionic strength are capable to physically adsorb the proteins and, when they are adsorbed, are able to be covalently linked by the glutaraldehyde molecules. As was related with MANAE supports, when laccase was offered to glutaraldehyde activated supports the enzyme was completely immobilized after 1 hour. This immobilization is produced in a first step by a physical adsorption and finally the covalent immobilization is produced. Final activity of these derivatives was better than in the case of immobilization by physical adsorption. This activity was 84% if compare with soluble enzyme.

5.3.4 Immobilization on glyoxyl agarose 10 B-CL support (different conditions)

While the immobilization of the laccase on glutaraldehyde supports has enabled its complete immobilization with a high percentage of recovered activity, in general stabilizations obtained by immobilization on these supports are not often the best possible because these supports are quite unstable specially at alkaline pH. At this pH the support is extremely reactive not allowing long protein-support contact times. For this reason, immobilizations are performed at neutral pH so that in general the degree of the obtained multipoint covalent attachment and therefore the rigidification of the 3D structure are not the highest possible. Probably, the best way to increase the multipoint covalent attachment degree is the use of glyoxyl supports.

The 10 B-CL agarose gel has been chosen as support for immobilization of laccase because of several reasons: it is very easily handled and activated, its chemical structure presents good possibilities to obtain a strong enzyme-support multipoint attachment, it can be assayed and studied by spectrophotometric methods, and it can be used in batch reactors because it is compressible and resistant to mild stirring. The overall activation (etherification to obtain glyceryl groups from glycidol, and periodate oxidation to produce aldehyde groups from glyceryl groups) was performed in mild experimental conditions to obtain intact agarose gel containing monolayers of identical aldehyde groups. By controlling the amount of periodate added, the quantity of aldehyde groups in the support can be determined. In this case, about 220 μmoles $NaIO_4$ ml^{-1} gel (75% of total amount) are consumed which means a much better probability of enzyme-support multiinteraction.

As it was already commented in introduction, these supports only are able to link proteins if the formation of several linkages between protein and support are produced. This makes that these supports perform their immobilization through the

richest place in lysines (Guisàn, 1988; Mateo et al., 2005) allowing obtaining a high multipoint covalent attachment degree and consequently the stabilization values of the obtained derivatives by using this methodology are in many cases the best reported (Blanco and Guisán, 1989; Alvaro et al., 1990; Guisán et al., 1991). The need to perform simultaneously several imine linkages makes that immobilization processes must be performed at around pH 10 where most of the lysines will be deprotonated and therefore be reactive with aldehyde groups.

The interaction between the enzyme and the support is determined by the surface area of the support, the number of aldehyde residues on the support, and the external area of the enzyme. The surface area of agarose 10 B-CL is ≈66 m^2/mL and the surface density of aldehyde groups is expressed as number of residues of aldehyde groups per gel surface = 6.02 × aldehyde concentration/surface area (μmoles/mL per m^2/mL). In the multi point derivative, the aldehyde concentration is 220 μmoles/ml:

$$6.02 \times 220\ (\mu mol/mL)/\ 66\ (m^2/mL)$$

which is ≈20 residues per 1000$Å^2$.

On the other hand, the total external area of laccase (MW 80,000 Da, Stoke's radius = 39.04) must be ≈20000$Å^2$. Considering that about 10% of the external area of the enzyme structure can get in contact with the support and considering the external area of laccase that gets in contact with the support, the possibilities of enzyme-support multiinteraction in these conditions are very high.

Thus, laccase was incubated in the presence of glyoxyl supports at pH 10. However the enzyme was immobilized very slowly, with about 10% of the total enzyme immobilized after 24 hours of incubation with the support (table 3). This result is coherent after studying the aminoacid sequence of the protein where it can be seen how this enzyme only have 9 lysine moieties. (Pedersen et al, 1998). This explains the low rate of immobilization on glyoxyl supports because taking into account the view of 3D structure; lysines are distributed homogeneously through the enzyme surface and hardly are able to form several linkages simultaneously.

A possible solution to this problem consists in aminating the aspartic and glutamic acid to convert them into new amine groups that can react with the support. For this proposal it is proposed the reaction of these aminoacids with the bifunctional reactive ethylenediamine in the presence of a carboxyl activator as carbodiimide. Under these conditions the correspondent amide is synthesize and it has a primary amine group in the extreme of the formed molecule. These amino groups have a pK around 9 because of that are more reactive than the lysines being able to react at pH lower than 10. This reaction was studied in order to optimize the degree of substitution of the acid aminoacids of the laccase evaluating the influence on the enzyme activity. This reaction is performed using high concentrations of ethylenediamine and the limiting reactive is the carbodiimide. The concentrations of carbodiimide assayed were 10 and 50mM. To check the degree of amination in both conditions, the aminated preparations were adsorbed to a cationic exchanger to practically determine the amination degree. After 1 hour of incubation in the presence of these supports the enzyme aminated with 50mM of carbodiimide was almost completely adsorbed on the support while only 60% of the enzyme aminated with 10mM of carbodiimide was immobilized under the same conditions. This means that the first treatment was more efficient than the second one. In addition it could be observed as the residual activity in both cases was similar to the wild enzyme. After this

experiment all the aminations were performed using 50mM of carbodiimide. The previously aminated enzyme was incubated in the presence of glyoxl supports at pH 9. Under these conditions 87% of immobilization was obtained after two hours. No immobilization of the wild enzyme was obtained at this condition confirming the hypothesis (figure 19).

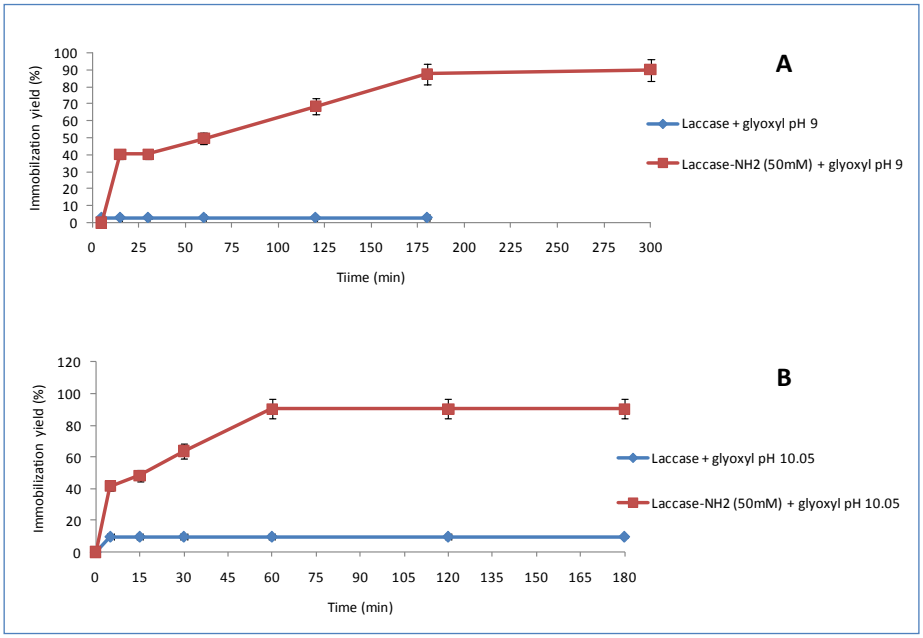


Figure 19. Immobilization course of the aminated and not aminated laccase on glyoxyl support at pH 9.0 and 10.05. The immobilization was conducted in 100mM bicarbonate buffer at pH 9 **(A)** and pH 10.05 **(B)**.

