Johann Faccelo Osma Cruz

PRODUCTION OF LACCASES BY THE WHITE-ROT FUNGUS *TRAMETES PUBESCENS* FOR THEIR POTENTIAL APPLICATION TO SYNTHETIC DYE TREATMENT

DOCTORAL THESIS

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Supervised by Dr. Susana Rodríguez Couto and Dr. José Luis Toca Herrera

Department of Chemical Engineering

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I, Dr. Susana Rodríguez Couto, IKERBASQUE Research Professor of the CEIT-IK4 Research Alliance (Universidad de Navarra), I, Dr. José Luis Toca Herrera, I3 Research Professor of the Center for Cooperative research in Biomaterials (CIC biomaGUNE) and I, Azael Fabregat Full Professor of the Department of Chemical Engineering of the Universitat Rovira i Virgili,

CERTIFY:

That the present work entitled "Production of laccases by the white-rot fungus *Trametes pubescens* for their potential application to synthetic dye treatment", presented by Johann Faccelo Osma Cruz to obtain the degree of doctor by the University Rovira i Virgili, has been carried out under my supervision at the Chemical Engineering Department, and that it fulfills the requirements to obtain the Doctor European Mention.

Tarragona, 31st of August of 2009

Dr. Susana Rodríguez Couto   Dr. José Luis Toca Herrera   Dr. Azael Fabregat

Acknowledgments

List of abbreviations

AAO	aryl alcohol oxidase (EC 1.1.3.7)
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
Alg	alginate
ALR	airlift reactor
BC	before Christ
BP	before present
CI	colour index
cm	centimeter
CO_2	carbon dioxide
COD	chemical oxygen demand
Cu	copper
DAH	1,6-diaminohexane
e.g.	(*exempli gratiā*) for example
EC	enzyme commission
EDC	endocrine disrupting chemicals
EGDMA	ethyleneglycol dimetacrylate
EPR	electron paramagnetic resonance
et al.	*(et alia)* and others
EU	european union
EWA	european water association
FDH	formate dehydrogenase
g, mg, µg	gran, milli-gram, micro-gram
GLOX	glyoxal oxidase (EC 1.2.3.5)
GMA	glycidyl methacrylate
H_2O	water
H_2O_2	hydogen peroxide
HBT	1-hydroxybenzotriazole
HNNS	2-nitroso-1-naphthol-4-sulfonic acid
HPLC/MS	high performance liquid chromatography / mass spectrometry
i.e.	(*id est*) that is

INE	instituto nacional de estadística
kDa	kilo-Dalton
L, mL	liter, milli-liter
LCA	low-cost adsorbent
LiP	lignin peroxidase (EC 1.11.1.14)
LMS	laccase mediator system
m^2, cm^2	square meter, square centimeter
MG	methyl green
MnP	manganese peroxidase (EC 1.11.1.13)
NIPA	N-isopropylacrylamide)
NNDS	1-nitroso-2-naphthol-3,6-disulfonic acid
ODC	oxalate decarboxylase (EC 4.1.1.2)
OMW	olive mill wastewater
P(AAm)	poly(-acrylamide)
P(HEMA)	poly(hydroxyethylmethacrylate)
PUF	polyurethane foam
PW	pine wood
PZ	promazine
RB5	reactive black 5
RBBR	remazol brilliant blue R
RDR	rotating drum reactor
RITA ®	récipient à immersion temporaire automatique
SmF	submerged fermentation
SSF	solid-state fermentation
STR	stirred tank reactor
TEMPO	2,2,6,6- tetramethylpiperidin-1-yloxy
U	unit
UV	ultraviolet
VA	veratryl alcohol
VIO	violuric acid
VP	versatile peroxidase (EC 1.11.1.16)

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CHAPTER 1. Introduction

1   White-rot fungi

White-rot fungi are a physiological group of fungi capable of biodegrading lignin. The name white-rot derives from the white appearance of the wood when is attacked by these fungi, where the wood gets this bleached appearance due to lignin removal (Pointing 2001). Though some white-rot fungi are Ascomycete, taxonomically talking, most of them are Basidiomycetes (Eaton and Hale 1993). They can grow in a wide range of temperatures and withstand a wide range of pH (Verma and Madamwar 2002), but no growth has been observed below 10 ºC.

White-rot fungi are the only organisms able to degrade lignin efficiently (Heinzkill *et al.* 1998) metabolizing it to CO_2 and H_2O (Tien and Kirk 1983, Kirk and Farrell 1987, Kaal *et al.* 1995). This ability is correlated to the capacity of these organisms to synthesise extracellular lignin-degrading enzymes. The non-specific nature of such enzymes allows them degrading a wide variety of persistent environmental pollutants (Barr and Aust 1994), including dyes (Robinson *et al.* 2001a, Wesenberg *et al.* 2003).

Lignin is an heterogeneous, complex and stable polymer composed of various aromatic monomers connected to each other by various bonds carbon-carbon and ethers (Gellerstedt and Northy (1989) (Fig. 1). The most common bond is the β-aryl ether (β-O-4) linkage, which may represent 50% of the intermonomeric bonds in softwood lignin and 60% in hardwood lignin (Sjöström 1993). Due to the similarity between the lignin structure and the chemical structure of several dyes, the use of white-rot fungi and their enzymes for the degradation of dyes had been considered (Paszczynski *et al.* 1991, Zhou and Zimmermann 1993).
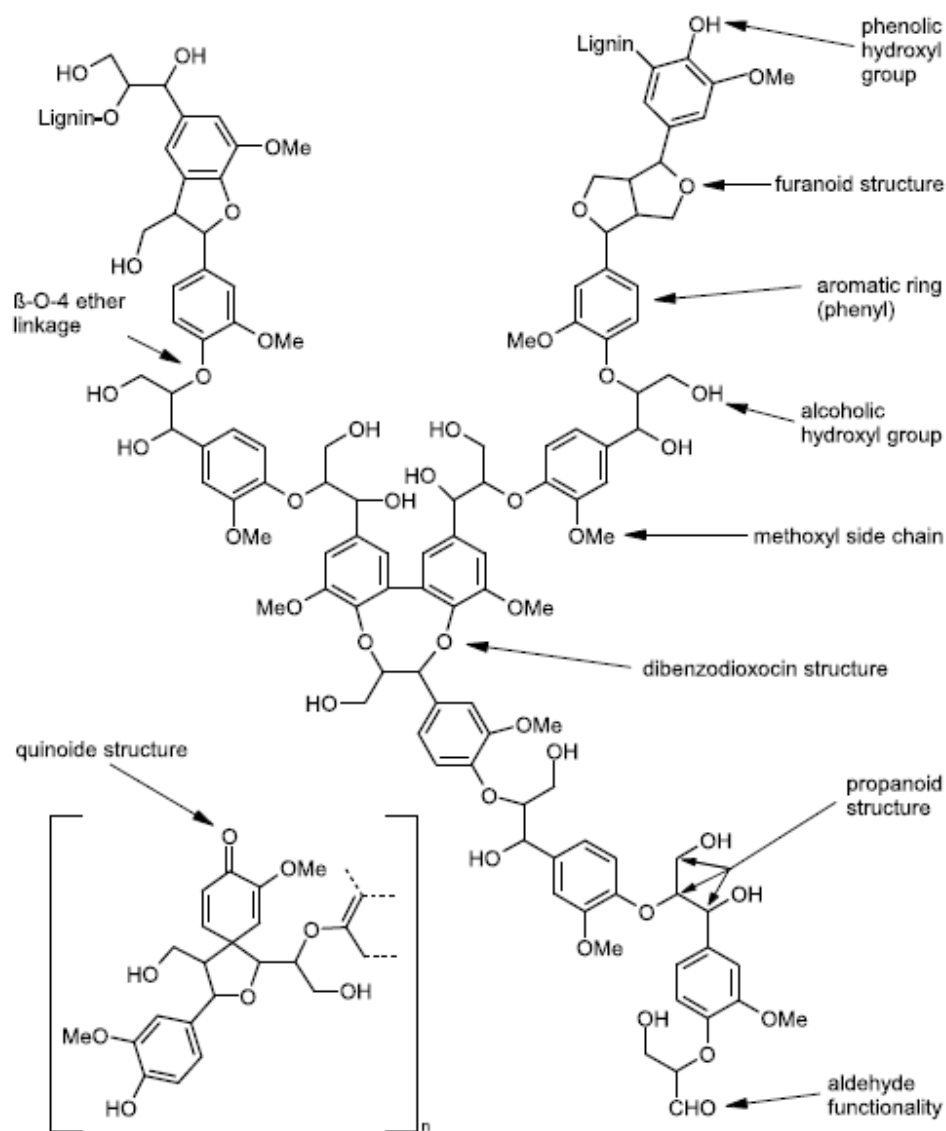
Figure 1. Representative part of the lignin polymer (Brunow 2001)

The extracellular enzymatic system of white-rot fungi generally consists of lignin peroxidase (LiP, E.C. 1.11.1.14), manganese-dependent peroxidase (MnP, E.C. 1.11.1.13) and laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2I). LiP catalyzes the oxidation of non-phenolic aromatic compounds such as veratryl alcohol (VA). MnP preferably oxidizes Mn^{2+} to Mn^{3+} which is able to oxidize many phenolic compounds. Laccase is a copper-containing enzyme that catalyzes the oxidation of phenolic substrates

with the concomitant reduction of oxygen to water (McMullan *et al.* 2001, Wesenberg *et al.* 2003). A third group of peroxidases, versatile peroxidase (VP, E.C.1.11.1.16), has been further recognized in species of *Pleurotus* and *Bjerkandera* (Heinfling *et al.* 1998a, Heinfling *et al.* 1998b). Also, accessory enzymes such as H_2O_2-forming glyoxal oxidase (GLOX), aryl alcohol oxidase (AAO), oxalate producing oxalate decarboxylase (ODC), NAD-dependent formate dehydrogenase (FDH) and P450 monooxygenase have been isolated from many white-rot fungi strains (Wesenberg *et al.* 2003, Aguiar *et al.* 2006).

