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Laccases from new fungal sources
and some promising applications

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Department of Biotechnology
Doctoral Thesis
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Academic thesis which, by due permission of the Faculty of Engineering of Lund University, will be publicly defended on Monday, February 28 at 13:00. in Lecture Hall B, at the Center for Chemistry and Chemical Engineering, Sölvegatan 39, Lund, for the degree of Doctor of Philosophy in Engineering.

Faculty opponent: Professor Helena Maria Pinheiro, Centro de Engenharia Biológica e Química, Portugal.

Title and subtitle
Laccases from new fungal sourcesand new promising applications

Abstract

Fungi are a group of organisms with the ability to produce different types of enzymes in order to get access to nutrients. Among the enzymes, oxidoreductases have the ability to degrade lignocellulosic material via an oxidative mechanism, facilitating the uptake of cellulosic material, which will be metabolized using other enzymes to provide the required nutrients to the fungi. Ecologically, oxidoreductases play an essential role in the mobilization of carbon into the ecosystem. Laccase is an oxidative enzyme that has the ability to oxidize lignin using molecular oxygen, which is reduced to water. The oxidative ability of laccases is employed in a number of industrial and environmental applications including bioremediation, food technology, nanotechnology, medicine, pulp and paper industry, and cosmetology. Owing to its versatility, laccase is continuously under investigation for new applications.

The present thesis reports on new sources of fungal laccase as well as novel laccase applications.

Fusarium sp. BOL35 is an ascomycete fungus, isolated from the Bolivian Amazonas. It was found to produce laccase when it was cultivated to degrade benzo[a]pyrene (a polycyclic aromatic hydrocarbon) indicating a possible potential of laccase in the degradation of xenobiotic compounds.

Galerina sp. HC1 was also isolated from the Bolivian Amazonas and was selected as a laccase producer. This fungus is easy to grow in submerged cultivation as well as in solid state fermentation for laccase production. *Galerina* sp. HC1 laccase has been shown to have a potential application in dye decolorization as well as in demethylation of lignin, which opens up the possibility to use this fungal laccase in various biotechnological applications.

Trametes versicolor laccase has been used to decolorize textile dyes in a membrane reactor system. The enzyme is capable of catalyzing fast decolorization reactions in the presence of natural mediators. Moreover, it is able to catalyze decolorization in the presence of an immobilized mediator, opening up the possibility of recycling both the enzyme and the mediator.

Trametes versicolor laccase has in the presence of a mediator (TEMPO) been shown to catalyze oxidation of primary alcohols on polysaccharide gel to produce the corresponding aldehydes. The oxidized Sepharose was applied for protein immobilization and was found to be as effective as the gel oxidized with perioda te.

Key words
Fungi; ligninases; laccase applications; dye decolorization; modification of lignin; modification of primary alcohols

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List of Papers

The thesis is based on the following papers, referred to in the text by their Roman numerals. The papers are attached as appendices at the end of the thesis. Reprints are published by kind permission of the editors/publishers concerned.

I. Characterization of *Fusarium* sp. strain BOL35 as *Fusarium santarosense* sp. nov. isolated in the Bolivian jungle and its ability to remove benzo[a]pyrene in oil polluted soil.

   Enrique Terrazas, Teresa Alvarez, Laura Mendoza, Alberto Gimenez, Mattiasson Bo

   To be submitted

II. Laccase from *Galerina* sp. HC1: production and application in dye decolorization

   Laura Mendoza, Victor Ibrahim, Teresa Alvarez, Gashaw Mamo, Rajni Hatti-Kaul

   To be submitted

III. Blue laccase from *Galerina* sp.: Properties and potential for Kraft lignin demethylation

   Victor Ibrahim, Laura Mendoza, Gashaw Mamo, Rajni Hatti-Kaul

   Process Biochemistry (2011) 46: 379-384

IV. Decolorization of dyes by laccase/mediator system in a membrane reactor

   Laura Mendoza, Maria Jonstrup, Rajni Hatti-Kaul, Bo Mattiasson

   Submitted for publication

V. Laccase mediator system for activation of agarose gel: application for immobilization of proteins

   Laura Mendoza, Gashaw Mamo, Ninoska Flores, Alberto Gimenez and Rajni Hatti-Kaul

   Journal of Molecular Catalysis B: Enzymatic (2011) 68: 270-274

My Contribution to the Papers

Paper I.   I collected, isolated and maintained the strain, and assisted in important aspects of the experimental work. I participated in the corrections of the manuscript.

Paper II.   I collected and isolated the strain from the Amazonas region from Bolivia. I planned the work together with the co-authors, designed and performed the experimental work together with Victor Ibrahim. The results were discussed with the co-authors. I wrote the first draft of the manuscript and I participated in the corrections.

Paper III.   Victor Ibrahim planned the work. I did part of the experimental work and participated in writing and correcting the manuscript.

Paper IV.   I planned the work with Maria Jonstrup. I and Maria divided the experimental work as well as the calculations of the data amongst us. The results were discussed with all the co-authors. I wrote the first draft of the manuscript.

Paper V.   I planned the work with Rajni Hatti-Kaul based on her idea, and did all the experimental work. The results were discussed with the co-authors. I wrote the first draft of the manuscript and was responsible for correcting it after comments by the co-authors.

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1

Introduction

Fungi are a vast group of eukaryotic organisms including yeast, molds and mushrooms that have a significant role in nature by breaking down organic material, and have importance for humans as producers of important antibiotics, enzymes, foods, etc. on one hand, and as agents of food spoilage, plant and animal diseases on the other. They are the only organisms that are able to completely mineralize lignocellulose, the most abundant recalcitrant renewable material available in nature. They do so by producing several sets of enzymes for breaking down polysaccharides, celluloses and hemicelluloses as well as lignin, a natural aromatic polymer. Ligninases or ligninolytic enzymes constitute a group of oxidoreductases that are highly specialized in polymerization as well as in the degradation of lignin. These enzymes are mostly produced by the so called white-rot fungi and litter-decomposing fungi.

Laccase is one of the three main ligninases, and differs from the others in its ability to catalyze the oxidation of lignin components using molecular oxygen as the electron acceptor, which in turn is reduced to water. In order to bring about the degradation of complex structures, laccases make use of small molecules as electron mediators both in nature and in the applications developed by humans. Laccase is among the oldest known enzymes. Laccase activity was first discovered in 1883 in the sap of the Japanese lacquer tree *Rhus vernicifera*, leading to its polymerization and the product is used commonly as Japanese lacquer varnish. Since then, the oxidative activity of laccase has been used in a number of industrial and environmental applications. Although laccases originate from different sources, those from fungal origin are used in most of the applications.

Laccases from a large number of fungi have been studied. These enzymes offer great variability in terms of induction mechanisms, degree of polymorphism, expression of different isoenzymes, and physico-chemical and catalytic properties. This variability determines the application for the isolated laccases, and depends on whether the enzyme has a potential to oxidize a substrate, or whether it is robust enough to be used in a certain application. Hence, the search for fungal laccases with different properties and potential applications is still on-going. It is expected that new isolated enzymes may catalyze old known processes in a better way (i.e., remediation of organic pollutants) or may oxidize new substrates for novel applications.

1.1 Scope of the thesis

The present thesis describes new sources of fungal laccase as well as some novel potential applications for laccases.

Chapter 2 provides a description of Fungi, their applications as well as their capacity to produce extracellular enzymes and their role in the modification of lignocellulosic material. The chapter focuses on the principal enzymes involved in lignin modification.

Chapter 3 addresses laccase and includes a description of reported sources of laccase. It also presents the fungal laccases that were used in this thesis and describes the properties and structure of the enzyme, which is related to its catalytic mechanism. Furthermore, the chapter deals with the mechanisms of laccase and a laccase/mediator system, which is the basis for understanding the possible applications of this class of enzymes. It also contains strategies for laccase production based on submerged cultivation and solid state fermentation.

Chapter 4 describes the existing and potential applications of the laccase and/or laccase-mediator system. Decolorization of dyes and oxidation of alcohol groups on polysaccharides are primarily addressed. The laccase application in these two processes is compared to traditional methods, in order to determine the advantage of using the enzyme. In addition, the chapter describes a system for laccase and mediator recycling during decolorization of dyes as well as a potential application of the oxidized alcohol-containing substrate for the immobilization of ligands.

Finally, chapter 5 includes conclusions based on the work performed in this thesis and some future perspectives.

Fungi – A Useful Source of Oxidative Enzymes

Fungi constitute a kingdowm including eukaryotic organisms such as mushrooms, yeasts and molds. The principal characteristics include the presence of cells walls, basically containing chitin and glucans, their heterotrophic behavior and the ability to produce extracellular hydrolytic enzymes in order to obtain the nutrients (Barr 1992), which can be absorbed through the cell wall and cell membrane. From an evolution point of view, fungi are believed to be closer to animals than plants, since they have chitin rather than cellulose structures, store nutrients such as glycogen and produce spores (similar to gametes) (Barr 1992). Their life cycle includes the formation of spores, germination, the development of hyphae and mycelia and the growing of fruit body (Figure 2.1) (Carlite et al 2001).
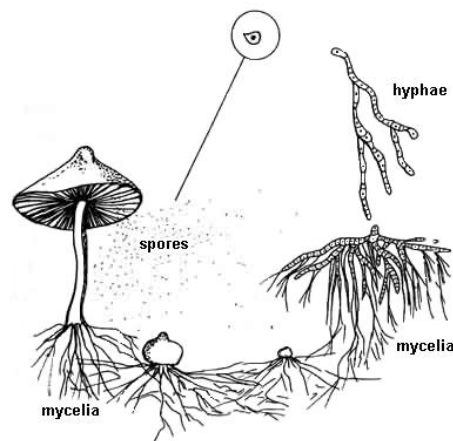
Figure 2.1 Life cycle of fungi.

The above-mentioned characteristics of fungi determine their expansion over considerably long distances and even their vast distribution all over the world, including in extreme conditions such as deserts, salty environments and deep sea sediments. Around 70 000 species of fungi have been described; however, it is estimated that 1.5 million species might exist. Most of the fungal species occur as saprophytes (which use carbon fixed by other organisms) in wood, soils, leaf litter and animals, while others are biotrophs, which means that they form symbiotic associations with plants (mycorrhizae and endophytes), animals, algae (lichens) and prokaryotes.

As far as is known, fungi include 7 phyla (or divisions): Microsporidia, Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Glomeromycotina (before Zygomycota), Ascomycota and Basidiomycota (Hibbett et al 2007). Ascomycota are among the principal fungal pathogens of humans and plants, but they are also the principal source of chemical compounds with high commercial value. On the other hand, Basidiomycota include around 37% of all the described species of fungi and can participate in the decay of dead organic matter, which represents a key step of the carbon cycle in the ecosystem. At the same time, it is a negative economic factor for humans when some species of Basidiomycota attack wood in buildings and other structures (Carlite et al 2001).

Fungi are among the most useful organisms for industry and biotechnology. First of all, they are the main agents responsible for decomposition of lignocellulosic material and thus for mobilizing the carbon into the ecosystem. They are also involved in bread and alcohol production (Kavanagh 2005), cultivation of edible mushrooms (Barbado 2003), production of compounds with biological activity like antibiotics (cephalosporin and penicillin from *Cephalosporium acremonium* and *Penicillium chrysogenum,* respectively), enzymes (Table 2.1), cholesterol-lowering agents (lovastatin from *Monascus ruber* and mevastatin from *Penicillium citrinum*), organic acids (itaconic, malic and fumaric acids from *Candida* spp.), vitamins (riboflavin and ascorbic acid from *Candida* spp.), amino acids (lysine, tryptophan and phenyalanine from *Saccharomyces* sp., *Hansenula* sp. and *Rhodoturula* sp., respectively), recombinant therapeutics (insulin using *Saccharomyces cerevisiae*), recombinant enzymes, and immunosuppressing drugs (cyclosporin A from *Tolypocladium inflatum*) (Murphy & Horgan 2005).

2.1 Lignocellulose degrading fungi

Certain groups of fungi are able to efficiently degrade lignocellulosic material. These are divided into four categories: stain fungi, soft-rot fungi, brown-rot fungi and white-rot fungi (Martínez et al 2005).

Stain fungi and soft-rot fungi are ascomycetes and are unable to degrade lignin. Brown-rot fungi are exclusively basidiomycetes (Martínez et al 2005) and produce enzymes to break down cellulose and hemicelluloses, and partially also lignin of soft-wood (Tanesaka et al 1993). Generally, brown-rot fungi initiate the depolymerization of structural polysaccharides of lignocellulose with the production of extracellular reactive oxygen species (ROS), which reduce Fe^{3+} and subsequently produce degradative hydroxyl radicals (Wei et al 2010). Once the lignocellulosic structure is partially broken, brown-rot fungi produce cellulases to degrade cellulosic material. That is the reason why the remaining substrate is left as a brown shrunken

tissue. All these catalytic mechanisms for degradation make the brown-rot fungi useful in a variety of applications, e.g., in the treatment of textile wastewater (Gomaa et al 2010), in the degradation of pesticides (Purnomo et al 2010) and in the production of metabolites from cellulose (Tewalt & Schilling 2010).

Finally, white-rot fungi include basidiomycetes and ascomycetes and are able to produce enzymes to act on the cell walls and lignin of hard- and soft-woods. Consequently the decayed material will be seen as soft, moisturized and spongy white tissue (Tanesaka et al 1993; Martínez et al 2005). The fungi in this group are able to secrete a bulk of enzymes, which are directly involved in lignin modification. These enzymes are named ligninases or usually only referred to as Lignin Modifying Enzymes (LME) and belong to the group of oxidoreductases. They are described in Section 2.3 and Chapter 3.

2.2. Lignin: understanding the role of fungal oxidative enzymes

Lignin is the second most abundant polymer on Earth. It is mostly concentrated in the middle lamella of plants, where it acts as cement between wood fibers, but it can also be found in the cell wall, forming an amorphous matrix together with hemicelluloses (Hammel et al 1997). It is basically composed of p-hydroxyphenyl, guaiacyl and syringyl units (Figure 2.2a) combined with monomers of lignin (Figure 2.2b). The complexity and recalcitrance of lignin among the species will depend on how these units are combined as well as the degree of their polymerization. Softwoods (gymnosperms) have the highest lignin content, and their lignin is mostly composed of guaiacyl. On the other hand, hardwoods (angiosperms) consist mainly of syringyl, guaiacyl and low traces of p-hydroxyphenyl (Martínez et al 2005).
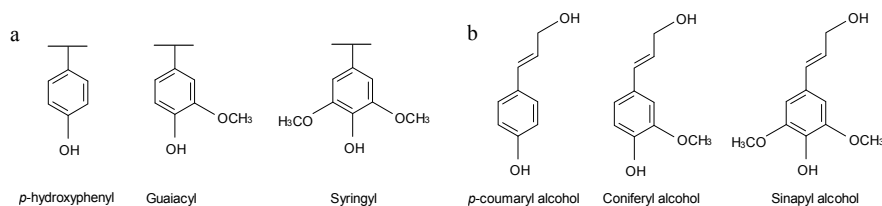
Figure 2.2 Basic structures for lignin biosynthesis. a) Structural units of lignin; b) Monomers of lignin or monolignols.

Table 2.1 Fungal enzymes and their applications in industry and biotechnology

Enzyme	Source	Fungal application	Industrial application	Reference
Acylase	*Aspergillus niger*	Not determined	Deacylation of penicillin	Bashir et al. 2008
Glucoamylase	*Saccharomyces cerevisiae*	Hydrolysis of carbohydrates to obtain glucose	Starch modification for ethanol production	Yan et al 2011
Catalase	*Aspergillus phoenicis*	Mechanism of defense against hydrogen peroxide	Removal of hydrogen peroxide used for bleaching	Kacem-Chaouche et al 2005
Cellulase	*Trichoderma spp.*	Hydrolysis of carbohydrates to obtain glucose	Production of bioethanol	Schuster & Schmoll 2010
Glucose oxidase	*Aspergillus niger*	Generation of hydrogen peroxide	Food additive in dairy, baking and alcohol fermentation, production of gluconic acid and glucose sensors.	Ming et al 2008
Lactase	*Kluyveromyces lactis*	Hydrolysis of lactose	Degradation of lactose in dairy products	Coenen et al 2000
Lipase	*Rhizomucor miehei*	Hydrolysis of lipids	Chemical synthesis: esterification of fatty acids and resolution of chiral compounds	Alcántara et al 1998
Protease	*Rhizopus SMC*	Hydrolysis of protein	Milk processing for the hydrolysis of casein and tannery industry to produce smoother and shiner leather	Ramamurthy et al 1991
Ligninases	*Anthracophyllum discolor, Fusarium* sp.BOL35 *Trametes versicolor* and *Galerina sp.*HC1	Modification of lignin	Biodegradation of polycyclic aromatic hydrocarbons	Acevedo et al 2011
			Decolorization of textile dyes and aldehyde production	**Paper I-V**

Lignin decomposition is an indispensable step to recycle carbon in the ecosystem and this is where fungi can play an important role.

2.3 Lignin modifying enzymes

Among the fungal enzymes, ligninases are oxidoreductases that are involved in humification of soils, and catalyzing the oxidative transformation of plant debris and lignin (Zavarzina et al 2011). This ability has been used in biotechnology to modify a number of substrates.

The oxidoreductase mechanism involves exchange of electrons or redox equivalents between donor and acceptor molecules (Xu 2005). The major fungal redox enzymes involved in lignin modification are lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), other peroxidases, laccases and tyrosinase. Most of these enzymes can perform polymerization of lignin; however, in the presence of mediators they can catalyze its degradation. Table 2.2 shows a comparison of the principal characteristics of these fungal oxidoreductases.

Table 2.2. Some properties of fungal oxido-reductases.

Property	LiP	MnP	Peroxidase	Laccase	Tyrosinase
Redox Potential (V)	1.2-1.5	≈1.1	≈1.0	0.4-0.9	0.26-0.35
pH optimum	2.5-3.5	4.0-4.5	≈5.5	3.0-6.0	5.0-7.0
pI	3.2-4.7	2.8-7.2	≈3.5	≈4.0	4.5-8.5
MW (kDa)	38-46	38-50	40-45	40-130	30-105
Native mediators	Veratryl alcohol	Mn^{2+}; Mn^{3+}	--	3-HAA	--
Synthetic mediators	None	Thiols, unsaturated fatty acids	--	ABTS, HBT Syringal dazine	--
Main producers	←---------------------White-rot basidiomycetes---------------------→				
		←-- Litter-decomposing basidiomycetes, ectomicrorrhizae --→			
				←Basidiomycete, Ascomycete, lichens→	

3-HAA: 3-hydroxyanthranilic acid; ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); HBT: 1-hydroxybenzotriazole

Tyrosinase (EC 1.14.18.1) is an oxidoreductase enzyme, capable of acting as two types of catalysts using molecular oxygen: monophenol oxidase and catecholase. As monophenol oxidase (phenolase), it can oxidize phenols to *o*-quinones and produce *o*-diphenols, and as catecholase (diphenolase), *o*-diphenols are oxidized to *o*-quinones. Some biotechnological applications include biosensors for phenol, catechol and *p*-cresol detection (Rajesh &

Kaneto 2005), synthesis of antioxidants highly valuable in food applications, removal of phenols in wastewaters (Halaouli et al 2005), and modification of polymers by addition of quinones to chitosan (Demolliens et al 2008).

Laccases (EC 1.10.3.2) (*p*-diphenol: dioxygen oxidoreductases; benzenediol dioxygen oxidoreductases) are oxidative enzymes using molecular oxygen as the electron acceptor, which oxidize the phenolic units of lignin (Claus 2004; Riva 2006). Laccases will be described in detail in Chapter 3.

Peroxidases (EC 1.11.1) are hemoproteins, widely distributed in nature, produced by a number of microorganisms besides fungi (Azevedo et al 2001). They catalyze the oxidation-reduction reaction with hydrogen peroxide as the catalyst (Durán & Esposito 2000). Fungal peroxidases include lignin peroxidase (LiP), which has a redox potential higher than other types of peroxidases, and manganese peroxidase (MnP) that catalyzes the oxido-reduction reaction by generating Mn^{3+} from Mn^{2+}. Versatile peroxidase (VP) combines the catalytic properties of LiP and MnP, and is hence able to oxidize compounds in the presence or absence of Mn^{2+} ions (Rodríguez et al 2004). Haloperoxidases are the largest and most extensively studied group of peroxidases since they are able to catalyze halogenation of organic substrates (Preobrazhenskaya et al 2006).

Other oxidoreductases indirectly involved in lignin degradation include glyoxal oxidase, superoxide dismutase, glucose oxidase, aryl alcohol oxidase, aryl alcohol dehydrogenases and quinone reductase. The former four enzymes supply hydrogen peroxide for catalysis by the peroxidases (Barr & Aust 1994; Lan et al 2006). Aryl alcohol dehydrogenase reduces intermediates that are used to generate hydrogen peroxide, whereas quinone reductase participates in the production of oxygen reactive species to generate hydroxyl free radicals to attack lignin.

Figure 2.3 describes the participation of peroxidases, laccases and other oxidoreductases in the modification of lignin. Peroxidase and laccase are involved in the generation of aromatic radicals (1), which evolve in different non-enzymatic reactions like demethoxylation (2) and cleavages on Cα-Cβ (3), aromatic (4) and C4-ether (5) bonds. The aromatic structure released in (3) can be re-synthesized de novo by other fungal enzymes (6, 7) to generate hydrogen peroxide. On the other hand, the aromatic structures released from C4-ether breakdown (5) can re-polymerize on lignin (8), if they are not first reduced by dehydrogenases (9) to form phenolic compounds. The latter can be re-oxidized by laccases or peroxidases (10). The aromatic structures produced in (5) can also suffer non-enzymatic Cα-Cβ breakdown (11) ending with the formation of *p*-quinones. The produced quinones contribute to

oxygen activation in redox cycling reactions involving quinone reductases, laccases and peroxidases (12). The activated oxygen generates the reduction of Fe^{3+} (13), which leads to the activation of hydrogen peroxide to form hydroxyl free radicals (OH·) (14) that are strong enough oxidizers to attack the lignin (15) (Martínez et al 2005).

The ability of these enzymes to directly or indirectly modify a complex structure like lignin makes them very attractive for the modification/degradation of different kinds of substrates. The high redox potential and the reduction of peroxides at the expense of electron-donating substrates render peroxidases useful in a number of biotechnological applications such as bioremediation of soil and water, and biosensors to detect toxins and other analytes (Regalado et al 2004). However, one of the principal limitations when it comes to using peroxidases in biotechnology is related to the additional cost of hydrogen peroxide as well as its negative effect on the enzyme stability (Torres et al 2003). On the other hand, most of the other oxidoreductases mentioned above need co-factors in order to catalyze reactions. The high cost of such co-factors poses a major disadvantage and limitation for the application of these enzymes in large-scale processes. Consequently, laccases are highly attractive for utilization in biotechnology since they employ oxygen to oxidize the substrates and produce water as the only by-product.
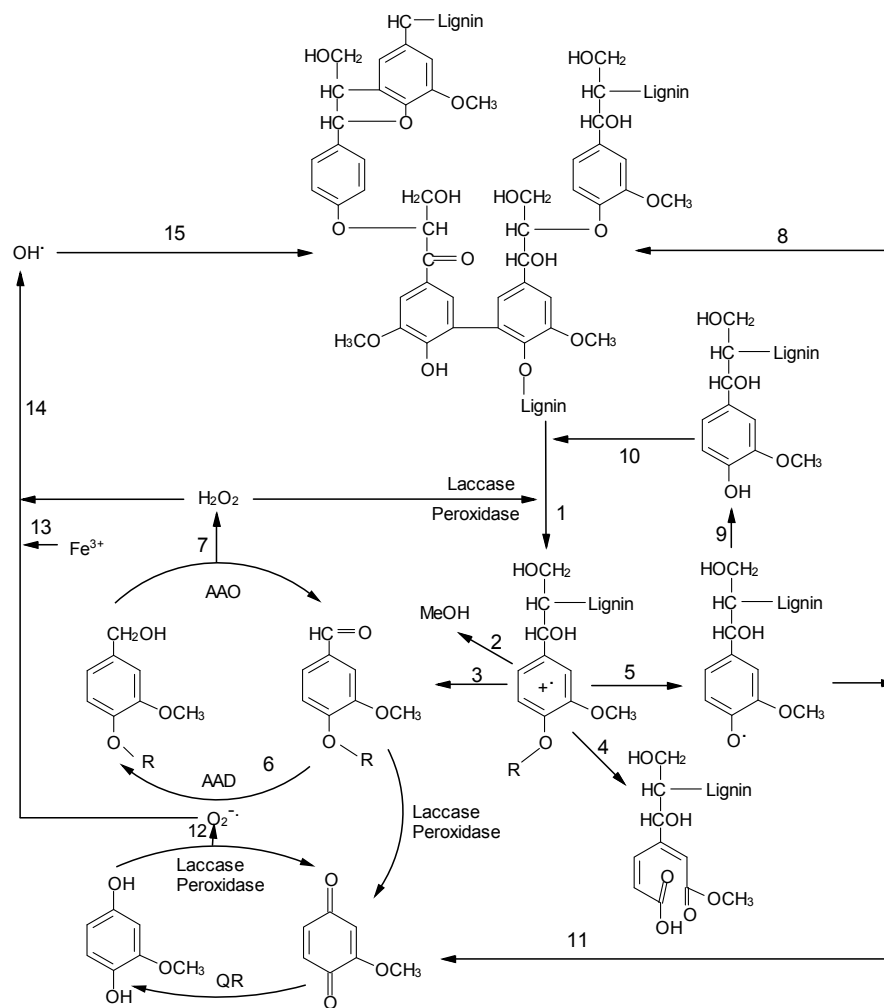
Figure 2.3 Modification of lignin by laccase, peroxidase and other enzymes. AAO: aryl-alcohol oxidase; AAD: aryl-alcohol dehydrogenases; QR: quinone reductases (QR). Modified from Martínez et al 2005.

Laccase: An Important Member of Oxidoreductases

Laccases are ubiquitous enzymes present in higher plants, bacteria, fungi, insects and lichens (Arakane et al 2005; Riva 2006; Lisov et al 2007). The first report dates from 1883 when Yoshida detected laccase-like activity in *Rhus vernicifera* (O´Malley et al 1993). However, it was not until 1962 that laccase was designated a *p*-diphenol oxidase (Benfield et al 1964), and accepted as part of the lignification process in plants (O´Malley et al 1993).

3.1 Sources
3.1.1 Plants
Structural analysis of plants together with the development of new techniques over the years have helped to confirm that plant laccases participate in the early steps of lignification by catalyzing the oxidation of monolignols (Figure 2.2b). They are however not able to act on structures with higher complexity such as phenolic configurations with multiple aromatic rings (O´Malley et al 1993). Besides lignification, plant laccases play an important role in wound healing and in the mechanism of defense against external conditions (Dwivedi et al 2011).

Some examples of plant sources from which laccases have been isolated include sycamore maple (*Acer pseudoplatanus*), loblolly pine (*Pinus taeda*) (O´Malley et al 1993), *Rhus vernicifera* (Benfield et al 1964) and *Populus euramericana* (Ranocha et al 1999).

3.1.2 Bacteria
In bacteria, the first reported laccase was found in *Azospirrullum lipoferum*, where laccase was associated with the melanin production for cell pigmentation (Faure et al 1994). In other bacterial species, it was related with morphogenesis (Endo et al 2002) or the resistance of spores against hydrogen peroxide and UV (Held et al 2005; Sharma et al 2007). Characterization of bacterial laccases has revealed that they have a low redox potential (0.45-0.54 V) (Durão et al 2006) but that they are active and stable at high temperatures (66 h at 60°C), pH (7-9) and salt concentrations (Held et al 2005; Dwivedi et al 2011). These characteristics represent advantages for industrial applications, since many processes are carried out under similar conditions where other types of laccases might easily be inactivated. The application of bacterial laccases in dye decolorization (Dawkar et al 2010) and textile wastewater treatment (Jadhav et al 2010) confirms the industrial potential of these bacterial enzymes.

3.1.3 Fungi

Fungal laccases are involved in delignification of lignocellulosic material, protection against toxic compounds, formation of fruiting body, fungal morphogenesis, sporulation (Dwivedi et al 2011) and synthesis of molecules with virulent activity (i.e., melanin) to cause fungal diseases (Riva 2006).

The number of fungal laccase producers is immense (Rodríguez Couto & Toca-Herrera 2006a; Morozova et al 2007) and they belong to the basidiomycetes and ascomycetes. Laccase activity has never been reported in the lower fungi belonging to the zygomycete (glomeromycete) and chytridiomycete.

Among the basidiomycetes, *Trametes versicolor* (formerly known as *Coriolus versicolor* or *Polyporus versicolor*) is the most characterized and studied fungus for production of laccase. It is one of the principal white-rot decaying fungi since it is able to produce other oxidoreductases besides laccase (Mikiashvili et al 2005). *Trametes versicolor* laccase has been applied in a number of processes ranging from baking to bioremediation as well as modification of polymers (**Paper V**). Other laccase producers of *Trametes* include *T. pubescens* (Galhaup et al 2002), *T. hirsuta* (Rodríguez Couto et al 2003) and *T. gallica* (Dong et al 2005).

Galerina sp. HC1 was isolated from the Amazon region in Bolivia and also belongs to the basidiomycetes. It is one of the few described species that has been isolated in South America (Gulden et al 2005; Tortella et al 2008). The fungus produced laccase, lignin peroxidase and manganese peroxidase activities (**Paper II**). *Galerina* sp. laccase was isolated, characterized and evaluated for different biotechnological applications (**Paper II-IV**).

Myceliophthora thermophila belongs to the ascomycetes and is reported to be related to fatal human infections (Bourbeau et al 1992; Farina et al 1998). However, laccase production is achieved by gene expression in *Aspergillus oryzae* avoiding the production of toxic molecules (Olempska-Beer 2004).

Fusarium sp. BOL35 also belongs to the ascomycetes and has been isolated from the Bolivian Amazon region. The naphthoquinones and laccase production by the fungus is probably part of its pathogenicity mechanism. Production of *Fusarium* sp. laccase under laboratory conditions could be achieved only when 8 mg/l of Cu^{2+} was present in the cultivation medium (**Paper I**). The capability of the fungus to produce laccase was related to its ability to degrade benzo[a]pyrene (**Paper I**).

3.2 Physico-chemical properties and structure

Laccases often occur as isoenzymes or monomers that oligomerize to form multimeric complexes (Kunamneni et al 2008a,b). Each isoenzyme has four copper atoms and is able to individually carry on the catalytic mechanism of laccases; *Galerina* sp. HC1 was able to produce 4 isoenzymes (**Paper III**), whereas *Fusarium* sp. BOL35 laccase was seen as a single protein (Terrazas 2005). The molecular mass of the laccase monomers ranges from 40 to 130 kDa with a covalently linked carbohydrate content of 10–25% in fungi and 20-45% in plants. The carbohydrate moiety typically consists of mannose, N-acetylglucosamine and galactose, which may contribute to the high stability of the enzymes (Claus 2004; Kunamneni et al 2008a,b).

The isoelectric point of laccases is around 4 and the optimal pH depends on the substrate that is used to test the enzyme activity. Usually, fungal laccases exhibit pH optima in the range of 3-5 and bacterial laccases in the range of 5-6, when the substrate is a hydrogen atom donor compound (i.e., ABTS). When the substrate is a phenolic compound (e.g., syringaldazine), on the other hand, the optimal pH is shifted to 6-7. This shift in pH is a result of the balance of redox potentials between the substrate and the inhibition of the T2/T3 copper site by binding of an OH^- ion. The optimal temperature differs with the source of laccase, being 40 °C for *Fusarium* sp. BOL35 laccase (Terrazas 2005), 50 °C for *Trametes versicolor* (Han et al 2005), 60 °C for *Galerina* sp HC1 (**Paper III**), and 70 °C for *Myceliophthora thermophila* (Olempska-Beer 2004). The redox potential ranges from 0.4-0.5 V in plants and bacteria (Gianfreda et al 1999; Durão et al 2006) to 0.4-0.9 in fungal laccases (Wesenberg et al 2003).

Three-dimensional structural analysis of several fungal, bacterial and plant laccases reveals that all are composed of three sequentially arranged cuprodoxin-like domains; each of them with a greek key β-barrel topology, highly related to small copper proteins such as azurin and plastocyanin (Dwivedi et al 2010; Giardina et al 2010). The multiple alignment of primary sequences of laccases shows that the copper binding motifs are highly conserved in all sequences, which reflects a common mechanism for copper oxidation and oxygen reduction (Dwivedi et al 2010). However, putative binding pocket analysis reveals that bacterial laccases have larger binding cavities when compared to those from plants and fungi (Dwivedi et al 2010).