After finishing this immobilization process at milder pH, it was performed an incubation at pH 10 to maximize the degree of multipoint covalent attachment of the enzyme and the support. After this incubation the final recovered activity of the immobilized preparation was around 65% compared with the wild enzyme. The decrease of the activity must be produced by the multiinteraction between protein and support, but this decrease of the activity generally is correlated with a great increase of the stability in the obtained preparation (table 3). Finally the derivative was reduced using $NaBH_4$ to transform the Schiff bases into permanent secondary amine groups and to convert the remanent aldehyde groups into inert hydroxyl groups (Blanco and Guisan, 1989). This treatment did not alter the final activity of the obtained derivatives.

Laccase derivative	Immobilization yield (ψ), (%)	Expressed activity (%)
Laccase-glyoxyl pH 10	10	15
Laccase-NH_2 50mM-glyoxyl pH 9, 10	87	65

Table 3. Immobilization yield and expressed activity of laccase-glyoxyl derivatives.

These derivatives were incubated at 60ºC and pH 7 and compared with the unipunctual CNBr-agarose and with the derivatives obtained after immobilization on MANAE and glutaraldehyde supports. In these incubation conditions the half life of the glyoxyl preparations was similar to the unipunctual derivatives. The half life was around 3 hours of incubation in all cases. Curiously in the case of immobilized derivatives on MANAE and glutaraldehyde supports stabilization factors of 9 and 5.3 were obtained respectively (table 4).

Laccase derivative	Thermal stabilization fold[a]
Lacc-NH_2 (50mM) + Glyoxyl pH 9, 10	(pH 7, 60°C) 2.6
Lacc + MANAE	(pH 7, 55°C) 9
Lacc + MANAE dimmer Glutaraldehyde	(pH 7, 55°C) 5.3

Table 4. Stabilization fold, [a] calculated respect to CNBr agarose derivative in the same conditions.

5.3.5 Immobilization on glyoxyl-heterofunctional supports

Although in principle glyoxyl supports must be the most stabilizing because the enzyme is orientated through the richest region in lysine of the enzyme surface and the possibility of establish multipoint covalent attachment is maximal; in some cases there are different regions such as unstable loops or other regions highly sensitive to the stabilization processes. In fact, for this laccase this hypothesis was confirmed when using MANAE or glutaraldehyde supports, where the stabilization factors were higher than using glyoxyl support (table 4). For this, it was proposed the immobilization on a new generation of heterofuncional glyoxyl supports recently developed in the laboratory of enzymatic engineering in Madrid because as was previously commented in introduction they allow the immobilization of the protein through different regions of the enzyme surface.

These supports have two kind of functional groups: groups that are able to promote the physical adsorption of the proteins via different mechanisms and through different regions of the surface and many glyoxyl groups capable to establish covalent linkages. Thus, the immobilization is produced by a first adsorption process through the groups introduced in the support. This first process can be performed in very mild conditions such as neutral pH or low ionic strength. After this first step that controls the orientation of the immobilization, an incubation at alkaline pH is proposed. This is to increase the degree of multipoint covalent attachment. Different heterofunctional glyoxyl supports were used: with ionic groups to promote the immobilization through an ionic exchange at neutral pH and low ionic strength and supports activated with metal groups to promote the formation of metal chelate with the enzyme (figure 20).

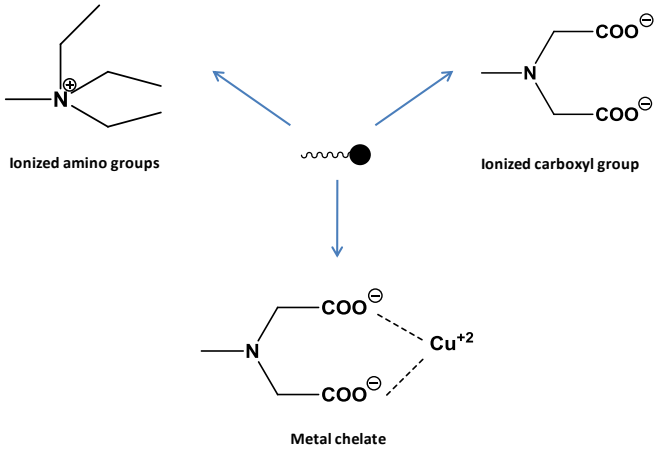


Figure 20. Different ligands capable to adsorb proteins at neutral pH.

As it could be seen previously, a good orientation may be that where it is rigidified the place of the enzyme richest in negative charges (MANAE and glutaraldehyde supports) because of that it was proposed the immobilization of the non-aminated

laccase on amine-glyoxyl supports (TEA) prepared incubating the epoxy-agarose support with triethylamine (figure 21).

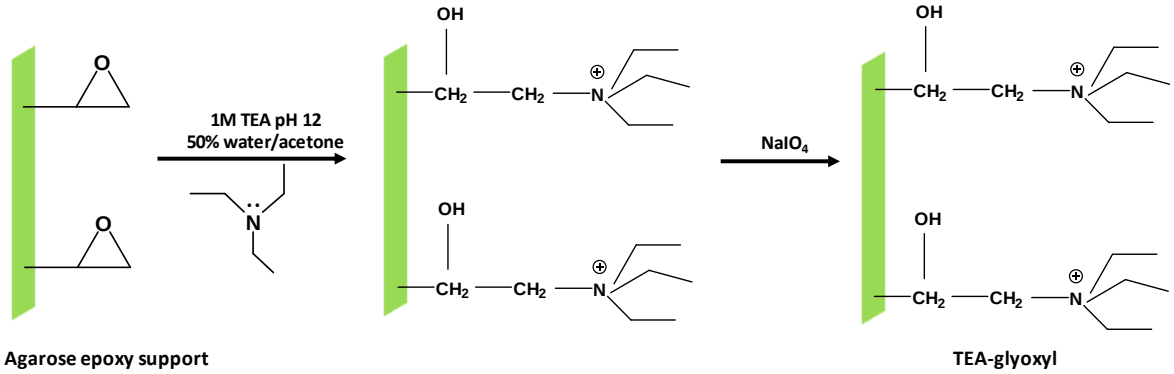


Figure 21: Preparation of the amine-glyoxyl support (TEA).

Under conditions of neutral pH and low ionic strength the enzyme was completely immobilized in 1 h. To check the immobilization mechanism the enzyme was immobilized on these supports at high NaCl concentration and no immobilization was detected after incubation in 500 mM of this salt (data not show). This confirms the hypothesis that the first immobilization step is promoted by the ionic adsorption of the enzyme on the support. Then, the previously adsorbed enzyme was incubated at alkaline pH to promote the reaction of the lysines with the glyoxyl groups of the support.