Table 1. Ligninolytic enzymes and their main reactions (after Hatakka 2001)

Enzyme and abbreviation	Cofactor	Substrate, mediator	Reaction
Lignin peroxidase, LiP	H_2O_2	VA	Aromatic ring oxidized to cation radical
Manganese-dependent peroxidase, MnP	H_2O_2	Mn, organic acids as chelators, thiols, unsaturated fatty acids	Mn(II) oxidized to Mn (III); chelated Mn(III) oxidizes phenolic compounds to phenoxyl radicals; other reactions in the presence of additional compounds
Versatile peroxidase, VP	H_2O_2	Mn, VA, compounds similar to LiP and MnP	Mn(II) oxidized to Mn (III), oxidation of phenolic and non-phenolic compounds, and dyes
Laccase	O_2	Phenols, mediators, e.g., HBT[a] or ABTS[b]	Phenols are oxidized to phenoxyl radicals; other reactions in the presence of mediators
Glyoxal oxidase, GLOX		Glyoxal, methyl glyoxal	Glyoxal oxidized to glyoxal acid; H_2O_2 production
Aryl alcohol oxidase, AAO		Aromatic alcohols (anisyl, VA)	Aromatic alcohols oxidized to aldehydes; H_2O_2 production
Other H_2O_2-producing enzymes		Many organic compounds	O_2 reduced to H_2O_2

[a] HBT: 1-hydroxybenzotriazole

[b] ABTS: 2,2´-azino-bis 3-ethylbenzothiaoline-6-sufonic acid

During the last decades, the scientific interest in the utilization of white-rot fungi and their ligninolytic enzymes for bleaching processes, environmental applications, synthesis of complex compounds and biofuel production has largely increased (Luisa *et al.* 1996,

Asgher *et al.* 2008). This interest is justified by the presence of the above-mentioned microorganisms in a good number of natural and contaminated environments.

1.1 *Trametes pubescens*

Figure 2. *Trametes pubescens* grown in nature (Primulakisoana website. Photo kindly provided by Dr. Masato Ohtani, Forestry and Forest Products Research Institute, Japan)

The genus *Trametes* belonging to the kingdom of fungi, phylum Basiomycota, class Basidiomycetes, subclass Agaricomycetidae, order Polyporales and family Polyporaceae, is assumed to be one of the main laccase producers (Oh *et al.* 1999). *Trametes* is probably one of the most widely investigated Basiodiomycota for ligninolytic enzyme production and application (Nyanhongo *et al.* 2007). Like other white-rot fungi, *Trametes* genus is attractive due to the extracellular secretion of non-specific ligninolytic enzymes and the possibility of growing on cheap media such as lignocellulosic wastes (Nyanhongo *et al.*

2007). Among them, *Trametes pubescens* has been described as a promising laccase producer (Galhaup and Haltrich 2001). *T. pubescens* normally grows in nature in circular clusters of 1.5-5 cm across, 2.5-8 cm wide and 0.3-l cm thick, often overlapped (Fig. 2). It presents a white or grayish yellow color when fresh and grayish or yellow when dry. Its spores are cylindrical and smooth of about 5-8 x 2-2.5 µm. It lives on dead wood of deciduous trees and is found in Europe and widely distributed in North America, especially in the season from June to October (The Oakes Museum, USA).

2 Laccases

2.1 Description

Laccases are N-glycosilated multi copper oxidases belonging to the group of the blue copper proteins (Thurston 1994, Xu 1996). Laccases are widely found in fungi and higher plants (Messerschmidt and Huber 1990) and also in a lower proportion in insects and bacteria.

Laccase was first described by Yoshida in 1883 when he extracted it from the exudates of the Japanese lacquer tree *Rhus vernicifera*, from which the name laccase was derived (Thurston 1994, Levine 1965). In 1896, Bertrand and Laborde demonstrated the presence of laccases in fungi (Bertrand 1895, Levine 1965, Thurston 1994). Since then, the presence of laccase was shown in Ascomycetes, Deuteromycetes and Basidiomycetes. Moreover, laccases have been particularly found in many white-rot fungi involved in the lignin metabolism (Galhaup and Haltrich 2001, Thurston 1994, Xu 1996a,b).

Laccases have a lower redox potential (450-800 mV) than those of ligninolytic peroxidases (> 1 V), so it was initially thought that laccases would only be able to oxidize phenolic substrates (Kersten *et al.* 1990). However, the range of substrates oxidized by laccases can be increased through a mediator-involved reaction mechanism. Mediators are low molecular weight compounds that are easily oxidized by laccases producing, in some cases, very unstable and reactive cationic radicals, which can oxidize more complex substrates before returning to their original state. The electrons taken by laccases are finally transferred back to oxygen to form water (McGuirl and Dooley 1999, Wong and Yu 1999).

More than 100 fungal laccases have been purified and somehow characterized until now. So far, the three-dimension structure of five fungal laccases and one from a bacterium have been reported: *Trametes versicolor* (Fig. 3) (Bertrand *et al.* 2002, Piontek *et al.* 2002), *Pycnoporus cinnabarinus* (Antorini *et al.* 2002), *Melanocarpus albomyces* (Hakulinen *et al.* 2002), *Rigidoporus lignosus* (Garavaglia *et al.* 2004), *Coprinus cinereus* (in a copper Type 2-depleted form) (Ducros *et al.* 1998) and CotA laccase from *Bacillus subtilis* (Enguita *et al.* 2003 and 2004).
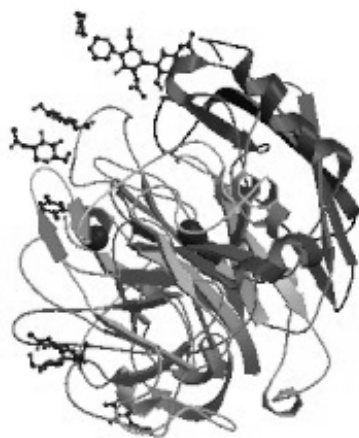
Figure 3. Three-dimensional structure of laccase from *T. versicolor* complexed with 2,5-xylidine (Bertrand *et al.* 2002)

The fungal laccase molecule usually contains four copper (Cu) atoms, although some of them contain only 3 Cu atoms in its structure. Laccase has a molecular mass from about 50 to 100 kDa and when using ABTS as a substrate its optimum pH is in the range 3.0-5.0 (Heinzkill *et al.* 1998). Laccases can be polymeric, and the enzymatically active form can be a monomer, dimer, trimer or tetramer. Laccases presents a high level of glycosylation that may contribute to the high stability of the enzyme through the covalent link of carbohydrate moieties that can reach from 10 to 45% of the total weight (Duran *et al.* 2002, Kunamneni *et al.* 2007).
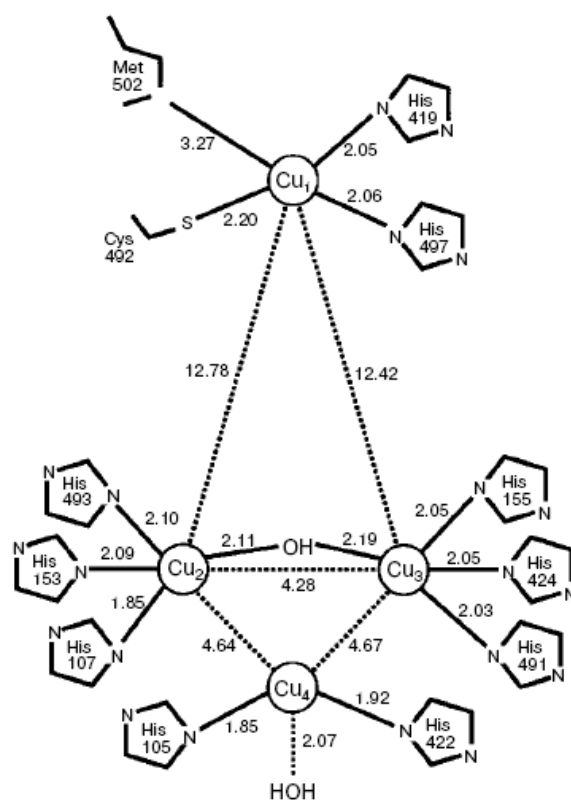
Figure 4. Illustration of the active site of laccase showing the relative orientation and distances of the copper atoms of a CotA laccase from *B. subtilis* (Enguita *et al.* 2003, Morozona *et al.* 2007)