Generally, laccase contains four copper atoms (Figure 3.1), which have been classified into three groups based on the absorption and Electronic Paramagnetic Resonance spectra. Type 1 (T1) paramagnetic "blue" copper has an intense absorption at 600-610 nm, which is caused by the covalent copper-cysteine bond and confers the typical blue color to the multicopper

proteins. The T1 copper has a trigonal coordination with two histidines and one cysteine; in bacterial laccases the axial ligand is conformed by methionine and in fungal laccase by leucine or phenylalanine (Witayakran & Ragauskas 2009). Type 2 (T2) paramagnetic "non-blue" copper has no visible absorption spectrum and is coordinated by two histidines. Type 3 (T3) is a diamagnetic coupled binuclear copper center, with an absorption band at 330 nm. It is coordinated by six histidines (Claus 2004; Witayakran & Ragauskas 2009). Nevertheless, it is possible to find non-blue laccases in nature (Palmieri et al 1999); the "white" laccases, as they are called, have been structurally characterized and atypically show the presence of one copper, one iron and two zinc atoms per molecule.
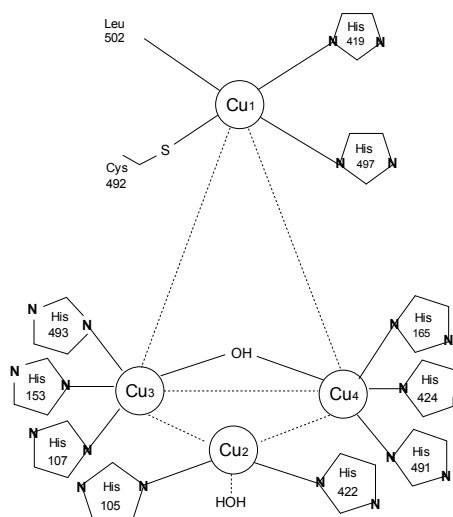
Figure 3.1 Schematic representation of copper centers in fungal laccase. Modified from Claus 2004.

Structural analysis of *Trametes versicolor* laccase and site-directed mutagenesis in *Bacillus* sp. laccase have revealed that the axial ligand in T1 copper is responsible for displaying the redox potential; T1 copper has no axial ligand in *Trametes versicolor* laccase and this has given rise to a modest elevation of its redox potential to 0.78 V (Piontek et al 2002). Moreover, mutations of *Bacillus* sp. laccase have been used to confirm that modifications in the axial ligand of T1 (methionine was replaced by phenylalanine or leucine) allowed changes in the redox potential (the change of amino acids led to an increase of 0.06-0.1 V of the redox potential as compared to the wild type) (Durão et al 2006). The redox potential is directly related to how good a laccase will catalyze oxido-reduction reactions.

Different compounds have been reported as inhibitors of laccase. Among them, anions like azide, cyanide and fluoride inhibit laccase by binding T2/T3, thus preventing electron transfer from T1. Other inhibitors like metal ions, fatty acids and quaternary ammonium detergents replace or chelate the copper centers and may also denature the protein (Witayakran & Ragauskas 2009). *Trametes versicolor* laccase is inhibited by copper-chelating agents like sulphamic acid, oxalic acid, hydroxylammonium chloride, malonic acid, citric acid and EDTA (Lorenzo et al 2005), whereas *Fusarium* sp. BOL35 and *Galerina* sp. HC1 laccases are barely inhibited by EDTA (Terrazas 2005; **Paper III**).

3.3 Catalytic mechanism

Figure 3.2 illustrates the catalytic mechanism of laccase where molecular oxygen follows a reduction to water. Starting from the native intermediate, the substrate reduces the T1 site, which transfers the electron to the trinuclear cluster T2/T3. Here, two possible mechanisms for reduction of the trinuclear cluster are conceivable: either T1 and T2 sites together reduce T3, or each copper on the cluster is sequentially reduced by electron transfer starting from T1. Once the enzyme is completely reduced, one oxygen atom is bound with the T2 and T3 copper ions, and the other oxygen atom is bound with the other copper ion of T3, forming the peroxide intermediate. Subsequently, the peroxide bond (O-O) is broken to produce a native intermediate (fully oxidized form), which will end the catalytic cycle with the reduction of oxygen to water. Sometimes the native intermediate is converted to a completely oxidized cluster called the "resting" form, where the T2 copper is isolated from the coupled T3 coppers. In this form, the T1 can still be reduced by the substrate, but the electron transfer is too slow to be significant (Durán et al 2002; Witayakran & Ragauskas 2009).

The use of molecular oxygen as the oxidant and the fact that water is the only by-product are very attractive catalytic features, rendering laccases as excellent 'green' catalysts (Riva 2006).

3.3.1 Natural substrates

The natural substrates of laccase include phenols like *ortho-* and *para-*diphenols, aminophenols, polyphenols, polyamines and aryl diamines (Rodríguez Couto & Toca-Herrera 2006a; Wells et al 2006). The oxidation of these molecules is represented in Figure 3.3. Here, laccase oxidizes the molecule with a simultaneous radical formation, which can spontaneously rearrange to cleave the aromatic rings or promote their polymerization (Kunamneni et al 2008a). These phenolic compounds are typical substrates for laccase due to their low redox potential (0.5-1 V); however, other non-

phenolic structures (including some phenolic compounds) might have a higher redox potential, which determines the low efficiency of laccase towards the substrate.

3.3.2 Laccase/mediator systems

The oxidation potential of laccases can be broadened by using small molecules known as "mediators", which have the capacity to change the redox potential during the oxidation (Bourbonnais et al 1998; Zille et al 2004) i.e., from 0.78 V to 1.084 V (Zille et al 2004). Consequently, the oxidation of non-natural substrates and/or with high redox potentials (like lignin) is possible. The catalytic mechanism involves the oxidation of the mediator, which can diffuse away from the enzyme, oxidize the substrate and return to the catalytic cycle as a reduced specie (Wells et al 2006; Riva 2006). Mediators can oxidize the substrate by different mechanisms; those containing N-OH oxidize the substrate via hydrogen atom transfer pathway (Figure 3.4a), whereas others (i.e., ABTS) do it via electron transfer (Figure 3.4b).

Reduced Form

Oxidized "Resting" Form

H_2O
|
T_2 —— Cu^{1+} ——

OH
|
—— Cu^{2+} —— T_2

slowly
$+4e^-$

T_3 Cu^{1+} Cu^{1+}

T_1 Cu^{1+}

Cu^{2+} — O — Cu^{2+} T_3
H

Cu^{2+} T_1

$+ O_2$

$+4e^-$
rapidly

Catalytic Cycle

$-H_2O$

$-H_2O$

H_2O
|
T_2 —— Cu^{2+} ——
O — O
O

OH
|
—— Cu^{2+} —— T_2
O — H

T_3 Cu^{1+} Cu^{2+}

T_1 Cu^{1+}

Cu^{2+} O Cu^{2+} T_3
H

Cu^{2+} T_1

O-O bond
cleavage

Peroxide intermediate

Native intermediate
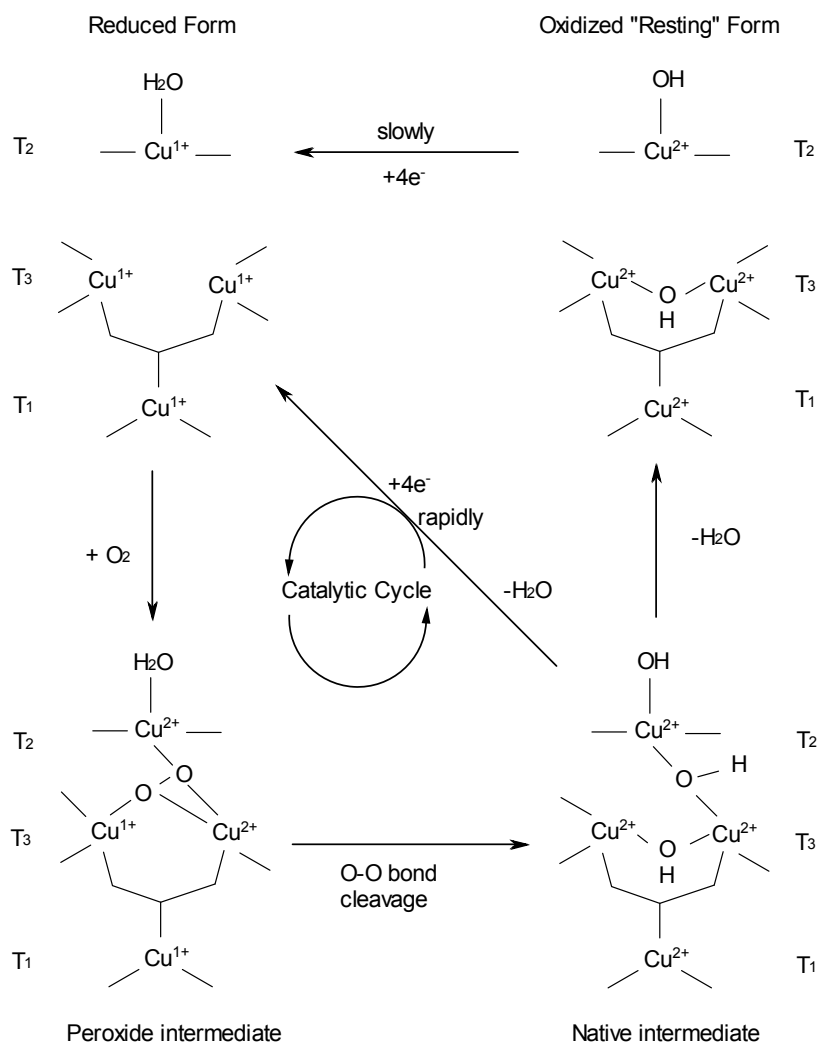
Figure 3.2 Mechanism of four-electron reduction of molecular oxygen to water in the catalytic cycle of laccase. Modified from Witayakran & Ragauskas 2009.
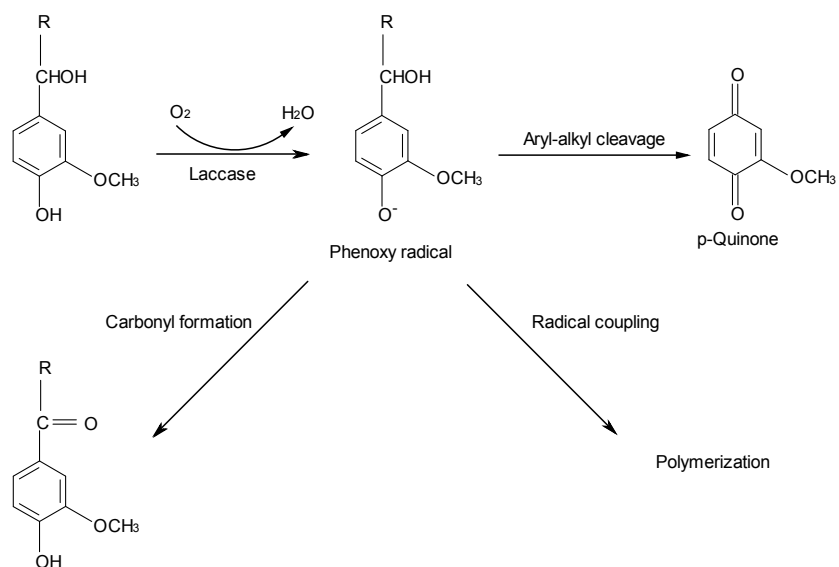
Figure 3.3 Oxidation of a phenolic compound (natural substrate) by laccase. Modified from Madhavi & Lele 2009.

The proper selection of the mediator plays a key role in the applications of laccase-mediator systems. Parameters such as the stability of the generated radicals, their redox potential, their ability to oxidize substrates (González et al 2009), the kinetic parameters such as k_{cat} of the laccase for the mediator, their effect on the enzyme stability (Li et al 1999; **Paper IV**) and possible environmental concerns should be taken into consideration. The oxidation efficiency of the mediators increases proportionally with their redox potential. However, values higher than 0.9 V can determine the decrease of the oxidation capacity on polycyclic aromatic hydrocarbons (Johannes & Majcherczyk 2000). In mediators of the type N-OH (Table 3.1), the efficiency has been demonstrated to be the opposite: the lower their redox potential, the better is the oxidation capacity (Xu et al 2000).

a

$$>N\text{-}OH \xrightarrow{\text{Laccase}} >N\text{-}OH^{\cdot +} \xrightarrow{-H^+} >N\text{-}O\cdot$$

$$>N\text{-}O\cdot$$

$$ArCH_2OH \longrightarrow ArCH(^\cdot)OH \longrightarrow ArCHO$$

$$>N\text{-}OH$$

b

$$Med_{ox} \xrightarrow{\text{Laccase}} Med_{red}$$

$$Med_{ox}$$

$$ArCH_2OH \longrightarrow ArCH_2OH^{\cdot +} \xrightarrow{-H^+} ArCH(^\cdot)OH \longrightarrow ArCHO$$

$$Med_{red}$$

c

$$R\text{-}OH \xrightarrow{\text{Laccase}} R\text{-}O^\cdot + H^+$$

$$R\text{-}O^\cdot + ArCH_2OH \longrightarrow R\text{-}OH + ArCH(^\cdot)OH \longrightarrow ArCHO$$

$$R\text{-}OH$$
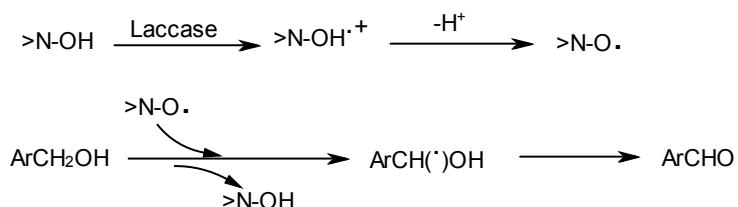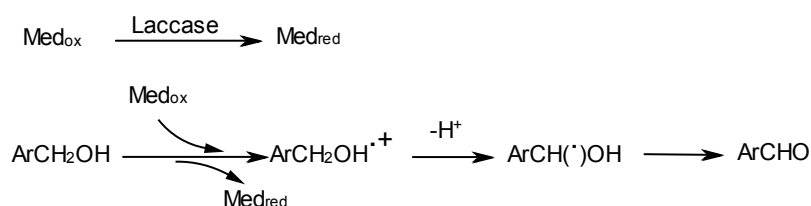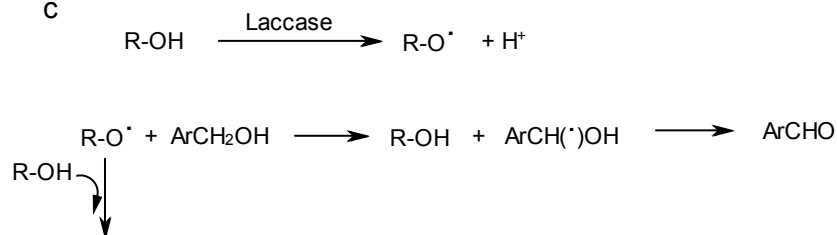
Oxidative phenol coupling

Figure 3.4 Laccase-mediated oxidation of substrates in the presence of synthetic and natural mediators. $ArCH_2OH$ is the substrate and represents an aromatic alcohol. a) A type of N-OH as the synthetic mediator showing the H-atom transfer pathway; b) a synthetic mediator showing the electron transfer pathway; c) a phenolic compound as the natural mediator. Modified from Fabbrini et al 2002a and Calcaterra et al 2008.

Several organic and inorganic compounds have been reported as effective mediators (Table 3.1). In 1990, the diammonium salt 2,2'-azino*bis* (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was found to mediate and enhance the laccase activity (Morozova et al 2004). Later, 1-hydroxybenzotriazole (HBT), N-hydroxyacetanilide (NHA), violuric acid, N-hydroxyphthalimide (HPT) and 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO) (and its derivatives) were reported to act as mediators (Arends et al

2006; Wells et al 2006). The latter was proven to be the most effective towards lignin degradation (Kunamneni et al 2008a,b).

The application of these mediators can be limited due to their high cost as well as their toxicity. Nevertheless, this can be overcome by the application of immobilized mediators, which allows their recyclability and facilitates their disposal by using membranes that retain the immobilized molecule (Pozzi et al 2004; Benaglia et al 2005; **Paper IV**).

On the other hand, molecules like vanillin, *p*-coumaric acid, acetovanillone, methyl vanillate, syringaldehyde, acetosyringone (Table 3.1) and some dyes are reported as "natural" mediators (Camarero et al 2005). They have been shown to catalyze reactions as effectively as the other type of mediators (Figure 3.4c; Camarero et al 2007; **Paper II; Paper IV**). Additionally, they can be easily produced from lignin (Villar et al 2001).

3.4 Laccase production

The demand for laccase for different applications requires the production of the enzyme in large amounts (Songulashvili et al 2006). Gene expression (Kiiskinen et al 2004), the modification of culture conditions, or a combination of both strategies are utilized to cover the demand.

3.4.1 Heterologous production

Heterologous expression of the genes encoding the laccases is carried out in order to increase their production and to be able to apply them in large-scale processes/applications. However, the expression of laccase genes in different hosts (Kojima et al 1990; Saloheimo et al 1991; Yaver et al 1999; Olempska-Beer 2004; Madzak et al 2005; Lu et al 2009; Abyanova et al 2010) has not shown any significant enhancement in the production up until now. Nevertheless, heterologous production can help in the characterization of individual laccase isoenzymes (Yaver et al 1999) as well as in avoiding additional production of toxic compounds besides the laccase (Olempska-Beer 2004).

3.4.2 Cultivation parameters for laccase production

3.4.2.1 Nutrients. Carbon, nitrogen and copper sources are the main nutritional parameters studied for laccase production due to the fact that they can regulate the level of gene transcription for laccase expression (Collins & Dobson 1997). The concentration of these nutrients is also determinant for the level of enzyme production. The diversity and number of carbon sources for laccase production is as large as the number of reported laccase producers.

Table 3.1 Mediators used in the laccase oxidation system

Mediator	Structure	Redox potential (V)	Reference
ABTS		0.472; 0.885	Bourbonnais et al. 1998; **Paper II**
HBT		0.878	Bourbonnais et al. 1998
Viorulic acid		0.663	González et al. 2009
HPT		0.785	González et al. 2009
TEMPO		0.492	González et al. 2009 **Paper V**
NHA		0.660	Xu et al. 2000
PEG-TEMPO		n.d.	**Paper IV**
Syringaldehyde		0.542	González et al. 2009; **Paper II & IV**
Acetosyringone		0.534	González et al. 2009; **Paper IV**
Acetovanillone		n.d.	Camarero et al., 2005
Vanillin		n.d.	Camarero et al., 2005
p-Coumaric acid		n.d.	Fillat et al. 2010
Methylvanillate		n.d.	Camarero et al., 2005

The first seven mediators (ABTS through PEG-TEMPO) are grouped under **Synthetic mediators**. The remaining mediators (Syringaldehyde through Methylvanillate) are grouped under **Natural mediators**.

Among them, glucose is the easiest carbon source for fungi to metabolize but its effect on laccase production will depend of the fungal strain. Although it has been found to enhance the production in *Galerina* sp. HC1 (**Paper II**), it repressed the production in *Trametes pubescens* (Galhaup et al 2002), *Trametes versicolor* (Tavares et al 2005) and *Phlebia* sp. (Arora & Rampal 2002).

Fructose was shown to be a good carbon source for laccase production in *Pleurotus sajor-caju* (Bettin et al 2009), cellobiose in *T. pubescens* (Galhaup et al 2002), and lactose or glycerol in *Pseudotrametes gibbosa*, *Coriolus versicolor* and *Fomes fomentarius* (Revankar & Lele 2006). On the other hand, while low nitrogen levels (as yeast extract) improves the laccase production in *Pleurotus ostreatus* (Prasad et al 2005), *Coriolus versicolor* (Revankar & Lele 2006) and *Pycnoporus sanguineus* (Pointing et al 2000), high concentrations are needed for *Trametes pubescens* (Galhaup et al 2002), *Trametes gallica* (Dong et al 2005) and *Galerina sp*. HC1 (**Paper II**).

Casein, another nitrogen source was successfully used for the production of laccase in *Pleurotus sajor-caju* (Bettin et al 2009). In *Trametes versicolor* and *Coriolopsis polyzona* the laccase production was significantly improved when NH_4NO_3, $(NH_4)_2SO_4$, KNO_3 and peptone were used as supplementary nitrogen sources (Elisashvili et al 2008). On the other hand, agricultural residues can be used as carbon and nitrogen sources for laccase production (Table 3.2). They are cheap substrates and may have additional nutritional metabolites. Cultivation of *Galerina* sp. HC1 on bagasse and orange peels demonstrated the potential of the agro residues as substrates for laccase production: the production was increased 1- and 4-fold, respectively when compared to cultivation in glucose containing medium (**Paper II**).

Copper can have a toxic effect in many organisms (Xing et al 2006); however, low concentrations are required for laccase production (**Paper I**). The growth of *Galerina* sp. HC1 was highly favored when 0.01 g/l copper sulphate was added to the cultivation. Interestingly, the fungus was able to tolerate and produce the enzyme at copper concentrations as high as 0.1 g/l when the yeast extract concentration was increased up to 35 g/l (**Paper II**).

3.4.2.2 Additives. The addition of chemicals to the cultivation can enhance the laccase production based on their ability to induce the expression of the different isoforms (Marques de Souza et al 2004). *p*-Coumaric acid, α-benzoin oxime and 2,5-xylidine highly improved laccase production in *Galerina* sp. HC1, whereas lignin or lignin-related structures only enhanced the production slightly (**Paper II**). Other lignin-related chemicals such as ferulic acid and/or vanillin proved to increase the laccase production up to 10

times in *Pleurotus pulmonarius* (Marques de Souza et al 2004); vanillin induced the production in *Phanerochaete flavido-alba* (de la Rubia et al. 2002) and caffeic acid in *Coprinus comatus* (Lu & Ding 2010). Vitamins like biotin, riboflavin and pyridoxine hydrochloride as well as amino acids such as methionine, tryptophan, glycine and valine stimulated laccase production in *Cyathus bulleri*, whereas cysteine inhibited the production (Dhawan & Kuhad 2002). Antibiotics like apramycin sulfate stimulated laccase production in *Cyathus bulleri* and *Pycnoporus cinnabarinus* (Dhawan et al 2005). Metals like Mn^{2+} led to a 4.5-fold increase in the laccase production by *Coprinus comatus* (Lu & Ding 2010).

3.4.2.3 Operational conditions. Other important parameters include the temperature, pH and aeration level during the cultivation. Most reports indicate that an initial pH of 4.5-6 and a temperature between 25-30 °C are suitable for laccase production. However, the effect of aeration varies between species; growth of some fungi is highly favored with aeration, while others can suffer from stress caused by oxygen. In addition, as aeration can involve mechanical stirring, this may cause stress on the cells by rupturing them (Brijwani et al 2010).

3.4.3 Submerged cultivation

The process of submerged cultivation involves the growth of microorganisms in a liquid medium rich in nutrients under aerobic conditions. In order to achieve high production, the studies are first focused on the optimization of nutritional and operational conditions (Dong et al 2005; Revankar & Lele 2006). *Galerina* sp. HC1 produced high laccase activity under optimized conditions in batch submerged fermentation (Figure 3.5) (**Paper II**).

Submerged cultivation can be carried out by utilizing cheap materials considered as "waste" and which are produced in large amounts. These materials can contain considerable concentrations of soluble carbohydrates, nitrogen, minerals and vitamins, and even inducers for enzyme production (Table 3.2).

The industrial production of enzymes is mainly achieved by submerged cultivation. One of the disadvantages of this method is the excessive growth of mycelium, which affects the production yield due to mass transfer and metabolic rate limitation. The excessive growth can also affect the mechanical set up of the used reactor. In other words, the mycelia can wrap around the impellers, cause blockage of the system and increase viscosity; however, this has been overcome by immobilization of the cells in various supports (Table 3.2) or by using fed-batch cultivation for controlling the fungal growth (Galhaup et al 2002).

Table 3.2 Production of laccase in submerged cultivation using agricultural and synthetic materials.

Source	Support	Production (U/l)	Reactor	Reference
Ganoderma adspersum	Corn bran, soy bran, chicken feathers, wheat bran, kiwi fruits, banana peels, mandarin peels, ethanol production residue and cotton stalks	600-3400	Batch	Songulashvili et al 2006.
Phellinus robustus		700-4000		
Lentinus edodes	Malted barley (brewing process)	100	Batch	Hatvani & Mécs 2001
Pleurotus eryngii	Dried ground mandarin peels	999	Batch	Stajić et al 2006
Trametes versicolor (CBS 100.29)	Grape seeds, grape stalks and barley bran	200-600	Batch	Lorenzo et al 2002.
Galerina sp. HCl	Orange peels	2000	Batch	**Paper II**
	Bagasse	600		
Pycnoporus cinnabarinus	Cubes of sponge	280	Packed-bed reactor	Rodríguez Couto & Herrera 2007
Neurospora crassa	Capillary membrane supports	10000	Batch	Rodríguez Couto & Herrera 2007
Trametes hirsuta	Alginate beads	1043	Air lift reactor	Rodríguez Couto & Herrera 2007
Pleurotus ostreatus	Immobilized in polyurethane foam	1403	Packed bed reactor	Rodríguez Couto & Herrera 2007
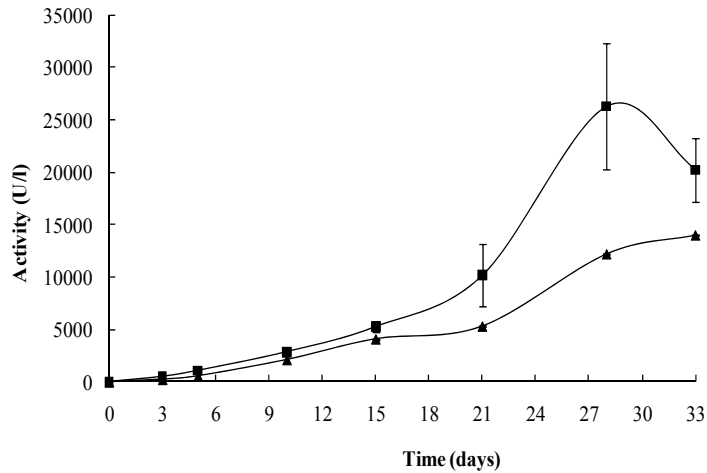
Figure 3.5 Time course of laccase production by *Galerina* sp under optimized conditions: 30 g/l glucose, 10 g/l yeast extract, 0.01 g/l copper sulphate, 2mM 2,5-xylidine (squares), 2mM α-benzoin oxime (triangles) (**Paper II)**.

3.4.4 Solid state fermentation

Solid state fermentation (SSF) is defined as a cultivation technique where additional liquid can be nearly or totally absent (Singhania et al 2009). During the cultivation, synthetic or natural substrates can be used as the solid support (Table 3.3). A possible reason for the high level of enzyme production obtained by SSF is that it "simulates" the conditions of natural growth for fungi (Hatvani & Mécs 2001; Singhania et al 2009). In addition, the low cost of the substrates, reduced risk of bacterial contamination, low energy requirements as well as downstream processing of the enzyme are considered as the principal advantages of implementing this type of cultivation (Singhania et al 2009; Robinson & Nigam 2003).

The principal disadvantages for growing microorganisms in SSF are related to the low transfer of oxygen, nutrients, moisture, temperature and regulation of pH (Rodríguez Couto & Toca-Herrera 2007). However, the magnitude of the disadvantage will greatly depend on the type of reactor and the characteristics of the substrate used (Table 3.3) (Rodríguez Couto et al 2003).

Table 3.3 Production of laccase under solid state fermentation

Source	Support	Reactor	Production (U/l)	Reference
Trametes versicolor	Nylon sponge	Immersion	229	Rodríguez Couto et al 2003
Trametes versicolor	Barley bran	Immersion	600	Rodríguez Couto et al 2003
Trametes versicolor	Nylon sponge	Expanded bed	229	Rodríguez Couto et al 2003
Trametes versicolor	Nylon sponge	Tray	343	Rodríguez Couto et al 2003
Trametes versicolor	Barley bran	Tray	3500	Rodríguez Couto et al 2003
Pycnoporus cinnabarinus	Sugarcane bagasse	Packed reactor	10 000	Meza et al 2006
Trametes hirsuta	Nylon sponge	Tray	6898	Rodríguez Couto et al 2006
Trametes hirsuta	Grape seeds	Tray	18 715	Rodríguez Couto et al 2006
Trametes hirsuta	Orange peels	Tray	12 000	Rosales et al 2007
Trametes hirsuta	Kiwi fruits	Cotton-plugged Erlenmeyer flasks	5399	Rosales et al 2005
Trametes pubescens	Banana skin	Cotton-plugged Erlenmeyer flasks	1570	Osma et al 2007
Galerina sp. HC1	Orange peels	Cotton-plugged Erlenmeyer flasks	*720	**Paper II**

* The value is an estimation of 6 U per gram of substrate reported in **Paper II**

Laccase Applications

Laccases have been used in a variety of industrial and environmental applications since they can oxidize a number of natural and synthetic substrates using oxygen, producing water as the only by-product. Laccases are versatile enzymes able to oxidize recalcitrant compounds like lignin, which makes them attractive for use in various biotechnological processes.

4.1 Decolorization of dyes

4.1.1 Textile dyes

Textile dyes are crucial, widespread contaminants of industrial wastewaters. They are discharged in the effluents as part of the manufacturing process in textile industries where they represent 10-15% (Trella et al 2006; Cristovao et al 2009; Gopinath et al 2009) and even 90% of the used dyestuffs (Abadulla et al 2000). Once discharged, the dyes can confer toxic and carcinogenic properties to the water and can contribute to the total organic loading (Dos Santos et al 2004; Cristovao et al 2009). In addition, the coloring of the water will affect the normal absorption of sunlight for many aquatic organisms (Cristovao et al 2009).

Structurally, a dye molecule consists of two parts: the chromophore and the auxochrome. The first is responsible for generating the color and the second provides water solubility and the affinity to attach to textile fibers (Gupta & Suhas 2009). Dye classification is mostly based on the chemical structure of the chromophore group (Figure 4.1).

4.1.1.1 Azo dyes. Azo dyes are the most extensively used colorants in the textile and dyestuff industries (Martins et al 2001; Steffan et al 2005). It is estimated that they represent 65–70% of the total amount of produced dyes (Gupta & Suhas 2009), thus constituting the predominant colorants in wastewaters (Moreira et al 2000). Structurally, they contain azo groups (-N=N-) mostly linked to benzene or naphthalene rings, and occasionally to aromatic heterocycles or to enolizable aliphatic groups (Zollinger 2003). They are classified as anionic monoazodyes, acid dyes, disperse dyes, azoic dyes, cationic dyes, direct dyes and reactive dyes, and each one is used in a different dyeing process (Table 4.1).

Table 4.1 Classification of the azo dyes with regard to principal characteristics and substrate to be dyed. Modified from Zollinger 2003.

Azo dye	Characteristic/property	Substrate
Anionic monoazo dye	Soluble in water due to the presence of sulfonate groups	Paper and leather
Acid dye	Anionic monoazodye with 1 to 3 sulfonate groups. It requires acidic conditions during the dyeing of materials	Wool and silk
Azoic dyes	Water-insoluble monoazo dyes	Cellulosic fibers
Disperse dyes	Partly soluble in water, and thus suspended in aqueous solutions	Polyester fibers
Cationic azo dyes	Need mordant compounds	Paper
Metal-complex azo dye	Metal complexed dye	Ink-jet printing
Direct dyes	Do not need mordant compounds	Cellulosic fibers
Reactive azo dyes	Covalent attachment between dye and substrate	Protein fibers

Wastewaters containing these dyes are usually resistant to biological treatment due to the presence of the N=N bond (Figure 4.1a) and/or sulfonate groups (SO_3H) (Martins et al 2001; Steffan et al 2005). In addition, most of the azo dyes are considered toxic, since they can form aromatic amines (anilines), which are carcinogenic and/or mutagenic (Martins et al 2001; Yang et al 2009).