The previously aminated enzyme was also immobilized on a different heterofunctional supports. When we used heterofunctional supports activated with cationic exchangers (IDA-CHO) aminated laccase was completely immobilized in less than 5 minutes. In this support the epoxy-agarose was treated with iminodiacetic acid (IDA), (figure 22).

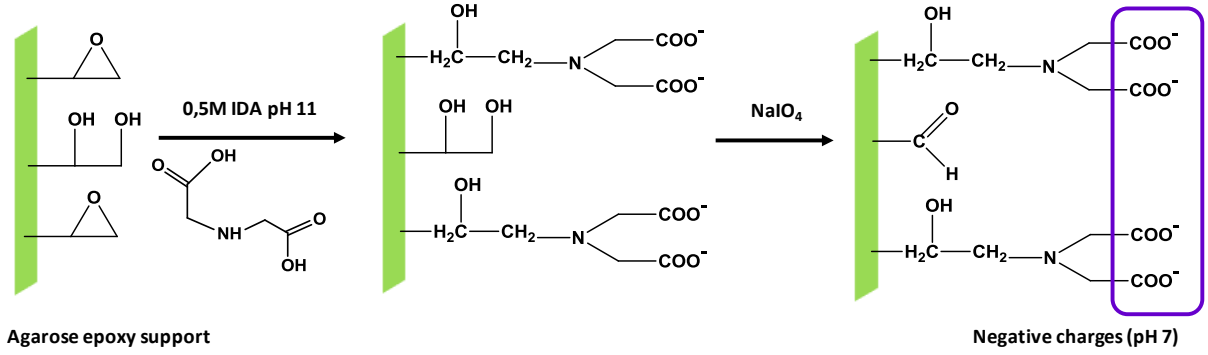


Figure 22: Preparation of the cationic exchanger support (IDA-CHO).

In this case the adsorption mechanism is the same, but ionic intermolecular interaction takes places between the positive charges of the enzyme surface and the negative ones of the support. At the immobilization pH the amino groups of lysine (pK 10.7), the ones introduced with chemical amination (pK 9.2) and the guanidine lateral chain (pK 12.48) are protonated and positive charged.

As well as before the immobilization was performed in the presence of different concentrations of NaCl. The immobilization yield decreased when ionic strength increased immobilizing only around 30% in the presence of 500mM of salt and confirming again the mechanism of the first adsorption of the enzyme (figure 23).

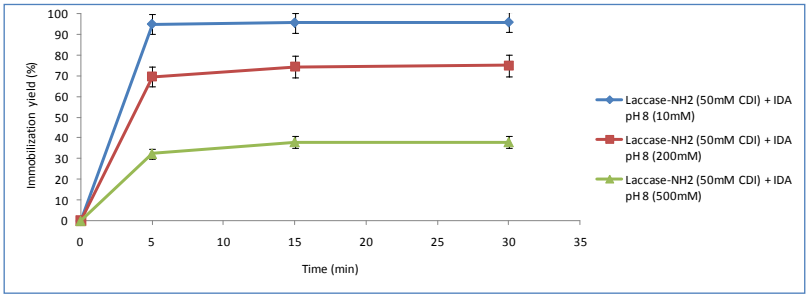


Figure 23. Immobilization course of the aminated laccase on cationic exchanger support (IDA-CHO) at different ionic strength. The immobilization was conducted in phosfhate buffer pH 8 (10, 200 and 500mM).

Moreover, the anionic support was modified with a solution of metallic salt ($CuSO_4$) to obtain the chelate-glyoxyl support (IDA-Cu), (figure 24).

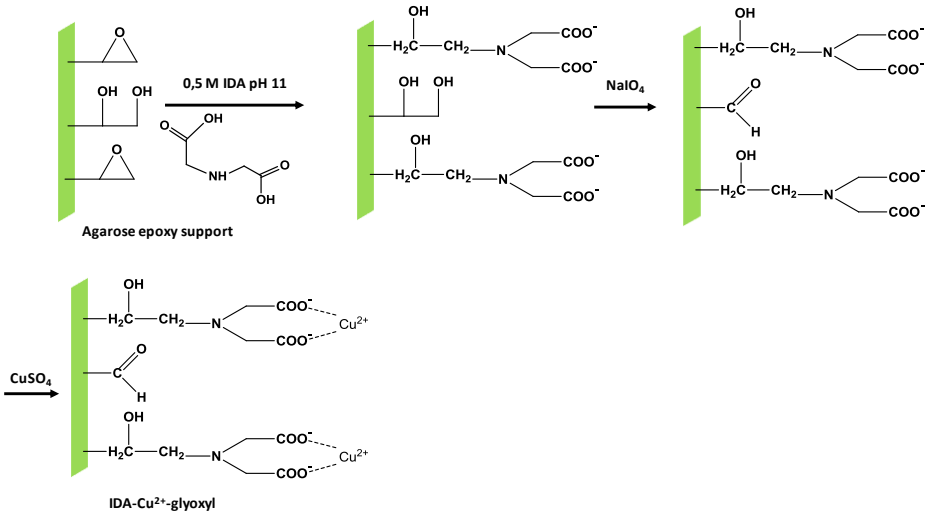


Figure 24: Preparation of the chelate-glyoxyl support (IDA-Cu).

In this support the cationic metal makes the coordination complex with the hystidine residues, after that, the glyoxyl groups take places to the covalent interaction. When the aminated enzyme was incubated in the presence of this support again the laccase was completely immobilized in less than 5 minutes. Similarly enzyme and support were incubated in growing salt concentrations. Enzyme was immobilized in less than 5 minutes in all salt assayed concentrations (figure 25).

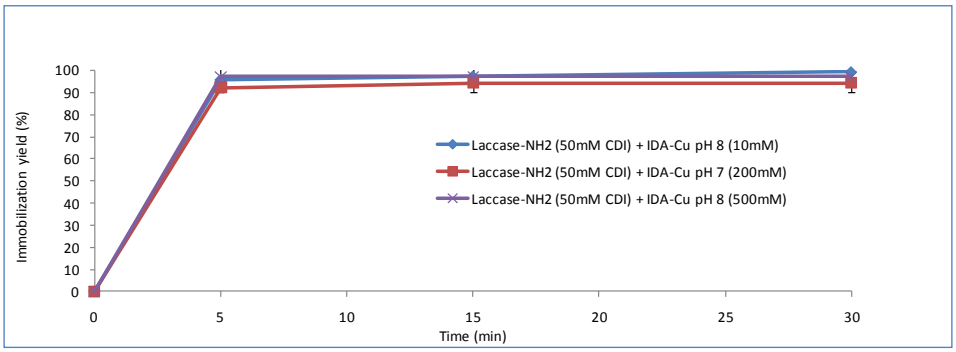


Figure 25. Immobilization course of the aminated laccase on chelate glyoxyl support (IDA-Cu) at different ionic strength. The immobilization was conducted in phosfhate buffer pH 8 (10, 200 and 500mM).