The four copper ions of laccase are classified into three types, referred to as Type 1 (T1), Type 2 (T2) and Type 3 (T3) (Yaropolov *et al.* 1994) (Fig. 4). The T1 copper is responsible for the blue color of the enzyme and has a characteristic absorbance around 610 nm. The T2 copper cannot be detected spectrophotometrically; however, it generates a characteristic electron paramagnetic resonance (EPR) signal (Leontievsky *et al.* 1997, Koroljova-Skorobogat'ko 1998). The bi-nuclear T3 copper is diamagnetic. It displays a spectral absorbance shoulder in the region of 330 nm and also displays a characteristic fluorescence spectrum (Shin and Lee 2000). T2 and T3 copper atoms form a trinuclear cluster, where the reduction of molecular oxygen and release of water take place (Gianfreda *et al.* 1999). The T1 copper centre is involved in the oxidation of the reducing substrate and transfers electrons to the T2 and T3 copper. T1 copper atom has a trigonal coordination with two

histines and a cysteine, while the four position varies between a methionine in the bacterial (CotA) and a leucine or phenylalanine in fungal laccases (Fig. 4). Moreover, the redox potential of the T1 site has been determined for different laccases and ranges from 430 mV up to 780 mV (Xu *et al.* 1996, Schneider *et al.* 2001, Gianfreda et al 1999). It was found that the catalytic efficiency of laccases for some reducing substrates depends linearly with the redox potential of the T1 site. Thus, laccases with a high redox potential in the T1 site are of special interest for biotechnological applications.
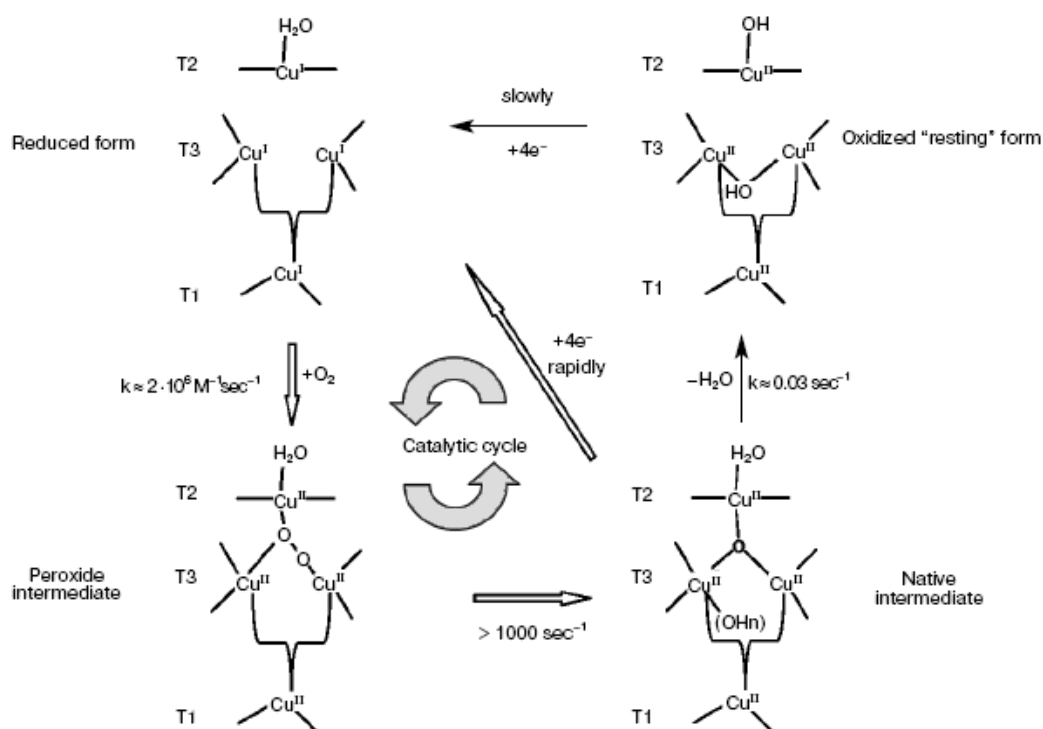
Figure 5. Catalytic cycle of a four-copper laccase (Shleev *et al.* 2006)

The catalytic cycle of the laccase (with four copper atoms), described by Yaropolov *et al.* in 1994 and Shleev *et al.* (2006), shows the reduction of molecular oxygen into two water molecules (Fig. 5). In order to reduce molecular oxygen to water, laccase stores electrons from individual oxidation reactions similar to the operation of a battery. Substrate oxidation by laccase is a one-electron reaction that generates a free radical. Thus, the oxidation of four reducing substrate molecules is necessary for the complete reduction of the molecular oxygen to two water molecules. However, the details of the molecular oxygen reduction have not been fully elucidated and continue to be studied.

2.2   Laccase mediator system (LMS)

Redox mediators, first described by Bourbonnais and Paice (1990), allow laccases oxidizing non-phenolic compounds expanding, thus, the range of substrates that can be oxidized by these enzymes. Redox mediators are low molecular weight compounds that speed up the reaction rate by shuttling electrons from the biological oxidation of primary electron donors or from bulk electron donors to the electron-accepting organic compounds (Husain and Husain, 2008). Once the mediator (M) is oxidized by the laccase, it forms a short-lived intermediate cation radical (M+) (co-mediator) that diffuses away from the enzyme and co-oxidizes any substrate (S) that was not able to enter into the active site of the enzyme due to its size (Fig. 6).
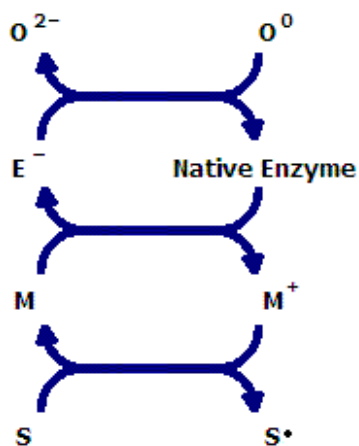
Figure 6. Catalytic process of a substrate (S) by laccase (E) and a redox mediator (M)

(Adapted from Bourbonnais *et al.* 1998 and Wesenberg *et al.* 2003)
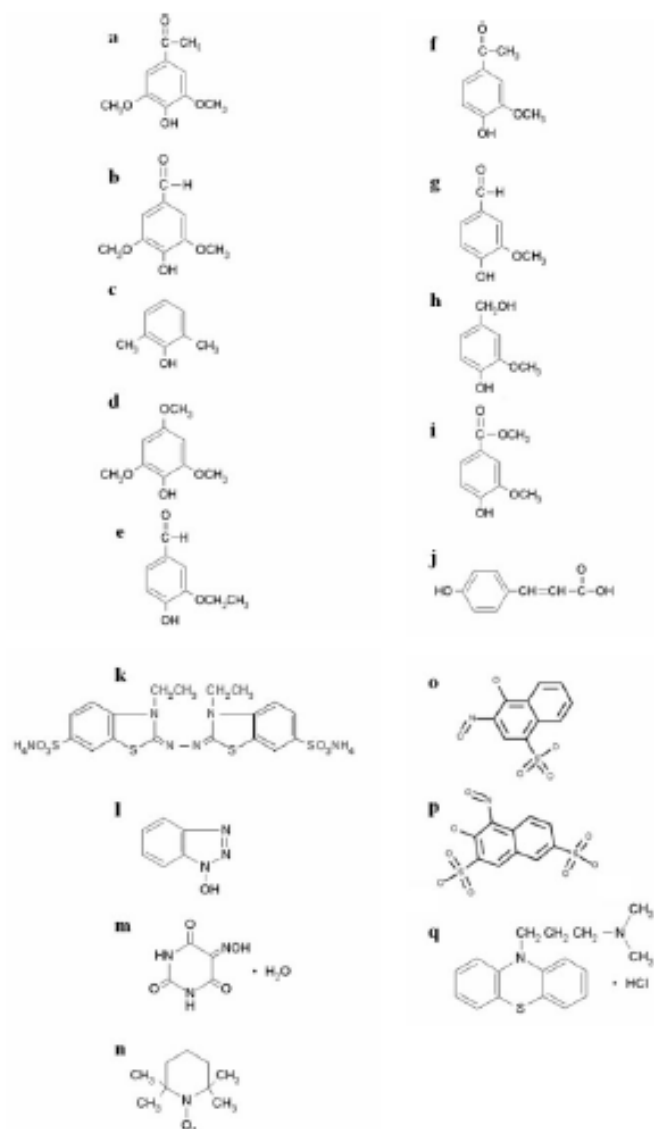
Figure 7. Chemical structures of natural (a to j) and synthetic mediators (k to r). (a) acetosyringone; (b) syringaldehyde; (c) 2,6-dimethylphenol; (d) 2,4,6-trimethoxyphenol; (e) ethyl vanillin; (f) acetovanillone; (g) vanillin; (h) vanillyl alcohol; (i) methyl vanillate; (j) p-coumaric acid; (k) ABTS; (l) HBT; (m) VIO; (n) TEMPO (2,2,6,6-tetramethylpiperidin-1-yloxy); (o) HNNS (2-nitroso-1-naphthol-4-sulfonic acid); (p) NNDS (1-nitroso-2-naphthol-3,6-disulfonic acid); (q) PZ (promazine) (extracted from Camarero *et al.* 2005)