4.1.1.2 Triphenylmethane dyes. Triphenylmethane dyes are used extensively in the textile industry (Ayed et al 2009) as they can provide a large variety and coloration range for dyeing cotton, wool, silk, nylon, etc. (Ayed et al 2009; Hamza et al 2009). They are also employed in the paper, leather, plastics, varnish, fat and wax industries (Casas et al 2009) as well as in the analytical and medical sector (Casas et al 2009; Hamza et al 2009). This determines their presence in many colored industrial wastewaters (Ayed et al 2009; Hamza et al 2009). Triphenylmethane dyes are very recalcitrant to chemical or biological degradation (Ayed et al 2009; Hamza et al 2009) and their presence in waters causes perturbation of the aquatic life (Ayed et al 2009) as well as severe human and animal health problems, such as skin and eye damage, irritation and pain by ingestion (Hamza et al 2009). The basic structure is shown in Figure 4.2b.
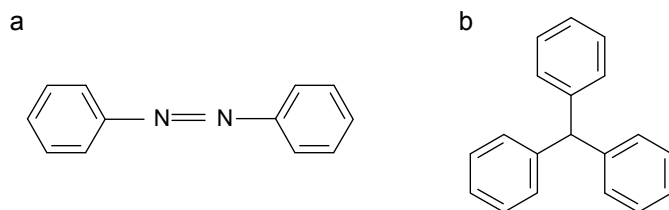
Figure 4.1 Basic structures for (a) an azo dye and (b) a triphenylmethane dye. Adapted from Rodríguez Couto 2009.

4.1.2 Physical and chemical methods for treatment of dye-containing waters

Adsorption involves the concentration of molecules on a solid surface. Among the supports used for adsorption, those based on carbon are the oldest (Forgacs et al 2004; Gupta & Suhas 2009). However, the method is expensive since large amounts of material are required for the treatment of dye wastewaters. Organic supports are relatively cheap and usually originate from renewable sources or from wastes from industrial processes (Saravanabhavan et al 2007; Singh et al 2009). Examples include chitosan, chitin, orange peels, banana pith, lignin, sawdust, etc. (Forgacs et al 2004; Bukallah et al 2007).

Sedimentation is a basic form of a primary treatment. It can be significantly improved with the addition of certain chemicals, such as aluminum, calcium or ferric ions, to the process stream (Gupta & Suhas 2009).

Filtration techniques include microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. This method is based on the use of a coated membrane that will retain the dyes thus producing colorless water. Although the method has been shown to be efficient for the removal of dyes (Mo et al 2008), its application is still limited due to problems of membrane clogging, generation of high operating pressures, significant energy consumption, high membrane costs, relatively short membrane lifetimes (Gupta & Suhan 2009) and the required pretreatment of some dyes with coagulants (Mo et al 2008).

Electrochemical methods include electro-oxidation and electro-coagulation. These methods are effective since decolorization of dyes with the concomitant reduction of the chemical oxygen demand (COD) can be achieved. However, the rate of dye removal depends on the material of the anode as well as its working potential, which represent the main drawbacks due to high electricity costs (Gupta & Suhas 2009).

Sonochemical techniques employ ultrasonic waves to produce an oxidative environment. The mechanism involves formation, growth and collapse of cavitation bubbles, generating high local temperatures (above 4500 ˚C) and pressures (over 1000 atm). Such extreme conditions will make possible the dissociation of chemical compounds including water and oxygen to generate small amounts of ˙OH and ˙OOH radicals which can combine to produce hydrogen peroxide and contribute to the oxidation and organic destruction of molecules (Ghodbane & Hamdaoui 2009). The addition of chemicals, such as chloroalkanes and carbon tetrachloride, increase the decomposition efficiency and reduce the time required for removing the pollutants (Ghodbane & Hamdaoui 2009).

Photocatalysis is a series of advanced oxidation steps where light energy excites an electron from the catalyst (zinc oxide, zirconium oxide, etc), which will initiate a chain of reactions resulting in the formation of hydroxyl radicals. These have high oxidizing potential and can therefore attack most organic structures. The degradation of dyes depends, among other factors, on the presence of electron acceptors such as hydrogen peroxide and ammonium persulfate (Gupta & Suhas 2009).

Although most of these treatment methods are effective, their disadvantages include high costs, the formation of hazardous by-products and elevated energy requirements (Ramsay & Nguyen 2002; Ramya et al 2007). In addition, some of them have short operational lifetimes (Kaushik & Malik 2009), which will determine the color remaining in the wastewater (Dos Santos et al 2004). They can also generate a significant amount of polluted or non-polluted sludge (Ramsay & Nguyen 2002; Ramya et al 2007).

4.1.3 *Biological methods for treatment of dyes*
4.1.3.1 Bacteria. Bacterial treatment of azo dyes can be performed under anaerobic and aerobic conditions (Gopinath et al 2009). Under anaerobic conditions, the decolorization of azo dyes is achieved by reductive cleavage of the azo bond to produce amines (Martins et al 2001), which can be toxic and/or carcinogenic. Bacterial anaerobic treatment of dyes, as in any other process, has both drawbacks and advantages. Among the disadvantages, the production of amines represents the most important limitation; however, bacteria remain the most applied organism for treatment of azo dyes (Saratale et al 2011) since high yields of decolorization can be achieved, with the concomitant reduction of the chemical oxygen demand (COD) (Brás et al 2005). In addition, bacterial treatments can be coupled to other methods to degrade the produced aromatic amines. So far, aerobic approaches (Lourenço et al 2006), electrochemical techniques (Carvhalo et al 2007) and cultivating

a mixture of bacterial strains (Saratale et al 2011) have been shown to be effective in degrading aromatic amines.

The mechanism of how the azo dyes are cleaved by anaerobic bacteria seems to be related to the presence of azoreductase (Feng et al 2010; Dawkar et al 2010). However, studies with *Bacillus* sp. VUS revealed that other enzymes (laccase and lignin peroxidase) could also participate in the reductive cleavage of the azo-dye (Dawkar et al 2010). The role of bacterial laccase in decolorization of azo dyes was described by the application of *Bacillus*. sp. CotA laccase (Pereira et al 2009). In this enzymatic pathway, azo dyes are oxidized non-specifically via a free radical mechanism, forming phenolic-type compounds, able to undergo polymerization.

Aerobically, bacteria are able to produce mono-oxygenases and di-oxygenases to catalyze the incorporation of oxygen into the aromatic structure prior to cleavage, whereas other species catalyze the reductive cleavage using oxygen to produce aromatic amines, which are used as carbon sources by certain bacteria.

Bacteria are also able to decolorize/degrade triphenylmethane dyes with concomitant toxicity reduction under aerobic conditions (Ayed et al 2009). The decolorization involves demethylation of the dye (Sarnaik & Kanekar 1999) and the degradation mechanism is based on the reductive cleavage of the dye *via* Michler´s ketone (Chen et al 2008)

4.1.3.2 Fungi. Fungi are applied for treatment of dyes in two ways. The first involves the mycelial adsorption of dyes by application of living or dead cells (Fu & Viraraghavan 2001; Kaushik & Malik 2009), while the second includes the application of living cells and their extracellular enzymes (Kapdan et al 2000; Wesenberg et al 2003). The efficiency of the treatment will depend on the fungal source as well as on the type of dye to be treated (**Paper II** and **IV**).

Extracellular fungal enzymes demonstrate advantages over the living whole cells since they provide lower mass transfer limitations (Kaushik & Malik 2009). Among them, laccases offer the possibility of decolorizing/degrading dyes in the presence or absence of mediators (Wesenberg et al 2003; **Paper II** and **IV**). Laccases are able to decolorize dyes based on the mechanism of free radical formation that can end up in the polymerization (Zille et al 2005) or cleavage of the dyes (Chivukula & Rengahatan 1995). The laccase decolorization efficiency depends among other things on the type of dye, the amount, source and status of the enzyme (free or immobilized) (**Paper II** and **IV**; Lu et al 2007), the presence, amount and nature of the mediator (Figure

4.2; Figure 4.3; Zille et al 2004; Camarero et al 2005; **Paper II** and **IV**), as well as on operational conditions like pH (**Paper IV**), temperature and reaction time (Zille et al 2005).

The treatment of azo dyes and triphenylmethane dyes, for example, are decolorized by laccase in different modes. Azo dye decolorization depends primarily on how the structure of the azo dye is substituted (Chivukula & Rengahatan 1995), i.e., 2,6-dimethoxy, 2,6 dimethyl and 2-methoxy substituted azo dyes can be efficiently decolorized by laccase, whereas chloro-, nitro-, 2-methyl-, 2,3-dimethyl and 2,3-dimethoxy substituted azo dyes have been found to be poorly decolorized (Chivukula & Rengahatan 1995). The affinity of laccase for methoxy- or methyl- substituted dyes could be based on the enzyme's affinity for the same substituents on lignin (Smirnov et al 2001).

The decolorization of azo dyes is mainly based on phenoxy radical formation with further cleavage of the azo linkage to produce quinone- and sulfophenyldiazene-containing structures. The latter is unstable in the presence of oxygen and is oxidized to produce a phenyldiazene radical (Chivukula & Rengahatan 1995), which can either become stabilized or catalyze polymerization reactions (Zille et al 2004). Triphenylmethane dye decolorization by laccase is performed through demethylation of the structure (Murugesan et al 2009) followed by oxidation of the methyl carbon of the dye structure, which can lead to its cleavage (Casas et al 2009). However, in the presence of the mediator, the breakdown takes place by oxidation of the carbinol form of the dye followed by demethylation (Chandra et al 2009).

However, decolorization of both dyes, either by laccase alone or with mediators, leads to high decolorization (or degradation) yields (Figure 4.2; Table 4.1; Murugesan et al 2009; Katuri et al 2009; **Paper II**; **Paper IV**) as well as to a reduced toxicity (Murugesan et al 2009; Abadulla et al. 2000).

The immobilization of laccase renders possible the recycling of the enzyme and protection against deactivation. Thus, the immobilization procedure determines how effective and stable the enzyme will become. Chitosan is a polymer of amino-D-glucose groups produced from deactylation of chitin. Chitosan is a non-toxic and biodegradable polymer, soluble in water under acidic conditions and insoluble in neutral and alkaline environments (Chiou et al 2003). It has been applied for the treatment of dyes due to its high capacity of adsorption and its reusability (Forgacs et al 2004). Its mechanical property and low cost render it suitable to be applied as a matrix for the immobilization of proteins (Lu et al 2007; **Paper II**). Laccase immobilized on chitosan demonstrated higher yields of decolorization of an azo- and triphenylmethane

dye as well as a faster decolorization of the azo-dye when compared to the free enzyme (**Paper II**). In addition, it allowed re-use of the laccase to catalyze decolorization of 3-7 batches.
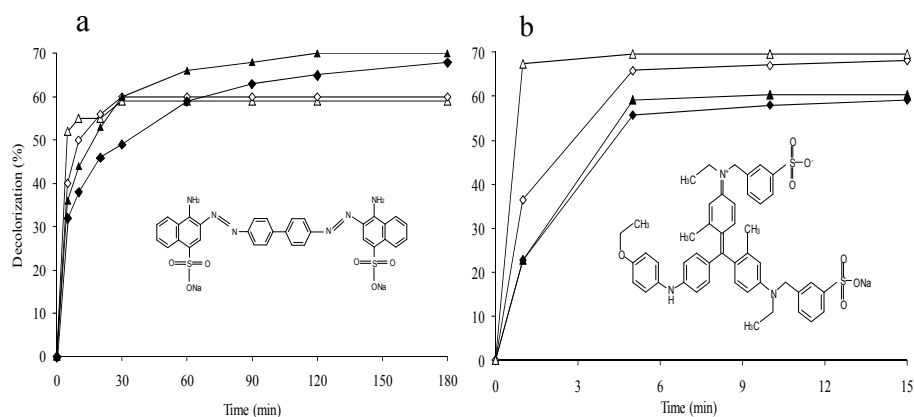
Figure 4.2 Decolorization of (a) Congo red and (b) Coomassie Brilliant blue G-250 using *Galerina* sp. HC1 (*diamonds*) and *Trametes versicolor* (*triangles*) laccases in the presence of ABTS (*filled symbols*) and syringaldehyde (*open symbols*) as mediators (**Paper II**).

On the other hand, a membrane reactor system renders possible the retention and recycling of the free enzyme in a number of cycles of decolorization (Table 4.2). The membrane reactor also permits the recycling of TEMPO coupled to polyethylene glycol, enabling the re-use of both laccase and the mediator (Figure 4.3).

Table 4.2 Decolorization efficiency after repeated applications of the laccase from *T. versicolor* using a membrane filtration device and syringaldehyde as the mediator. **Paper IV**

Number of batches	Red FN-2BL			Red BWS			Remazol Blue RR			Blue 4BL		
	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)
1	95	100	0.1	84	100	1.1	94	100	2.7	71	100	1.6
2	94	72	0.15	74	82	1.2	94	73	2.3	72	80	1.8
3	94	33	0.17	72	88	0.8	94	64	2.8	61	83	1.6
4	94	24	0.22	69	75	1.0	94	63	3.3	68	80	1.8
5	93	16	0.3	67	73	1.0	94	44	2.8	66	64	1.3
6	92	13	0.4	67	67	1.2	94	38	2.8	64	66	1.5
7	93	8	0.5	62	70	1.4	94	37	3.2	63	65	1.8
8	94	6	0.7	62	52	1.2	94	34	3.0	62	56	1.2
9	92	5	0.9	59	59	1.2	94	21	3.0	63	56	1.6
10	91	5	1.0	60	53	1.2	93	21	3.0	61	49	1.6
11				55	30	1.3	94	14	3.2			
12				55	37	1.5	93	16	3.7			
13				56	31	1.3	93	12	3.7			
14				55	29	1.5	94	11	4.5			
15				52	26	1.6	94	8.0	4.3			
16							93	7.0	5.0			
17							93	7.0	4.7			
18							93	6.0	6.7			
19							93	5.0	7.0			
20							85	4.0	6.3			

Figure 4.3 Continuous use of *Trametes versicolor* laccase and PEG-TEMPO to decolorize Remazol Blue RR in a membrane reactor. The symbols denote: ($\diamond$) absorbance of the dye, and ($\blacksquare$) laccase activity (**Paper IV**).

Laccase can even be used in the textile industry to replace traditional chemical methods like the alkaline conditions in scouring of flax material (Ossola & Galante 2004), the polluting stone-sodium hypochlorite during the denim finishing of fabrics, as well as hydrogen peroxide in cotton bleaching and in the anti-shrink treatment of wool (Rodríguez Couto & Toca-Herrera 2006b; Lantto et al 2004).

Moreover, laccase can perform synthesis of biodegradable dyes; the enzyme has been found to catalyze the oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone with methoxyphenol to produce red-colored azo dyes (Setti et al 1999) and the polymerization of hydroquinone, catechol and ferulic acid for wood dyeing (Shin et al 2001; Hadzhiyska et al 2006).

4.2 Removal and degradation of other organic pollutants
Laccase can be used for removal/degradation of a number of environmental pollutants besides dyes. The enzyme alone has been able to catalyze the removal of phenolic compounds (Dec & Bollag 1990; Ullah et al 2000), endocrine disrupting chemicals (Cabana et al 2007) and estrogens from wastewater samples (Auriol et al 2008). Laccase mediator systems have been employed in the degradation of dibenzothiophene, a sulphur-containing compound (Villaseñor et al 2004), organophosphorus pesticides (Amitai et al 1998) and a number of polycyclic aromatic hydrocarbons (PAH) (Dodor et al 2004; Cho et al 2002) under laboratory conditions. Moreover, it was

demonstrated that laccase and a mediator were able to degrade PAH directly from contaminated soil (Wu et al 2008).

4.3 Modification of lignin

Advantage can be taken of the ability of laccases to catalyze demethylation (Murugesan et al 2009; Bourbonnais & Paice 1992) and to form reactive radicals for further polymerization in order to modify lignin by the coupling of targeted molecules and subsequently produce polymers with novel properties. For instance, adhesion of fibers to lignin is utilized to obtain lignocellulosic fiberboards with decent mechanical properties (Rodríguez Couto & Toca-Herrera 2006a). Laccase catalyzed demethylation of lignin is a mild procedure, which could represent an advantage over other methods that either require high temperatures (Yang 1981) or the presence of strong oxidants (Marton et al 1963). *Galerina* sp. HC1 laccase was able to demethylate lignin in the presence of several mediators; syringaldehyde (a natural mediator found in lignin) was among the most effective (Figure 4.4; **Paper III**).
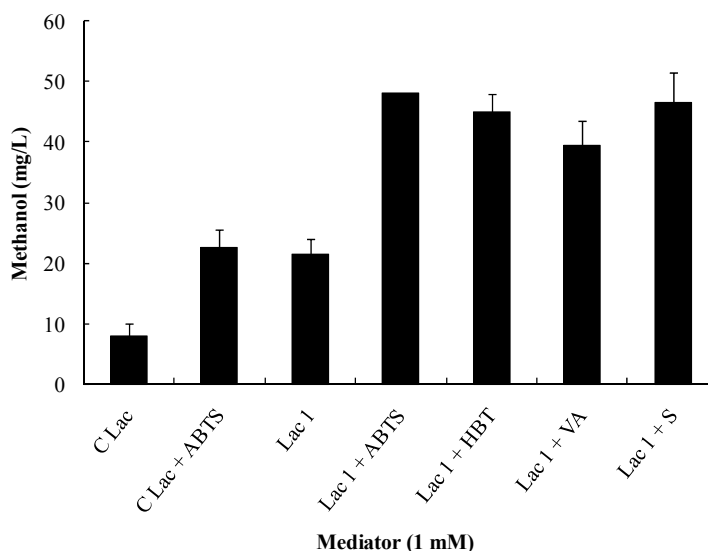
Figure 4.4 Methanol release on oxidation of lignin by laccase/mediator. ABTS: 2,2'-azino*bis* (3-ethylbenzothiazoline-6-sulfonic acid; HBT: Hydroxybenzotriazole, VA: viorulic acid and S: syringaldehyde. **Paper III**.

Laccase can also be used to enhance the auto-adhesion of fibers, thus improving the wet strength and hydrophobicity of the final product (Felby et

al 2004). Moreover, the enzyme has been used in a pilot-scale production of fiberboards. The procedure showed promising results, especially concerning the possibility of avoiding the toxic chemical mixture of urea-formaldehyde (Felby et al 2002; Conner 1996). However, further investigation is required due to the fact that additional steps are needed to enhance the cross-linking between fibers (Felby et al 2002).

4.4 Modification of polysaccharides: Oxidation of alcohol groups
Oxidation of alcohols to aldehydes (or ketones) is a reaction used in chemical synthesis for racemic resolution of molecules (Kroutil et al 2004). It is also applied in the derivatization of carbohydrates (Kim et al 2000), which can be used for the preparation of macromolecular pro-drugs (Bruneel 1993) and for protein immobilization (Guisán 2006; **Paper V**). In addition, it can help to analyze the structure of carbohydrates (Kim et al 2004).

4.4.1 Chemical catalysis
Periodate and sodium bromide/TEMPO/sodium hypochloride are among the most used chemical methods for catalyzing the oxidation of carbohydrates (Kim et al 2000; Guisán 2006; Saito et al 2006; Bragd et al 2004; Marzorati et al 2005).

Periodate catalysis involves the action of a periodate ion, which acts on a molecule with one or more vicinal diols. Subsequently, the C-C bond between the diols is broken to produce two aldehyde groups (Figure 4.5) (Sussich & Cesàro 2000; Vold & Christensen 2005). Periodate is a strong oxidant, which can further oxidize the aldehydes to carboxylic acids (Puchtler et al 1975). It can also cause severe physical modifications of the substrate (Vold & Christensen 2005), thus increasing the susceptibility to biodegradation (Balakrishnan et al 2005).
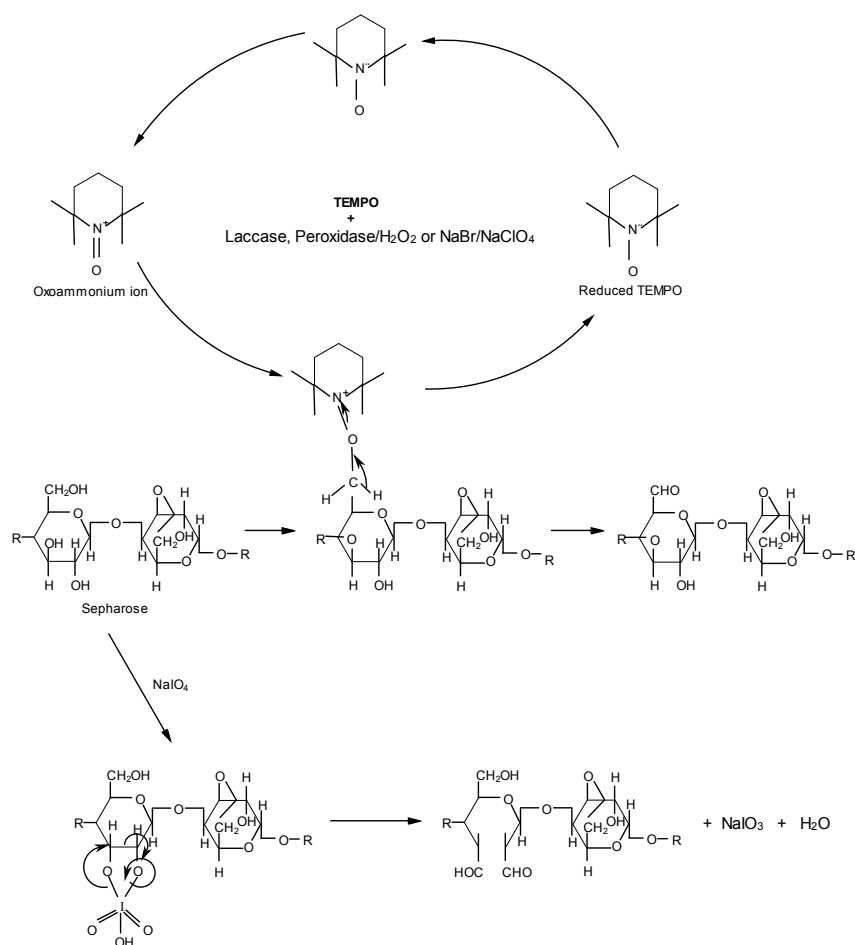
Figure 4.5 Oxidation of agarose by various catalysts: Laccase/TEMPO, NaBr/TEMPO/NaClO$_4$ and NaIO$_4$. Modified from Galli & Gentilli 2004.

On the other hand, sodium hypochlorite-TEMPO-sodium bromide oxidation involves activation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) to produce an oxoammonium ion, which oxidizes the substrate (Figure 4.5) (Bragd et al 2004). Sodium hypochlorite-TEMPO-sodium bromide system, like periodate, is a strong oxidant and thus performs further oxidation of aldehydes to carboxylic acids (Tahiri & Vignon 2000; Fraschini & Vignon 2000). In addition, the system employs toxic and corrosive reagents, which is not very attractive from environmental and safety perspectives. Recent publications have reported on a free sodium bromide oxidation; however, this reaction is performed at high temperatures and utilizes sodium hypochlorite (Bragd et al 2000), which can produce toxic gas emissions.

Other chemical methods to oxidize alcohols involve chromium-based reagents, dimethylsulfoxide, Dess-Martin periodinane, ruthenium and Fétizon´s reagent. These techniques are however of limited use due to their harmful and toxic properties, their high reactivity and low stability, in addition to their high cost (Tojo & Fernández 2006).

4.4.2 Enzymatic catalysis
The application of enzymes, especially oxidoreductases, can be considered as a cleaner way to oxidize alcohols (Bragd et al 2004; **Paper V**).

4.4.2.1 Peroxidase-mediator-hydrogen peroxide system. This oxidation involves the use of a catalytic amount of nitroxyl compounds (i.e., TEMPO). Once the compound is oxidized by peroxidase and hydrogen peroxide, it is able to catalyze the oxidation of primary alcohols at the carbon 6 in a similar way as $NaBr/TEMPO/NaHClO_4$ and laccase (Figure 4.5; Bradg et al 2004). The method has been used for the oxidation of oligosaccharides and polysaccharides (Jetten et al 2000; Jetten et al 2004).

4.4.2.2 Laccase-mediator system. Laccase and different types of mediators are reported to catalyze the oxidation of primary (Fabbrini et al 2002b; Arends et al 2006) and secondary alcohols (Fabbrini et al 2001) on various substrates to produce the respective aldehydes. The efficiency of the system has been tested with mono- and disaccharides (Baratto et al 2006), aromatic alcohols (Majcherczyk et al 1999), allylic alcohols (Fritz-Langhals & Kunath 1998) and Sepharose (**Paper V**).

The simplified mechanism of oxidation is described in Figure 4.5. Laccase activates the mediator, which oxidizes the primary alcohol at the carbon 6 of a sugar molecule to produce the corresponding aldehyde. Oxidation of an insoluble agarose gel, Sepharose CL-6B by laccase/TEMPO gave an aldehyde content of 55 µmol per gram of gel, which was comparable to a periodate-mediated oxidation (with a yield of 72 µmol per gram of gel) (**Paper V**).

Furthermore, the aldehydes produced by laccase/mediator showed good stability upon storage (Fritz-Langhals & Kunath 1998; **Paper V**)**.**

4.4.3 Immobilization of ligands on aldehyde-activated matrices
Schiff base formation (Scheme 4.1) is a spontaneous reaction that gives rise to a double bond between an aldehyde and an amino group. This reaction is applied in order to immobilize ligands or proteins to aldehyde-containing supports (Guisán 2006; **Paper V**). The stability of the bond can be increased by the application of reducing agents, like sodium borohydride or sodium

cyanoborohydride, which transform the double bond into a simple covalent one.
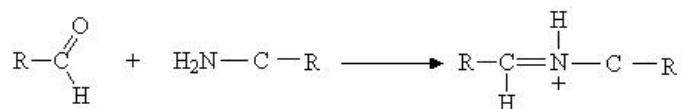

Scheme 4.1 Mechanism of the Schiff base formation between an aldehyde and an amino-containing group

Laccase/mediator-activated Sepharose was found to be comparable to a periodate-activated Sepharose in terms of immobilization of the protein and the retention of activity (Figure 4.6), as well as the stability during recycling of the immobilized protein (Figure 4.7).
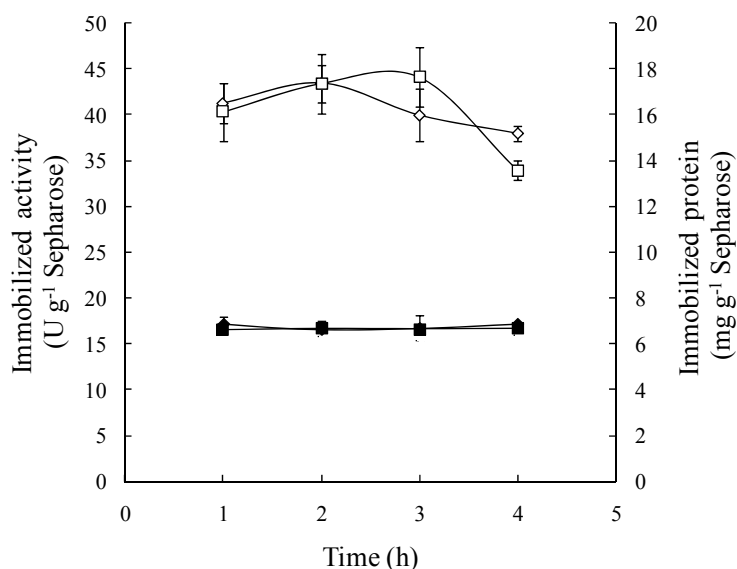
Figure 4.6 Immobilization of trypsin on Sepharose CL-6B beads activated by laccase/TEMPO ($\diamondsuit$) and periodate ($\square$), respectively. The immobilized protein is denoted by filled symbols, while the activity is shown with open symbols. (**Paper V**)
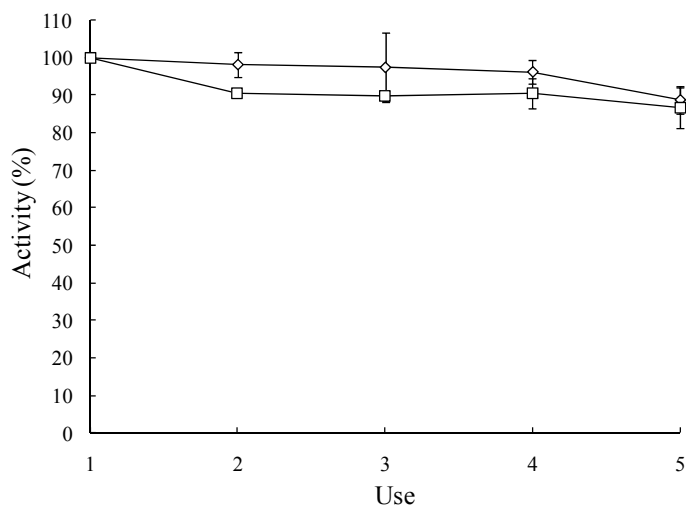
Figure 4.7 Re-use of immobilized trypsin on laccase/TEMPO-
(◇) and periodate- (□) activated Sepharose. (**Paper V**).

4.5 Pulp and paper industry

Laccase/mediator, laccase/peroxidase and laccase/xylanase systems have been applied for paper bleaching and can thus replace the use of chlorinated compounds (Kunamneni et al 2008a; Valls et al 2010). The delignification of eucalyptus Kraft pulp material catalyzed by laccase and mediator was found to reach values up to 50%, whereas with laccase/mediator/xylanase could reach up to 60% (Rochefort et al 2004; Valls & Roncero 2009). In addition, the laccase/mediator treatment was shown to improve the burst, tensile strength (Chandra et al 2004) and brightness (Camarero et al 2007; Valls & Roncero 2009), while the pulp quality was not modified (Kunamneni et al 2008a). On the other hand, laccase mediator systems can also be applied to remove pitch and dyes from wood-based materials (Widsten & Kandelbauer 2008).

4.6 Food industry

Laccase application in the food industry is based on its ability to polymerize molecules (Table 4.3)

Table 4.3. Application of laccase in the food industry for improvement of the quality of the products

Industry	Substrate	Improved parameter	Reference
Wine, Juices, Beer	Phenolic compounds	Clarification, aroma, flavor and taste	Minussi et al 2007; Alper & Acar 2004
Ripe-olive processing	Phenolic compounds	Debittering and darkening	Xu 2005
Sugar beet pectin gelation	Feruloyl groups	Thermostability of the gel	Minussi et al 2002
Bakery	Probably on glutenins, gliadins and arabinoxylans	Softness, strength, machinability of the dough.	Minussi et al 2002; Rodríguez Couto & Toca-Herrera 2006a

Alternatively, laccase participates in the oxidation of residual molecules produced during the processing of food (Yesilada et al 1998; Jaouani et al 2005).

4.7 Other applications

Immobilized laccase can be coupled to physical transducers to build biosensors. The detection of O_2 (Mousty et al 2007), anilines (Xu 2005), antioxidants (Kulys & Bratkovskaja. 2007), toxic substances (Mousty et al 2007) and other phenolic compounds (Jarosz-Wilkołazka et al 2004; Gamella et al 2006) has been reported by such laccase-mediated biosensors.

Laccase has been applied in an immunoassay to improve the detection of molecules (Bier et al 1996; Kunamneni et al 2008a). Here, it was coupled to an enzymatic system in order to regenerate analytes, which helps to amplify the cycles during the immunoassay tests.

Laccase is known to catalyze the oxidation, transformation and cross-linking of various precursors (phenols and anilines), which are used in hair dyeing processes. In personal hygiene, laccase can oxidize thiol, sulfide, ammonia and amine compounds that cause bad breath (Xu 2005).

Laccase or laccase/mediator systems have found applications in the synthesis of molecules with commercial value. Some examples are presented in Table 4.4, where some of the molecules have antitumor, anti-inflammatory and antioxidant properties and are used in the medical field.

Table 4.4 Laccase-catalyzed synthesis of molecules and their applications

Compound	Application	Reference
Salicylic esters	Functionalized biaryls	Ciecholewski et al 2005
Hydroxystilbenes	Analogue to phytoalexin resveratrol, with cardiovascular health properties and cancer chemoprotective agent	Ponzoni et al 2007
Benzofurans	Anticancer, antimicrobial, antioxidant and anti-HIV activities	Witayakran et al 2007a; Hajdok et al 2007
Napthoquinones	Antitumor, wound healing, anti-inflammatory, antimicrobial and antiparasitic activity	Witayakran et al 2007b; Witayakran & Ragauskas 2007
Pinoresinol	Antihypertensive activity	YunYang et al 2007
Para-dihydroxylated benzoic acid incubated with 4-aminobenzoic acid	Importance in pharmaceuticals synthesis	Manda et al 2005
Polyaniline	Conducting materials	Karamyshev et al 2003
Poly (catechol)	Redox polymer exploited in a number of biosensor applications	Aktaş & Tanyolaç 2003
Urushiol	Polymeric films	Kobayashi et al 2001
Tyrosine and tyrosine-containing peptides	Synthesis of "proteins"	Mattinen et al 2005
Antioxidants	Improvement of antioxidant capacity	Ncanana & Burton 2007; Desentis-Mendoza et al 2006

Interestingly, laccase isolated from *Tricholoma giganteumis* showed a significant inhibitory effect against HIV-1 reverse transcriptase (Wang & Ng 2004), which could help to prevent the development of the disease.