In a similar way it was performed the same experiment but in presence of imidazol. Imidazol is a competitor with the hystidines in the formation of chelate groups with the metals of the support. In presence of 500mM of imidazol only around

20% of immobilization of laccase was produced confirming that in this case the mechanism that controls the immobilization was not the ionic adsorption but the metal chelate formation (figure 26).

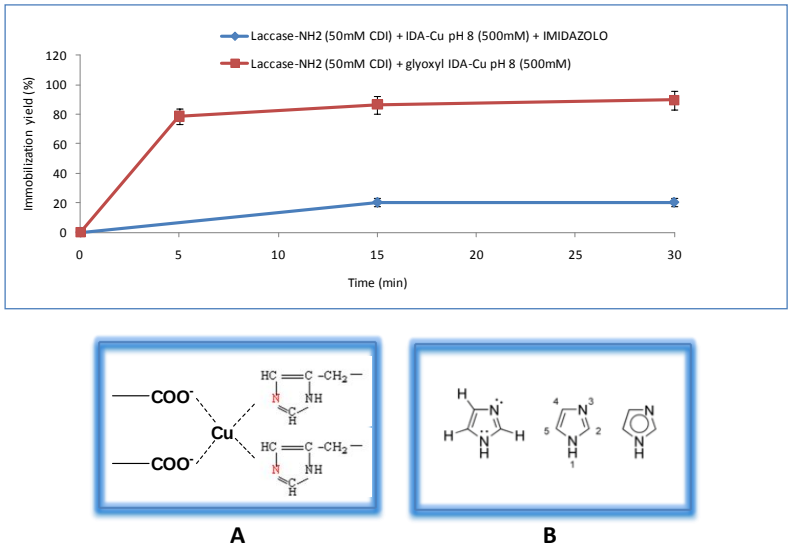


Figure 26. Immobilization course of the aminated laccase on bifunctional chelate-glyoxyl support al in presence of imidazole. The immobilization was conducted in 500mM phosfhate buffer pH 8 and in presence and assence of 100mM imidazole. **(A)** Structure of metal chelate. **(B)** Structure of imidazole.

In all cases the preparation with the adsorbed enzyme was incubated at alkaline pH in order to increase the reactivity of the amine moieties and increase the formation of multipoint covalent linkages as intense as possible. In all cases the recovered activity after incubation at pH 10 was similar to the immobilized one (table 5).

Support	Immobilization yield (ψ) (%)	Expressed activity (%)
Amine-glyoxyl (TEA)	100	100
Carboxylic-glyoxyl (IDA)	100	100
Chelate-glyoxyl (IDA-Cu)	100	100

Table 5. Immobilization yield and expressed activity of laccase-glyoxyl- heterofunctional derivatives.

In addition to the study of the recovered activity of the immobilized enzyme on different heterofunctional glyoxyl support the stability of them at different pH was studied. The availability of very stable derivatives in different conditions as pH is very important because of the great variability of the reaction media in the industry. It would be interesting to have derivatives with high stability in all kinds of conditions. In this way, the previously obtained derivatives were incubated under different conditions such as different pHs and temperatures. After derivatives incubation at high temperature and different pHs the most stable derivative resulted that where the enzyme was immobilized on agarose-IDA-glyoxyl. This derivative was more stable than the rest and much more stable than the unipunctual at neutral pH as well as at acid pH. However, at pH 9 the stability is similar to that obtained for the derivative immobilized on glyoxyl support (figure 27).

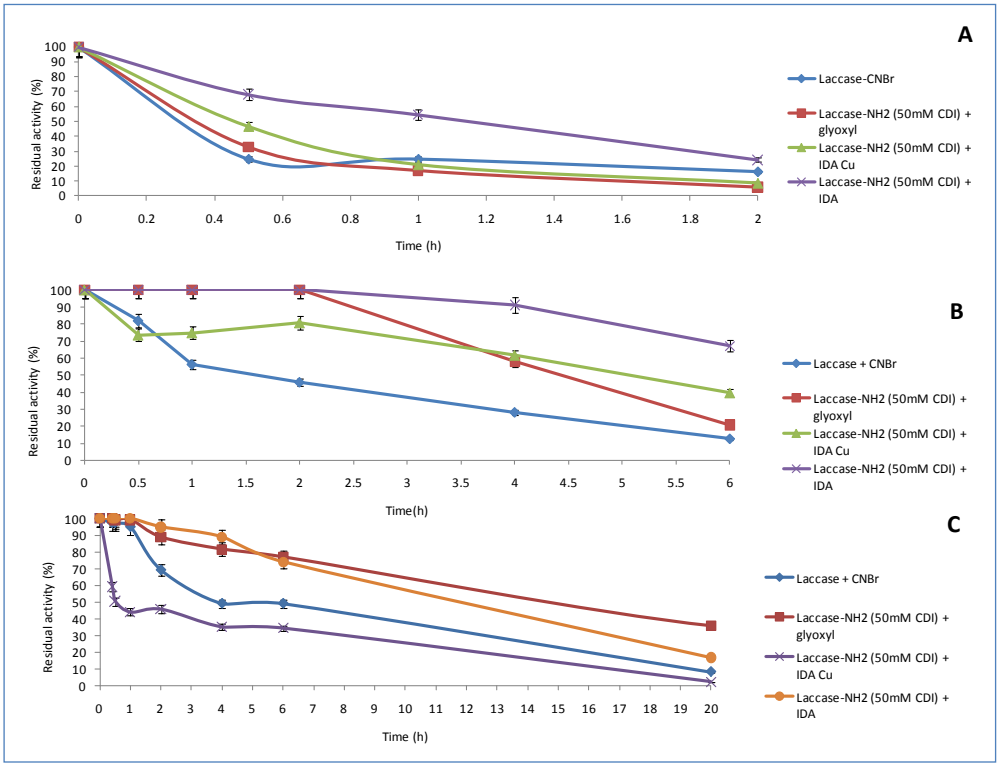


Figure 27. Time-courses of thermal inactivation of different derivative. (A) Inactivation was performed in 25mM sodium acetate at pH 5 and 60°C. **(B)** Inactivation was performed in 25mM sodium phosphate at pH 7 and 60°C. **(C)** Inactivation was performed in 25mM bicarbonate buffer at pH 9 and 55°C.

Summarizing the stabilization factors can be observed in all conditions in table 6. The most stable derivative resulted that of the aminated enzyme immobilized on heterofunctional IDA glyoxyl support there at pH 7 their half-life resulted around 35 times higher than the soluble enzyme.

Laccase derivative	Thermal stabilization fold[a]		
	pH 7 60°C	pH 5 60°C	pH 9 55°C
Lacc-NH_2 (50mM) + Glyoxyl	2.6	1.3	3.6
Lacc-NH_2 (50mM) + IDA	35.3	4	3
Lacc-NH_2 (50mM) + IDA-Cu	3.1	1	0
Lacc + TEA	7.6	N.D.	N.D.