Three types of mediators have been proposed: (1) an electron transfer route for mediators such as ABTS; (2) a radical hydrogen atom transfer route for mediators of the –NOH type such as HBT and violuric acid (VIO). First, laccase extracts hydrogen and NO$\cdot^-$ radicals are formed. Then the NO$\cdot^-$ radicals extract a hydrogen atom from the substrate (Spadarry *et al*. 1994, Fabbrini *et al.* 2002, Baiocco *et al.* 2003, Minussi and Pastore 2007) and (3) phenolic compound mediators such as the phenol compounds syringaldehyde and acetosyringone, whose mediating mechanism is the same as HBT except that intermediate is a phenoxy radical. The NOH-type mediators were found to be very effective in the discoloration of dyes (Soares *et al.* 2001, Soares *et al.* 2002, Rodríguez Couto and Sanromán 2007).

LMS has not yet been applied at large scale due to the cost of mediators and their toxicity. The use of naturally-occurring laccase mediators would present environmental and economic advantages. Recently, Camarero *et al*. (2005) reported that several lignin-derived phenols (such as syringaldehyde and acetosyringone) represented ecofriendly alternatives to synthetic mediators for the degradation of different types of dyes and other recalcitrant compounds by laccase in terms of both efficiency and velocity of oxidation. Figure 7 shows the structure of different synthetic and natural mediators of laccases.

3   Fermentation techniques

Fermentation techniques can be divided into two main groups: solid-state fermentation (SSF) and submerged fermentation (SmF). The difference between these two techniques consists in the quantity of free flowing liquid present in the system. SSF involves the growth of microorganisms on solid materials in the absence or near-absence of free flowing water, whereas in SmF the microorganisms grow on a continuous liquid phase (Marques 2005).

In SSF, the moisture necessary for microbial growth exists in an absorbed state or complex within the solid matrix. This solid matrix can be either a natural support (e.g. lignocellulosic materials) or an inert support (e.g. plastic foams) (Pandey *et al.* 2000). Although most researchers consider solid-state and solid-substrate fermentation essentially one and the same, Pandey et al. (2001) have distinguished these two as separate processes. According to them, solid-substrate fermentation includes those processes in which the

substrate itself acts as the carbon source, and occurring in the absence or near-absence of free flowing water, whereas SSF is defined as any fermentation process occurring in the absence or near-absence of free flowing water, using a natural substrate or an inert substrate as solid support.

Due to the small quantity of water present in the SSF, the formation of specific products, which are not produced under SmF, can take place. For the products that can be obtained using both techniques, SSF presents higher volumetric productivity and better performance than SmF (Moo-Young *et al.* 1983). In general, SSF shows several biotechnological advantages (listed in table 2).

SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi (Moo-Young *et al.* 1983, Pandey *et al.* 1999a) since they reproduce the natural living conditions of such microorganisms (Pandey *et al.* 1999b). One of the advantages often cited for SSF processes is that enzyme titres are higher than in SmF, when comparing the same strain and fermentation broth (Viniegra-González *et al.* 2003). Viniegra-González *et al.* (2003) compared the productivity of three fungal enzymes, invertase, pectinase and tannase, using SSF and SmF techniques. They reported that the higher titres found in SSF than in SmF were due to SSF cultivation works as a fed batch culture with fast oxygenation but slow sugar supply.

Castilho et al. (2000) performed a comparative economic analysis of solid-state and submerged processes for the production of lipases by *Penicillium restrictum*. They found that for a plant producing 100 m^3 lipase concentrate per year, the process based on SmF needed a total capital investment 78% higher than the one based on SSF and its product had a unitary cost 68% higher than the product market price. These results showed the great advantage of the SSF due to its low cost.

Table 2. Advantages and disadvantages of SSF and SmF (Pérez-Guerra *et al.* 2003, Singhania *et al.* 2009)

Solid-State Fermentation	Submerged Fermentation
Advantages	
Minimum water consumption	All the substrate is equally accessible to the microbes as it is completely dissolved
No problems with foaming	
Low waste-water output	Aeration is normally not a problem in SmF
Lower demand on sterility due to the low water activity used in SSF	Better heat transfer in liquid phase
Low cost media, as substrates are often agricultural by-products or waste	Mixing is much easier in liquid substrate
	Microbial growth is faster in liquid broth
Higher fermentation productivity	Control of pH is easier in mixed SmF
Higher end-concentration of products	
Higher product stability	
Lower catabolic repression	
Disadvantages	
Partial accessibility of substrate by microbes	High water consumption
Aeration problem in the 3-phase system	Foam formation
Product recovery and purification processes are more expensive	High waste water output
	High water activities, increase risk of contamination
Pretreatment of the substrate is normally needed (Grinding, chopping, homogenization, physical, chemical or enzymatic hydrolysis, cooking or vapor treatment)	High cost media, as substrates are often expensive compare to SSF
	High demand on sterility due to higher water activity
Mixing problems of large amount of solid substrate	
Slower microbial growth on solid	
Control of process variables such as heat, pH, mass transfer, oxygen, is difficult.	

4   Laccase production under SmF and SSF

There are many contributions to the field of laccase production under SmF using different microorganisms, at different scales and with the possible use of immobilization supports and the addition of inducers. Some of the most remarkable results in terms of laccase activity were obtained by the *Trametes* genus: *T. pubescens* (Galhaup *et al.* 2002), *T. versicolor* (Font *et al.* 2003, Tavares *et al.* 2006) and *T. hirsuta* (Rodríguez Couto *et al.* 2006b). In almost all cases, the cultures were supplemented with laccase-inducing compounds. Galhaup *et al.* (2002) obtained a maximum laccase activity of 740,000 U/L by *T. pubescens* cultured in a 20-L stirred-tank reactor (STR) with a stirring speed of 100 rpm and with 2 mM Cu^{+2}. Font *et al.* (2003) obtained a maximum laccase activity of 16,000 U/L by free pellets of *T. versicolor* in a 0.5-L pulsed-bed reactor. Tavares *et al.* (2006) reported a maximum laccase activity of 11,403 U/L when cultured the same fungus on a STR of 1 liter, supplementing the medium with 30 µM of xylidine. Rodríguez Couto *et al.* (2006b) reported a maximum laccase activity of 19,400 U/L by culturing *T. hirsuta* in a 6-L airlift reactor (ALR) and supplementing the medium with glycerol and Cu^{+2}.

Laccase has also been produced under SSF, especially during the last decades. The use of natural solid substrates, especially lignocellulosic agricultural residues, as growth substrates for fungi has been studied for laccase production in recent years (Rodríguez Couto and Sanromán, 2005 and references therein). Furthermore, such residues contain cellulose, which act as an inducer of laccase activity.

The scarcity of bioreactor designs to perform solid-state processes together with the advantages offered by such processes promote the necessity of developing new bioreactor configurations or modifying the designs that already exist. These bioreactor designs should be able to operate in continuous mode with high enzyme productivity for prolonged periods of time without operational problems as well as permit the scale-up of the process. Thus, Rivela *et al.* (2000) developed a new bioreactor design for the production of ligninolytic enzymes under SSF conditions named immersion bioreactor. They attained high ligninolytic activities and, in addition to this, the bioreactor was able to operate in continuous mode (Rodríguez Couto *et al.* 2002b). Dominguez *et al.* (2001) developed a rotating drum reactor (RDR) for the production of ligninolytic enzymes under SSF

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conditions. This bioreactor was able to operate in batch and continuous mode. Also, Böhmer *et al.* (2006) reported the advantages of adapting the temporary immersion RITA®-System (Récipient à Immersion Temporaire Automatique) as a bioreactor for laccase production by white-rot fungi and its application to synthetic dye discoloration.

Rodríguez Couto *et al.* (2003) tested three bioreactor configurations (immersion, expanded-bed and tray) with different agitation systems (mechanical, pneumatic and static, respectively) for laccase production by *T. versicolor* under SSF conditions using an inert (nylon sponge) and a non-inert (barley bran) support. They found that the tray configuration with barley bran as support-substrate led to the highest laccase activities. More recently, they (Rodríguez Couto *et al.* 2006a) compared two bioreactor configurations (immersion and tray) for laccase production by *T. hirsuta* using grape seeds as support-substrate and found that much higher laccase activities were attained in the tray bioreactor. Also, they reported much higher laccase activities in a tray bioreactor than in a fixed-bed one for *T. hirsuta* grown on ground orange peelings (Rosales *et al.* 2007).