Concluding Remarks and Future Perspectives

Laccases are ubiquitous enzymes produced by many living species. Fungi, for instance, produce laccases to oxidize lignin in order to get access to nutrients. This oxidative capacity has been used for various applications, including the removal/degradation of recalcitrant organic pollutants and for the synthesis of organic compounds.

This thesis has shown that new fungal species with the ability to produce laccases and other oxido-reductases can provide enzymes that can be useful for a wide variety of applications, such as facilitating the removal of polycyclic aromatic hydrocarbons from soil, which would otherwise take a long time to get clean by natural processes.

Similarly, the remediation of organic pollutants in wastewaters is an important challenge. To cover this demand, numerous biotechnological procedures have been used; however, they are not always very efficient. For example, the treatment of azo dyes under anaerobic conditions results in high yields of decolorization and the reduction of the Chemical Oxygen Demand (COD). Nevertheless, the mechanism involves the formation of aromatic amines, which need further degradation before being released into the environment.

Other strategies involve the formation of sludge or require high energy costs. In this sense, laccase/mediator systems seem to be a better alternative since they render it possible to catalyze the decolorization/degradation without formation of aromatic amines or accumulation of sludge. Nevertheless, since some mediators are considered harmful for the environment, it would be possible to use natural mediators or macromolecular derivatives of mediators. The former are considerably cheaper and can be easily produced from lignin. On the other hand, macromolecular mediators can be employed together with laccase in membrane reactors. This novel approach seems to be a promising technique as it allows recycling of laccase as well as of the mediator.

Nevertheless, the use of chitosan for immobilization of laccase and the treatment of dyes also appears to be a promising approach, especially considering that chitosan is a cheap material that can adsorb dyes during the treatment, while a laccase/mediator system can catalyze the decolorization/degradation of the dyes. However, much work is still required to evaluate the full potential of these strategies, including further

investigation of immobilization methods for the mediators as well as the optimization of the processes for operating the reactors.

An increasing interest is being devoted to the modification of lignocellulosic material and its further utilization as an inexpensive raw material for the production of adhesives. This is due to the fact that lignin-based materials have decent mechanical properties and are highly resistant to physico-chemical conditions. Moreover, the breakdown of lignin represents the accessibility to cellulose, which is a sought-after substrate for the production of biofuels. In this sense, laccase or laccase/mediator systems offer advantages for the catalysis of the modification of lignin without affecting the properties of the cellulosic material and by avoiding the use of corrosive and toxic conventional methods. Nevertheless, further investigations are necessary in order to increase the feasibility of the enzymatic technique.

Likewise, the modification of other substrates for the synthesis of valuable molecules is an important process leading to the generation of new molecules with novel properties. Laccase catalysis offers the possibility to produce a wide range of new compounds with interesting characteristics, which can be applied in the medical and industrial fields.

The major bottlenecks of fungal laccases for large-scale applications are regarded to be their poor stability during long-term use and their low pH optima. The treatment of textile wastewaters, for example, requires enzymes with high stability and activity under alkaline conditions. Nevertheless, the biodiversity of different environments as well as laboratory evolution may provide new laccases - more powerful and specific - to be used in future applications.

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Paper I

Characterization of *Fusarium* sp. strain BOL35 as *Fusarium santarosense* sp. nov. isolated in the Bolivian jungle and its ability to remove benzo[a]pyrene in oil polluted soil.

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Abstract

Fusarium sp strain BOL35 was isolated on decaying wood substrate from the Bolivian jungle. The fungus was characterized taxonomically, phylogenetically and compared against *Fusarium solani*. The reverse of *Fusarium* sp strain BOL35 colony when grown on SNA usually had a brown color; the aerial mycelium was white to grayish and was short and slightly dense. The fungus sporulated in gray sporodochia. The conidia were relatively slender, seldom uniform in size, rather falcate, with a constricted apical cell and a distinct pedicillate basal cell, mostly 2 to 6 septate. The chlamydospores were observed after 20 days of incubation. The strain was able to ferment several sugars and oxidize ABTS. Laccase enzyme and naphthoquinone pigments were detected as phytopathogenic factors in fungal cultures; although, the fungus showed poor invasive and colonizing characters on wood. The strain was able to remove benzo[a]pyrene from oil polluted soil and in liquid medium. The phylogenetic tree using rDNA sequence, localize *Fusarium* sp strain BOL35 far from a natural cluster dominated by *F. solani*. Based in these observations a new species, *Fusarium santarosense* is proposed.

Key words: Phytopathogen; laccase; *Fusarium santarosense;* Bioremediation; PAHs.

Introduction.

The generation of hazardous wastes is a negative consequence of the expansion of oil and gas field activities that has been increased in these last years in Bolivia. The contamination emerges from oil producing zones, due to exploration activities and operation during the well perforation and in non-traditional zones, where oil pipeline spills exist and in refining plants that generate harmful wastes coming from the storage tanks in the process of sedimentation (Coaquira, 2004). Polycyclic aromatic hydrocarbons (PAH) are pollutants widely found in nature even in oil, they may be of antropogenic nature or from e.g. forest fires. They are listed as priority pollutants and identified as toxic, carcinogenic or mutagenic. Strategies for pollutant degradation is thus a high priority (Alexander, 2000).

Some basidiomycete fungi produce extracellular enzymes involved in lignin degradation. They are also capable of degrading pesticides and PAHs. These enzymes are known as manganese peroxidase (MnP), lignin peroxidase (LiP) and laccase (Cameron et al. 2000). The degradation of recalcitrant compounds in ascomycetes has been related more to the intracellular concentration of cytochrome P450 than to ligninolytic enzymes (Verdin et al. 2005).

In addition to ligninolytic enzymes, naphthoquinones has been considered as a phytopathogenic factor. These pigments are secondary metabolites and produced during cessation of fungal growth. Several pigments from *Fusarium spp.* have been well characterized physically and chemically (Medentsev and Akimenko, 1998). In *Pleurotus spp.* the production of quinones was related to the degradation of lignin (Guillen et al. 2000).

In Bolivia the fungal biodiversity in the jungle has not been studied much. Since microorganisms can be useful tools for biotechnological applications, the knowledge of their biological properties are considered worthwhile.

The aim of this study was to characterize *Fusarium* sp, strain BOL35 isolated from wood decaying in the jungle of La Paz, Boliva. Characteristics as morphology, sugar fermentation, production of lignin modifying enzymes, production of naphthoquinones, wood colonization, as well as its ability to remove benzo[a]pyrene in soil and liquid medium were tested. These characteristics were compared against *F. solani,* a related strain and *Bjerkandera* sp., a white rot fungus. Moreover, the phenotypic character of strain BOL35 was well supported with similarities between the species as seen by 18S/28S rDNA sequencing.

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Material and methods.

Chemicals.

Poly R 478, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), benzo[a]pyrene (BaP) were purchased from Sigma-Aldrich. 2,6-dimethoxyphenol (DMP) 99% came from Acros organics. The soil sample was obtained from a region polluted with oil in Oruro-Bolivia.

Fungi used in this study.

Fusarium sp. strain BOL35 (DSM 17503) and *Fusarium solani* strain BOLSTR (DSM 17502) under Gen Bank accession numbers (DQ 124200) and (DQ 139962) respectively, were isolated in Santa Rosa de Maravilla, San Buenaventura La Paz, Bolivia. The strains were picked up from decayed wood on the soil. *Bjerkandera* sp. strain BOL13 (AY 633927) was isolated in Oruro, Bolivia from an oil polluted site. The fungi were maintained in a medium with the following composition per liter: agar- agar, 15 g; yeast extract, 1 g; glucose, 1 g; KH_2PO_4 0.2 g and K_2HPO_4 0.1 g.

Colony morphology.

The growth patterns of *Fusarium* sp. strain BOL35 and *F. solani* were determined in 7 days old cultures the potato on dextrose agar (PDA). Fungal microscopic characteristics were studied in synthetic low nutrient agar (SNA) overlaid with a piece of 1x2 cm of sterile filter paper (Nirenberg, 1998). The media were inoculated with pieces of agar of 0.5 cm in diameter from a plate prepared 8 days before. They were incubated during 6-10 or until 20 days at 20 ºC in darkness. Pictures of the hyphae, conidiophores, phialides, conidia, chlamydospores were taken. Conidia to be measured were chosen depending of the quantity of septa.

Sugar fermentation.

The capacity to use carbohydrates as a carbon source was determined in solid Czapek's medium supplied with bromophenol blue. Degradation of monosaccharides, disaccharides and

polysaccharides were studied. The acidification of the media was detected by a change from blue to red or yellow of the indicator.

Determination of ligninolytic enzymes as phytopathogenic factor.

The presence of ligninolytic enzymes was studied as pathogenic factor. For qualitative detection, the fungus was grown in two culture media, supplied with 0.25 g/L of the dye poly R 478 and ABTS, respectively. In the medium with poly R 478 the activities of the enzymes were detected due to change in color from purple to yellow; while in the medium supplied with ABTS due to change from transparent to green. The quantitative determination of ligninolytic enzymes were performed in liquid media, in which different concentrations of copper and manganese were used as inducers. *Fusarium* sp. strain BOL35, *F. solani* and *Bjerkandera* sp. were inoculated in sterile conditions as mentioned above. The fungi were incubated for 15 days and the enzymes were detected in the culture medium for withdrawing 200 µL of sample. The determinations were carried out spectrophotometrically in 1 mL of reaction mixture, using 100 µL of sample and 400 µL of buffer. MnP was determined, for the oxidation of DMP to coerulignone ($\varepsilon = 27\ 500\ M^{-1}$ cm^{-1}) at 470 nm in 100 mM tartrate buffer pH 5; $MnSO_4$ 3 mM, 100 µL; DMP 1 mM, 100 µL and H_2O_2 0.5 mM, 100 µL added at the end. LiP was determined to 420 nm, for the oxidation of ABTS to radical cation ($\varepsilon = 29\ 500\ M^{-1}\ cm^{-1}$) using 100 mM succinate buffer pH 3; ABTS 10 mM, 100 µL; H_2O_2 0.5 mM, 100 µL; Laccase was determined with the same reaction used for LiP, but without the addition of hydrogen peroxide (Terrazas et al, 2005).

Isolation of laccase and naphthoquinones by chromatography.

A chromatography separation was performed in order to detect the enzyme and naphthoquinones produced by *Fusarium* sp. strain BOL35. A fungal culture (800 mL) was concentrated 10 X using a 10 kDa cut-off membrane (Sartorius). Then 5 ml of the concentrated fluid was loaded on a 10 ml column containing Q Sepharose beads, pre-equilibrated with acetate buffer 25 mM, pH 5.5. The compounds that bound weakly on the matrix were washed out with 5 column volumes of the same buffer. The enzyme and naphthoquinones were eluted by a salt gradient from 0 to 1 M of NaCl. Fractions of 1 ml at a flow rate of 1 ml min^{-1} were collected for 100 min. The enzyme activity and napththoquinones were detected by scanning spectra of the fractions. In order to determine the apparent molecular weight of laccase, a denaturing sodium dodecyl

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sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad, mini-protean 3cell) was performed on 12 % polyacrylamide gels according to Laemmli (1970). Protein bands were stained with Coomassie brilliant blue R-250. Precision plus protein (All Blue Standards, Bio-Rad) was used as molecular marker.

Pattern of growing in wood

The experiment was carried out using Petri dishes (20 cm in Ø). The fungi were inoculated on 10 g pieces of wood (0.5x 0.2 cm). The pieces of wood were previously sterilized and soaked in a solution of yeast extract, 1%. The pieces of wood were inoculated with 8 days old fungal cultures. Pictures were taken using an image capture system (Biorad Gel) after 15 and 30 days of incubation at 20 ºC.

Benzo[a]pyrene (BaP) removal in soil and in liquid medium.

In order to remove BaP from soil the, fungi were as mentioned above grown on wood for 8 days. Then, 50 g of soil sample with 500 mg Kg^{-1} of pollutant was added to the plates. The mycelia growth and the quantity of BaP removed were measured after 60 days. *F. solani* and *Bjerkandera* sp. were used as positive controls. In order to analyze BaP in the soil samples, this was recovered in a 500 mL flask. The remaining pollutant in soil was dissolved with 150 mL of acetonitrile containing fluorantene 60 mg L^{-1} as internal standard. The flask was put on the shaker at 100 rpm over night in the dark. Then 1 mL from the supernatant was filtered and analyzed for HPLC-UV spectrum. The detection was conducted at 254 nm in a "Varian System" equipped with a reverse phase column, Supelcosil LC-PAH. The elution was carried out in a mixture of acetonitrile and water (80:20 v/v) at 0.8 mL/min. The plates used as a control were treated in the same way.

For Benzo[a]pyrene removal in liquid medium, a stock solution of 1 g L^{-1} of BaP in acetonitrile was prepared. 500 μL BaP was adsorbed in the depth of a serum bottle of 100 mL. When acetonitrile was evaporated, 10 mL of culture medium used for laccase production was added to the bottle, achieving 50 mg L^{-1} of BaP in the medium. Three different cultures, grown at different nitrogen concentrations were tested. The medium was inoculated with the *Fusarium* sp strain BOL35 as mentioned above. Controls without BaP, but with fungal inocula and without inocula, but with BaP were prepared. The experiment was carried out in triplicate and the cultures were incubated for 30 days at 20 ºC in darkness. The remaining BaP in the bottles was

dissolved by addition of 30 mL acetonitrile to the entire culture. BaP was analyzed as mentioned above.

18S/28S rDNA sequencing and phylogenetic analysis

Fusarium strain BOL35 and *F. solani* were grown in the medium used for maintenance, but without agar. Approximately 1.5 ml of mycelium was withdrawn, washed twice with ultra pure water and placed into sterile Eppendorf tubes containing 0.3 g of glass beads, particle size 425-600 μm. To obtain chromosomal DNA, 0.2 ml of extraction buffer containing 100 mM Tris-HCl pH 8, 1% SDS, 2% Triton X-100, 10 mM EDTA, 100 mM NaCl were added plus 0.2 ml of phenol:chloroform:isoamylalcohol at a ratio of 25:24:1 respectively. The soluble fraction containing DNA was purified according to standard procedures (Sambrook et al. 1989). Partial sequence of the nuclear rDNA ribosomal genes and spacer regions 18S, ITS1, 5.8S, ITS2 and 28S were amplified using the primers (Gardes and Bruns 1993): ITS1- F (5´- CTT GGT CAT TTA GAG GAA GTA A-3´) and LR3 (5´-GGT CCG TGT TTC AAG ACG G-3´). For D1/D2 domain, the primers used were F63 (5´-GCA TAT CAA TAA GCG GAG GAA AAG-3´) and LR3, also named CTB6 and NL4 respectively (Altschul et al. 1997). PCR reactions were performed in a Gene Amp PCR System 9700. For ITS1 and NL4 primers, the PCR procedure had an initial denaturation at 94 °C by 4 min followed by 30 cycles of denaturation (94°C, 60 s), annealing (55°C, 60 s), extension (70°C, 60 s) and a final extension at 72 °C by 7 min. To amplify the D1/D2 domain, amplification conditions were an initial denaturation at 94 °C by 3 min followed by 30 cycles of denaturation (94°C, 60 s), annealing (55°C, 60 s), extension (72°C, 60s) and a final extension at 72°C by 7 min. Negative controls (excluding DNA template) were included in each reaction to ensure no contamination in experimental materials. PCR products were purified with a Gel extraction Kit (Cat. N. 28704 Qiagen). DNA sequencing of both strands was performed using the dideoxy chain termination method with an ABI prism 3100 DNA Analyzer, and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). The phylogenetic analysis based on 18S/28S rDNA was performed with the aid of DNAMAN v.4.03 (Lynnon Biosoft) software.

Results.
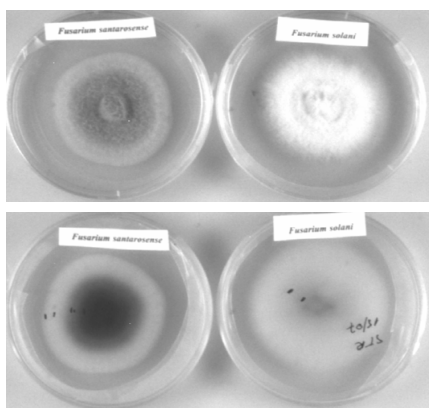
Figure 1. Colony characteristics in PDA medium. View from the top and reverse plate of *Fusarium sp strain BOL35* to the left and to the right *Fusarium solani*. The fungi were grown for 7 days in the dark at 20 C.

Morphology of *Fusarium* sp. strain BOL35

Colonies growing in concentric rings, reaching 5-6 cm of diameter in 7 days on PDA in the dark at 20 ºC, with entire margins (Figure 1). Short aerial mycelium, slightly dense, white to grayish. Colony reverse usually light brown. Sporulation in grey sporodochia starting after 10 days in the aerial mycelium, the conidia aggregate in false heads in SNA. Odor not perceptible. Conidiophores arising as single lateral phialides on hyphae, soon branching generously terminating in phialides (Figure 2B). Conidia relatively slender, not uniform in size, rather falcate, with a constricted apical cell and a distinct pedicillate basal cell, mostly 2-6 septate in the dark, SNA medium. Measuring, 6-septate (39.5-)41 - 46.2(-48.5) x 4-4.5 µm (Figure 2A). Chlamydospores in pairs or short chains observed after 20 days of incubation in SNA.

Occurrence: Wood decaying in shadow places of the jungle.

Delimitations: *Fusarium* sp strain BOL3 is a species slowly growing on decaying wood substrates, colony with entire margin, the sporodochial conidia with a constricted apical cell and a distinct pedicillate basal cell, mostly 2 - 6 septate.

Distribution: Bolivian jungle. La Paz: San Buenaventura, Santa Rosa de Maravilla. S 13° 57.076'; W 68° 00.276'; altitude 292 m. Substrate: decayed wood. 6 Aug 2003.

Description in Latin: Coloniae in agaro PD temperatura 20 C in 7 diebus circiter 5- 6 cm diametro, in obscuritate, margine integro. Mycelium aerium griseae. Color in parte reversa rubrae, Initium sporulation in micelio aerio praecox, conidiis typice in capitulis falsis agregatis. Sporodichia griseus post 10 dies formata. Odor non perceptibilis. Conidiophora simplicia vel ramosa cum phialidibus simplicibus, plerumque monophialidicae, raro poly poliphialidicae.

Conidia etiam accumulata in sporodochiis brunneis, hyaline, falciforma, apice acerosa, pedicellata, plerumque 6-septata, in agaro SN in obscuritate (39.5-)41 - 46.2(-48.5) x 4-4.5 µm. Chlamydosporae griseae singulares vel binae post 20 dies formata.

Habitat

Aera geographica: Bolivia, La Paz/San Buenaventura, Santa Rosa de Maravilla, 6 Aug 2003.

HOLOTYPUS: in culto agaro desiccatus Fusarii santig-rosense culturae BOL35, deposita DSMZ Germania.

A   B

C   D
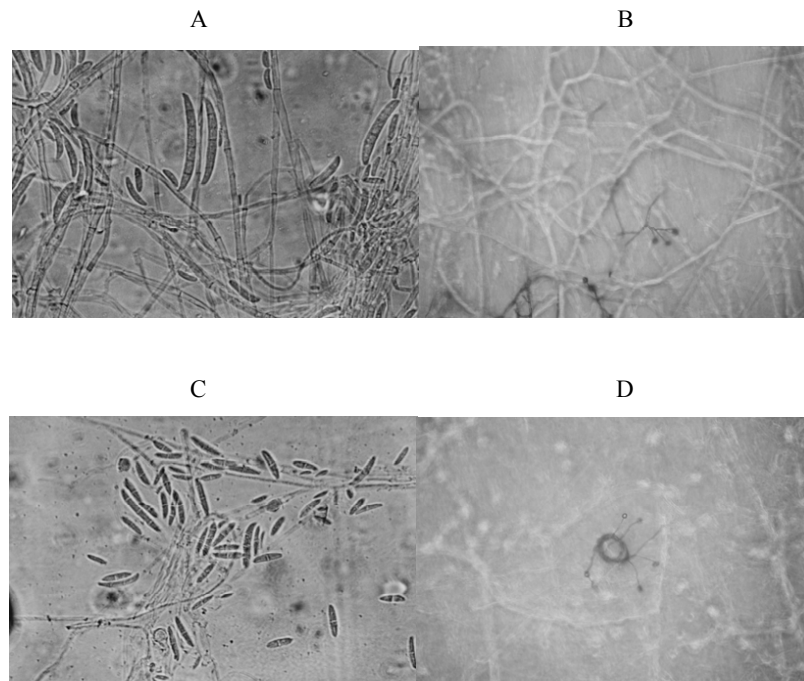
Figure 2. Microscope images of *Fusarium* in 10 days old cultures. *Fusarium* sp strain BOL35, (A) macroconidias and (B) conidiophores and phialides. *Fusarium solani* strain BOLSTR, (C) macroconidia and (D) conidiophores and phialides. The images were taken using a Nikon microscope, 400 X for macroconidia and 100 X for conidiophores and phialides.

Taxonomy of *Fusarium* sp. strain BOL35

Fusarium strain BOL35 was capable to ferment most of the sugars tested. The biomass formed in the culture medium was not dense. The pattern of sugar fermentation and growth was different compared to that of *F. solani* (Table 1).

Table 1. Carbohydrates fermentation by *Fusarium* sp. strain BOL35[a]

Strain	Carbohydrate[b]							
	Glucose	Xylose	Sucrose	Maltose	Lactose	Starch	Xylan	Cellulose
Fusarium BOL35	+	+	+	+	-	+	+	+
Fusarium solani	+	+	-	+	-	-	-	-

[a]The signs: (+) denote growth and acidification of the medium by the fungus, (-) means growth without acidification.
[b]The different sugars were assayed by triplicate in Czapek's medium at 30 g/L, supplied with bromophenol blue. The cultures were incubated for 7 days and observed every day during this period.

Fusarium sp. strain BOL35 was capable to produce lignin degrading enzymes. The strain BOL35 was unable to decolorize the dye poly R 478 in similar way as was *F. solani*; however, both strains were capable to oxidize ABTS in agar plates. In order to test if the ABTS oxidation was due to laccase, MnP or LiP, the fungus was cultivated in a liquid medium in which Mn^{2+} or Cu^{2+} was used as inducer. Indeed the laccase was the dominant enzyme found in the liquid medium with strain BOL35, only when Cu^{2+} was present at a concentration of 8 mg L^{-1}. Neither *Fusarium solani* nor *Bjerkandera* sp. strain BOL13 were capable to produce laccase under that condition. In presence of Mn^{2+}, *F. solani*, was producer of laccase, while *Bjerkandera* sp. strain BOL13 was producer of MnP (Table 2).

Table 2. Determination of lignin modifying enzymes in fungal cultures[a]

Strains [c]	Qualitative		Quantitative[b]			
	Laccase or MnP		Cu^{2+}		Mn^{2+}	
	Poly R478	ABTS	Laccase	MnP	Laccase	MnP
Fusarium sp strain BOL35	-	+	80±10	ND	ND	ND
Fusarium solani	-	+	ND	ND	50±15	ND
Bjerkandera sp.	+	+	ND	ND	ND	70±12

[a]The oxidation of Poly R478 and ABTS was carried out in presence of 1 g/L glucose; while for quantitative production of enzymes was used 5 g/L. The basal medium was composed of; NH_4NO_3, 40 mg; yeast extract, 2 g; KH_2PO_4, 0.2 g; K_2HPO_4, 0.1 g; $MgSO_4$ $7H_2O$, 0.5 g; $CaCl_2$ $2H_2O$, 0.01 g, $CuSO_4$ $5H_2O$, 8 mg or $MnSO_4$ 40 mg and distilled water, 1 liter.
[b]The laccase in *Fusarium* sp strain BOL35 was determined at pH 3, using 100 mM succinate buffer, while *Fusarium solani* laccase was assayed at pH 5 in acetate buffer 100 mM.
[c]*Fusarium solani* was the positive control for production of laccase and *Bjerkandera* sp. for production of MnP.

The laccase of *Fusarium* sp. strain BOL35 is a protein of 66 KDa. Indeed, the enzyme was isolated by column chromatography. The SDS-PAGE showed a single band and with an apparent molecular weight of 66 kDa (Figure 3). This enzyme oxidizes ABTS and DMP as traditional laccase does.
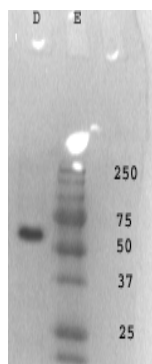

Figure 3. SDS-PAGE. The molecular weight of the protein was estimated by using a calibration curve with the migrated distance of the molecular markers. The protein was obtained by chromatography in column using Q-sepharose. The laccase appear isolated in the fraction 63 to 68, collected at rate of 1 ml/min for100 min.

In addition to laccase, naphthoquinones were found in the culture medium of *Fusarium* sp. strain BOL35. Indeed, during chromatography laccase was isolated, but also naphthoquinones. Two peaks characteristics of naphthoquinones were clearly detected by scanning spectra. The maximum absorbance of the fractions in the UV-visible spectrum was observed around 500 nm and 300 nm (Figure 4).

The pieces of wood were partially colonized by *Fusarium* sp. strain BOL35. After 14 days of growth on the wood the colonization was not total. The mycelium was slightly dense. The pattern of growth on wood was completely different to that of *Fusarium solani* (Figure 5). Indeed the mycelia of *F. solani* was very dense, the pieces of wood were colonized completely (invasive) and the wood remained colonized after 60 days. Similar results were found by using *Solanum tuberosum* leafs or wheat leaf sheaths as a fungal substrate (data not shown).
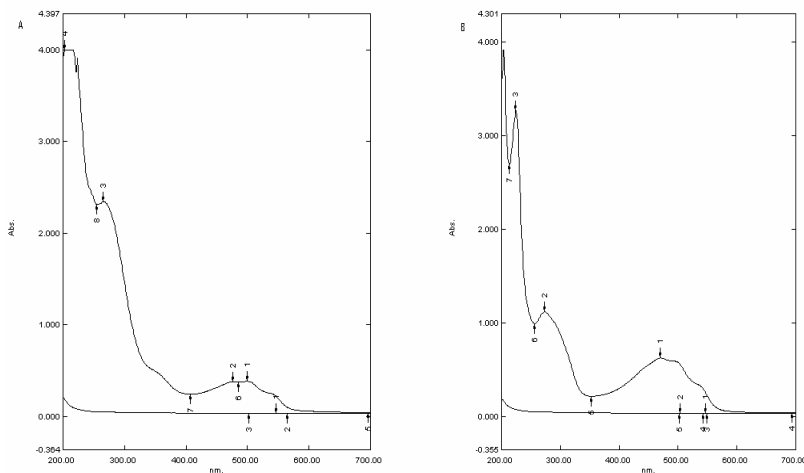
Figure 4. Scanning spectrum of naphthoquinones isolated by chromatography in column from cultures of *Fusarium* sp. strain BOL35. In A, spectrum for the fraction 74. In B the spectrum for the fraction 92. The chromatography was run at rate of 1 ml min^{-1} and fractions of 1 ml were collected for100 minutes.
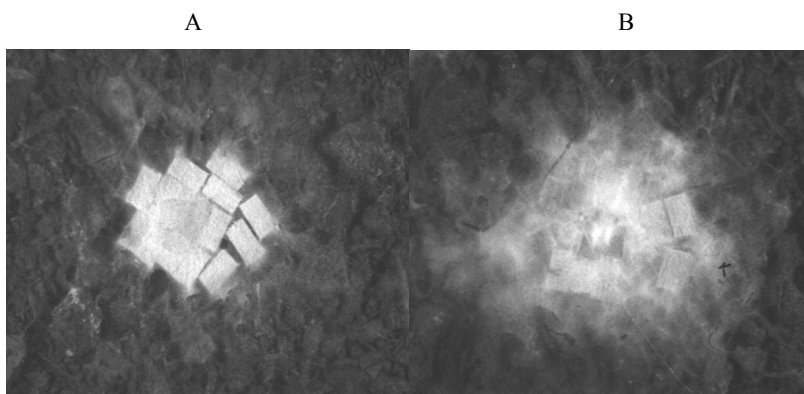
Figure 5. Growth pattern in pieces of wood and in oil polluted soil of *Fusarium* sp. strain BOL35 (A) and *F. solani* strain BOLSTR (B). The pieces of wood were sterilized and hydrated with a solution of yeast extract (1%). The pices of wood were inoculated with 8 days old fungal culture. A soil amended with benzo[a]pyrene, 500 mg/Kg was added to the plates after 8 days on incubation. The pictures were taken after 30 days of incubation at 20 C.

Fusarium sp. strain BOL35 was capable to remove benzo[a]pyrene when it grew on oil polluted soil. The growth of mycelia of strain BOL 35 was slightly dense on the surface with a visible growth in the depth of the soil sample (Figure 5). Approximately 30% of BaP was

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removed from an intentionally polluted soil sample. Removal of BaP was more efficient when the soil was inoculated with strain *F. solani*, or *Bjerkandera* sp. strain BOL13 (Table 3).

Table 3. Percentage of benzo[a]pyrene removal on oil polluted soil by *Fusarium*.

Treatment	*Fusarium* sp. strain BOL35	*Fusarium solani*	*Bjerkandera* sp.
Sterile soil plus fungus	40±7	60±15	75±18
Sterile soil non inoculated	10±5	5±3	14±4
Non sterile soil plus fungus	55±8	70±12	65±10
Non sterile soil without fungus	20±4	22±7	18±6

The soil was amended with benzo[a]pyrene 500 mg/Kg from a stock solution of 1% in acetonitrile. The plates were aerated every week and sprayed every two weeks with 2.5 mL of sterile water in order to supply humidity to the soil and to the fungi.

Removal of BaP in liquid medium was dependant of nitrogen concentration in the medium. Only in the medium with 40 mg L^{-1} of ammonium nitrate a significant removal of BaP (70%) was observed. In medium containing twofold of concentration of nitrogen or without any addition of nitrogen source the BaP disappearance decreased until 45 and 30% respectively (Figure 6A).
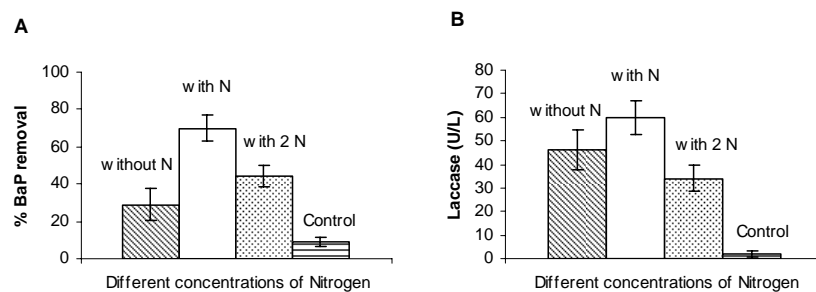
Figure 6. Degradation of benzo[a]pyrene (A) and laccase production (B) in liquid medium by *Fusarium* sp. strain BOL35. Three different media were used in order to remove BaP. The dashed columns, media without NH_4NO_3; white columns, media with 40 mg L^{-1} of NH_4NO_3; and doted columns, media with 80 mg L^{-1} of NH_4NO_3. The error bars show the standard deviation of triplicate measurement. The controls had BaP but were no inoculated with the fungus.

Phylogenetic characterization of *Fusarium* sp. strain BOL35

18S and 28S rDNA sequences of *Fusarium* sp strain BOL35 notably differs with the parent strain *F. solani*. Comparing in GenBank databases with closely related species, strain BOL35 was found to bear close relationship with *F. solani* strain voucher with 96% of sequence similarity. However, the phylogenetic tree obtained by neighbour joining method, showed that *Fusarium* sp. strain BOL35 has a low bootstrap values, 39% respect to the cluster dominated by *F. solani* cucurbitae and *F. solani* strain voucher (Figure 7). The rDNA sequence of strain BOL35 was deposited in the Gen Bank (NCBI) under the accession number DQ 124200.