Table 6. Stabilization fold. [a] calculated respect to CNBr agarose derivative in the same conditions.

5.3.6 Optimization of laccase carboxy-glyoxyl derivative

The thermal inactivation of the different immobilized preparations showed that the technique that permitted the best stabilization of the enzyme was the immobilization on carboxy-glyoxyl support (35.3 fold higher half-live, at pH 7 and 55°C). Thus we intended to prepare an optimixed carboxy-glyoxyl derivative with better stability. The treatment with H_2SO_4 for different times (1 and 3 hours) made the partial hydrolysis of the epoxy groups, then this hydrolyzed support was oxidized with $NaIO_4$ and the number of epoxy groups was calculated by the difference in periodate consumption between the hydrolyzed support and the initial epoxy support, before the treatment with iminodiacetic acid. Table 7 shows the percentage of glyoxyl and carboxy groups in the three different supports.

Support	Glyoxyl groups (%)	IDA groups (%)
IDA-initial	50	50
IDA-1h	58	42
IDA-3h	75	25

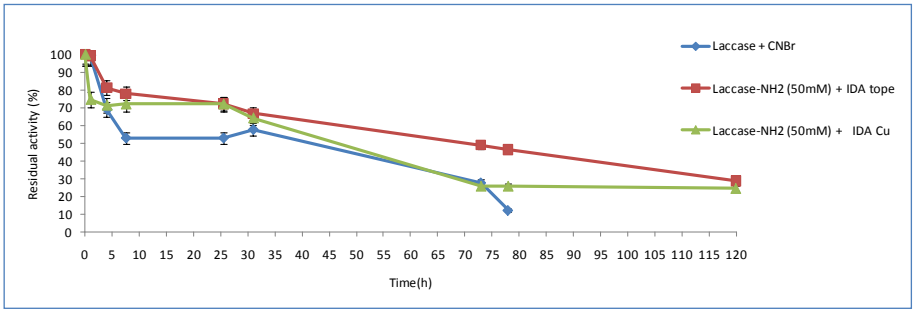
Table 7. Caracterization of the different gluoxyl-IDA support.

Than we immobilized the aminated enzyme on the support named IDA-3h because it was the most hydrolyzed support where the enzyme became adsorbed. In this immobilization both the yield and the recovered activity were 100%. The inactivation of the derivates was performed (IDA-initial and IDA-3h) to study the possible effect of having more glyoxyl reactive groups and consequently the higher multipoint covalent attachment degree on their thermal stability. At pH 7 and 60°C (data not show) both the derivatives had the same inactivation trend and this is an evidence that the bigger quantity of glyoxyl groups in the IDA-3h support was not involved in the derivative stability and not promote an increase of their stabilization.

Moreover, the effect in the stability of the derivatives induced by the incubation time under alkaline pH during the immobilization was also studied. For this proposal the previously adsorbed enzyme on IDA-glyoxyl supports was incubated at pH 10 during 1, 4 and 8 hours. As well as in the other cases, the thermal stability was not improved when assayed at pH 7 and 60°C. This result indicates that all covalent immobilization process possible is produced during the first hour and then there are not more possibilities to increase the multipoint covalent attachment degree.

5.3.7 Stability with denaturant agent: UREA 4M

In the context of the present thesis it was also considered very important the stability of the enzyme and their immobilized derivatives under incubation of other denaturant agents. One of the most traditional denaturant agents constitutes the chaotropic compounds such as urea or guanidine. Many studies have sought to explain the action of denaturants such as urea (Tanford, 1968). These studies suggest three possible mechanisms: (i) direct interactions of urea with the protein; (ii) indirect effects via perturbation of the solvent environment to favor solvation of the hydrophobic residues; and (iii) a combination of direct and indirect effects. Urea promotes unfolding by both indirect and direct mechanisms. Direct urea interactions consist of hydrogen bonding to the polar moieties of the protein, particularly peptide groups, leading to screening of intramolecular hydrogen bonds. Solvation of the hydrophobic core proceeds via the influx of water molecules, then urea. Urea also promotes protein unfolding in an indirect manner by altering water structure and dynamics, as also occurs on the introduction of non polar groups to water, thereby diminishing the hydrophobic effect and facilitating the exposure of the hydrophobic core residues. Overall, urea-induced effects on water indirectly contribute to unfolding by encouraging hydrophobic solvation, whereas direct interactions provide the pathway (Brian et al., 2003). In this case, both the derivatives did not present different stability against this distorting agent and the stabilization fold is quite low (figure 28).



Laccase derivative	Stabilization fold[a]
Lacc-NH_2 (50mM) + IDA	1.65
Lacc-NH_2 (50mM) + IDA-Cu	1.09

Figure 28. Time-course of inactivation in presence of 4M UREA and stabilization fold, [a] calculated respect to CNBr agarose derivative in the same conditions.

These results show that the denaturant agent has a drastic effect on the protein and the immobilization doesn't preserve the enzyme integrity.

5.3.8 Stability in organic solvents

Furthermore, in most of the cases the compounds oxidized by this enzyme are not water soluble and they are solved in organic media. The model solvent used for the inactivation was the dioxane because it is known the deleterious effect produced in most of the assayed and described enzymes (Leutrek et al., 1998; Rogalski et al., 1999; Pickard et al., 1999). In the case of inactivation in organic solvents is very important the use of the unipunctual derivative as initial reference solution because in general soluble enzymes trend to aggregate in the presence of cosolvents. The stability when the different preparations were incubated under 70% of dioxane and 4°C was only slightly improved when the enzyme was immobilized on agarose-IDA-glyoxyl support.

Laccase derivative	Stabilization fold (pH 7, 4°C)[a]
Lacc-NH_2 (50mM) + IDA	2.3
Lacc-NH_2 (50mM) + IDA-Cu	0

Table 8. Stabilization fold, [a] calculated respect to CNBr agarose derivative in the same conditions.

5.4 RESULTS AND DISCUSSION (*Trametes versicolor laccase*)

5.4.1 Purification and immobilization on CNBr activated sepharose

As well as in the case of the laccase from *Myceliopthora thermophila* the initial commercial extract is served with a high purity and a priori would not be necessary its purification at industrial level. However, from the academic point of view we thought it is of great interest the purification of the enzyme using a very simple protocol in order to avoid and eliminate different impurities added to stabilize the commercial preparation that could interfere during the immobilization process. For this the enzyme was incubated in the presence of an anionic exchanger such as DEAE. Thus, in conditions of low ionic strength the enzyme was completely adsorbed in a short time (less than 30 minutes) keeping its catalytic activity unaltered. Finally and after a washing to eliminate the not adsorbed components of the commercial extract the enzyme was desorbed in the presence of 200mM of NaCl. Under these

conditions the enzyme was desorbed from the support recovering 88% of the activity if compare with the initial one. By using the purification protocol in only one step, it was possible to obtain the enzyme with a high purity because besides the elimination of possible contaminant compounds that could interfere in the immobilization process it was possible to eliminate some protein bands present in the starting extract (figure 29).