Table 3 shows some of the results reported since the year 2000 for the production of laccase by different white-rot fungi at both reactor and flask scale under SmF and SSF conditions. Part of this table was extracted from the review by Rodríguez Couto and Toca-Herrera (2007).

Table 3. Maximum laccase activities obtained by the cultivation of different white-rot fungi under SmF or SSF at flask and reactor scale

Fungus	Type of reactor	Type of cultivation	Inducer	Max Laccase activity [U/L]	Reference
P. cinnabarinus	10-L packed-bed	SmF, immobilized on nylon cubes	10 mM VA	280	Schliephake *et al.* (2000)
Neurospora crassa	Capillary membrane	SmF, immobilized on membrane supports	1 μM cycloheximide	10,000	Luke and Burton (2001)
Phanerochaete chrysosporium	RDR	SSF immobilized on nylon cubes	0.05% Tween 80 and 2mM VA	56	Dominguez *et al.* 2001
T. pubescens	20-L STR (150 rpm)	SmF, free cells	2 mM Cu^{+2}	61,900	Galhaup and Haltrich (2001)

Coriolus hirsutus	10-L jar fermentor (160 rpm)	SmF, free cells, semi-continuous	0.25 g/L Cu^{+2}	83,830 (1st fermentation) 80,730 (2nd fermentation)	Koroleva *et al.* (2002)
Pycnoporus sanguineus	15-L Biostat C (8 L; 250 rpm)	SmF, free cells	16 mM VA	8131	Van der Merwe (2002)
Phanerochaete flavido-alba	Bioflo III (975 mLa; 70 rpm)	SmF, free cells	OMW [c]	72	Blánquez *et al.* (2002)
P. sanguineus	2-L Biostat C	SmF	16 mM VA	460	Van der Merwe (2002)
Trametes multicolor	STR	SmF	-	-	Hess *et al.* (2002)
T. pubescens	20-L STR (100 rpm)	SmF, free cells	2 mM Cu^{+2}	333,000	Galhaup *et al.* (2002)
	20-L STR (100 rpm)	SmF, free cells fed-batch	2 mM Cu^{+2}	740,000	Galhaup *et al.* (2002)
Irpex lacteus	Packed-bed (27 mL)	SmF (immobilized on PW) [a]	-	-	Kasinath *et al.* (2003)
I. lacteus	Packed-bed (27 mL)	SmF (immobilized on PUF) [b]	-	-	Kasinath *et al.* (2003)
Panus tigrinus	3-L STR (2 L; 250 rpm)	SmF, free cells	OMW [c]	4600	Fenice *et al.* (2003)
	20-L RDR	SSF (maize stalks)	OMW [c]	1309	Fenice *et al.* (2003)
	3-L airlift reactor (2.5 L)	SmF, free cells	OMW [c]	4300	Fenice *et al.* (2003)
Pleurotus ostreatus	Benchtop fermenter (3 L; 200 rpm)	SmF, free cells	OMW [c]	65	Aggelis *et al.* (2003)
T. versicolor	0.5-L fluidized-bed	SmF (pellets)	-	1187	Font *et al.* (2003)
	0.5-L pulsed-bed	SmF (pellets)	-	16,000	Font *et al.* (2003)
	ALR (2 L)	SmF free cells	Tween 80	1670	Rancaño *et al.* (2003)
	Biostat Q (4 reactors of 330 mL each)	SmF (pellets)	-	-	Font *et al.* (2003)
	Expanded-bed (300 mL)	SSF (barley bran)	Tween 80	600	Rodríguez Couto *et al.* (2003)
	Expanded-bed (300 mL)	SSF (nylon sponge)	Tween 80	126	Rodríguez Couto *et al.* (2003)
	Tray (1 L)	SSF (barley bran)	Tween 80	3500	Rodríguez Couto *et al.* (2003)
	Immersion (2.5 L)	SSF (barley bran)	Tween 80	600	Rodríguez Couto *et al.* (2003)

	Immersion (2.5 L)	SSF (nylon sponge)	Tween 80	229	Rodríguez Couto *et al.* (2003)
	Tray (1 L)	SSF (nylon sponge)	Tween 80	343	Rodríguez Couto *et al.* (2003)
	2-L STR (1.5 L)	SmF (immobilized on nylon mesh)	-	5.3	Sedarati *et al.* (2003)
	2-L STR (1.5 L)	SmF free cells	-	5.3	Sedarati *et al.* (2003)
Bjerkandera adusta	STR (5 L)	SmF (immobilized on plastic net)	-	4	Mohorčič *et al.* (2004)
P. cinabarinus	STR (12 L), 150 rpm	SmF	-	2800	Sigoillot *et al.* (2004)
P. ostreatus	Solid-substrate	Trickle-film processing (sugarcane bagasse)	Sugarcane bagasse	3500	Lenz and Hölker (2004)
T. hirsuta	1-L fixed-bed	SmF (immobilized on stainless steel sponges)	Cu^{+2}	2206	Rodríguez Couto *et al.* (2004a)
	0.5-L immersion	SSF	Cu^{+2}	4892	Rodríguez Couto *et al.* (2004b)
T. versicolor	Fluidized (1.5 L)	SmF (pellets)	-	1685	Blánquez *et al.* (2004)
P. ostreatus	Packed-bed (280 mL)	SmF (immobilized on PUF) [b]	Cu^{+2}	1403	Prasad *et al.* (2005)
T. hirsuta	ALR (2 L)	SmF (immobilized in alginate beads)	4 Mm VA	1043	Domínguez *et al.* (2005)
T. pubescens	ALR (3.5 L)	Pellets	Phenolic effluent	11,800	Ryan *et al.* (2005)
T. versicolor	Fluidized-bed with air pulses (10 L)	SmF pellets	-	2700	Blánquez (2005)
Funalia trogii	2-L STR	Immobilized on Na-alginate beads	-	1000	Park *et al.* (2006)
P. cinnabarinus	Vapor phase Bioreactor (300 mL; 18 L)	SSF (sugarcane bagasse)	Ethanol vapor	10,000	Meza *et al.* (2006)
P. ostreatus	ALR (5 L)	SmF pellets	OMW [c]	1200	Olivieri *et al.* (2006)
P. chrysosporium	Bench scale bioreactor (800 mL)	Batch fermentation	30 mM Cu^{+2}	30.2	Gnanamani *et al.* (2006)

T. hirsuta	Immersion (0.5 L)	SSF (grape seeds)	-	12,877	Rodríguez Couto *et al.* (2006a)
	Tray (0.2 L)	SSF (grape seeds)	-	18,715	Rodríguez Couto *et al.* (2006a)
	Tray (0.2 L)	SSF (nylon sponge)	-	6898	Rodríguez Couto *et al.* (2006a)
	ALR (6 L)	SmF free cells	Cu^{+2}, glycerol	19,400	Rodríguez Couto *et al.* (2006b)
T. versicolor	Fluidized-bed with air pulses (1.5 mL)	Pellets	-	1160	Blánquez *et al.* (2006)
	RITA® System	SSF (palm oil fiber)	-	138.6	Böhmer *et al.* (2006)
	RITA® System	SSF (pine wood chips)	-	54	Böhmer *et al.* (2006)
	Pulsed fluidized-bed (0.5 L)	Pellets	-	> 1500	Romero *et al.* (2006)
	STR (1 L)	SmF	30 μM xyldine	11,403	Tavares *et al.* (2006)
T. hirsuta	1.8-L tray (200 mL)	SSF (orange peels)	5 mM Cu^{+2}	12,000	Rosales *et al.* (2007)
	250-mL fluidized-bed (200 mL)	SSF (orange peels)	5 mM Cu^{+2}	3000	Rosales *et al.* (2007)
T. versicolor	Fluidized-bed with air pulses (1.5 mL)	Pellets	-	2123	Blánquez *et al.* (2007)
	5-L STR (1.25 L)	SmF (pellets)	-	1385	Thiruchelvam and Ramsay (2007)
Streptomyces psammoticus	Flask, 150 rpm.	SmF	Pyrogallol, para-anisidine	9200-9600	Niladevi and Prema (2008)
T. sp.	flask	SSF (60% rape stems, 20% peanut shells, 20% wheat bran)	-	2110 U/g of support	Xiao and Hong (2008)
P. ostreatus	Static flask (22 mL)	Semi SSF (Juncao *Miscanthus floridulus,* wheat bran, ammonium tartrate, yeast extract)	-	4475,000	Liu *et al.* 2009

Pseudomonas desmolyticum	Static flask (100 mL)	SmF	-	120 U/g of protein	Kalme *et al.* (2009)
S. psammoticus	Flask, 150 rpm	SmF (Coffee pulp)	Trace elements (MgSO$_4$, (NH$_4$)$_2$SO$_4$, CuSO$_4$)	15,000	Niladevi *et al.* (2009)