Discussion

The new species of *Fusarium* was isolated in the Bolivian jungle. It differs in several characteristics compared to its closely related strain *Fusarium solani*. The strain BOL35 showed relatively low growth rate, distinct colony morphology, colony consistence and color. The macroconidia of strain BOL35 was different to *F. solani* strain BOLSTR; however, it was similar to *F. torulosum* (Nirenberg, 1995) or *F. solani* A type (Matuo and Snyder 1973). The chlamydospores found in strain BOL35, resemble to those of *F. equiseti* rather than those of *F. solani*. This incongruence was expected, since the phenotypic character usually is affected by conditions of the culture medium and the environment.

Studying sugar fermentation pattern, additional differences between species were found. Several sugars were fermented by *Fusarium* sp. strain BOL35 contrary to *Fusarium solani*. Conversely, strain BOL35 showed a small biomass; moreover, this phenomenon was expected, since the fermentative metabolism of sugars does not yield high biomass.

Fusarium sp strain BOL35 might be phytopathogenic, as judged from the fact that laccase activity was detected in its culture media. *Fusarium* sp strain BOL35 was able to oxidize ABTS only in the medium supplied with glucose, but it was unable to oxidize poly R478. These properties were tested using *Bjerkandera* sp. strain BOL13 which oxidizes ABTS as well as the dye poly R478. The oxidation of ABTS and dyes can be done biologically by laccase, LiP and MnP. In liquid cultures of strain BOL35 and *F. solani* only laccase was detected. However, an
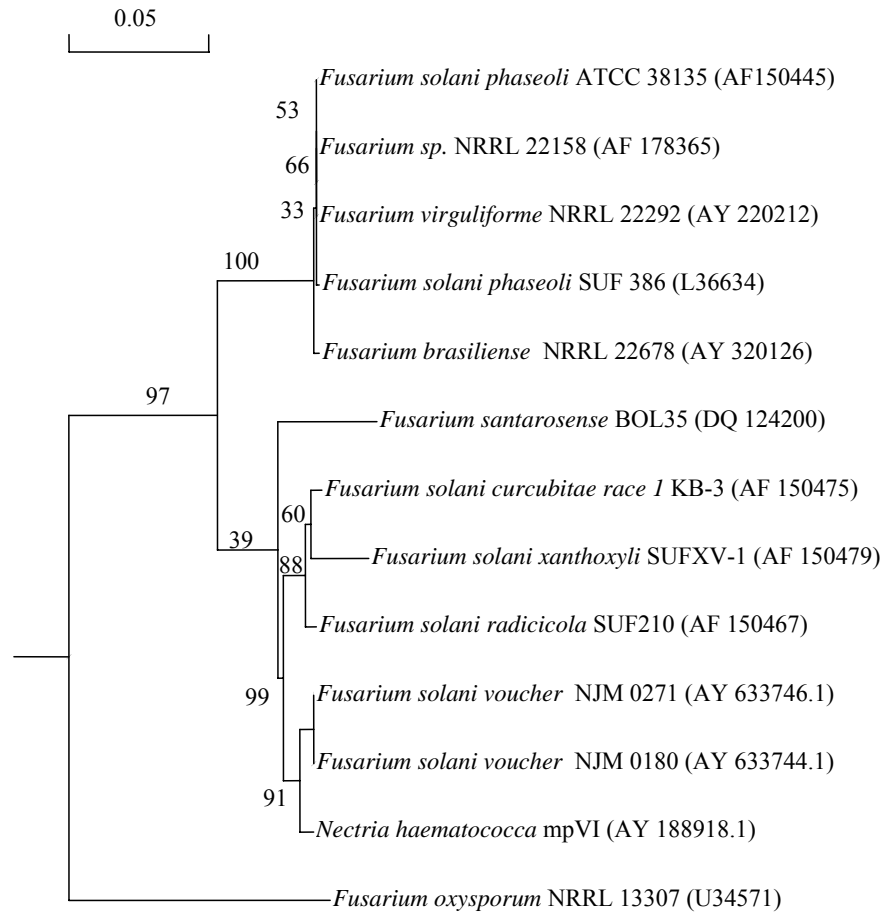
Figure 7. A neighbour - joining tree based on the rDNA-ITS region of *Fusarium* sp. strain BOL35 and related species. *Fusarium oxysporum* was used as out group to root the tree. Branch length was proportional to the distance represented by the bar. Frequencies occurrence in 100 or 500 bootstrap replicates are indicated on the internodes.

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interesting difference was detected when analyzing the samples. The laccase of strain BOL35 had higher activity at pH 3 than at pH 5 contrary to what is observed for traditional laccase of *Fusarium spp.* (Kwon et al. 2001). At physiological pH around 5 for fungi, this enzyme can be inactive. Probably this fact can be supported by the growth pattern on wood substrate, where the strain BOL 35 was not invasive at all; although, more studies must be done. The molecular weight and catalytic activity of the isolated laccase confirm its presence in cultures of strain BOL35.

Fusarium sp. strain BOL35 excreted naphthoquinones in the culture medium. The presence of naphthoquinones was easily evidenced, since they gave a characteristic red color to the cultures. At least two different spectra were detected by UV-visible scanning. The maximum peaks of the pigments were at 500 nm and 300 nm. This finding is in agreement with studies of Medentsev and Akimenko (1998) in which several naphthoquinones were detected from *Fusarium spp*. The physiological role of naphthoquinones is not completely clear; although, they are considered as a phytopathogenic factor of fungi due their cytotoxic effect.

Benzo[a]pyrene was removed by *Fusarium* sp. strain BOL35 in an oil polluted soil. Although the removal of the PAH was not high (30%), it can be significant, especially when the fungus is used on non sterile soil, where the removal increases approximately until 55%. It is interesting to note that, the strain BOL35 prefer to grow more in the sub surface rather than on the surface of the soil, showing a microaerophile character. The low removal would be explained by the unfavorable conditions offered to the fungus by the soil with regard to heavy metals, pH and predating microorganisms. In some cases, accumulation of an intermediate product has been observed during soil remediation (Andersson and Henrysson, 1996). The ability to remove BaP by strain BOL35 was confirmed when it was capable to degrade the pollutant in liquid medium, although the disappearance of BaP was dependent of the nitrogen concentration. A nitrogen deficient culture medium and a culture medium with high nitrogen concentration were unfavorable for BaP removal. The removal of BaP was well supported with laccase activity in the cultures (Figure 6B). Furthermore the presence of naphthoquinones was noticed once in the fungal cultures. Apparently laccase and naphthoquinones are constitutively produced by strain BOL35. It has been demonstrated that the nitrogen source in the cultures of some ascomycete, modulate the synthesis of naphthoquinones (Baker et al., 1981) and laccase.

The degradation of BaP by fungi has been related to production of laccase, MnP and LiP by white-rot fungi (Cameron, 2000). In ascomycetes and some basidiomycetes it is believed that the presence of intracellular cytochrome P450 can be involved in the disappearance of BaP. In *Pleurotus spp.* degradation of non-phenolic lignin has been attributed to free radicals production in which laccase and semiquinones play a key role (Guillen et al., 2000). Here two potential factors responsible for BaP degradation were found; laccase and naphthoquinones in cultures of *Fusarium* sp. strain BOL35. Hence BaP removal by this fungus can be due to free radical more than direct enzymatic oxidation.

Strain BOL35 can be a new species of *Fusarium*. The sequences comparison of the divergent domains, D1-D2, in the 28S rDNA, showed 99% of identity with *Sinosphaeria bambusicola*; currently called *Thyridium chrysomallum (Thyridiaceae)* (Eriksson et al., 1989); however, it was discarded for its morphological differences between both strains. The second strain with 94% of identity was *Fusarium* sp. NRRL 22354. Since, a second round of amplification of rDNA was done using ITS1 and NL4 primers, 1039 base pairs were sequenced to get a clear identity or differences with the closely related strains. Indeed, with the last sequence obtained from strain BOL35 and by comparison with related sequences showed 96% of identity with *Fusarium solani* voucher NJM 0271. Even more, the phylogenetic tree showed that strain BOL35 evolved from an common ancestor that gave origin to *F. solani*, however it does not appear clustered with the known species, lower bootstrap even by comparison of 400 or 600 bp (Mitchell et al., 1995; Suga et al., 2000). These differences were well correlated with morphological, physiological and biochemical characters between strain BOL35 and *F. solani*.

Based on the taxonomic and phylogenetic differences, we propose strain BOL35 as a new member of the genus *Fusarium*, with the name *Fusarium santarosense*. This strain differs from the related strain *F. solani* with respect to morphology, sugar fermentation, napththoquinones and laccase production. Two pathogenic factors were isolated, laccase enzyme and naphthoquinones. The phytopathogenic pattern with regard to wood showed that the strain is less invasive than the *F. solani*. It can remove benzo[a]pyrene from soil and liquid medium. Further studies must be done in order to establish the biotechnological potential of this novel strain.

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Paper II

Laccase from *Galerina* sp. HC1: production and application in dye decolorization

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Abstract

The laccase producing fungus, HC1 was isolated from a sample collected in the Bolivian Amazon region. Analysis of 5.8S and 28S rDNA, and internal transcribed spacer 1 & 2 sequences revealed that isolate HC1 belongs to genus *Galerina*. High production of laccase was achieved in basal salt medium supplemented with 30 g/l glucose, 10 g/l yeast extract, and 0.01 g/l copper sulphate. The enzyme production was further improved by addition of 2,5-xylidine, α-benzoin oxime and *p*-coumaric acid, but strongly repressed by sinapyl alcohol, 3-(dimethylamino)benzoic acid, hydroquinone, 1-naphthaleneacetic acid, 2,6-dichlorophenol and 3-methyl-2-benzothiazolinone hydrazone. Cultivation under optimum conditions in the presence of 2 mM 2,5-xylidine and 0.01 g/l copper sulfate, resulted in the enzyme yield of over 26 000 U/l. *Galerina* sp. HC1 could also produce laccase in media composed of orange peels both in submerged- and solid-state fermentations.

The laccase from *Galerina* sp. was able to decolorize over 60% of 35 μM Congo red in 2 h in the presence of 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt as mediator, and 65% of 30 μM Coomassie Brilliant Blue G-250 in 5 min using syringaldehyde as mediator. The laccase was immobilized on cross-linked chitosan and used efficiently to decolorize the dyes.

Keywords *Galerina* sp. - laccase - inducer - dye decolorization – immobilized enzyme

Introduction

Laccases (EC 1.10.3.2) are multicopper oxidases that are known to be the most abundant members of the oxidoreductase family, which catalyze the oxidation of various substrates using molecular oxygen as the electron acceptor. They are widely distributed among plants, fungi, bacteria and insects. In plants, laccases are known to be involved in the synthesis of lignin by catalyzing the free radical polymerization of *p*-coumaryl-, coniferyl- and sinapyl alcohols [31], while fungal laccases are involved in lignin degradation and pathogenesis [4], and are also considered to be necessary for fungal development and morphogenesis [50]. In bacteria and fungi, laccases are useful in synthesis of pigments such as melanin [23] and detoxification of toxic compounds [47]. In insects, the primary role of laccases is in cuticle sclerotization [37], a process where the cuticle stabilizes by cross-linking of reactive quinones.

Laccases are regarded as green catalysts for *in vitro* oxidation under mild conditions using molecular oxygen as oxidant without the need of any expensive cofactor unlike several other oxidoreductases. These enzymes have shown remarkably low substrate specificity catalyzing the oxidation of a fairly broad range of substrates such as phenolic compounds, diamines and aromatic amines with concomitant reduction of oxygen to water. The substrate range is increased in the presence of mediators – compounds that act as electron shuttles.

So far, laccases have been used in delignification of pulp, detoxification of industrial effluents, bioremediation of soils contaminated with herbicides, pesticides and certain explosives, synthesis of drugs and cosmetic ingredients, medical diagnostics, etc. [4,37]. They have even attracted attention for catalysing decolorization of dyes that are used in large amounts (10^9 kg annually) in leather, textile and printing industries and have caused considerable environmental concern [10]. Most of these dyes or their derivatives are mutagenic and carcinogenic [46], and about 10% of the total amount used in the industries is estimated to end up in effluents [21].

The widespread interest in laccases has led to efforts in search for organisms producing these enzymes, the great majority of which are fungi. Among the well known laccase producers, *Trametes versicolor*, *Agaricus bisporus, Coriolus* spp., *Pleurotus ostreatus*, *Phlebia radiata*, *Pycnoporus cinnabarinus* and *Coprinus cinereus* belong to basidiomycetes. Recently, we have purified and characterized an interesting laccase from a newly isolated fungus belonging to *Galerina* [24], a

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basidiomycete which often grow on dead bryophytes, on woody materials or on other plant debris. This paper reports studies on the production of laccase activity by this organism and its ability to decolorize a toxic azo dye and a triphenylmethane dye.

Materials and Methods

Chemicals

The following chemicals were purchased from Sigma-Aldrich (Germany): 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Sabouraud broth, yeast extract, coniferyl alcohol (C Al), sinapyl alcohol (S Al), sinapic acid (S Ac), 3,5- dinitrosalisylic acid (DNS), 2,6-dichlorophenol (DP), 1-naphthaleneacetic acid (1-Nac), nicotinic acid (N Ac), benzidine (BZD), hydroquinone (HQ), naphthalene (N), pyridoxine (P), pyrogallic acid (P Ac), caffeic acid (C Ac), α-benzoin oxime (B), 4-aminobenzoic acid (4AB), ferulic acid (F Ac), *p*-coumaric acid (*p*C) benzoic acid (B Ac), 3-methyl-2-benzothiazolinone hydrazone (MTBH), 3-dimethylaminobenzoic acid (DMBA), syringaldehyde (SA), 2,5-xylidine (X), veratryl alcohol (VA) and 1-napthol. Eucalyptus Kraft lignin was kindly offered by Innventia AB (Stockholm). Malt extract agar was obtained from HiMedia Laboratories Limited (Mumbai, India) while agar-agar was from Riedel-de-Haën (Germany). All salts and solvents used were of analytical quality and were obtained from standard sources.

Organisms, culture conditions and screening of laccase producers

Isolate HC1 was selected in a screening procedure involving 41 fungi collected in the upper Amazon River basin in Bolivia and grown on modified malt extract agar containing per liter: 45 g malt extract agar and 3 g peptone. The cultures were purified by repeated transfer to fresh agar plates, and grown at room temperature (around 22 ℃) for 12 days and then stored at 4 ℃, and sub-cultured on fresh agar plates every month. Screening for the presence of laccase activity was done by formation of green halos on solid ABTS medium containing per liter: 1 g glucose, 15 g agar-agar, 0.5 g yeast extract, and 2.5 g ABTS.

Identification of isolate HC1

Isolate HC1, was grown in Sabouraud broth. After incubation for 7 days at 25 °C, 1.5 ml of the culture was withdrawn, washed twice with sterile ultra-pure water before transferring the fungal mycelia to sterile Eppendorf tubes and vortexing with 0.3 g

glass beads (425-600 μm; Sigma) to break the cells. Chromosomal DNA was purified from the lysate according to Sambrook et al. [39] and used as template for the PCR reaction. Primers ITS1- F (5´-CTT GGT CAT TTA GAG GAA GTA A-3´) and ITS4-B (5´-CAG GAG ACT TGT ACA CGG TCC AG-3´) were used to amplify internal transcribed spacer regions 1 (ITS1) and 2 (ITS2), and the rDNA 5.8S and 28S as described by Gardes & Bruns [19]. The PCR reactions were performed in a Gene Amp PCR System 9700 (PE Applied Biosystems) and products were purified after electrophoretic separation using a gel extraction kit (Qiagen). The purified product was sequenced in both directions with the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, USA) following the manufacturer's protocol. Sequence reactions were electrophoresed using ABI 3100 DNA sequencer.

Laccase production by isolate HC1

Thirty millilitres of basal medium (pH 6) composed of 0.04 g NH_4NO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g K_2HPO_4, 0.2 g KH_2PO_4, and 0.01 g $CaCl_2 \cdot 2H_2O$ per liter was supplemented with varying concentrations of glucose, yeast extract and copper sulphate was autoclaved. After cooling, each bottle containing the medium was inoculated with 6 agar discs (0.5 cm in diameter) taken from 12 days old agar plate. The bottles were incubated at room temperature (22 °C) with continuous agitation at 100 rpm. Samples were taken after 10, 15 and 20 days of cultivation for determination of laccase activity.

To 30 ml basal medium supplemented with 4 g/l glucose, 4 g/l yeast extract and 0.05 g/l $CuSO_4 \cdot 5H_2O$ were added different chemical additives (1 mM) or lignin (1 g/l) and laccase production was followed over a period of 20 days. The additives used were C Al, S Al, S Ac, C Ac, DNS, DP, B Ac, 4 AB, DMBA, 1-Nac, N Ac, X, BZD, HQ, N, P, P Ac, B, VA, MeOH and EtOH, respectively.

In another experiment, dried orange peels and bagasse fibers, respectively, were used as inexpensive substrates for production of laccase by isolate HC1. Thirty milliliter of the basal medium containing 0.05 g/l of $CuSO_4 \cdot 5H_2O$ and 20 g/l of orange peels or bagasse fibers were inoculated with the fungus and incubated as described above. Samples were withdrawn at different time intervals to determine the laccase activity.

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Besides submerged fermentation, the enzyme production using orange peels was also studied by solid-state fermentation. The orange peels were soaked overnight in 0.2 M KOH, washed extensively until neutralization and lyophilized. About 3 g of lyophilized peels was added to 100 ml bottles containing 25 ml of the basal medium and autoclaved. Some bottles were supplemented with copper sulphate or 2,5-xylidine or both.

Immobilization of the *Galerina* sp. laccase on cross-linked chitosan

The fungal culture was filtered using filter paper and the enzyme in the filtrate was partially purified using a combination of anion exchange and hydrophobic interaction chromatographies as described earlier [24]. A medium molecular mass chitosan (750 kDa) was cross-linked with glutaraldehyde according to Zhang et al. [49] and extensively washed with water to remove the excessive glutaraldehyde. Then, 20 ml of the partially purified laccase (6 U/ml and specific activity of 75 U/mg) was added to 4 g of the cross-linked matrix and mixed on rocking table at 4 °C overnight. Subsequently, it was centrifuged and thoroughly washed to remove the unimmobilized enzyme. The preparation was stored in wet condition at 4 °C.

Dye decolorization using free or immobilized laccase

The efficiency of isolate HC1 laccase in degrading Congo red (azo dye, λ_{max} 500 nm) and Coomassie Brilliant Blue G-250 (triphenylmethane dye, λ_{max} 595 nm) was assessed using ABTS and syringaldehyde as mediators. The reaction mixture was composed of 25 mM sodium acetate buffer pH 5, 0.5 U/ml laccase, 35 μM Congo red or 30 μM Coomassie Brilliant Blue G-250, and 50 μM mediator in a total volume of 800 μl. The change in the absorbance of the reaction mixture was followed for 3 h at the respective wavelengths. For comparison, the dyes were also treated with commercially available *Trametes versicolor* laccase under the same conditions.

Dye decolorization by the immobilized laccase was studied by circulating 12 ml of the dye solutions over a packed column (5 cm high and 1cm diameter) with 500 mg of the preparation at a flow rate of 1.6 ml/min. As the dyes were adsorbed to the cross-linked chitosan (0.36 mg dye per g chitosan), the immobilized enzyme preparation was saturated with the respective dyes prior to studying the decolorization (the adsorbed dyes were not decolorized by laccase mediator system). When no further decolorization was achieved, a new dye solution (12 ml) and the respective mediator (50 μM) were pumped through the column.

One ml of the total dye solution was withdrawn at different time intervals to measure the residual absorbance at the specific wavelength, and returning the sample to the liquid stream. The decolorization yield was calculated as follow:

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where, A_0 is the absorbance of the untreated dye and A_t is the absorbance of the treated dye after time t.

UV-visible scans (200 to 800 nm) were made for both the laccase treated and untreated dye solutions using PerkinElmer (Waltham, USA) spectrophotometer.

Assays for ligninases

The laccase activity was assayed following the method of Dong et al. [11] using 1 mM ABTS (ε_{420nm} = 36000 $M^{-1}cm^{-1}$) as a substrate. Activity of the immobilized laccase was assayed in 20 ml reaction mixture with 5 mg of the enzyme particles. One unit of laccase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol ABTS per min. The values reported are averages of at least duplicate samples.

The activity of manganese peroxidase was determined according to the method of Castillo et al. [6]. One unit of manganese peroxidase activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of DMAB/MTBH per min (ε_{590nm} = 53000 $M^{-1}cm^{-1}$).

Lignin peroxidase (LiP) was assayed as described by Tien and Kirk [42]. One unit of LiP activity was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol veratryl alcohol per min (ε_{420nm} = 9300 $M^{-1}cm^{-1}$).

The versatile peroxidase activity was checked following the method described by Mohorcic et al. [30] using 1-napthol as substrate (ε_{255nm} = 12800 $M^{-1}cm^{-1}$).

Results and Discussion

Isolate HC1 identification and its ligninase activities

Analysis of 5.8S and 28S rDNA, and the internal transcribed spacer 1 & 2 DNA sequences revealed that isolate HC1 isolated from the Bolivian soil belongs to genus *Galerina*. This fungus is considered as a polyphyletic organism, and more than 300 species have originated from the Northern Hemisphere [22]. The sequence of isolate HC1 showed 99% similarity to the corresponding sequence of *Galerina clavata*

(sequence ID AY281021). However, as species delimitation in genus *Galerina* is mostly based on morphological and/or ecological characters, it needs further study to decide the taxonomic position of isolate HC1. *Galerina* sp. HC1 has been deposited in Deutsche Sammlung von Mikroorganismen und Zellkulturen culture collection with an accession number of DSMZ 22662. Although, involvement of *Galerina* spp. in lignin decomposition has been described [32] and the presence of laccase activity in the culture of *Galerina patagonica* is reported [43], there is no available report on production of laccase by any fungus that belongs to *Galerina*.

Galerina sp. HC1 was grown in liquid basal medium containing per liter: 4 g glucose, 4 g yeast extract and 0.05 g $CuSO_4 \cdot 5\ H_2O$, and ligninase activities were followed for 20 days (Fig. 1). Unlike most fungal cultures, which need more than five days of cultivation to produce detectable amount of laccase [2,18], laccase activity appeared on the 2nd day and peaked to about 265 U/l on the 8th day of isolate HC1 cultivation. The fungus was able to produce at least 4 laccase isoforms and the major laccase was purified to homgenity, characterized and used to modify lignin [24]. As shown in Figure 1, lower amounts of lignin peroxidase and manganese peroxidase were produced; however no versatile peroxidase activity was detected. The lignin peroxidase activity decreased drastically to very low level after of 16 days while manganese peroxidase activity remained stable with time. The presence of all the major lignin degrading enzymes, especially the lignin peroxidase that acts even on non-phenolic components of lignin, suggests that *Galerina* sp. HC1 could be an efficient lignin degrader [8,20].

Laccase production by submerged cultivation

Laccase production by different organisms is influenced by glucose, yeast extract and copper concentrations in the culture medium, the effect of each nutrient being dependent on the concentrations of the others [17]. Based on a preliminary study, three different concentrations of glucose, yeast extract and copper sulphate were chosen to study the effect on laccase production by *Galerina* sp. HC1 using a simple factorial design (data not shown). The highest laccase production was achieved at glucose-, yeast extract- and copper concentrations of 30 g/l, 10 g/l and 0.01 g/l, respectively. Increasing the glucose concentration to 60 g/l led to a decrease in the laccase activity in the medium. While supporting growth, glucose is known to repress expression of several genes encoding extracellular enzymes in fungi and yeast.

However, the effect of glucose on laccase production varies for different organisms; reduced production with increasing glucose concentration has been reported for *Trametes versicolor* [41] and *Phlebia* sp. [3], whereas improved yields have been seen in *Trametes pubescens* [18].

Production of laccase by *Galerina* sp. HC1 decreased with increase in the concentration of yeast extract (data not shown), as reported earlier also for *Pycnoporus sanguineus* [33], *Pleurotus ostreatus* [35] and *Coriolus versicolor* [36]. In contrast, the production of laccase by *Trametes gallica* has been stimulated by organic nitrogen source [11].

Microbial requirements for copper are usually satisfied by very low concentrations of the metal (1–10 μM), while high metal concentration exerts a toxic effect. Interestingly, *Galerina* sp. HC1 can grow at copper concentration as high as 1.2 g/l. Laccases are copper containing enzymes and often their production is stimulated by addition of copper salt to the culture medium [40]. The effect of copper on the production of laccase by *Galerina* sp. HC1 had a positive correlation with the concentration of yeast extract in the medium, implying that the laccase production was favoured with either increase or decrease in concentrations of both yeast extract and copper. Changes in their concentrations relatively inverse to each other led to reduction in the enzyme production. Yeasts are known to produce cysteine rich proteins - metallothioneins (MT) or other low molecular proteins that are capable of chelating relatively large quantities of copper ions [5]. Hence, the observed effect may be due to the binding of copper to the yeast extract components that in turn may reduce the availability of copper for the fungus. On the other hand, at low yeast extract concentration, the copper included in the medium would satisfy the need for growth and laccase production.

Laccase production by *Galerina* sp. was further investigated in the presence of a number of additives that were selected either due to their existence in wood or structural similarity to the natural substrate of laccase - lignin or its precursors. The effect of ethanol and methanol was also tested because their addition has resulted in increased laccase production by other fungal strains [29]. As summarized in Table 1, the best inducer was 2,5-xylidine followed by α-benzoin oxime, *p*-coumaric acid, lignin, pyridoxine and methanol. However, the toxic nature of most aromatics restricts their use in industrial applications. The induction achieved by lignin is economically attractive because of its availability in large amounts as a by-product of

paper pulp. Additives like 3-(dimethylamino) benzoic acid, sinapyl alcohol, 1-naphthaleneacetic acid, 2,6-dichlorophenol and 3-methyl-2-benzothiazolinone hydrazone hydrochloride strongly repressed laccase production by *Galerina* sp. HCl. The fungus did not grow in the presence of hydroquinone, which is a known antioxidant that efficiently inhibits the growth of several fungi [13].

The effect of different concentrations of the three best inducers, 2,5-xylidine, α-benzoin oxime and *p*-coumaric acid, respectively, on laccase production was further tested. Laccase production was the highest at the inducer concentration of 2 mM after 15 d of cultivation, amounting to over 5800 U/l in the presence of xylidine, 3800 U/l with α-benzoin oxime, and 3400 U/l with *p*-coumaric acid. Higher concentrations of xylidine (>2 mM) resulted in lower growth and enzyme production.

Laccase production was then studied in the medium under optimal nutrient and inducer conditions determined above, i.e. 30 g glucose, 10 g yeast extract and 0.01 g copper sulphate per liter and 2 mM xylidine or α-benzoin oxime. As high as 26 000 U/l laccase activity was produced in the presence of 2 mM xylidine after 28 days of cultivation (Fig. 2), which exceeded that reported for other fungi including several strains of *Trametes* [15,38]. The exception is *Trametes pubescens* that produced 740 000 U/l in a fed-batch cultivation at low level of glucose which avoids repression of the enzyme synthesis [17,18].

Agricultural residues as substrates for laccase production

Orange peels and sugarcane bagasse, the two agricultural residues available in abundance and also rich in sugars, vitamins and minerals [28,33], were tested as substrates for cultivation of *Galerina* sp. HC1 and production of laccase. Use of such renewable materials that may otherwise have nil or negative value would potentially reduce the enzyme production costs. At the residues concentration of 20 g/l that is determined to be equal to the chemical oxygen demand of 4 g/l glucose, the laccase production was 4 times higher than the maximum production achieved using glucose as carbon source (Fig. 3). Orange peel contains many aromatics especially *p*-coumaric acid, and soluble and insoluble carbohydrate compounds [14] that may stimulate laccase production. Bagasse, on the other hand, contains high amount of lignin (25%) [35] and polysaccharides that are difficult to access without pretreatment, and provided only slightly higher laccase activity than glucose.

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Orange peel was further considered as substrate for laccase production using solid-state fermentation that has many advantages over submerged fermentation in terms of low liquid volumes and -related processing costs [27]. The orange peels were soaked with the basal medium prior to inoculation with the fungus, and absorbed most of the liquid when suspended at a concentration of about 12% (w/v), resulting in a wet solid mass. Laccase production was fond to be about 0.5 U per gram of the dried orange peels, and supplementation of the peels with copper, 2,5-xylidine, and combination of the two, separately increased the activity 5, 6 and 10 fold, respectively (data not shown).

Decolorization of dyes using *Galerina* sp. HC1 laccase

The dye decolorization activity of the laccase from *Galerina* sp. HC1 was evaluated using Congo red and Coomassie Brilliant Blue G-250 as model dyes. Decolorization of both the dyes by the laccase in the presence of mediators was comparable to that achieved with *T. versicolor* laccase which has been reported to be the most efficient enzyme in decolorization of dyes (Fig. 4) [26]. The initial decolorization rate for Coomassie Brilliant Blue was faster, reaching more than 50% decrease in absorbance in less than 5 min, while similar degree of decolorization of Congo red took up to 30 min (Fig. 5). This could be due to the presence of electron donating methyl and methoxy groups on the triphenylmethane dye that have earlier been shown to enhance laccase activity [1]. Furthermore, decolorization was relatively faster with syringaldehyde as the mediator (Fig. 4), which is attractive due to its availability from lignin that is among the primary renewable resources [44]. While maximum decolorization of Coomassie blue was obtained in the presence of syringaldehyde (Fig. 4b), that of Congo red was achieved with ABTS on prolonged incubation to 2 h (Fig. 4a). The resistance of Congo red to decolorization by a laccase from *Trametes* sp. SQ01, an enzyme that efficiently decolorizes various azo-, triphenylmethane-, and anthraquinone dyes has been reported; only 47% of the dye was decolorized by the pure laccase after 24 h while complete decolorization was achieved by the fungus in the liquid culture [48]. This suggests the possible involvement of other *Trametes* sp. SQ01 enzymes for complete decolorization of Congo red. Thus, the decolorization achieved by the *Galerina* sp. HC1 laccase is encouraging. Screening different organisms and their enzymes for dye decolorization is beneficial as it may result in more efficient dye decolorizers than the commonly studied organisms [7].

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Decolorization of the dyes was further investigated using *Galerina* sp. laccase immobilized on cross-linked chitosan. Chitosan is deacetylated chitin and considered as cheap and abundant, and has been used in various applications. About 12 U of the *Galerina* sp. HC1 laccase was immobilized per gram of cross-linked chitosan, which was equivalent to 76% of the initial activity used in the immobilization process. Since chitosan has high dye binding capacity [9,45], the cross-linked chitosan–enzyme preparation was first saturated with respective dyes without adding any mediator in the solution. The dye solution was recirculated over a bed of immobilized enzyme preparation, and after no more dye was observed to bind to the matrix, the dye solutions containing mediators were used for laccase mediated decolorization. It was possible to decolorize more than 97% of the 84 ml of 35 μM Congo red solution (7 batches of 12 ml each), however only 36 ml of 30 μM Coomassie Brillant Blue G-250 solution (3 batches each with 12 ml) was decolorized and thereafter the decolorization efficiency of the immobilized enzyme declined (Fig. 5). Katuri et al [25] have earlier reported that laccase immobilized in chitosan was the most efficient method to treat dyes; in their work the immobilized laccase could be used for at least 20 batches for the decolorization of azo dyes.

UV-visible scan of the treated dyes showed disappearance of the band in the visible region and significant decrease in absorbance in the UV region (Fig. 6), which indicates significant modification of the dye compounds by the laccase-mediator system. Currently, we are investigating the exact structure of the decolorized product obtained.

Concluding remarks

This study demonstrates that *Galerina* sp. to be a potentially new source of laccase and even other oxidizing enzymes. Agricultural residues could serve as useful substrates for the fungus, instead of glucose and yeast extract, indicating potential for reducing enzyme production costs and also providing added value to the wastes. Further increase in enzyme yield should be possible e.g. by using fed-batch mode of cultivation.

The laccase produced by *Galerina* sp. HC1 showed the ability to extensively decolorize a toxic azo dye and a triphenylmethane dye, which represent groups making up the bulk of the commercial dyes. This shows that the enzyme potential for dye decolorization is very attractive. The availability of such enzymes make

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enzymatic dye decolorization more preferable to other relatively expensive methods such as radiation, Fenton oxidation, ozonation, photochemical oxidation, etc. Further studies on the mechanism of decolorization and determining the reaction end product(s) could be of interest for both basic and applied research.

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Table 1. Effect of additives on the production of laccase by *Galerina* sp. HC1. 100% laccase activity corresponds to 508 U/l. The laccase production was followed for 20 days and the activity stated is the maximum production achieved during the cultivation period.