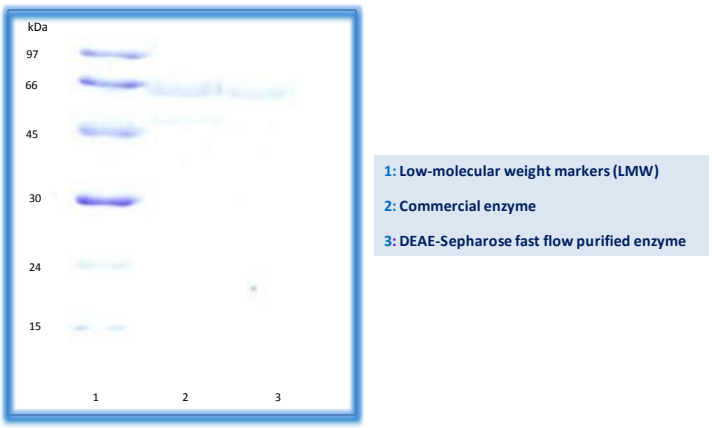


Figure 29. Electrophoretic profile of the purified and not purified soluble enzyme.

Furthermore as it was commented in paragraph 5.3.1 the soluble enzymes can suffer different processes as aggregation, proteolysis and so on that could alter their properties and when the enzymes are evaluated the properties would not correspond with the real ones. Thus as with the other lacasse the enzyme was immobilized under very mild conditions on CNBr activated supports. Thus the obtained derivatives have to keep similar properties compared to soluble enzyme. Under these conditions 40% of the activity was immobilized on the support conserving unaltered its catalytic activity.

5.4.2 Immobilization on glyoxyl supports

As it was commented before glyoxyl supports are in general appropriate for multipoint covalent attachment processes and therefore for stabilization of the 3D structure. Because of this laccase was immobilized on glyoxyl support at pH 10. At this pH most of the lysines of its structure are deprotonated and therefore are able to react with the glyoxyl groups of the support. As well as in the case of laccase from *Myceliopthora thermophila* immobilization was not produced using this support. Again when the aminoacid sequence was checked it was shown that it has 24 lysine moieties homogeneously situated through the entire surface. It was also confirmed that there is not an especially rich place in lysine moieties (figure 30).

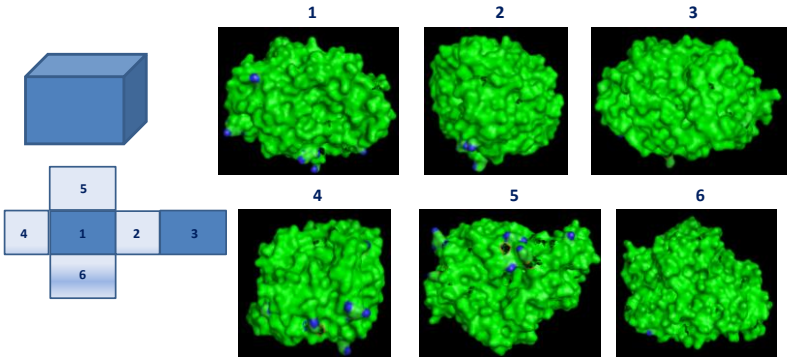


Figure 30. Schematic representation of *Trametes versicolor* surface before ammination process. Lysine residues are in blue on the surface.

In fact faces 4 and 5 are the parts richest in lysines but these lysines would not be able to be in the same plane difficult the immobilization. In addition this enzyme is glycosilated having seven sugar chains (Piontek et al., 2002). This fact difficult even more the immobilization processes. Taking into account this problem it was considered very important the amination of the enzyme promoting the enrichment of the surface into amine groups. Thus the immobilization may be simpler. Again the enzyme was aminated using different concentrations of the carboxile activating reactive, the carbodiimide. The found optimal concentration was 50 mM. Under these conditions the amination of the enzyme was maximal without loosing of catalytic activity. The use of this treatment allows obtaining a new enzyme with 69 aminated and reactive moieties from an enzyme with 24 lysines (figure 31).

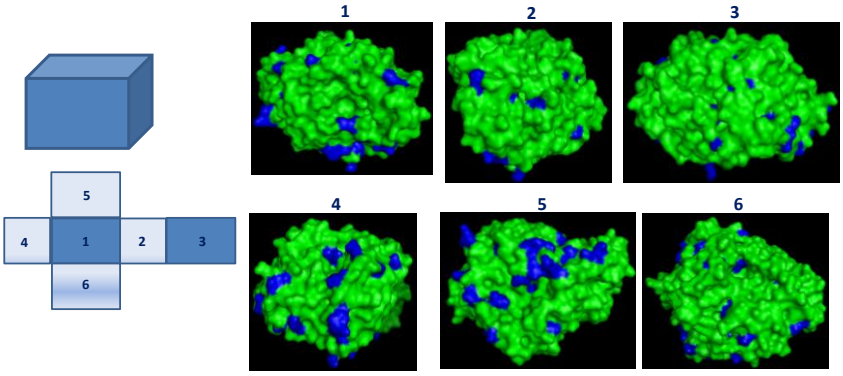


Figure 31. Schematic representation of *Trametes versicolor* surface after amination process. Lysine, aspartic and glutamic acid residues are in blue on the surface.

This new aminated enzyme was incubated at pH 10 in the presence of glyoxyl agarose supports. 80% of the activity was immobilized in less than 1 hour on glyoxyl supports under these conditions; similar results were obtained when enzyme was immobilized at pH 9 (data not shown). This is a normal value taking into account that the pK of the new amine groups is close to 9 and then at both assayed pHs some of the groups are deprotonated and subsequently reactive. The recovered activity after incubation at the indicated pHs was always higher than 60% if compare with the activity previously immobilized (table 9).

Laccase derivative	Immobilization yield (ψ) (%)	Expressed activity (UI/g)	Expressed activity (%)
BrCN-Sepharose®	46	4.38	100
Lacc-NH_2-Glyoxyl pH 10	80	2.9	73
Lacc-NH_2-Glyoxyl pH 9	80	6.6	92.2
Lacc-NH_2-Glyoxyl pH 9, 10	85.5	4.1	59.6

Table 9. Immobilization yield and expressed activity of laccase-glyoxyl derivatives.

Thermal stability was also studied. For this proposal the different preparations were incubated at different pHs and temperatures. In all cases glyoxyl derivatives resulted more stable that cyanogen bromide immobilized preparations. The most stable derivative was that immobilized on glyoxyl supports at pH 10, higher than that obtain for the derivatives previously immobilized at pH 9 (figure 32; table 10). This result indicated that the orientation of the immobilized enzyme was different depending of the immobilization pH because it can also be seen that when the derivative previously immobilized at pH 9 was incubated at pH 10 the obtained

derivative was less stable than the immobilized at pH 10. In addition, it can be obserbed that only when the inactivation was performed at acid pH the resulting stability of the aminated or not aminated cyanogen bromide was similar. On the other hand, at neutral pH as well as at basic pH a destabilization is promoted when the cyanogen bromide preparation was aminated. Similar results were obtained when soluble enzyme was used (data not shown).