[a] PW: pine wood

[b] PUF: polyurethane foam

[c] OMW: olive mill wastewater

5   Enzyme immobilization

The main drawbacks of many important enzymes for their use in industrial applications are their low stability and productivity and high production costs (Godfrey, 1996). The most frequently used stabilization method is immobilization, which in addition provides many other process benefits such as reduction of enzyme replacement, facilitation of separation and reuse of the catalyst and assistance of reaction control (Cao et al. 2003, Mateo et al. 2007). Moreover, it is well known (Tischer and Wedekind 1999, Cao et al. 2003, Mateo et al. 2007) that immobilization shifts the enzyme properties like: optimum values of pH and temperature, kinetics parameters and strengthens protein structure. Especially higher thermostability of the enzyme allows conducting the processes at higher temperatures and so it reduces reaction time. The advantages and disadvantages of enzyme immobilization are shown in Table 4 (van de Velde *et al.* 2002, Bornscheuer 2003).
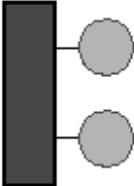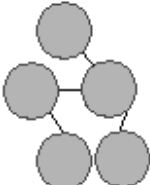
Enzyme immobilization was performed for the first time by Nelson and Griffin in 1916. They adsorbed the enzyme invertase on activated carbon and alumina with the retention of its catalytic activity. The development of enzyme immobilization processes took place from 1960. Thus, in 1969, in Japan the first industrial process using immobilized enzymes for the production of L-aminoacids was implemented followed by the implementation in 1972, in USA, of the process of glicose isomerization in fructose using immobilized glicose-isomerase. From then, new processes and new immobilization methods have been reported in the literature (Zanin and Moraes 2004).
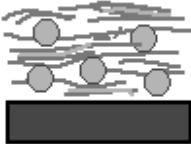
Table 4. Advantages and disadvantages of enzyme immobilization (van de Velde *et al.* 2002, Bornscheuer 2003)

Advantages
Easier separation and recuperation of the enzyme and products
Reusability
Increase of thermal stability and resistance against denaturalizing agents
Reaction can be stopped easier
Continuous operations can be easier to achieve
Higher flexibility in the design of bioreactors
Prevents the contamination caused by the protein in the final product
Microbial contamination is easier to control
Disadvantages
Lower enzymatic activity caused by the immobilization process
Increase of the Michaelis-Menten constant

Different methods based on physical and chemical mechanisms are used for enzyme immobilization on solid materials and gels (Duran *et al.* 2002, Giamberini *et al.* 1994). The chemical methods include covalent bonds between the enzyme and the support, cross-linking between the enzyme and the support and enzymatic cross-linking by multifunctional agents. The physical methods involve adsorption, entrapment of enzymes in insoluble polymeric gels (polymeric entrapment) or in micelles (encapsulation) (Duran *et al.* 2002). Table 5 shows a scheme of the classification methods used for enzyme immobilization with their particular advantages and disadvantages (adapted from Mateo *et al.* 2007).

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Table 5. Advantages and disadvantages of various enzyme immobilization methods (adapted from Mateo *et al.* 2007)

Immobilization Method	Advantages	Disadvantages	Model
Chemical			
Covalent bond	Enzyme molecules fully dispersed	Full interaction between the enzyme and any external interface Possible inactivation of the enzyme by any external agent	
Single covalent bond with cross-linker	No need for geometric congruence between the enzyme and the support The mass of the support can be comparable to the mass of the enzyme	Enzyme is attached, but there is no evidence of substantial increase of the enzyme stability Enzyme can present deactivation by conformational changes induced by distorting agents	
Multipoint covalent bond with cross-linker	Increase of the enzyme stability by reducing the conformational changes of the enzyme induced by any distorting agent (heat, organic solvents, extreme, pH values)	High need of geometric congruence between the enzyme and the support The mass of the support is considerably higher than the mass of the immobilized enzyme	
Enzyme cross-linking	The mass of the enzyme aggregate can be comparable to the mass of the enzyme Simplicity in operation	Interaction between any external interface and the enzyme is conditioned by its position in the aggregate Poor reproducibility and mechanical stability Handling problems	

Physical			
Adsorption	No need to functionalize the support (or a more simple method is needed) Immobilization takes place by electrostatic or hydrophobic binding, without the need of adding more compounds	If the substrate is very large or hydrophilic, the near presence of the hydrophobic support surface may generate some steric hindrances, reducing the activity of the enzyme Attachment is weaker than by covalent binding	
Entrapment			
Encapsulation	Retains the enzyme in its natural form in capsules of different size Reduces the contact of the enzyme with the external interface (depends on the diffusion of different compounds through the capsule)	Enzymatic retention hardly depends on the porosity of the capsule Enzyme can present deactivation by conformational changes induced by distorting agents	
Polymeric entrapment	Retains the enzyme in its natural form, useful for flat surfaces Reduces the contact of the enzyme with the external interface (depends on the diffusion of different compounds through the layers)	Enzyme can present deactivation by conformational changes induced by distorting agents Possible appearance of nanoenvironments that can alter the operation of the enzyme	

In order to enhance its industrial utility, laccase has been immobilized on different solid supports and using different techniques (Duran *et al.* 2002 and references therein). Different techniques can be used for laccase immobilization, mainly based on chemical or physical mechanisms such as those absorbed on porous glass (Rogalski *et al.* 1995), entrapped in alginate beads (Palmieri *et al.* 1994) and gelatin (Grecchio *et al.* 1995), or covalently attached on different inert and polymeric materials (Brenna and Bianchi 1994, Hublik and Schinner 2000). The immobilization of laccases by a covalent bond is a very common technique used by many authors. This technique normally begins with the

modification of the surface of the support or by an activation step. The activation of the surface with organic functional groups using an organo-functional silane reagent (silanization) is a widely used strategy for an initial surface modification of the support. The subsequent addition of a bi-functional compound (cross-linker), such as glutaraldehyde or glyoxal, forms covalent bonds with the modified support and with the large number of amine groups present in the enzymes, usually located on the surface (Duran *et al.* 2002, Mateo *et al.* 2000). In addition, the concurrent addition of free enzyme together with a cross-linking agent can result in the undesirable internal cross-linking of the enzyme during the covalent binding step (Cao *et al.* 2003). Table 6 summarizes the most recent results reported on laccase immobilization.

Table 6. Most recent results on laccase immobilization on different materials for different applications

Fungus	Support	Field	Immobiliza tion rate	Concentration [mg laccase/g support]	Half life (cycles)	Reference
Aspergillus oryzae	Chitosan microspheres	Rutin oxidation	-	0.55 U/mg [a]	> 930 sensing	Fernandes *et al.* 2008
Cerrena unicolor	carbon nanoparticles - electrode surface in a sol–gel film	Batteries (biocells)	-	2.7×10^{-10} mol / film [a]	-	Szot *et al.* (2009)
	Granocel	Batch reactions (bioprocess)	12%	-	> 20	Rekuć *et al.* (2009b)
	siliceous cellular foams - glutaraldehyde	Dye discoloration	50.7%	0.640 mg/mL [a]	26	Rekuć *et al.* (2009a)
	siliceous cellular foams – oxirane rings	Dye discoloration	< 2%	0.324-0.513 mg/mL [a]	-	Rekuć *et al.* (2009a)
	siliceous cellular foams divinyl sulfonate	Dye discoloration	0.3%	0.084 mg/mL [a]	-	Rekuć *et al.* (2009a)
Coriolopsis polyzona	Celite ® R-633-glutaraldehyde	Elimination of endocrine disrupting chemicals (EDC)	27%	-	>5	Cabana *et al.* (2009)
	Celite ® R-633-glutaraldehyde (with internal cross linking)	Elimination of EDC	14%	-	-	Cabana *et al.* (2009)

	Celite ® R-633-glyoxal	Elimination of EDC	14%	-	-	Cabana *et al.* (2009)
	Celite ® R-633-glyoxal (with internal cross linking)	Elimination of EDC	9%	-	-	Cabana *et al.* (2009)
Coriolus versicolor	chitosan - glutaraldehyde	Phenol removal	52%	-	6	Zhang *et al.* (2009)
Pleurotus sajor-caju	SBA-15 mesoporous silica	Phenolic compounds treatment	98%	-	> 14	Salis *et al.* (2009)
R. vernificera	poly(GMA/EGDMA) beads [c]	Dye degradation	53%	5.6	> 6	Arica *et al.* (2009)
	poly(GMA/EGDMA)-DAH [c,d] beads	Dye degradation	88%	4.9	> 6	Arica *et al.* (2009)
Trametes trogii	Gold surface – LbL	Biocells	-	0.7 g/cm^{2,a}	-	Szamocki *et al.* (2009)
T. versicolor	macroporous bead cellulose – NH$_2$ group	Biocatalytic applications (possible dye degradation)	1.25%[b]	-	-	Rotková *et al.* (2009)
	macroporous bead cellulose – OH group	Biocatalytic applications (possible dye degradation)	< 1%[b]	-	-	Rotková *et al.* (2009)
	Magnetic macroporous bead cellulose – NH$_2$ group	Biocatalytic applications (possible dye degradation)	5.25%[b]	-	-	Rotková *et al.* (2009)
	Magnetic macroporous bead cellulose – OH group	Biocatalytic applications (possible dye degradation)	1.83%[b]	-	-	Rotková *et al.* (2009)
	p(HEMA-g-GMA)-NH$_2$ films [e]	Phenolic compounds treatment	71%	139 µg/cm^{2,a}	> 6	Bayramoğlu and Arica (2009)