Additive	Structure	Activity (%)	Additive	Structure	Activity (%)	Additive	Structure	Activity (%)
No additive	---	100	*p*C		200	S Al		5
Lignin	Complex	194	VA		14	HQ		0
4AB		14	DMBA		0	Phenol		117
N Ac		109	B Ac		122	X		374
C Al		110	S Ac		49	P		152
F Ac		36	C Ac		70	BZD		72
P Ac		91	DNS		108	DP		0
N		108	B		233	EtOH		91
MBTH		1	1-Nac		<1	MeOH		135

Abbreviations: 4AB: 4 aminobenzoic acid; N Ac: nicotinic acid; C Al: coniferyl alcohol; F Ac: ferulic acid; P Ac: pyrogallic acid; N: naphthalene; MBTH: 3-methyl-2-benzothiazolinone hydrazone; *p*C: p-coumaric acid; VA: veratryl alcohol; DMBA: 3-dimethylaminobenzoic acid; B Ac: benzoic acid; S Ac: sinapic acid; C Ac: caffeic acid; DNS: 3,5-dinitrosalisylic acid; B: α-benzoin oxime; 1-Nac: 1-naphthaleneacetic acid; S Al: sinapyl alcohol; HQ: hydroquinone; X: 2,5-xylidine; P: pyridoxine; BZD: benzidine; DP: 2,6-dichlorophenol; EtOH: ethanol; MeOH: methanol.
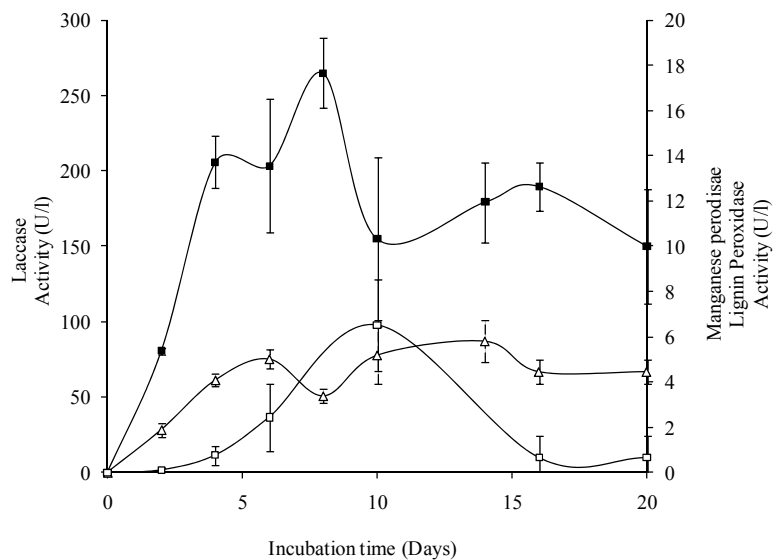
Figure 1 Production of laccase (■), manganese peroxidase (Δ) and lignin peroxidase (□) by *Galerina* sp. HC1 in a medium composed of (g/l): glucose, 4; yeast extract, 4; $CuSO_4 \cdot 5H_2O$, 0.05; NH_4NO_3, 0.04; $MgSO_4 \cdot 7H_2O$, 0.5; K_2HPO_4, 0.1; KH_2PO_4, 0.2 and $CaCl_2 \cdot 2H_2O$, 0.01. Cultivation was done at room temperature with continuous agitation at 100 rpm.
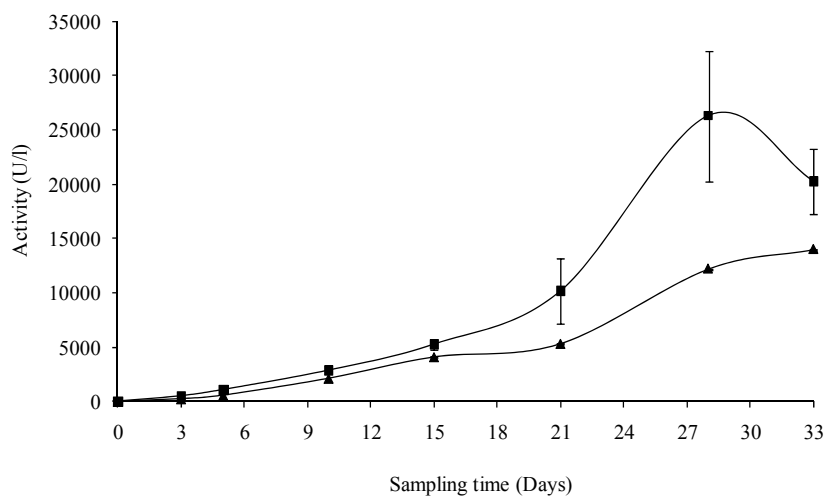

Figure 2. Time course of laccase production by *Galerina* sp. HC1 during cultivation under optimized conditions at 22 °C. The medium contained 30 g/l glucose, 10 g/l yeast extract, 0.01 g/l copper sulphate, and 2 mM 2,5-xylidine (■) or 2 mM α-benzoin oxime (▲).

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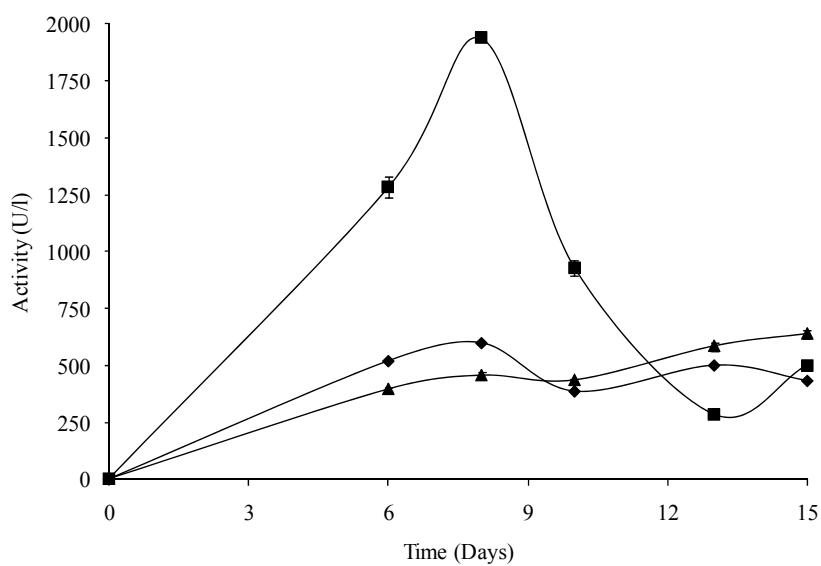
Figure 3. Production of laccase by *Galerina* sp. HC1 using sugarcane bagasse (♦), orange peels (■) and glucose (▲), respectively as substrates during submerged cultivation at 22 °C. The experimental details are given in the text.

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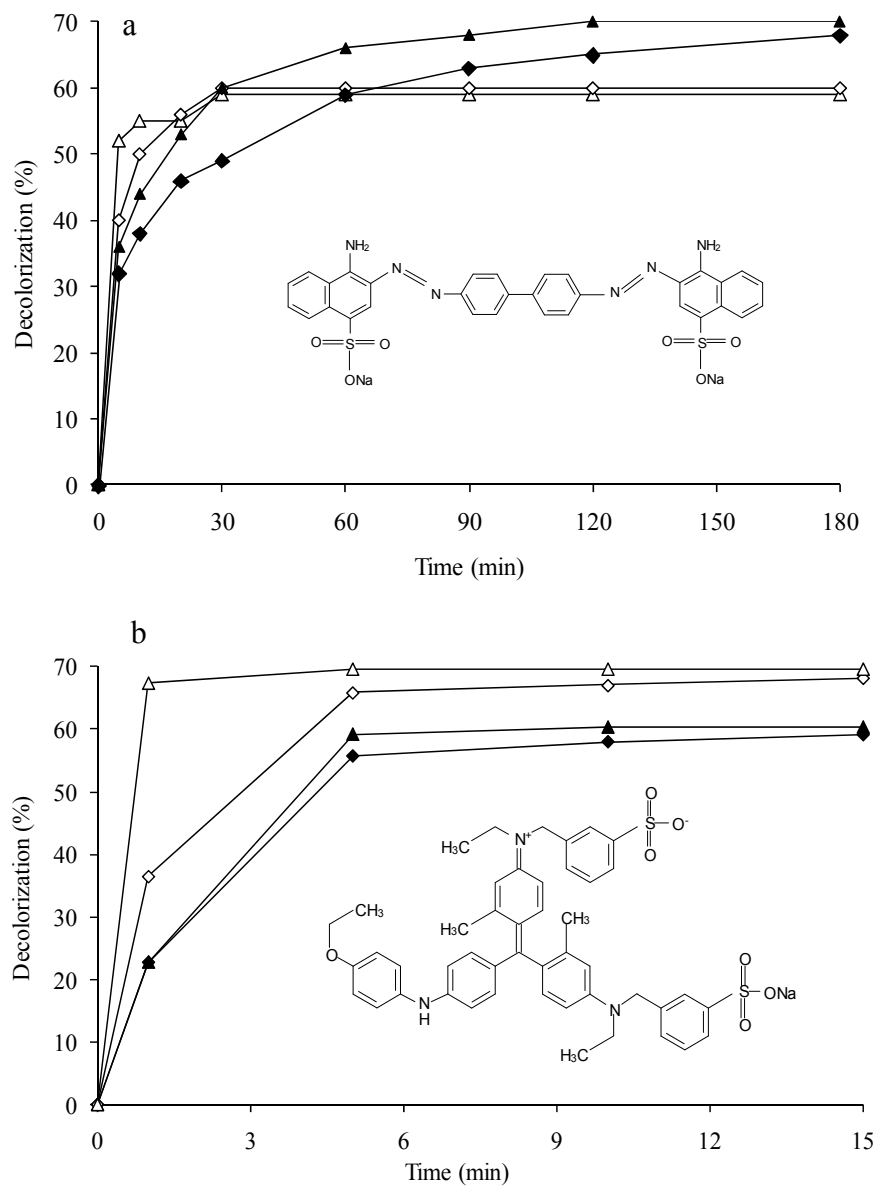
Figure 4. Decolorization of (a) Congo red and (b) Coomassie Brilliant Blue G-250 using *Galerina* sp. HCl (*diamonds*) and *T. versicolor* (*triangles*) laccases in the presence of ABTS (*filled symbols*) and syringaldehyde (*open symbols*) as mediators.

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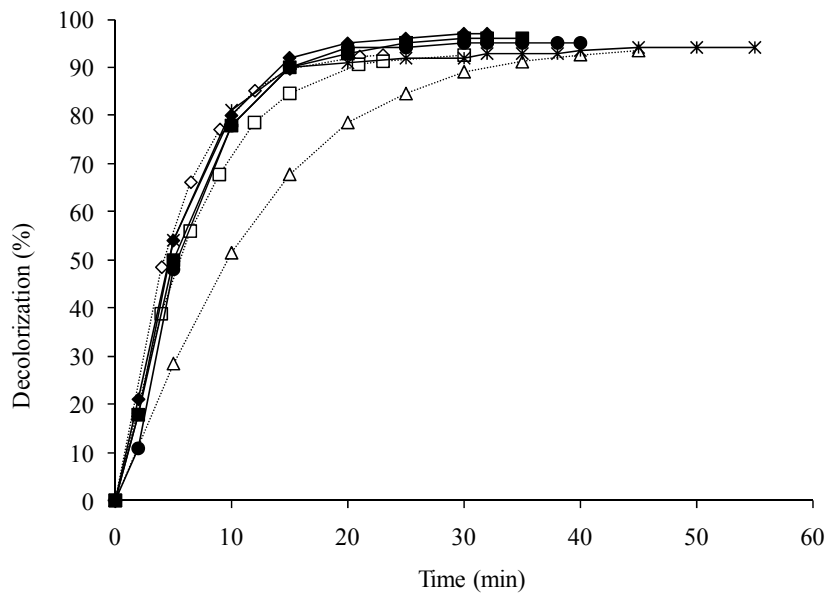
Figure 5. Re-use of immobilized laccase for decolorization of Congo Red (filled symbols and continuous line) and Coomassie Brillant Blue G-250 (open symbols and dashed lines). The used mediators are ABTS and SA, respectively. Symbols represent: (*diamonds*) first batch, (*square*) second batch, (*triangle*) third batch, (*circle*) fourth to sixth batch, and (*cross*) seventh batch.

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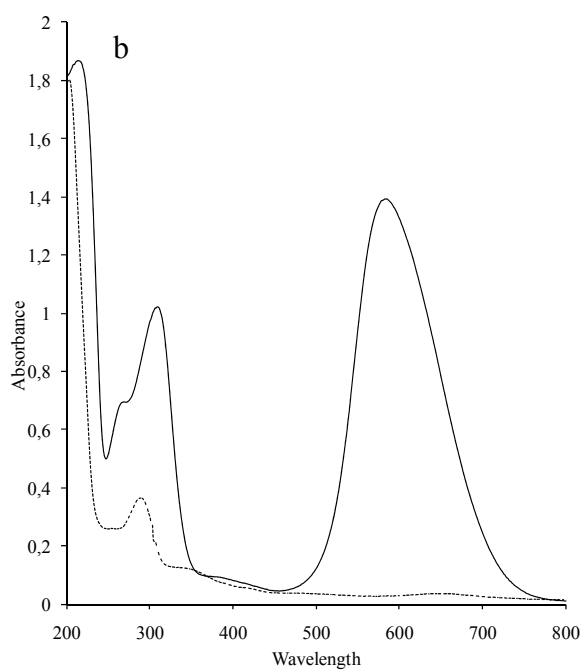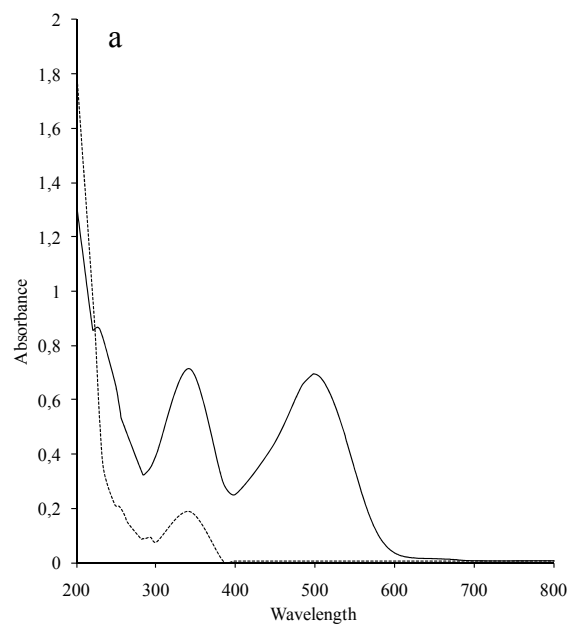
Figure 6. UV-visible scan of (a) Congo red and (b) Coomassie Brilliant Blue G-250 before (*continuous line*) and after (*dashed line*) treatment with laccase and the respective mediator.
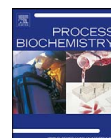
Paper III

Short communication

Blue laccase from *Galerina* sp.: Properties and potential for Kraft lignin demethylation

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A B S T R A C T

We purified a laccase isoenzyme, Lac1 from *Galerina* sp. HC1 using a combination of anion exchange- and hydrophobic interaction chromatography. Lac1 has a molecular mass of 64 kDa, an isoelectric point of 4, and 3.35 copper atoms/enzyme molecule. The enzyme has features typical of fungal blue laccases. The sequences of two internal peptides were highly similar to reported laccase sequences from other fungi such as *Trametes* sp. Lac1 exhibited optimal activity on substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at pH 3 and 60 °C. It had high stability at ≤20 °C and at high pH. Lac1 exhibited high substrate affinity and the highest catalytic efficiency reported among laccases for ABTS, and among the lowest for syringaldazine. The most potent inhibitors of Lac1 were sodium azide, sodium cyanide, disulfide reducing agents, and metal ions in the order $Li^+ > Sn^{2+} > Hg^{2+}$. The laccase efficiently catalyzed demethylation of eucalyptus hard wood Kraft lignin.

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1. Introduction

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are the most widely distributed (from bacteria to insects) enzymes of the multicopper oxidase family [1,2]. Laccase-like activity has even been found in some bacterial spores [3]. The most commonly studied laccases are from white rot fungi belonging to *Trametes* sp. However, a wide variety of other species have shown laccase levels equal to or greater than those of *Trametes* sp. [4].

The oxidation catalyzed by laccases or laccase-mediator systems (LMS) is regarded as a "green" reaction; it uses molecular oxygen as the acceptor of electrons that are abstracted from the substrate and generates four corresponding reactive radicals and two water molecules as by-products [5,6]. The oxidative abilities of laccases have been employed in a broad range of applications and products including the removal of pollutants from waste water and contaminated soil [7,8], the degradation of dyes (especially substituted phenolic azo dyes) [9,10], pulping processes [11,12], cosmetic products such as hair dyes [13], and dermatological preparations [14]. Additionally, a series of applications have indicated a promising role for laccase in synthetic chemistry [15,16].

Lignin, one of the most abundant natural resources, has attracted a great deal of attention owing to increasing interest in the use of renewable resources for production of specialty materials and energy. Lignin is a structurally complex molecule and is heavily methylated; removal of the methyl or methoxyl groups is required to enhance lignin's reactivity for its application, e.g. as a bio-adhesive. It has been suggested that lignin demethylation by laccase is a crucial step in pulp biobleaching [17]. Laccase can oxidize phenolic subunits of lignin and LMS widens the oxidation spectrum to non-phenolic subunits [18]. Intermediates of this oxidation, such as phenolic radicals or *ortho*-quinones, lead to spontaneous polymerization or degradation of the modified lignin. The demethylation of lignin has been directly correlated with methanol release and a decrease in the pulp's kappa number [18–20].

The existing and potential environmental and biotechnological applications of laccases have promoted the search for additional laccase producing organisms. Genus *Galerina* includes basidiomycetous fungi that grow as saprophytes on dead bryophytes, woody materials, and other plant debris. As such, they are expected to produce lignin-degrading enzymes. However, reports on such enzymes from this genus are quite limited. It is only recently that laccase activity from *Galerina patagonica* was reported [21]. We have lately investigated the production of laccase from a *Galerina* sp. HC1 that was isolated from a sample of decaying litter in the tropical region of Bolivia. The production was achieved in submerged and solid state cultivations. In submerged cultivation, laccase levels as high as 26,000 units per liter were achieved (Mendoza et al., manuscript submitted).

The current investigation focuses on the purification and characterization of a laccase from *Galerina* sp. HC1 and its use for demethylation of hardwood Kraft lignin.

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2. Materials and methods

2.1. Organism and culture conditions

Galerina sp. HC1 was isolated in our laboratory and deposited in the culture collection of Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSMZ (DSM 22662). The fungus was maintained on a solid medium composed of 30 g malt extract, 15 g agar, and 3 g peptone (all from Merck) per liter. An inoculum of the fungus was transferred to 500 mL of liquid medium (in a 2 L flask) composed of 2 g glucose, 5 g yeast extract, 0.04 g ammonium nitrate, 0.2 g potassium dihydrogen phosphate, 0.1 g dipotassium hydrogen phosphate, 0.01 g calcium chloride dihydrate, 0.5 g magnesium sulfate heptahydrate, and 0.08 g copper sulfate pentahydrate per liter. The culture was grown at room temperature (22 °C) with continuous shaking (120 rpm). After seven days of cultivation, the culture was harvested and the supernatant containing laccase activity was collected by vacuum filtration (MUNETELL filter paper).

2.2. Purification of laccase from Galerina sp. HC1

A three-step procedure was used to purify laccase from the crude culture filtrate. All steps were performed at room temperature. Five hundred milliliters of the culture filtrate were centrifuged at 5500 × g, 4 °C for 25 min, and Tris–HCl buffer (pH 8) was added to a final concentration of 10 mM. The liquid was loaded onto a 9 mL Streamline QXL anion exchanger (10 cm height column pre-equilibrated with 10 mM Tris–HCl buffer (pH 8)) at a flow rate of 1.5 mL/min using a Fast Protein Liquid Chromatograph (BioLogic Duo-Flow System from BIO-RAD). The bound proteins were eluted using a linear gradient of 60–200 mM NaCl in the binding buffer. The laccase active fractions were pooled and supplemented with 600 mM ammonium sulfate, and the solution was loaded on a Phenyl Sepharose column (9 mL bed volume in a 10 cm height column pre-equilibrated with 600 mM ammonium sulfate in 10 mM Tris–HCl (pH 8)) at a flow rate of 1.5 mL/min. Bound proteins were eluted with a decreasing linear gradient of 500–150 mM ammonium sulfate in 10 mM Tris–HCl buffer (pH 8). Subsequently, active fractions were pooled and dialyzed (Spectra/Por® 3 dialysis membrane of 3.5 kDa cutoff) overnight at 4 °C against deionized water. The dialyzed sample was centrifuged at 5500 × g, 4 °C for 20 min, and the pH was adjusted to 8.5 with Tris–HCl buffer prior to loading onto another strong anion exchanger matrix (MonoQ HR 5/5 column, from Amersham Pharmacia Biotech, pre-equilibrated with 10 mM Tris–HCl buffer (pH 8.5)) at a flow rate of 0.6 mL/min. A gradient of 140–170 mM NaCl in the Tris–HCl buffer (pH 8.5) was used for the elution. The eluate fraction containing laccase activity was repurified on a Mono Q column with a narrower gradient of 140–150 mM NaCl in the Tris–HCl buffer (pH 8.5) to obtain the major isozyme (Lac1) in a pure form.

2.3. Laccase catalyzed demethylation of lignin

A 1.5 mL reaction mixture containing 10 mg/ml of hardwood lignin (obtained from the Kraft pulping of eucalyptus wood chips, provided by Innventia AB – Stockholm) and either pure Lac1 (0.1, 0.5 or 1 U/mL) or crude laccase (CLac, 0.1 U/mL) with or without 1 mM ABTS in 20 mM sodium acetate buffer (pH 5) was incubated for 5 h at room temperature on a rocking table. Aliquots were withdrawn at different time intervals and analyzed to determine the amount of methanol released.

We also evaluated the effect of other mediators, such as hydroxybenzotriazole (HBT; Fluka), violuric acid (VA; Fluka), and syringaldehyde (S; Aldrich), at concentration of 1 mM each on lignin demethylation by 0.1 U/mL Lac1.

2.4. Determination of laccase activity

The laccase activity was determined by spectrophotometry or oxygen consumption at 25 °C. The spectrophotometric assay, unless otherwise stated, was based on the enzyme-catalyzed oxidation of ABTS. Briefly, 100 μl of 10 mM ABTS and 100 μl enzyme sample were added to 800 μl of 25 mM sodium acetate buffer (pH 3), and the absorbance was followed at 420 nm using an Ultrospec 1000 UV/visible spectrophotometer (Pharmacia Biotech). The molar extinction coefficient (ε) of oxidized ABTS was taken as 36,000 M^{-1} cm^{-1} [22]. One unit of enzyme activity was defined as the amount of the enzyme that produces 1 μmol of product per minute under the reaction conditions used.

Kinetic parameters were determined by spectrophotometrically following the reaction rates with ABTS (0.02–0.5 mM, 2 min at 420 nm) and syringaldazine (0.1–25 μM, 3 min at 530 nm), respectively as substrates using a UV-1650 PC UV–visible spectrophotometer (Shimadzu). The molar extinction coefficient (ε) of syringaldazine was defined as 65,000 M^{-1} cm^{-1} and used for the calculations [23]. K_m and V_{max} values were calculated using the Lineweaver–Burk transformation of the Michaelis–Menten equation.

Oxygen consumption during substrate oxidation was determined using a dissolved oxygen meter (HI 964400, HANNA Instruments). Laccase activity was determined in a reaction consisting of 0.5 mM substrate and 10 μg (1.57 U) enzyme in 20 mM acetate buffer (pH 3) in a total volume of 4 mL. The dissolved oxygen meter was calibrated to 0% with degassed water and to 100% with air at 25 °C. The amount

of consumed oxygen is expressed in μmol/min per mg of enzyme and was calculated based on the assumption that 100% of dissolved oxygen corresponds to a given amount in ppm or mg/L at a certain temperature and altitude.

The substrate specificity of laccase and the effect of inhibitors on laccase activity were determined using the oxygen consumption assay. For substrate specificity determination, the assays were performed at the optimum pH for the substrate examined; the optimum pH for each substrate was determined by spectrophotometric analysis. The wavelengths for maximum absorbance (λ_{max}) were determined by scanning the oxidized products between 200 and 700 nm.

The inhibitory effect of different compounds on the laccase activity was determined by incubating the enzyme with each compound for 10 min at 25 °C and then assaying activity with ABTS as the substrate.

2.5. Protein content determination and gel electrophoresis

Protein content was determined by the bicinchoninic acid (BCA) method [24]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% (w/v) polyacrylamide gel to determine the purity and molecular mass of the purified laccase. Protein bands were visualized by silver staining [25]. The gel was documented using a Gel Doc 2000 from BIO-RAD, and the molecular mass was estimated using Quantity One software. Native polyacrylamide gel electrophoresis (Native-PAGE) was performed to determine the presence of laccase isoforms in the samples. Activity staining was done using 1 mM ABTS in 20 mM sodium acetate buffer (pH 3) after washing and incubating the gel in the same buffer for at least 30 min.

2.6. Isoelectric point (pI), copper content and spectral analyses

The pI of Lac1 was determined by isoelectric focusing using a BIO-RAD "Rotofor Cell" system in a pH range of 3–5 created by ampholytes (Bio-Lyte 3/5 from BIO-RAD). The pH of the active fraction corresponded to the pI of the protein.

Atomic absorption spectroscopy (Graphite furnace spectrophotometer, GBC 932 AA from GBC scientific) was used to determine the copper content of a pure sample of laccase (2 mg/mL), which was washed extensively with millipore quality water and concentrated by ultrafiltration (Macrosep 10K Omega from Life Sciences). A spectrum of Lac1 solution (0.4 mM) was obtained by scanning the sample between 260 and 660 nm.

2.7. Sequencing of internal peptides

The purified Lac1 protein band was cut from the gel and in-gel digestion was performed using sequencing grade modified trypsin (V5111 from Promega). The peptides were subjected to mass spectrometry analysis using a Micromass Q-Tof Ultima^{TM} API mass spectrometer connected to a Waters CapLC HPLC. The spectra were edited manually, and the public databases were searched using the BLAST algorithm to identify similar sequences of known laccases.

2.8. Determination of methanol content

The samples from the demethylation reaction were diluted 1/10 in millipore quality water and extracted with an equal volume of 1-butanol (Sigma). The butanol top phase was filtered using 0.22 μm hydrophilic PES filters (Millipore) into 2 mL gas chromatography capped vials, and the methanol content of the extract was measured using a Varian 430-GC under the following conditions: a FactorFour^{TM} VF-1ms column from Varian (15 m length, 0.25 mm internal diameter, and 0.25 μm film thickness), a linear velocity of 85 cm/min, a split ratio of 1/20, an injector and detector set to 250 °C, an initial oven temperature of 33 °C for 0.5 min that increased to 233 °C at a rate of 50 °C/min, an injection volume of 1 μL, and a run time of 4.5 min. A calibration curve was plotted using 0.0005–0.1% (v/v) methanol (corresponding to 3.95–790 mg/L), prepared in the same way as the experimental samples but without the enzyme.

3. Results and discussion

3.1. Purification of Galerina sp. laccase

Among the ligninolytic enzymes, *Galerina* sp. HC1 produced mainly laccase and very low amounts of manganese peroxidase and lignin peroxidase (Mendoza et al., manuscript submitted). Native-PAGE of the culture supernatant identified four laccase isoforms. It is not yet clear if the different enzyme isoforms are products of one gene or are encoded by different genes. Purification of the enzyme led to the recovery of 21% of total laccase activity with 64-fold higher specific activity and 30-fold concentration (Table 1). The purified sample contained all four laccase isoforms (Fig. 1a). The major isoform, Lac1, was isolated after repeated chromatogra-

Table 1
Summary of the purification of *Galerina* sp. HC1 laccases.

Purification step	Volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification factor (fold)
Crude	500	670.4	272	2.5	100	1
Streamline Q XL	123	444.8	40	11.2	66	5
Phenyl sepharose	24.2	356.1	6	60.6	53	25
Mono Q	16.6	138.5	0.88	157	21	64
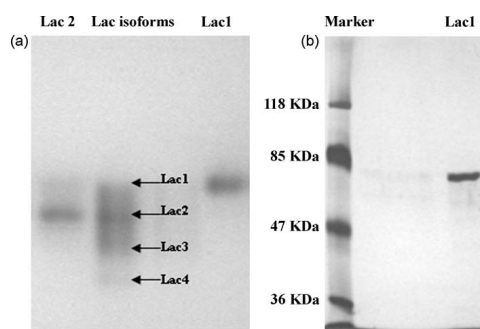
Fig. 1. Gel electrophoresis of the purified laccases from *Galerina* sp. HC1 on 10% polyacrylamide: (a) zymogram of a native-PAGE showing different laccase isoforms in a mixture and separated Lac1 and Lac2, and (b) SDS-PAGE developed by silver staining.

phy using a narrower salt gradient on a Mono Q column and was used for further investigations (Fig. 1b).

3.2. Galerina sp. Lac1 molecular features

Lac1 has a molecular mass of 64 kDa and p*I* of 4. The copper content of the enzyme as determined by atomic absorption spectrometry was 3.35 copper atoms per molecule of Lac1. UV/visible spectrum of the enzyme showed a broad peak close to 600 nm corresponding to type I or blue copper atom and a weak shoulder at 330 nm indicating a type III binuclear copper [2] (data not shown). A_{280nm}/A_{600nm} ratio of 28.9 was obtained from the spectral analysis, which is in the range typical for blue laccases [26].

In-gel digestion of pure Lac1 with trypsin followed by mass spectrometry led to the detection of several peptides, two of which had sequences of GPSTDLVIGNK and GFDGGIDSAILR, respectively (deposited in UnitProt knowledgebase under the accession number P86351). The peptides are designated as "N" and "C", respectively, as they had up to 72% and 91% similarity with sequences in proximity to N- and C-termini of known laccases from other fungal species such as *Trametes* sp. and *Pleurotus* sp. (Table 2).

Table 3
Substrate specificity of *Galerina* sp. Lac1.

Substrate	λ_{max} (nm)	Optimal pH	Oxygen uptake (μmol/min/mg Lac1)
ABTS	420	3	18.05
Syringic acid	360	4	7.31
Caffeic acid	380	5	4.31
Syringaldazine	530	5	2.38
Vanillic acid	390	5	1.19
Catechol	390	5	0.79
L-DOPA	460	6	0.21

3.3. Biochemical properties of Galerina sp. Lac1

Like several fungal laccases, Lac1 was most active in a relatively acidic environment [27,28]. With ABTS as the substrate, Lac1 exhibited optimum activity (157 units/mg) at pH 3, and the activity decreased sharply as pH increased such that no activity was observed at pH 7 (Fig. 2a). The enzyme exhibited a higher optimum pH with other substrates, e.g. pH 4 for syringic acid, pH 5 for caffeic acid, syringaldazine, vanillic acid and catechol, and pH 6 for Levo-dihydroxyphenylalanine (L-DOPA) (Table 3).

The effect of pH on stability of Lac1 was determined by incubating the enzyme in 50 mM buffers (pH 2–9) at room temperature for 33 h, followed by measuring the activity at optimum pH with ABTS as the substrate. As seen in Fig. 2b, the laccase was completely inactive within 3 h at pH 2, but the stability increased as pH increased. The enzyme retained about 20% and 90% of its original activity after 24 h incubation at pH 3 and pH 9, respectively. The stability of laccases at high pH has been attributed to hydroxide anion mediated protection of the enzyme from auto-oxidation [29].

The optimum temperature of Lac1 activity at pH 3 was 60 °C. However, the enzyme exhibited a broad activity profile in a lower temperature range, with 50% of the maximal activity at 20 °C (Fig. 3a). In contrast to the temperature optima of laccases, which typically range from 50 to 60 °C, their stability at different temperatures varies considerably, e.g. the half-life at 50 °C ranges from a few minutes [30] to 70 h [31]. Lac1 was more labile at high temperatures at pH 3; it had a half-life of 53 min at 50 °C, 23 min at 60 °C, and less than 2 min at 70 °C (Fig. 3b). The enzyme could be stored for several days at 22 °C before all activity was lost, while 10% of residual activity was retained after 1 year of storage at 4 °C. It seems that using Lac1 at ambient temperature would be an opti-

Table 2
Multiple alignments of peptide N and peptide C from Lac1 of *Galerina* sp. HC1 with peptide sequences of laccases from other sources.