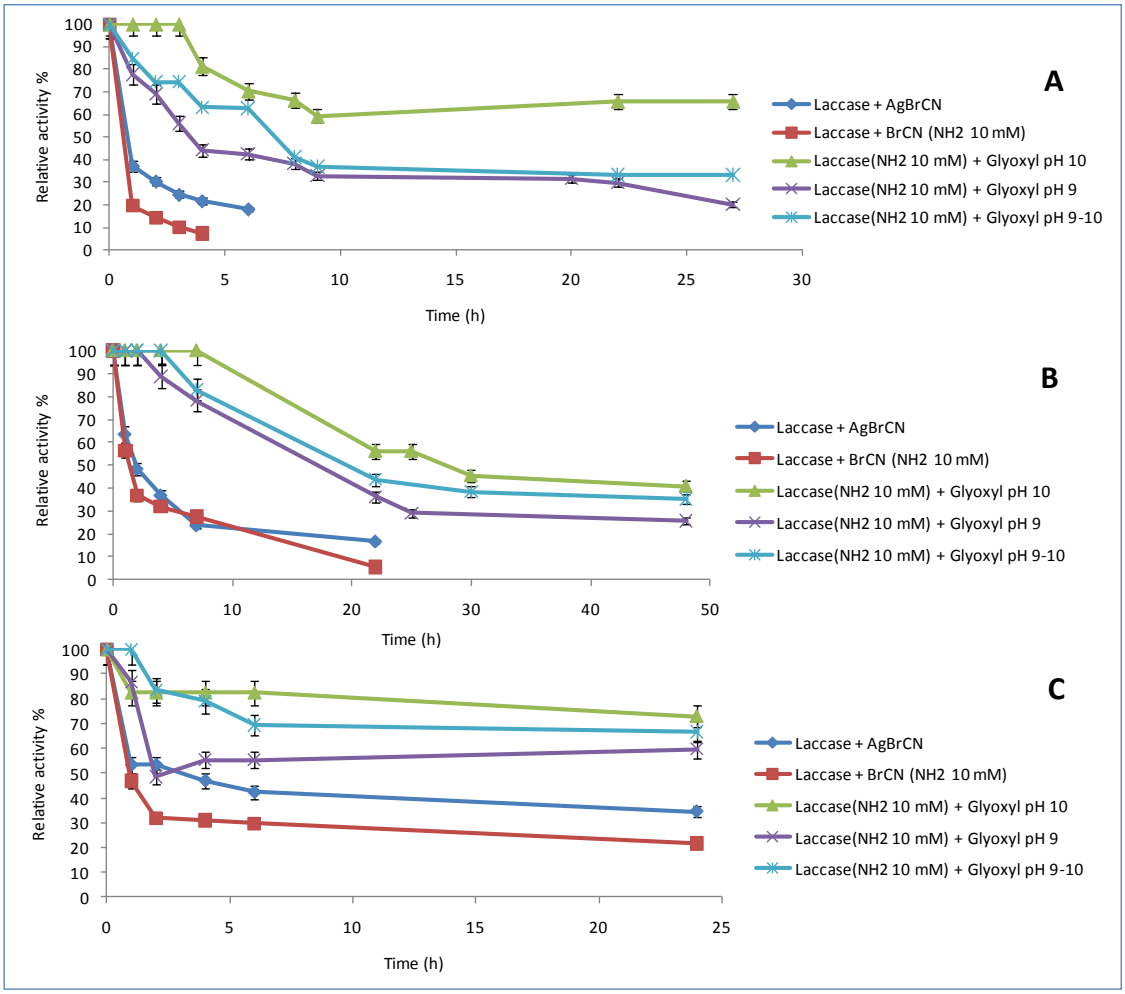


Figure 32. Time-courses of thermal inactivation of different derivative. (A) Inactivation was performed in 25mM sodium phosphate at pH 7 and 55°C. **(B)** Inactivation was performed in 25mM sodium acetate at pH 5 and 50°C. **(C)** Inactivation was performed in 25mM bicarbonate buffer at pH 9 and 40°C.

Laccase derivative	Thermal stabilization fold[a]			Stabilization fold dioxane 70%
	pH 7 55°C	pH 5 50°C	pH 9 40°C	
Lacc-NH_2-Glyoxyl pH 10	57.6	14.6	18.9	2
Lacc-NH_2-Glyoxyl pH 9	4.2	9.1	14	2
Lacc-NH_2-Glyoxyl pH 9, 10	8.4	10.4	15.7	2

Laccase derivative	Thermal stabilization fold[b]			Stabilization fold dioxane 70%
	pH 7 55°C	pH 5 50°C	pH 9 40°C	
Lacc-NH_2-Glyoxyl pH 10	73.5	20.7	77	2
Lacc-NH_2-Glyoxyl pH 9	5.4	13	50	0
Lacc-NH_2-Glyoxyl pH 9, 10	10.8	15	56	2

Table 10. Stabilization fold, [a] calculated respect to CNBr agarose derivative in the same conditions, [b] calculated respect to aminated CNBr agarose derivative in the same conditions.

5.5 CONCLUSIONS

The immobilization of these two laccases presented in this chapter allowed obtaining heterogeneous catalysts with good perspectives to be used at industrial level in different processes of oxidation of products highly contaminants such as effluents from oil factories, treatment of residual waters with recalcitrant compounds and so on. The use of different techniques of immobilization allowed having catalysts with different activities and stabilities for their use in processes in which different conditions of pH, temperature or the presence of denaturant agents (unfolding agents or organic cosolvents) are required.

- The immobilization of laccase from *Myceliopthora thermophila* was performed using different supports due to which it were obtained catalyst with different activities and stabilities.
- Non aminated enzyme only could be immobilized on supports capable to immobilize through the richest place in negative charges as aminated or glutaraldehyde supports.
- The amination of this laccase was performed in a very simple way keeping this enzyme its catalytic activity practically unaltered if compare with soluble not aminated enzyme.
- The immobilization of the aminated enzyme allowed obtaining derivatives more stable than that obtained with the non aminated enzyme. The most stable derivatives in all assayed inactivation conditions were those where the aminated enzyme was immobilized on heterofunctional supports activated with IDA and glyoxyl groups. These derivatives were immobilized through the richest place in positive charges. These derivatives were more than 30 times more stable than soluble enzyme in some of the assayed conditions and in addition resulted slightly more stable against the presence of organic cosolvent or in the presence of unfolding reagents (urea).
- *Trametes versicolor* laccase was able to be purified almost completely in only one step of adsorption-desorption using anionic exchanger supports.
- Previously aminated enzyme was immobilized on glyoxyl supports obtaining derivatives with different stabilization factors depending of the incubation conditions during the immobilization process. The best obtained stabilizations were obtained after incubation at pH 10 where it was possible to obtain stabilization factors over 70 times if compared with the stability of the soluble enzyme or the correspondent one point immobilized derivative (cyanogen bromide support). In addition the best derivatives were at least twice more stable against organic solvents.