[a] Concentrations given in different units due to characteristics of the support

[b] Data calculated using information provided in the article

[c] Poly(GMA/EGDMA): poly(glycidyl methacrylate/ethyleneglycol dimetacrylate)

[d] DAH: 1,6-diaminohexane

[e] p(HEMA-g-GMA): Poly(hydroxyethylmethacrylate-glycidyl methacrylate

6 Synthetic dyes

Dyes has been used for thousands of years (Christie 2007), it is believed that Neanderthal man, about 180,000 years ago, was the first user of colorants. However, the first known organic colorant, the blue indigo, was used in the wrappings of mummies in Egyptian tombs about 4000 years ago (Gordon and Gregory 1983). All dyes and colorants used until

1856 were natural and prepared on small scale, mainly extracted from plants, insects and mollusk. In 1856 the English chemist William H. Perkin discovered the first synthetic dye, mauveine, and since then dyes were manufactured synthetically and on a large scale (Hunger 2003, Venkataraman 1965). The automation of the process brought a revolution to the textile industry raising also the dyestuff industry.
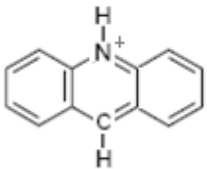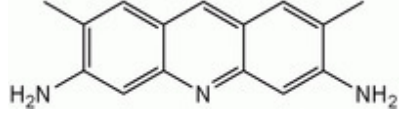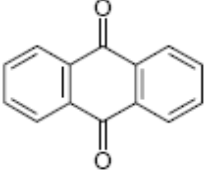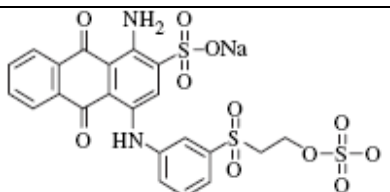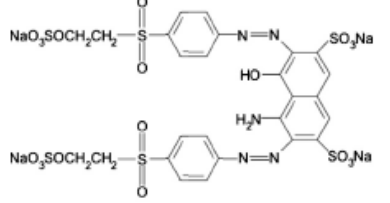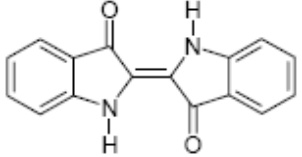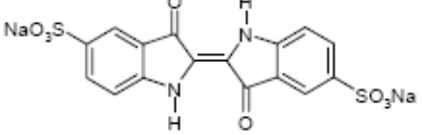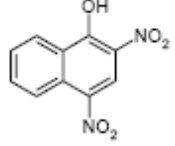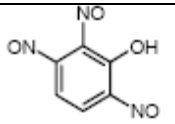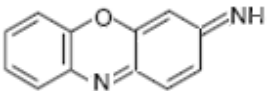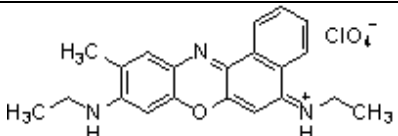
6.1 Dye classification
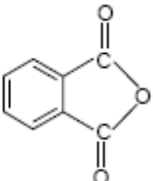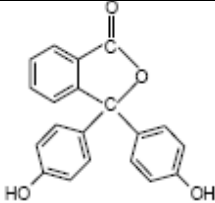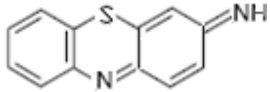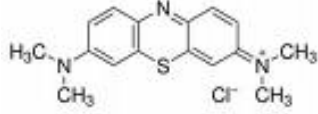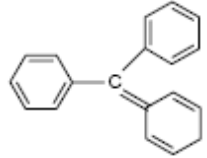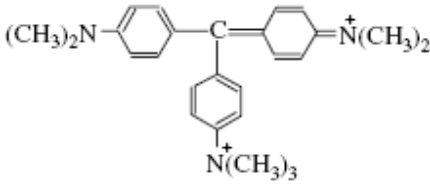
The international classification system is the *Colour Index* (CI), a publication from the *Society of Dyers and Colourists* launched in 1924 (O´Neill *et al.* 1999). Dyes are classified by a generic name determined by their application characteristics followed by a CI number based on their chemical structure. In addition, dyes can be classified according to their chemical structure or to the application method:

According to their chemical structure

Dyes are composed of a group of atoms responsible for the dye color called chromophores and electron withdrawing or donating substituents called auxochromes (Christie 2001), which intensify color and confers high solubility in water to the dyes. Some auxochromes also increase the dye affinity for the fiber (natural or synthetic). The most important chromophores are azo ($-N=N-$), carbonyl ($-C=O$), methyne ($-CH=$), nitro ($-NO_2$) and quinoid groups. The most important auxochrome groups are hydroxyl ($-OH$), sulfonate ($-SO_3H$), carboxyl ($-COOH$) and amino ($-NH_3$). Common classes of dyes, based on the chromophore present, are shown in Table 7.

Table 7. Classification and examples of dyes according to the chromophore present

Class	Chromophore	Example
Acridine		Acridine Yellow G
Anthraquinonic		Remazol Brilliant Blue R (RBBR)
Azo	—N═N—	Reactive Black 5 (RB5)
Indigoid		Acid Blue 71
Nitro		Acid Yellow 24
Nitroso	—N═O	Fast Green O
Oxazine		Oxazine 170 perchlorate

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Phenolphthalein		Phenolphtaleine
Thiazine		Methylene Blue
Triphenylmethyl		Methyl Green (MG)

According to the application way

Acid dyes: they are applied to fibers such as nylon, wool, silk, modified acrylics, and also to some extent to paper, leather, ink-jet printing, food and cosmetics, but not substantive to cellulosic fibers. Attachment to the fibers is partly attributed to bond between anionic groups in the dyes and cationic groups in the fibers. The main chemical classes of these dyes are azo (including premetallized), anthraquinone, triphenylmethane, azine, xanthene, nitro and nitroso. They are generally soluble in water.

Cationic (Basic) dyes: they are mainly applied to paper, polyacrylonitrile, modified nylons, modified polyesters, cation dyeable polyethylene terephthalate and to some extent in medicine, however they were originally used for wool, silk and tannin-mordanted cotton. Usually acetic acid is added to the dye bath to help the uptake of the dye onto the fiber. The main chemical classes are diazahemicyanine, triarylmethane, cyanine, hemicyanine, thiazine, oxazine and acridine.

Direct dyes: they are applied to cotton, rayon, paper, leather, wool, silk and to some extent to nylon. They are also used as pH indicators and as biological stains. Direct dyeing is normally carried out in a neutral or slightly alkaline dye bath, at near the boiling point with the addition of sodium sulfate (Na_2SO_4) or sodium chloride (NaCl). Generally the dyes

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belonging to this class are polyazo compounds, along with some stilbenes, phthalocyanines and oxazines.

Disperse dyes: they were originally developed for the dyeing of cellulose acetate but they are mainly applied to polyester and to some extent to nylon, cellulose and acrylic fibers. They are substantially water insoluble non-ionic dyes used for hydrophobic fibers from aqueous dispersion. These dyes are finely ground in the presence of a dispersing agent and sold as a paste, or if dried with spray, sold as a powder. They generally contain azo, anthraquinone, styryl, nitro, and benzodifuranone groups.

Mordant dyes: they are used for wool (30% of dyes for wool), leather and natural fibers after pre-treating with metals and anodized aluminum. They are very useful for black and navy shades. Most natural dyes are mordant dyes and there is a large literature base describing their dyeing techniques. Many mordant dyes, particularly the heavy metal category, can be hazardous to health. Some mordant dyes have azo and anthraquinone groups.

Vat dyes: they are mainly used for cotton. Vat dyes are insoluble in water and incapable of dyeing fibers directly. However, the leuco form of the dye, produced by the reduction in alkaline liquor (alkali metal salt), is water soluble and has an affinity for the textile fibers. Subsequent oxidation reforms the original insoluble dye. These dyes group contain anthraquinone (including polycyclic quinones) and indigoids.

Reactive dyes: they are generally used for cotton and other cellulosics, but also in a small extent for wool and nylon. These dyes use a chromophore attached to a substituent that is able to react directly with the fiber. These dyes form covalent bonds with the natural fiber, making them one of the most permanent dyes. Some reactive dyes such as Procion MX, Cibacron F, and Drimarene K, can be used at room temperature and are called "Cold" reactive dyes. Reactive dyes contain groups such as azo, anthraquinone, triarylmethane, phthalocyanine, formazan, oxazine, etc. Their chemical structures are simpler than other dyes; their absorption spectra show narrower absorption bands and the final dyeing color is brighter than when using direct dyes. They are, by far, the most common choice for dyeing cotton and other cellulose fibers at home or in art studios.

Sulfur dyes: they are used for cotton and rayon and have limited use with polyamide fibers, silk, leather, paper and wood. They have intermediate structures but their low cost and good wash fastness properties make them an important dye class from an economical point of view. Actually, Sulfur Black 1 is the largest selling dye by volume. These dyes have a double process when dyeing. The initial bath gives a yellow or pale chartreuse color, but after sulfur treatment a dark black color is produced, commonly used in socks.