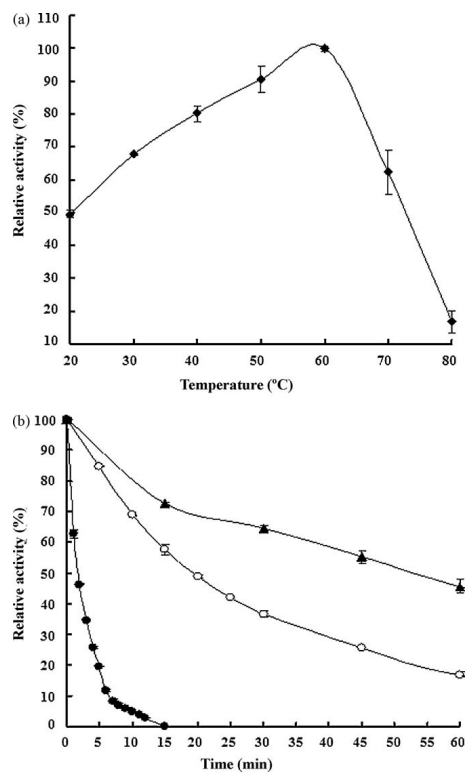|			
Galerina sp. HC1	Peptide N	GPSTDLVIGNK	100
Coriolopsis gallica	AAK02068.1	26 GPKTDLHIVNK 36	72
Ganoderma lucidum	ABK59822.1 XP_001881925.1	24 GPKTDLTISN 33	70
Laccaria bicolor	CAM12361.1	24 GPKTDLTISN 33	70
Trametes versicolor		25 GPVTDLTISN 34	70
Galerina sp. HC1	Peptide C	GFDGGIDSAILR	100
Trametes hirsuta	ACC43989.1	290 GFDGGINSAILR 301	91
Pleurotus ostreatus	AAR21094.1	302 GFDGGINSAILR 313	91
Pleurotus eryngii	CAO79914.1	302 GFDGGINSAILR 313	91
Pleurotu pulmonarius	AAY41065.1	85 GFDGGINSAILR 96	91
Trametes versicolor	BAD98307.1	207 FDGGINSAILR 217	90

Fig. 2. Effects of pH on *Galerina* sp. Lac1 activity (a) and stability (b). Optimum pH was determined using the spectrophotometric assay with ABTS as substrate in a pH range of 1.2–9 (pH 1.2 and 2: 20 mM glycine–HCl buffer; pH 3, 4, 5 and 6: 20 mM sodium acetate buffer; pH 7, 8 and 9: 20 mM Tris–HCl buffer) at 25 °C. The pH stability was followed by incubating Lac1 in 50 mM buffers at room temperature during 33 h, in a pH range of 1.2–9 (1.2 (♦); 3 (□); 5 (▲); 7 (○); 9 (●)) and then measuring the residual activity with ABTS at pH 3.

Fig. 3. Effects of temperature on *Galerina* sp. Lac1 activity (a) and stability (b). The optimum temperature was determined by assaying the enzyme in 20 mM acetate buffer pH 3 in a temperature range of 20–80 °C. Thermal stability of Lac1 was determined by incubating 3.5 μg/mL of the enzyme solution at 50 (▲), 60 (○) and 70 °C (●), respectively, and measuring the residual activity at pH 3.

mal choice for extending the operational life of the enzyme, which would further minimize energy consumption.

Activity of Lac1 with different substrates and in the presence of different inhibitor compounds, respectively, was determined by measuring the rate of oxygen consumption. The spectrophotometric assay has certain limitations for such measurements. For example, it would require determination of the molar extinction coefficients for each oxidized substrate, under respective optimal assay conditions. Also certain compounds such as sulfhydryl reagents affect the color development more than the enzyme activity [32]. Lac1 showed the highest activity towards ABTS, a non-phenolic heterocyclic compound, while the activity with the phenolic substrates was much lower and was in the order: syringic acid > caffeic acid > syringaldazine > vanillic acid > catechol > L-DOPA (Table 3). There was nearly a 400-fold difference between the turnover number for ABTS and syringaldazine, the two commonly cited laccase substrates. The enzyme had low K_m values for both the substrates, while the catalytic efficiency, K_{cat}/K_m was among the highest reported for ABTS (only *Trametes pubescens* laccase has

a higher value) and among the lowest for syringaldazine (Table 4).

While laccases are inhibited by a wide variety of compounds [33,34], the effect of 7 known inhibitor compounds on the activity of Lac1 was studied. The enzyme was incubated in the presence of different compounds for 10 min prior to measuring the activity (data not shown). Complete inhibition of Lac1 activity was observed at 0.01 mM sodium azide (a known inhibitor for metalloenzymes), 5 mM each of β-mercaptoethanol, dithiothreitol, thioglycolic acid (disulfide reducing agents) and sodium cyanide (affecting oxygen utilization), respectively. In contrast, a relatively high concentration of EDTA (25 mM) was required to completely inhibit Lac1 activity, indicating extremely high affinity of the enzyme for Cu^{2+}. Hydrogen peroxide was the least inhibitory; the enzyme retained more than 50% of its original activity in 50 mM H_2O_2. Such a high resistance to inactivation by hydrogen peroxide might be attributed to a structural adaptation of the laccase for its function as an oxidative catalyst. H_2O_2 is a very strong oxidizing agent in the presence of copper [35], and has been reported to bind the type II copper of laccase creating a new absorption band at 400 nm [36].

Table 4
Comparison of the kinetic properties of *Galerina* sp. HC1 laccase with other fungal laccases.

Laccase source	Substrates	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (s^{-1} mM^{-1})	Reference
Galerina sp. HC1	ABTS	14	592	42,286	Present work
	Syringaldazine	0.8	1.51	1888	
Pleurotus pulmonarius	ABTS	210	1520	7230	[33]
	Syringaldazine	12	654	54,500	
Agaricus blazei	ABTS	63	21	340	[41]
	Syringaldazine	4	5	1228	
Melanocarpus albomyces	ABTS	280	75	268	[42]
	Syringaldazine	1.3	78	60,000	
Trametes pubescens	ABTS	14	690	48,000	[43]
	Syringaldazine	6	280	47,000	
Basidiomycete C30	ABTS	10.7	55.8	5214	[44]
	Syringaldazine	1.8	30	16,667	
Xylaria polymorpha	ABTS	20	550	27,504	[45]
	Syringaldazine	3.5	199	56,809	
Trichophyton rubrum LKY-7	ABTS	45	10.3	233	[28]
Coriolopsis rigida	ABTS	12	77	6410	[46]
Trametes hispida	ABTS	16	16	986	[47]

Owing to the application of laccases for treatment of environmental samples, it is important to know the effect of metal ions on the enzyme. Among the metal ions tested (Na$^+$, K$^+$, Li$^+$, Hg^{2+}, Ca^{2+}, Mn^{2+}, Sn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}), Li$^+$ and Sn^{2+} were the most potent inhibitors leading to 92% and 88% inhibition, respectively, of Lac1 activity at metal salt concentration of 0.1 mM. Hg^{2+} resulted in 58% inhibition at 0.1 mM concentration and 97% at 1 mM, suggesting the presence of important thiol groups in the enzyme. Inhibition by Mn^{2+} and Fe^{2+} was stronger at 0.1 mM (67% and 75%, respectively) than at 1 mM (27% and 61%, respectively) metal salt concentration. An activity inducing effect of Cu^{2+} and Mn^{2+} has also been reported for laccase from the bird's nest fungus, *Cyathus bulleri* [37]. Lac1 activity was inhibited by about 40–42% with 0.1–1 mM Cu^{2+}. Although copper forms an important part of the active site of laccase, inhibition by excess of Cu^{2+} and also Fe^{2+} may partly be due to the metal catalyzed oxidation of some important amino acid residue in the enzyme. Inhibition by other metal ions was minor when used at 0.1 mM concentration but led to varying degree of inhibition (between 20 and 50%) at 1 mM. Information on interaction of metal cations with laccases is scarce. Further work is needed to fully understand the effect of metal ions on the activity of Lac1 and laccases in general.

3.4. Lignin demethylation

Lignin modifying enzymes are known to be important wood-decay enzymes, and the demethylation of lignin is a crucial step in this natural process. Thus, it was interesting to study the ability of *Galerina* sp. HC1 laccase to catalyze the demethylation of lignin. Hardwood lignin obtained from the Kraft pulping process of eucalyptus was used as a substrate. Enzymatic treatment at low concentrations of Lac1 was found to be more efficient in the presence of ABTS (46.7 and 125 mg/mL methanol released at 0.1 and 0.5 U/mL, respectively) than in its absence (21.7 and 86.7 mg/L, respectively) (Fig. 4a). However, the treatments were equally efficient at 1 U/mL of Lac1. Moreover, the results show an obvious correlation between methanol release (demethylation rate) and enzyme dose. The highest concentration of methanol released was about 142 mg/L, which corresponds to 13.7 mg of methoxyl group released per 1 gram of the treated lignin. Although the extent of demethylation observed with 0.1 U/mL Lac1 is comparable to levels of activity obtained with other extensively studied laccases

from white-rot fungi, the reaction with Lac1 is much faster (highest methanol release is achieved during the first hour at room temperature) while the reactions with the other enzymes require either higher temperature or longer incubation time [18,38–40]. Demethylation of lignin was also possible using the crude laccase preparation. However, the reaction was less efficient than with the purified enzyme (Lac1) (Fig. 4b).
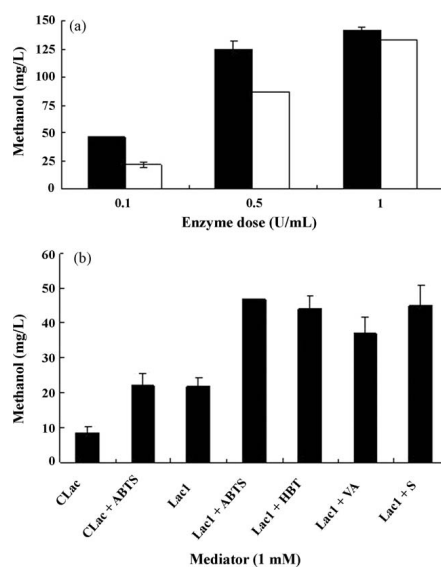
Fig. 4. Methanol released on demethylation of hardwood Kraft lignin from eucalyptus (a) using varying concentrations of pure Lac1 in the presence (■) and absence (□) of 1 mM ABTS, respectively; and (b) using 0.1 U/mL Lac1 or crude laccase (CLac) with 1 mM of different mediators, ABTS, hydroxybenzotriazole (HBT), violuric acid (VA) and syringaldehyde (S). The values correspond to 1 h reaction time.

The demethylation reaction was seen to occur with a nearly equal efficiency in the presence of different redox mediators including the natural mediator, syringaldehyde (Fig. 4b). Although being useful for the application, these observations seem to be in contrast to the significant differences in Lac1 activity obtained above with different substrates (Table 3).

We conclude that *Galerina* sp. laccase is among the very few enzymes described from litter-decomposing fungi. Although many of its properties resemble those of the known fungal laccases, it exhibited remarkable kinetic properties with non-phenolic substrate and very low activity with phenolic substrates. Nevertheless, it seemed to be the most efficient enzyme for catalyzing demethylation of hardwood Kraft lignin. This has led to further investigations regarding its potential in applications related to lignin modification.

Acknowledgements

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Paper IV

Decolorization of dyes by laccase/mediator system in a membrane reactor

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Abstract
A laccase/mediator system was developed for decolorization of textile dyes. By screening different laccases and mediators, *Trametes versicolor* laccase and syringaldehyde provided the best system for decolorization. Decolorization yields of 100%, 85%, 80% and 78% were obtained for the dyes Red FN-2BL, Red BWS, Remazol Blue RR and Blue 4BL, respectively. The pH and the ionic strength of the buffer had a significant effect on the decolorization. Over 95% decolorization of Red FN-2BL and 50% decolorization of the other dyes was achieved at sodium chloride concentration as high as 100 mM. A membrane reactor was set up for dye decolorization in a batch mode with reuse of the enzyme. Between 10-20 batches could be run with decolorization yields from 95 to 52% depending on the dye type. The reactor was run using TEMPO coupled to polyethylene glycol to study the possibility of also reusing the mediator. At least 9 batches were run for the treatment of Remazol Blue RR; decolorization yields of 96-78% were obtained. Cost analysis of the processes showed that the costs of laccase/syringaldehyde or laccase/TEMPO were almost equal when running 20 batches. The cost increased when using PEG-TEMPO; however, the reusability could be considered as a significant advantage.

Keywords: Laccase/mediator system; syringaldehyde; PEG-TEMPO; ultrafiltration membrane; cost analysis.

1. Introduction

Water is a valuable natural resource; however, the levels of pollution in water sources have increased during the last decades [1] Discharge of dyes from textile industries constitutes a major source of contamination, with about 10% of the used dyestuffs ending up in effluents [2].

Azo dyes are the most widely used colorants in textiles industries and are hence predominant in the wastewater. As these dyes have been developed to resist fading, they are quite recalcitrant and consequently the wastewater needs to be treated before being released into the environment. The conventional methods for treatment of textile wastewaters include adsorption and/or precipitation [3], which have the disadvantage of creating large amounts of hazardous waste since they only remove the dyes from the water phase without degrading them. Physicochemical methods, which can contribute to complete dye degradation include sonolysis [4], oxidation using Fenton´s reagent, ozonation [5], photochemical oxidation and electrochemical destruction [6]. Biological systems have the advantage of providing cheaper, milder and cleaner processes, and different fungi and bacteria are known to catalyze dye decolorization [7].

Bacterial treatment of azo dyes is usually based on a combined anaerobic-aerobic process. The anaerobic step results in reductive cleavage of the azo bond leading to decolorization and formation of aromatic amines, many of which are known carcinogens and their complete mineralization in the aerobic step cannot always be guaranteed [8].

Basidiomycete is a group of fungi able to produce different oxidoreductases, which can oxidize a wide range of toxic compounds [9]. The advantage of fungal treatment compared to bacterial treatment is that the reductive cleavage step can be avoided, and can be achieved either using whole fungi or with isolated enzymes. The latter involves higher costs associated with extraction; this might however be overcome by the advantage of faster reactions [10].

Laccases (benzenediol:oxygen oxidoreductase; EC 1.10.3.2) are increasingly being used in a variety of industrial and environmental applications as they catalyze the oxidation of a broad spectrum of compounds with phenolic or aromatic groups using molecular oxygen as electron acceptor. The range of substrates can be broadened using low molecular weight mediators [11], which act as electron acceptors during the oxido-reduction reaction, and participate in the oxidation of substrates that due to their high redox potential or size cannot be oxidized by laccase alone. Laccases or laccase/mediator systems have been used for decolorization of many different dyes and the results are highly promising. The main limitation however is the cost of the enzyme and mediators and in some cases their toxicity [12].

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Enzymes are used in an immobilized form in several large-scale applications to enable their reuse and in turn lower the process cost. An interesting approach would be to immobilize also the mediator so that it can be recycled providing further cost reduction as well as better management of disposal to avoid further process stream contamination. Immobilized mediators have previously been used for oxidation of alcohols to carbonyl compounds [13,14].

In the present paper, we have examined the decolorization of four azo dyes used in Bolivian and Indian textile industries using a laccase/mediator system. The possibility of recycling the enzyme and mediator by use of a membrane reactor was investigated and a cost analysis was conducted to evaluate the competitiveness of the developed enzyme/mediator process.

2. Materials and methods
2.1. Materials

Remazol Blue RR and Cibacron Red FN-2BL were supplied by a textile industry in India. Blue 4BL and Red BWS were purchased from Química Alemana (La Paz, Bolivia). Laccase from *Trametes versicolor*, *N*-hydroxyphthalimide (HPT), 3-hydroxyanthranilic acid (HAA), syringaldehyde (SA), acetosyringone (AS), acetovanillone (AV), syringic acid (Sac), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), *p*-coumaric acid (C), 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO), vanillin (V), viorulic acid (Vac) and poly(ethylene glycol)-bis-TEMPO (PEG-TEMPO) were purchased from Sigma-Aldrich (Schnelldorf, Germany). *Myceliophthora thermophila* laccase was obtained from Novozymes (Denmark) and *Galerina* sp. laccase was produced in our laboratory. EDTA was obtained from Merck (Darmstadt, Germany). The ultrafiltration polysulfonate membrane 10 KDa cut off was purchased from Sartorius AG (Goettingen, Germany).

2.2. Treatment of dyes using laccase/mediator system
2.2.1. General procedure

Stock solutions of Remazol Blue RR (λ_{max} 613nm), Red FN-2BL (λ_{max} 526nm), Blue 4BL (λ_{max} 604nm) and Red BWS (λ_{max} 513nm) were prepared in water at concentrations of 1 g/L.

Experiments for determination of optimal conditions for decolorization of the dyes were performed in 1 ml cuvettes at room temperature. The decolorization was determined spectrohotometrically by monitoring the decrease in absorbance at λ_{max} of each dye, and was calculated according to the following equation:

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 is the absorbance of the untreated dye and A_t is the absorbance of the treated dye after certain time.

2.3. Screening of optimal conditions for dye decolorization by laccase-mediator system

To select an efficient laccase-mediator system, laccase from three different sources (*T. versicolor, M. thermophila* and *Galerina sp*. strain HC1) and several mediators (HPT, HAA, SA, AS, AV, Sac, ABTS, C, TEMPO, V and Vac) were compared for decolorization of the different dyes.

Reaction conditions including dye- (25, 50 and 100 mg/L), enzyme- (0.1-1 U/mL) and mediator (50, 100 and 500 μM) concentrations, pH (sodium acetate buffer pH 5-6, sodium phosphate buffer pH 6-7) ionic strength (0-50 mM at pH 5) and salt concentration (0-100 mM NaCl) were tested with *T. versicolor* laccase and syringaldehyde to select the optimal conditions for decolorization. The effect of buffer ionic strength was also evaluated when 25 μM TEMPO was used as the mediator.

Each dye at a concentration of 50 mg/L in water was incubated with laccase (0.1 U/mL) and varying concentrations of PEG-TEMPO (0.05, 0.1, 0.5, 0.75 mg/mL) corresponding to 25, 50, 250 and 375 μM TEMPO (λ_{max} 248 nm). The optimal enzyme concentration (0.1, 0.5 and 1 U/mL) was then determined using the optimal mediator concentration.

2.4. Laccase stability

To investigate the effect of the mediator on the stability of the enzyme,5 mL of 0.1 U/mL *T. versicolor* laccase solution in water was incubated with 25 μM SA or TEMPO. A sample of 50 μL was taken at regular time intervals for determining the residual laccase activity according to [15].

2.5. Scale up of the process and reusability of the laccase/mediator system

2.5.1. Reusability of the enzyme. A 500 mL reactor with 300 mL working volume was set up for decolorization of the dyes in a batch mode. The reactor was coupled to a polysulfonate ultrafiltration membrane (SARTORIUS) with a nominal molecular weight cut-off of 10 KDa, which permitted retention of the soluble enzyme in the reactor for use in a subsequent batch. The reactor was operated for decolorization of 50 mg/L dye using 0.1 U/mL T. versicolor laccase and 25 μM SA. The enzyme was added at the start of the experiment and the reaction was run until no further decolorization was achieved, after which the reactor was emptied using a peristaltic

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pump set at 50 mL/min. A new 300 mL batch was started by adding new dye solution (50 mg/mL) and syringaldehyde (25 µM). At the start of each batch, an aliquot of 50 µL was collected for measuring residual laccase activity in the reactor.

2.5.2. Reusability of the laccase/mediator system. In order to investigate the recyclability of even the mediator when using PEG-TEMPO (25 µM), the reactor was set up as described above to decolorize Remazol Blue RR (50 mg/L), with 1 U/mL *T. versicolor* laccase. The new batch was started by adding only the dye solution.

3. Results and Discussion
3.1. Screening of laccases and mediators

Preliminary studies with the different laccases showed the enzyme from *T. versicolor* to be the most efficient enzyme for decolorization of all the dyes at room temperature. Red FN-2BL was completely decolorized in 1 min and 5 min with SA and AS, respectively, as mediators. Other mediators such as TEMPO, V and ABTS led to 98-95% decolorization in 60 min. Decolorization of Red BWS was relatively less efficient - 85-88% in 60 min with AS, ABTS or SA as mediator.

SA and AS turned out to be suitable mediators even for laccase treatment of the blue dyes – maximum decolorization of about 80% was achieved for Remazol Blue RR in 5 min, and 73-78% for Blue 4BL in 10 min. The high efficiency of SA and AS as mediators observed in this study corresponds well with the earlier reports of Camarero et al. [16] and Murugesan et al. [17]. According to the latter report, decolorization of malachite green in the presence of syringaldehyde was also accompanied by reduction in its toxicity. The high oxidative capacity of these mediators is related to the electron donor transfer of the methoxy substituents at the benzenic ring [16].

UV-visible spectrum of the dye solutions after treatment with *T. versicolor* laccase-syringaldehyde system showed complete decolorization only for Red FN-2BL (Fig. 1). The rate and extent of decolorization is dependent on dye structure and in particular on the functional groups in the aromatic region and their interactions with the azo bond [18]. Incomplete decolorization could also be attributed to the heterogeneous quality of the dye formulations, since intermediates are usually not removed during production and some dyestuffs can additionally be mixtures of several different dye types, leading to differences in recalcitrance of the dye molecules in the final product.

For the blue dyes, relatively high decolorization yields (55-80 %) could also be achieved with *M. thermophila* and *Galerina sp.* strain HC1 laccases in combination with SA, AS or ABTS after 60 min of reaction. The different laccase efficiencies may be attributed to their redox potential; in case of laccase from *T.*

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versicolor is reported to be 0.78 V [19], and laccase from *M. thermophila* 0.46 V [20].

The application of laccase for dye decolorization is highly advantageous compared to other conventional methods. The required treatment time is shorter than when using whole fungal cells; laccase from *T. versicolor* decolorized Remazol Blue RR and Red FN-2BL in minutes, while the fungal cells needed over 10 days to reach similar decolorization yields (unpublished data). In addition, the reaction can be run under non-sterile conditions with the isolated enzymes [10]. The advantage compared to bacterial treatment, is that accumulation of aromatic amines with concomitant increase in toxicity after treatment of dyes, is avoided [7]. Furthermore, in contrast to the microbial treatment, there is no accumulation of sludge [21]. Finally, when compared to chemical or physical treatments, the costs, accumulation of sludge and undesired side reactions can be reduced or avoided.

3.2. Optimization of parameters using *T. versicolor* laccase and SA

Based on the above results, the decolorization studies were continued with laccase from *T. versicolor* and SA as mediator. The latter has the advantage of having a renewable source of production and being considerably cheaper than other mediators [22].

Optimal conditions with respect to dye, laccase and SA concentrations varied for achieving maximal decolorization of the four dyes (data not shown). The decolorization of Red FN-2BL was dependent on the mediator concentration; the highest decolorization yields were reached using 50 µM of SA and 0.5-1 U/mL laccase, independently of the dye concentration. The decolorization of Red BWS was mainly dependent on the laccase and mediator concentrations. Increasing the enzyme concentration from 0.1 to 1 U/mL led to higher decolorization, while mediator concentration higher than 50 µM led to a lower decolorization.

In the case of Remazol Blue RR, the decolorization yield was increased with increase in the laccase concentration to 0.5 U/mL. The decolorization was comparable at 25-50 mg/mL dye and 50-100 µM mediator, but reduced at higher mediator and dye concentrations. Decolorization of Blue 4BL was more dependent on the dye and mediator concentrations than on the laccase concentration. The highest decolorization (approx. 88 %) was reached at 0.1 U/mL of laccase and 50 µM mediator and the decolorization was negatively affected by further increase in the dye and mediator concentrations.

Since higher mediator concentrations led to lower decolorization, due to loss of laccase activity, 50 µM SA was chosen for further experiments. The lowest laccase concentration (0.1 U/mL) providing high decolorization was chosen to minimize costs. It was also shown that acceptable decolorization could be achieved at

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all concentrations in the range of 25-100 mg/L of dye even if the yield was lower at the highest concentration.

pH had significant effect on the level of decolorization; pH above 5 led to a decrease in decolorization yield. At pH 6 the decolorization yields reached 73 %, 20 % 56 % and 34 %, for the dyes Red FN-2BL, Red BWS, Remazol Blue RR and Blue 4BL, respectively, and at pH 7, the decolorization yields only reached 2-4 % (data not shown). This is attributed to the low pH-optimum for laccase activity [23] and possibly low syringaldehyde stability at higher pH [16]. The ionic strength of the buffer had no effect on the decolorization of Red FN-2BL and Remazol Blue RR; the reaction was equally efficient in water adjusted to pH 5 as in 10-50 mM acetate buffer at the same pH. On the other hand, the decolorization of Red BWS and Blue 4BL was significantly lower in water, but ionic strength of the acetate buffer as low as 10 mM highly improved the decolorization yields.

The textile wastewaters can contain salt at concentrations between 50 and 115 mM [24]. Evaluating the effect of NaCl concentration in the present system showed that decolorization of Red FN-2BL was maintained over 95% at 0-100 mM NaCl. The other dyes did however show decreasing decolorization with increasing NaCl concentration (Figure 2). As reported earlier [25], NaCl could have an inhibitory effect on the laccase activity; nevertheless, over 50% decolorization of all the dyes was still achieved up to 100 mM NaCl.

Syringaldehyde was however found to affect the activity of *T. versicolor* laccase within 30 min when used at a concentration of 50 µM. At 25 µM SA the enzyme maintained activity during a longer period of incubation (Figure 3). Although the residual activity after first three days decreased to 56%, compared to 81% without the mediator, continued incubation up to 6 days resulted in similar residual activity of about 50% in both cases. TEMPO, on the other hand, led to a higher degree of inactivation. The mediator-induced loss of enzyme activity has been attributed to the degradation of essential amino acids residues or the glycosyl moieties on the periphery of the enzyme [26-28].

3.3. Decolorization of dyes using *T. versicolor* laccase and syringaldehyde in a membrane reactor

3.3.1. Laccase and PEG-TEMPO system

The optimized conditions were applied for dye decolorization in a membrane reactor to enable retention and recycling of the laccase. The enzyme (0.1 U/mL) could be recycled for 10-20 batches, however the reaction time required to obtain maximal decolorization depended on the dye (Table 1).

Red FN-2BL showed the fastest decolorization; the first batch needed only 8 min to reach 95% decolorization while 1 h was required during the 10th batch to reach 91% decolorization. The enzyme activity was however almost

completely lost after the 10th batch (Table 1). The possibility to stabilize the enzyme was investigated by including 1mM EDTA in the reaction. EDTA plays a protecting role for proteins, especially under oxidative stress [29]. When using EDTA it was possible to run 19 batches in a shorter period of time, although 85 % decolorization in the 10th batch and 69 % in the 19th batch with the enzyme retained 20% of its activity was determined (data not shown). Formation of precipitate, which did not pass through the membrane, may have some inhibitory effect on the decolorization.

The rate of decolorization of Red BWS was slower (1-1.6 h) than the above dye for 15 runs. The extent of decolorization did however decrease from 84 to 52%, probably due to accumulation of polymerization products.

Higher decolorization yields (94 to 85%) were obtained for Remazol Blue RR during 20 batches, even though only 4 % of the laccase activity remained during the last batch. The decolorization of this dye required longer reaction time – 2-3 hours for the initial batches and almost 7 hours for the last batches.

Ten batches could be run for treatment of Blue 4BL, resulting in 72-61% decolorization in 1.2 to 1.8 h. The enzyme activity decreased to 49% of the initial activity after the 10th batch. Although the residual enzyme activity and the decolorization yield were satisfactory, the experiment was terminated due to accumulation of blackish products retained by the membrane.

The lignin oxidizing enzymes including peroxidases and laccase are known to catalyze the oxidation of compounds by generation of free radicals, which can further initiate polymerization or degradation. Goszczynski et al. [30] and Chivukula and Renganathan [31] proposed that azo compounds can be "degraded" asymmetrically and symmetrically by ligninases. The latter can lead to formation of products that can further polymerize and produce a precipitate. Precipitation due to polymerization has also been observed during laccase treatment of other dyes [17]. The precipitate could also arise from dye degradation products reacting with protein forming coloured conjugates.

3.3.2. Laccase and PEG-TEMPO system

The feasibility of recycling the mediator modified in a way so as to increase its molecular size to be retained by the ultrafiltration membrane was tested. Such an approach has been applied for enzymatic reactions involving cofactor regeneration, where cofactors are coupled to polymers without notable effect on the kinetic constants of the reactions [32]. The cofactor-polymer conjugates such as NAD^+-polyethyleneimine, NAD^+-polylysine, PEG-$NADP^+$ have been used in continuous enzyme reactors. High product yields have been reported for long periods of incubation with low leakage loss of the cofactor [33-35], which can reduce the cost of the process significantly. Yet another advantage is the reduced cofactor inactivation by dimerization [35].

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TEMPO coupled to PEG (PEG-TEMPO) with an average molecular weight of approx. 5KDa is commercially available, and was used to test the concept for decolorization of the dyes even though TEMPO was not the optimal mediator in the present study [36,37]. The reaction using laccase and PEG-TEMPO could be performed in water for most of the dyes, except for Blue 4BL whose decolorization was improved in 50 mM sodium acetate buffer pH 5. Hence, further experiments were run without buffer (data not shown). At laccase concentration of 0.1 U/mL, the optimum PEG-TEMPO concentration for treatment of Red FN-2BL, Blue 4BL and Red BWS was 250 µM, while only 25 µM was needed for decolorization of Remazol Blue RR. The latter dye was decolorized faster and the decolorization yield increased to 95% with increase in laccase concentration to 1 U/mL (data not shown).

The possibility of reusing both the laccase and PEG-TEMPO in a membrane reactor was evaluated using Remazol Blue RR, since high degree of decolorization could be achieved with a low concentration of the mediator. Although 0.05 mg/mL PEG-TEMPO showed lower rates of decolorization (0.0003 Abs min^{-1}) than both syringaldehyde (0.006 Abs min^{-1}) and free TEMPO (0.0016 Abs min^{-1}) (Figure 4), it was possible to run 9 batches without adding extra enzyme or mediator during repeated batches (Figure 5). After the last batch, the reaction became slow due to loss of laccase activity. As shown in Figure 3, laccase activity was reduced to approximately 30% of its original value after 6 days when incubated with 25 µM TEMPO. So far, no other reports about the reuse of PEG-TEMPO (or any other mediator) for dye decolorization have been found, but it has been reported that PEG-TEMPO could be recycled at least 6 times in the synthesis of carbonyl compounds with conversion yields from 99 to 74 % [14]. These results open the possibility for further development of the concept and optimization of the system, e.g. by using different polymers with varying molecular weights, and other mediator compounds. Other supports that have earlier been used for immobilization of TEMPO include silica, polystyrene and sol-gel [38], and poly(ethyleneimine) [39].(Kulbe et al, 1987).

3.4. Cost analysis

The analysis of costs for treatment of dyes using the laccase/mediator system is shown in Table 2. The process costs are quite similar for syringaldehyde or TEMPO as mediators. The costs for running 20 batches would be 0.07 vs. 0.06 Euros per gram of dye for syringaldehyde and TEMPO, respectively; however, syringaldehyde would be preferred since it leads to faster decolorization than TEMPO, and leads to less inactivation of laccase. The application of PEG-TEMPO would cost more than the other mediators (1.08 Euros per gram of decolorized dye) based on current prices. This mediator requires even longer time to catalyze the decolorization. The advantage is however that PEG-TEMPO is retained in the system and so its disposal can be better managed than for the free mediators.

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PEG-TEMPO should however only be considered as a model compound to prove the possibility of mediator reuse. If the "immobilized" mediator were to be implemented, the production cost of the immobilized form would probably be lowered, e.g. it would not be necessary to use highly pure preparations for wastewater treatment. Other macromolecular mediators should also be screened to improve the cost efficiency.

4. Conclusions

It is encouraging to realize that coupling to a soluble polymer does not prevent the mediator from functioning as mediator, although the kinetic constants are affected. The possibility of reusing the enzyme and the mediator is important in order to promote their use in large-scale reactions. In addition they could have a negative impact on the environment if not properly disposed.