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6. OVERALL CONCLUSIONS

The application of polymerin, the polymeric organic fraction recovered from olive oil mill wastewater (OMW), as a bio-filter for remediation of point source-polluted waters is investigated. Althought polymerin has good sorption properties against the three investigated hercicides, cyhalofop acid, MCPA and simazine, its use as a biosorbent presents some technological and economical limitations, because of its high solubility in water and relative low molecular weight, an ultrafiltration system is necessary for a remediation processes. Polymerin doesn't appear suitable for complete purification of waters individually contaminated with the herbicides and many sorption cycles are required for the abatement of the concentration of toxic compounds, leaving still a residue.

Ferrihydrite allows the almost total removal of CyA from contaminated waters by two sorption cycles. In addition, it can be prepared more easily and at lower cost than ferrihydrite-polymerin complex and polymerin.

Among the new generation of investigated mesoporous mineral matrices, Al_2O_3, because of its chemical composition and mesoporous structure, results a sorbent highly efficient than polymerin and Fe_2O_3 to remediate wastewaters contaminated with MCPA and simazine allowing the total removal of MCPA by four sorption cycles and a significant removal of simazine after only two sorption cycles.

An additional benefit arising from the use of mineral matrices is the employment of a simple centrifugation procedure for the separation of purified waters; moreover Al_2O_3, being regenerated by incineration, can be considered for small-scale treatment systems and industrial scale.

A further innovative strategy, aimed to enhance the valorisation of OMW, concerns their use as grow media for oleaginous microorganisms, for potential biodiesel production, so this use could be a convenient strategy to avoid the negative environmental impact associated with its discharge. Microbial conversion of OMW study has highlighted that the accumulation of lipids by *L. starkeyi* may occur in the presence of OMW with no preliminary treatments and without external organic supplement. The growth of the yeasts is associated with a significant reduction of both the TOC and the total phenols content. The increase of the GI of the OMW after the biological treatment demonstrates the environmental benefits that can be achieved by this process. The use of diluted wastewaters increases the fraction of the organic compounds (TOC, total phenols, and proteins) removed, although the kinetics of the biomass growth is slower. The lipid concentration (between 20 and 30%) in the microorganisms increases in the course of the OMW treatment, particularly in the presence of the preliminary diluted feedstock. The fatty acids distribution demonstrates the usability of the lipids accumulated in the *L. starkeyi* as feedstock for biodiesel synthesis.

Finally, we have investigate the effect of immobilization of two laccase, from the fungi *Myceliophthora thermophila* and *Trametes versicolor*, on different supports. These enzymes are able to degrade a wide array of substrates, abundant in olive oil mill wastewaters, such as phenols, aromatic amines, benzenethiols, hydroxyindoles and phenothiazinic compounds. The use of different techniques of immobilization has allow having catalysts with different activities and stabilities for their use in processes in which different conditions of pH, temperature or the presence of denaturant agents (unfolding agents or organic cosolvents) are required.

The immobilization of laccase from *Myceliopthora thermophila* was performed using different supports by which catalyst with different activities and stabilities were obtained. Non aminated enzyme only could be immobilized on supports capable to immobilize through the richest place in negative charges as aminated or glutaraldehyde supports. The amination of this laccase was performed in a very simple way, so the immobilization of the aminated enzyme allowed obtaining derivatives more stable than that obtained with the non aminated enzyme. The most stable derivatives in all assayed inactivation conditions were those where the aminated enzyme was immobilized on heterofunctional supports activated with iminodiacetic acid (IDA) and glyoxyl groups.

As regards *Trametes versicolor* laccase, the enzyme was first aminated and than immobilized on glyoxyl supports obtaining derivatives with different stabilization factors depending of the incubation conditions during the immobilization process. The best stabilizations were obtained after incubation at pH 10 where it was possible to obtain stabilization factors over 70 times if compared with the stability of the soluble enzyme or the correspondent one point immobilized derivative (cyanogen bromide support). In addition the best derivatives were at least twice more stable against organic solvents.

In conclusion, in the present thesis different strategies aimed at a biotechnological employment of olive oil mill wastewaters have been proposed, in order to remediate water polluted from pesticide and, at the same time, to valorize this waste using it for potential biodiesel production. Moreover, among the technologies developed to disposing of OMW, the use of laccases immobilized on new generation supports is an innovative and great important strategy.

APPENDIX

ATTIVITA' DI RICERCA SVOLTE IN LABORATORI STRANIERI

- Stage presso il Consejo Superior de Investigaciones Cientìficas (CSIC), Instituto de Catàlisis y Petroleoquìmica, Campus Universidad Autònoma, Madrid, tra il 01/09/2009 e il 31/07/2010, direttori della ricerca: prof. Jose M. Guisàn e dott. Cèsar Mateo.

PUBBLICAZIONI SU RIVISTE SCIENTIFICHE INTERNAZIONALI

- Filomena Sannino, Marianna Iorio, **Veria Addorisio**, Renato Capasso. (2009). Comparative Study on the Sorption Capacity of Cyhalofop Acid on Polymerin, Ferrihydrite, and on a Ferrihydrite-Polymerin Complex. Journal of Agricultural and Food Chemistry , 57, 5461-5467.

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PARTECIPAZIONI A CONVEGNI

- PhD Schools in Scienze Molecolari e Biotecnologie Agrarie, Alimentari e Ambientali "Genetic resources and physiology for a sustainable production in agriculture", Parco tecnologico padano, Lodi, 24-27 Giugno 2008
  "Biotechnological employment of olive mill wastewaters for the decontamination of polluted waters from agrochemicals and the production of biodiesel through unicellular algae"
  Veria Addorisio. B1
- Società Italiana di Chimica Agraria, XXVI Convegno Nazionale "Il sistema suolo-pianta tra emergenze ed opportunità", Palermo 30 Settembre - 03 Ottobre 2008.
  "Adsorbimento del Cyalofop acido su ferridrite e un complesso organo-minerale $Fe(OH)_X$-polimerina"
  Sannino Filomena, **Addorisio Veria**, Iorio Marianna, De Martino Antonio, Capasso Renato. Pag. 128

- Società Italiana di Chimica Agraria, XXVI Convegno Nazionale "Il sistema suolo-pianta tra emergenze ed opportunità", Palermo 30 Settembre - 03 Ottobre 2008.
  "Adsorbimento di erbicidi su sostanza oragnica solubile e su nano particelle di Al_2O_3 e Fe_2O_3"
  Addorisio Veria, Sannino Filomena, Iorio Marianna, De Martino Antonio, Capasso Renato. Pag. 102
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  Filomena Sannino, **Veria Addorisio**, Antonio De Martino, Renato Capasso. Pag. 296
- Società Italiana di Chimica Agraria, XXVII Convegno Nazionale, Matera 15-18 Settembre 2009.
  "Decontaminazione di acque inquinate da MCPA e simazina mediante adsorbimento su nano particelle di Al_2O_3"
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  Veria Addorisio, Serena Esposito, Filomena Sannino. Pag. 50