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Table 1. Decolorization efficiency after repeated uses of laccase from *T. versicolor* using a membrane reactor. Syringaldehyde (25 µM) was used as mediator.

| Number of batches	Red FN-2BL			Red BWS			Remazol Blue RR			Blue 4BL		
	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)	Decolorization (%)	Activity (%)	Time (h)
1	95	100	0.1	84	100	1.1	94	100	2.7	71	100	1.6
2	94	72	0.15	74	82	1.2	94	73	2.3	72	80	1.8
3	94	33	0.17	72	88	0.8	94	64	2.8	61	83	1.6
4	94	24	0.22	69	75	1.0	94	63	3.3	68	80	1.8
5	93	16	0.3	67	73	1.0	94	44	2.8	66	64	1.3
6	92	13	0.4	67	67	1.2	94	38	2.8	64	66	1.5
7	93	8	0.5	62	70	1.4	94	37	3.2	63	65	1.8
8	94	6	0.7	62	52	1.2	94	34	3.0	62	56	1.2
9	92	5	0.9	59	59	1.2	94	21	3.0	63	56	1.6
10	91	5	1.0	60	53	1.2	93	21	3.0	61	49	1.6
11				55	30	1.3	94	14	3.2			
12				55	37	1.5	93	16	3.7			
13				56	31	1.3	93	12	3.7			
14				55	29	1.5	94	11	4.5			
15				52	26	1.6	94	8.0	4.3			
16							93	7.0	5.0			
17							93	7.0	4.7			
18							93	6.0	6.7			
19							93	5.0	7.0			
20							85	4.0	6.3			

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Table 2. Cost analysis for dye decolorization using laccase/mediator treatment. The cost is expressed in Euros per gram of treated dye.

Number of batches	[a]Syringaldehyde +Laccase	[a]TEMPO +Laccase	[b]PEG-TEMPO +Laccase
1	0.86	0.85	21.59
5	0.20	0.19	4.32
10	0.12	0.10	2.16
20	0.07	0.06	1.08

Mediator concentration: 25 µM; [a] 0.1U/mL laccase; [b] 1 U/mL laccase
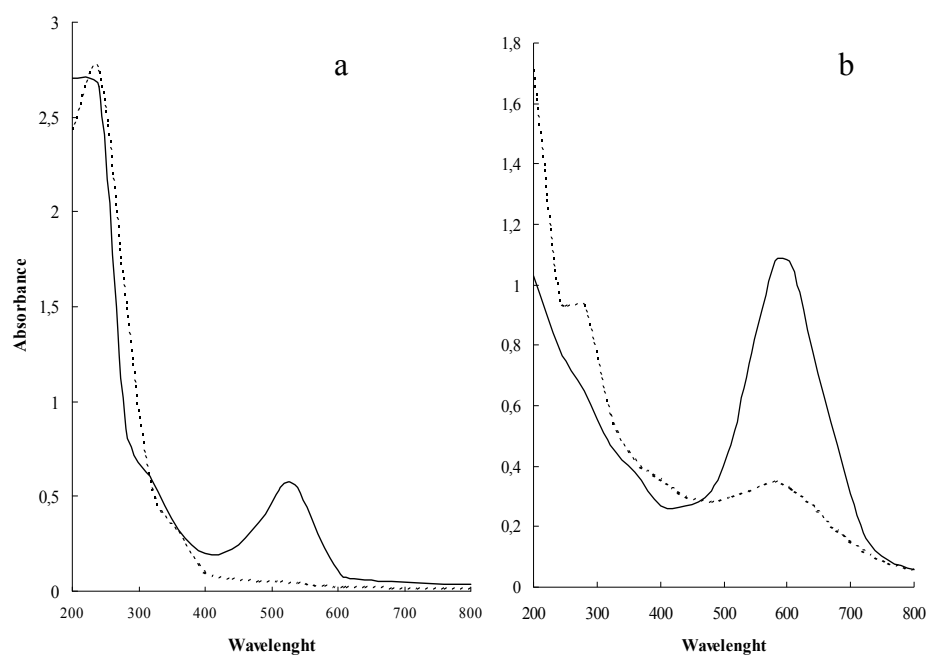
Fig. 1. UV-visible scan of dyes, (a) Red FN-2BL and (b) Red BWS before (*continuous line*) and after (*dotted line*) treatment with 0.5 U/mL *T. versicolor* laccase and 50 µM syringaldehyde.

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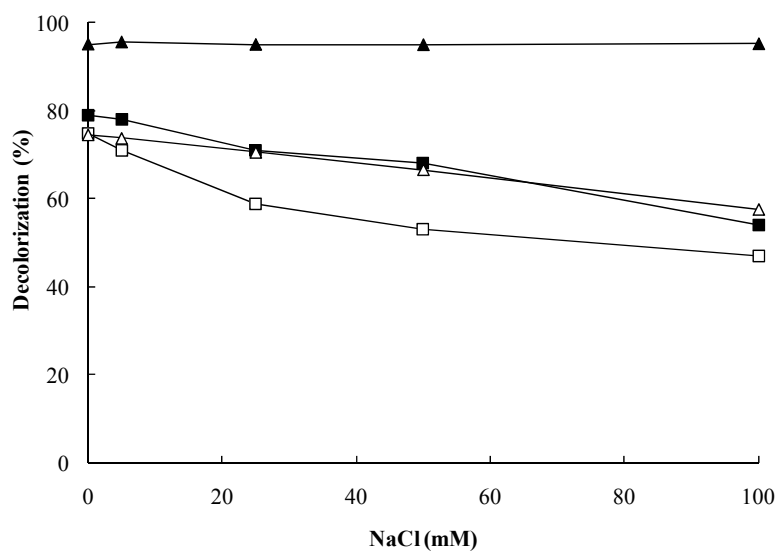
Fig. 2 Effect of NaCl concentration on the decolorization yield of Remazol Blue RR (■), Blue 4BL (□) Red FN-2BL (▲) and Red BWS (Δ) using 0.1 U/mL laccase and 50 μM syringaldehyde.
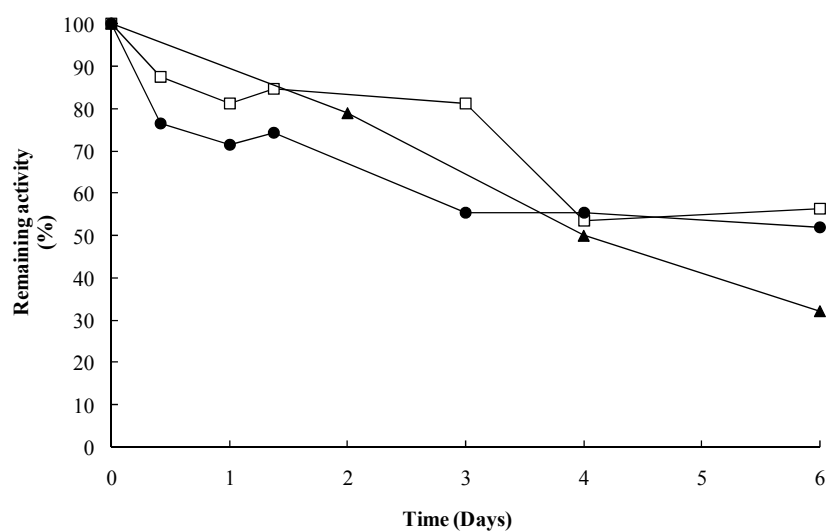
Fig. 3. Residual laccase activity during incubation with syringaldehyde (●), TEMPO (▲) and no mediator (□).
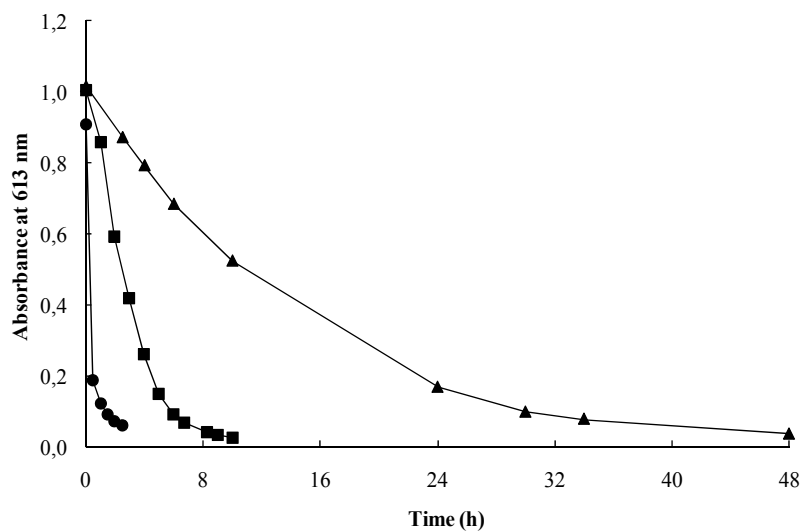
Fig. 4 Decolorization of Remazol Blue RR using 0.1 U/mL of *T. versicolor* laccase and 25 µM syringaldehyde (●), TEMPO (■) or PEG-TEMPO (▲).
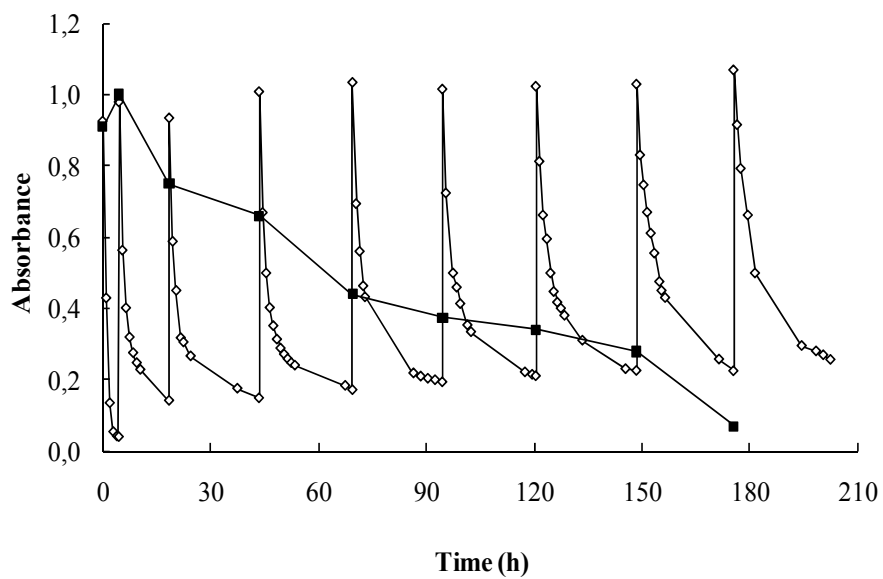
Fig. 5 Reusability of the laccase-mediator system for decolorization of Remazol Blue RR. The reactor was run with 1 U/mL laccase and 25 µM PEG-TEMPO. Absorbance at 613 nm (◊); laccase activity (■).

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Paper V

Laccase mediator system for activation of agarose gel: Application for immobilization of proteins

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ABSTRACT

Cross-linked Sepharose beads were treated with laccase–TEMPO system for oxidation of the primary alcohol groups on the sugar moieties. Optimal activation conditions using *Trametes versicolor* laccase were at pH 5 and 22 °C, giving an aldehyde content of 55 μmol g^{-1} Sepharose with 28 units g^{-1} of laccase and 12.5 mM TEMPO. The activated Sepharose was used for immobilization of trypsin as model protein. Highest degree of immobilization was obtained at pH 10.5 but the activity yield was only 31% of that loaded on the gel. The yield of gel bound trypsin activity was increased to 76% (corresponding to about 43 U g^{-1} Sepharose) when the immobilization was performed in the presence of trypsin inhibitor, benzamidine. The immobilization yields were comparable to that obtained on the matrix activated using sodium periodate (containing 72 μmol aldehyde per g Sepharose). Recycling and storage of the immobilized trypsin preparations showed high stability of the enzyme bound to laccase–TEMPO activated gel.

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1. Introduction

Laccases (p-diphenol:oxygen oxidoreductase, EC 1.10.3.2) are enzymes belonging to a family of multicopper oxidases, which catalyse the oxidation of various substrates with simultaneous reduction of molecular oxygen to water. White-rot fungi constitute the major source of laccases; however these enzymes are also produced by plants, bacteria and insects [1–3]. Laccases are rather non-specific with respect to the reducing substrates and are able to oxidize a number of organic compounds including phenolic compounds, anilines, benzenethiols, etc., and some inorganic ions [1,4]. The repertoire of substrates that can be oxidized by laccases is broadened in the presence of small redox mediators – compounds that are first oxidized by laccase and the oxidized form in turn acts as an oxidant for the substrates. As the mediator can function as a diffusible electron carrier, oxidation of macromolecular structures is also enabled. The laccases and laccase mediator systems (LMS) have offered an environmentally benign means of oxidation, which has been used in a range of applications including decolorization of dyes, bioremediation, paper pulp bleaching, cosmetics, and organic syntheses [4,5].

Earlier studies have demonstrated oxidation of alcohols to carbonyl compounds by laccase in the presence of different mediators such as TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy), ABTS (2,2′-azinobis-bis (3-ethylbenzthiazoline-6-sulfonic acid)), 3-hydroxyanthranilic acid, viorulic acid, and 1-hydroxybenzotriazole [6,7]. Later, this chemo-enzymatic approach was used for regio-selective oxidation of the primary alcohols of sugar derivatives, natural glycosides, and partial oxidation of water-soluble cellulose [8,9]. Selective oxidation of the hydroxyl groups on sugar derivatives is useful for further modifications of carbohydrates to bioconjugates and biopolymers [10].

Polysaccharides are commonly used as insoluble matrices for chromatography of proteins and also for immobilization of proteins and affinity ligands. Agarose (a polymer of (1 → 3)-β-D-galactopyranose-(1 → 4)-3,6-anhydro-α-L-galactopyranose) based gels are among the most widely used matrices, having the advantages of being hydrophilic and compatible with biomolecules. Crosslinking of gels further increases their mechanical- and chemical stability. Immobilization of ligands to such matrices requires activation of the hydroxyl groups on the sugar components of the polysaccharide. Oxidation of the alcohol groups to aldehyde is one of the activation methods used and a ligand containing amino group(s) is then coupled to the oxidized groups by Schiff base reaction [11,12].

In this paper, we demonstrate the potential of LMS system for activation of a commercially available cross-linked agarose matrix, Sepharose CL-6B, for subsequent immobilization of proteins or other ligand molecules bearing amino groups. This approach has

been compared with the conventional method for matrix activation via sodium periodate oxidation.

2. Experimental

2.1. Chemicals

Sepharose CL-6B was purchased from GE-Healthcare (Uppsala, Sweden). Laccase from *Trametes versicolor* (24 U mg^{-1}; one unit of laccase is defined as the amount of enzyme that catalyses the oxidation of 1 μmol 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid per min), trypsin (EC 3.4.21.4) from bovine pancreas, TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl), bicinchoninic acid, bovine serum albumin, N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA), benzamidine and 1,3-dinitrophenylhydrazine (DNPH) were obtained from Sigma–Aldrich (Germany). All chemicals used were of analytical quality and were obtained from standard sources.

2.2. Laccase–TEMPO activation of Sepharose CL-6B

Sepharose CL-6B was washed a few times with 25 mM sodium acetate buffer pH 5, centrifuged at 500 × g for 2 min and then the liquid was removed using a Pasteur pipette. One gram wet weight gel was suspended in 0.5 ml of the buffer in 15 ml tubes, supplemented with about 28 units of laccase (57 U ml^{-1}) and 12.5 mM TEMPO, and mixed on a rocking table at room temperature. Treatment with LMS was stopped at a defined time interval (up to a period of 48 h) by centrifuging the tubes and removing the liquid. The gel beads were analysed for determination of carbonyl groups.

The effect of pH (3–8) and temperature (20–45 °C) on laccase–TEMPO catalysed oxidation of Sepharose was examined. The effect of laccase (0–100 units per g of Sepharose) and mediator (0–25 mM) concentrations was evaluated by a factorial design analysis using the program statistic version 6.0.

2.3. Sodium periodate activation of Sepharose CL-6B

Oxidation of hydroxyl groups on Sepharose using sodium periodate was based on the modified method of Miron and Wilchek [13]. Sepharose CL-6B (1 g wet wt) was treated with 0.1 M sodium periodate (3 ml) solution in water for 3 h at room temperature. Subsequently, the gel was washed with water and analysed for determination of carbonyl groups.

2.4. Immobilization of trypsin to activated Sepharose CL-6B

To 0.5 g wet wt activated Sepharose CL-6B in a 15 ml tube was added 2 ml of a solution of trypsin (2.5 mg ml^{-1}, 5.7 U mg^{-1}) in 20 mM sodium phosphate buffer (pH 7), sodium bicarbonate (pH 8) and sodium carbonate buffer (pH 10.5), respectively. The tubes were placed on a rocking table, mixed for 2 h at room temperature, and then centrifuged at 500 × g for 2 min. Samples of the supernatant were withdrawn to quantify unbound protein concentration and trypsin activity. The gel with the bound enzyme was treated for 3 h with 2 mmol sodium borohydride dissolved in 10 ml of the respective buffers used for immobilization. Sepharose–trypsin was washed with 20 mM phosphate buffer, pH 7 and stored at 4 °C prior to analysis.

Immobilization of trypsin was also performed in the presence of 20 mM benzamidine. After immobilization, the gel was centrifuged to remove the supernatant, treated with sodium borohydride and washed with phosphate buffer, pH 7 as mentioned above. The effect of enzyme loading (2.5–10 mg ml^{-1}) and incubation time on the degree of enzyme immobilization was studied. The amount of immobilized protein (mg per g Sepharose) was estimated by the

difference between the original amount used for immobilization and that left in the supernatant.

2.5. Determination of carbonyl groups on the activated gel

2.5.1. Aldehyde content

Quantitative analysis of the aldehyde content of the activated Sepharose was performed by reacting the aldehyde groups with DNPH and measuring the unreacted DNPH by HPLC (Perkin Elmer, Norwalk, CT) on a RP-18 column (4.6 × 250 mm, pore diameter 5 μm, Merck, Germany). Fifty milligram of chemically or enzymatically activated Sepharose was incubated with 50 μl 0.1 M DNPH stock solution (prepared in a solvent system composed of 1:1 2 N HCl:acetonitrile) for 20 min. The solution was extracted with 250 μl ethyl acetate and 100 μl of the extract solution was dried and dissolved in 500 μl acetonitrile for injecting into HPLC. The column was eluted by a gradient of solvent A (30% (v/v) acetonitrile in water) and B (100% acetonitrile) starting from 90 vol A:10 vol B to 10 vol A:90 vol B for 15 min, and then 10 vol A:90 vol B for 5 min at a flow rate of 1 ml min^{-1}. Elution was monitored at 356 nm (RT$_{DNPH}$ ≈ 7 min). The consumption of DNPH was calculated from the peak areas and compared with a standard curve of 0–0.1 M DNPH prepared and analysed under the same conditions. One mol of aldehyde corresponds to one mol of DNPH consumed [14].

2.5.2. Carboxylic group

Activated Sepharose samples were dried overnight at 60 °C and then ground in a mortar. The powder (approximately 0.1 g) was mixed with 10 ml of 0.1 N NaOH containing 0.2 M NaCl, and the reaction mixture was mixed for 2 h on a rocking table at room temperature. The insoluble fraction was separated by centrifugation (2500 × g, 10 min), and 5 ml of the supernatant was removed and titrated with 0.1 M HCl until the pH reached a value of 6.9. The content of carboxylic acid was calculated using the equation:

$$\text{moles per gram} = \frac{C_{HCl} \times (V_{NaOH} - V_{HCl})}{m} \tag{1}$$

where V_{HCl} (in l) is the volume of 0.1 N HCl needed for titration and V_{NaOH} represents the volume of the NaOH used, and m is the amount of the Sepharose powder.

2.6. Trypsin assay

The activity of trypsin was assayed by measuring the rate of hydrolysis of BAPNA at 22 °C [15]. The assay was performed in a cuvette in 1 ml reaction volume containing 35 mM Tris–HCl buffer pH 8, 43 mM CaCl$_2$, 0.1 mM HCl, 0.56 mM BAPNA and 0.1 ml of free enzyme. The p-nitroaniline released was measured spectrophotometrically at 410 nm. One unit of trypsin was defined as the amount of enzyme that catalysed the production of 1 μmol p-nitroaniline per min per volume of reaction. The extinction coefficient of p-nitroaniline at 410 nm is 8800 M^{-1} cm^{-1} [15].

Fifty milligrams of the immobilized protein was mixed with the 900 μl assay mixture in an eppendorf tube and mixed on a rocking table for 2 min, centrifuged at 3800 × g for 2 min and absorbance of the products accumulated in the supernatant was determined as in case of the free enzyme system.

3. Results and discussion

Oxidation of primary alcohols using TEMPO proceeds through an oxoammonium ion, which is attacked by the substrate as a nucleophile, followed by removal of α-proton to give an oxidation product and reduced-TEMPO (N–OH), which is oxidized back to TEMPO (N–O) and then to the oxoammonium ion by the catalyst [16] (Fig. 1).
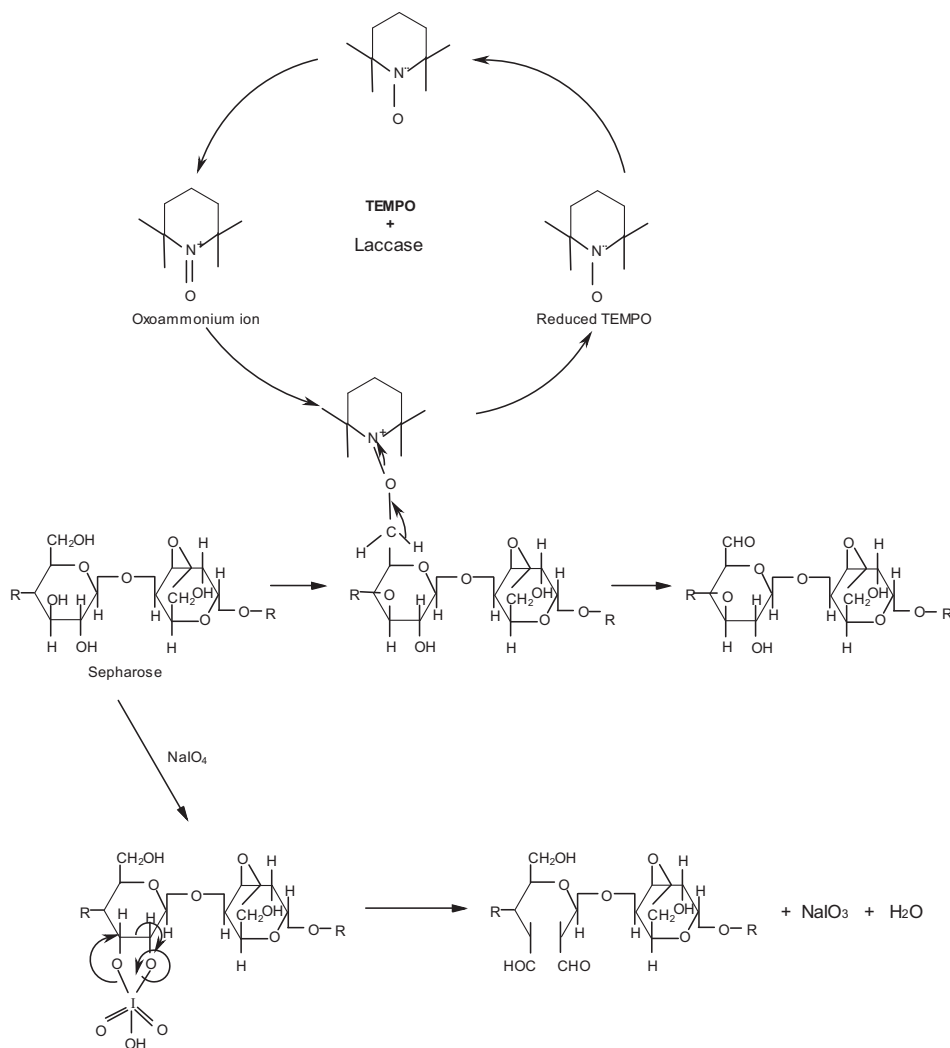
Fig. 1. Activation of agarose by oxidation using different catalysts: laccase/TEMPO and NaIO$_4$. Adapted from Galli and Gentilli [16].

Oxidation of Sepharose CL-6B using TEMPO was investigated using *T. versicolor* laccase as catalyst. Initially, Sepharose CL-6B was treated with 28 units of laccase per g (wet wt) matrix and 12.5 mM TEMPO for different periods of time and the carbonyl content of the gel was analysed. The aldehyde- and carboxyl groups generated were determined by direct measurement on the gel. Maximum aldehyde content of about 55 μmol and carboxylic acid content of 10 μmol per g (wet wt) Sepharose was obtained after 10–12 h at pH 5 and 22 °C. The degree of activation was lower when laccase treatment was performed at pH values below or above pH 5, which corresponds to the optimum pH for activity of the laccase. It has also been reported recently that carboxylic acids in the buffer systems inhibit laccase activity [17]; hence choosing an appropriate buffer (e.g. succinate or citrate buffer) could further reduce the degree of enzyme inhibition and provide the desired activation of the matrix in a shorter time or with a lower enzyme concentration.

Table 1
Immobilization of trypsin on laccase/TEMPO activated Sepharose CL-6B at different pH.

	Phosphate pH 7		Bicarbonate pH 8		Carbonate pH 10.5	
	Protein (mg g^{-1})	Activity (U g^{-1})	Protein (mg g^{-1})	Activity (U g^{-1})	Protein (mg g^{-1})	Activity (U g^{-1})
Buffer	3.6	3.6	5.1	12.3	4.5	17.4
Buffer + benzamidine	2.2	6.8	7.5	41.1	6.7	43.4

57 U of trypsin (specific activity of 5.7 U mg^{-1}) per g wet wt Sepharose was used for immobilization in the presence of 2 mM benzamidine.

Temperature higher than 22 °C also reduced the degree of oxidation by LMS, which is due to increasing loss of enzyme activity (data not shown). Activation of the gel was thus continued at pH 5 and 22 °C with varying laccase and TEMPO concentrations; aldehyde content of the gel was increased to 75 μmol per g (wet wt) Sepharose with 85 units of laccase and 12.5 mM TEMPO. At higher laccase (100 units per g) and mediator (25 mM) concentrations, there was a decrease in the aldehyde content. No laccase activity was detected on the Sepharose, and hence no laccase immobilization occurred during the activation period probably due to the low pH used that is not suitable for the coupling of the amino group.

The laccase/TEMPO activated Sepharose (with 55 μmol aldehyde per g wet wt gel) was then tested for immobilization of trypsin, which has 16 lysine residues besides the N-terminal residue that potentially contribute primary amino groups for the reaction with aldehyde groups on Sepharose. The coupling occurs through a Schiff base reaction which is subsequently stabilized by reduction with sodium borohydride [10]. The immobilization yield was dependent on the pH as well as the buffer used during the immobilization (Table 1). The highest yield of immobilized trypsin was obtained when carbonate buffer, pH 10.5 was used; the immobilized protein content was higher (45%) than the activity (31%) suggesting some loss of activity during the process most likely due to multipoint attachment. At pH 8, comparable yield (slightly higher than at pH 10.5) of immobilized protein was obtained, but with slightly lower activity. Blanco and Guisán [12] have earlier reported high immobilization yields for trypsin on glyoxyl agarose at pH 10, but the enzyme could also be immobilized at pH 7 due to the presence of some low pKa amino groups [18].
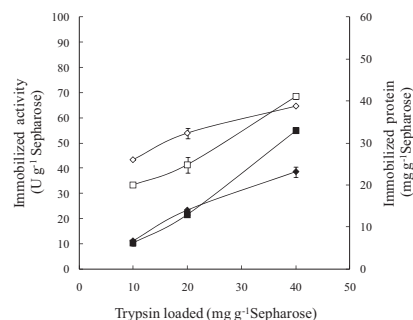
When the immobilization was performed in the presence of a trypsin inhibitor, benzamidine (2 mM), the activity yields of the immobilized enzyme were significantly improved to 43.4 U g^{-1} (corresponding to 76% retention) (Table 1). Benzamidine is known to protect the active site during immobilization, preventing the amino acid residues present therein from participating in coupling to the matrix, and also protects the protease from auto-digestion [19].

The immobilization procedure using laccase activated gel was compared with the commonly used sodium periodate activation procedure for immobilization of ligands with primary amino groups [13,20,21]. Periodate oxidizes the vicinal-diols in the sugar residues to aldehydes [22] (Fig. 1). Normally, the activation by sodium periodate is measured by determining the periodate remaining in the supernatant and based on that aldehyde content on the gel is calculated [20,23]. But a significant fraction of the aldehyde groups gets further oxidized to the carboxyl groups during the processing and handling of the gel prior to immobilization. The periodate-activated gel was found to contain 72 μmol aldehyde and 20 μmol carboxylic acid per g Sepharose. Comparable yields of the immobilized trypsin (6.6 mg, 43 U g^{-1} wet wt gel) were obtained after 2 h of incubation with this gel.

Immobilization of trypsin on laccase- and periodate-activated Sepharose was followed over a period of time. As seen in Fig. 2, the immobilized protein remained constant during incubation for 1–4 h, however the activity yield of the immobilized enzyme
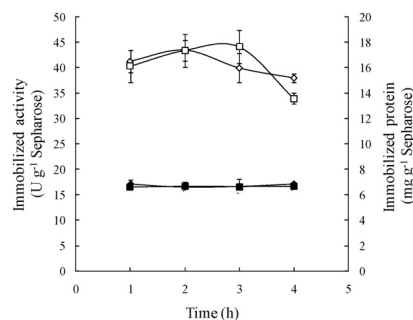

Fig. 2. Effect of reaction time on immobilization of trypsin on Sepharose CL-6B beads activated by 85 U laccase per g wet wt gel and 12.5 mM TEMPO (diamonds) and periodate (squares), respectively. Immobilized protein is denoted by filled symbols, while activity is shown by open symbols. The amount of trypsin used for immobilization was 10 mg per g gel, in a solution containing 2.5 mg ml^{-1} enzyme in 20 mM carbonate buffer, pH 10.5 containing 2 mM benzamidine.

Fig. 3. Profiles of immobilized trypsin activity (open symbols) and protein (filled symbols) with respect to the initial concentration used for immobilization. The immobilization was performed for 2 h in 20 mM carbonate buffer, pH 10.5 with 2 mM benzamidine. The specific activity of soluble trypsin used was 5.7 U mg^{-1}. The symbols used for immobilization on LMS activated Sepharose are diamonds and on periodate activated gel are squares.

increased slightly with time up to 2 h and 3 h on the laccase and periodate activated Sepharose, respectively, and then decreased. Further incubation led to decrease in the yields of the immobilized trypsin activity on both kinds of activated supports probably due to the extremely high pH to which the enzyme is exposed. The amount of trypsin immobilized with respect to the initial concentration of the enzyme was also similar in both cases (Fig. 3). Although the amount of immobilized protein and -activity increased with the increase in trypsin concentration applied, there was a decrease in the percent yield.
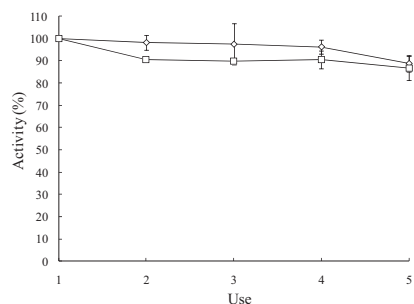
Fig. 4. Re-use of trypsin immobilized on Sepharose CL-6B beads activated using laccase/TEMPO (◊) and periodate (□).

The stability of trypsin immobilized to Sepharose, using the different activation procedures was tested during repeated use for BAPNA hydrolysis. As shown in Fig. 4, trypsin immobilized on laccase and periodate activated gel exhibited good stability, the former retained highest activity. The stability of the laccase-TEMPO immobilized trypsin was further tested during storage in 1 mM HCl containing 20 mM $CaCl_2$ at 4 °C. The enzyme retained complete activity during 4 weeks of storage, while the soluble enzyme had lost over 40% of activity during the same time period (data not shown).

4. Conclusions

This paper indicates the potential of using laccase-mediator system as an alternative method for activation of polysaccharide matrices for immobilization of proteins. The system would also be applicable for immobilization of affinity ligands bearing amino functionality. Further investigations are needed to determine the full scope of its applications. The method avoids the use of any toxic reagents and involves mild conditions, and the immobilization yields are comparable to the conventional chemical method used for the matrix activation. Treatment conditions for modification and immobilization would need to be optimized for different lig-ands to achieve optimal binding and specific activity. Laccase from different sources and different mediators are among the important parameters that would influence the activation of the matrix. Evaluating the potential of mediators originating from natural resources such as syringaldehyde could also be interesting from the point of view of lowering the cost and toxicity.

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