Solvent dyes: they are mainly used for plastics, gasoline, lubricants, oils, and waxes. They are insoluble in water and generally non polar or little polar. The predominant groups are azo and anthraquinone but phthalocyanine and triarylmethane are also used.

Dyes are used in textile industry, leather tanning industry, paper production, food technology, agricultural research, light-harvesting arrays, photoelectrochemical cells, hair coloring and cosmetics. Due to the large amounts used, the most significant industrial use is in textile dyeing.

6.2   Textile industry

Textile industry is a sector that presents a very complicated industrial chain and a high diversity in terms of raw materials, processes, products and equipment. The impact on the environment by the textile industry has been recognized in terms of the discharge of pollutants and of the consumption of water and energy (Lacasse and Baumann, 2006).

Textile industry includes four main activities: treatment of raw materials, production of textiles, dyeing and finishing processes and cloth production. Main pollution in textile wastewater comes from dyeing and finishing processes. The textile finishing includes the bleaching, dyeing, printing and stiffening of textile products in the different processing stages (fiber, yarn, fabric, knits, finished items). The purpose of finishing is in every instance the improvement of the serviceability and adaptation of the products to meet the ever-changing demands of fashion and function (Savin and Butnaru 2008). To achieve the desired effects, a range of chemicals, dyes and auxiliaries are used (Savin and Butnaru 2008).

6.2.1 Environmental impact of effluents from textile industry

The textile industry accounts for two-thirds of the total dyestuff market (Riu *et al.* 1998) and consumes large volumes of water and chemicals for wet processing of textiles. The discharges of wastewater are the main cause of the negative environmental impact of the textile industry. Its biggest impact on the environment is related to primary water consumption (80–100 m^3/ton of finished textile) and wastewater discharge (115–175 kg of COD/ton of finished textile, a large range of organic chemicals, low biodegradability, color and salinity) (Savin and Butnaru 2008). Therefore, reuse of the effluents represents an economical and ecological challenge for the overall sector (Li Rosi *et al.* 2007).

The chemical reagents used in the textile processing are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products and depend on the nature of the raw material and product (Mishra and Tripathy 1993, Banat *et al.* 1996, Juang *et al.* 1996, Aslam *et al.* 2004). The effluents resulting from these processes differ greatly in composition due to differences in the processes, used fabrics and machinery (Bisschops and Spanjers 2003).

Main pollution in textile wastewater comes from dyeing and finishing processes. These processes require the input of a wide range of chemicals and dyestuffs, which generally are complex organic compounds. Major pollutants in textile wastewater are high suspended solids, chemical oxygen demand (COD), heat, color, acidity, and other soluble substances (Venceslau *et al.* 1994, World Bank 2007). Color, for example, is noticeable at a dye concentration higher than 1 mg/L and an average concentration of 300 mg/L has been reported in effluents from textile manufacturing processes (Gonçalves *et al.* 2000, O'Neill *et al*. 1999). Actually, about 800,000 tons of dyes are produced annually worldwide, 40% of which are produced in Europe (Hessel *et al.* 2007). During the dyeing processes, 2-60% of the initial used dyes is not bound to the fabric and is lost in the effluent (Table 2 from Hessel *et al.* 2007).

The presence of dyes in aqueous ecosystems reduces sunlight penetration into deeper layers diminishing photosynthetic activity, deteriorates the water quality, lowering the gas solubility which causes acute toxic effects on aquatic flora and fauna (Nilsson *et al.* 1993).

In addition to their visual effect, adverse impact in terms of COD and their toxic, carcinogenic and genotoxic effects have made textile industry as one of the main sources of severe environmental problems worldwide (Vandevivere *et al.* 1998). Consequently, rigorous government legislations are holding textile industries with increasingly higher standards of treatment regarding waste effluents.

In Spain, Decret 130/2003 states that color is a contaminant parameter difficult to treat in wastewater treatment plants, thus, the color concentration in discharged wastewater is limited to be unappreciated at 1/30 dilution. In the European Union the legislation about industrial wastewater has been continuously updated, making wastewater treatment obligatory (European Water Framework Directive 2000/60/EC).

In 1999 a total of 1.2 million m^3 of water was used by the textile industry in Spain, generating 1.16 million m^3 of wastewater. From the 1.2 million m^3 of water, 1.12 millions were spent in Catalonia; however only 0.9% was recycled in industrial processes (INE website). In Catalonia, many of the textile and dyestuff industries are placed close to urban nuclei. Based on their wastewater management they are divided into two groups. The first one is composed by the industries that are allowed to dispose their wastewater directly to a communal water treatment system that treats both urban and industrial wastewater. The second group is composed by those industries that are forced to treat the wastewater before disposal until reaching the limits established by law of toxic and environmental hazardous compounds. However, without having a characterization of textile process effluent streams is difficult to develop strategies for a proper water treatment and reuse. To optimize treatment and reuse possibilities, textile industry waste streams should be, in principle, considered separately. When the characteristics of the separate streams are known, it can be decided which streams may be combined to improve treatability and increase reuse options (EWA 2005, Li Rosi *et al.* 2007).

6.3 Methods of dye removal

The treatment of dye wastewater was not seriously considered before the decade of 1980. Actually, more than half of the chemical structures of the dyes used in the industry at that time were unknown. However, with the growing concern about health issues and the

possible environmental consequences, dye manufacturers, users and governments have begun to pay more attention to this topic (Gupta and Suhas 2009). Thus, different methodologies have been proposed for the treatment of dye wastewater which are classified into three categories: physical, chemical and biological.

6.3.1 Physical methods

Adsorption

Adsorption refers to a process where a substance or material is concentrated at a solid surface from its liquid of gaseous surrounding. There are two types of adsorption based on the type of attraction between the solid surface and the adsorbed molecules. If this attraction is physical in nature is referred as physical adsorption (physiosorption) and van der Waals forces are the driving force in the process. Thus, the adsorption is weak and results in a reversible process. Otherwise, if the attraction forces are due to chemical bonding, the process is called chemical adsorption (chemisorption). Due to the higher strength of the chemical bond it is difficult to remove the chemisorbed molecules from the solid surface.

Adsorption methods are strongly used due to the high efficiency in the removal of recalcitrant toxic compounds difficult to treat with other methods. Adsorption is not only used for dye removal but also has a wide applicability in wastewater treatment (Mantell 1951, Weber Jr. *et al.* 1970, Mattson and Mark 1971, Liapis 1987, Freeman 1989, Pirbazari *et al.* 1991, Danis *et al.* 1998, Quignon *et al.* 1998, Imamura *et al.* 2002, Bansal and Goyal 2005).

Activated carbon

The use of carbon adsorption for water purification dates back to ancient times (Cheremisinoff 2002). As early as 1550 B.C., adsorption on porous carbon was described in an Egyptian papyrus, but it was also described by Hippocrates and Pliny the Elder. In 1173 Scheele observed the phenomenon of adsorption of gases exposed to carbon, whereas Lowitz, in 1785, observed the reversible removal of color and odor producing compounds from water by wood charcoal (Mantell 1951, Tien 1994). Nowadays, activated carbon is the

most used adsorbent material for the dye removal being very effective for the adsorption of cationic, mordant and acid dyes (Nasser and el Geundi 1991). However, in order to improve the removal speed of dyes a massive dose of activated carbon must be used (Robinson *et al.* 2001b). The use of activated carbon is an expensive method, also, carbon has to be regenerated (loosing between 10 and 15% of initial adsorption capacity) and the disposal of the final residue has to be considered. Many authors have used activated carbon for removing different types of dyes (DiGiano and Natter 1977, Walker and Weatherley 1999, Pelekani and Snoeyink 2000, Al-Degs *et al.* 2001).

Alumina

Alumina is a synthetic porous crystalline gel available in the form of granules. The surface area of the granules goes from 200 to 300 m^2/g (Do 1998). Alumina has been studied by various workers for the removal of dyes (Adak *et al.* 2005, 2006, Huang *et al.* 2007), however, not always successfully.

Silica gel

Silica gel is a porous and non crystalline granule of different sizes prepared by the coagulation of colloidal silicilic acid. Compared to alumina, silica gel shows a higher surface area (Do 1998) ranging from 250 to 900 m^2/g. This material is effective for the removal of basic dyes (Allingham *et al.* 1958, Alexander and McKay 1977); however, its high price and the possible side reactions make this material not very suitable to be used commercially (McKay et al. 1999).

Zeolites

Zeolites are natural microporous adsorbents materials, though they can also be synthetically prepared. They are considered selective adsorbents and show molecular adsorption (Kesraouiouki *et al.* 1994, Curkovic *et al.* 1997, Caputo and Pepe 2007) and ion exchange properties (Kesraouiouki *et al.* 1994, Menon and Komarneni 1998, Adebajo *et al.* 2003). Many zeolites have been used for the removal of dyes, however, not always successfully (Handreck and Smith 1988, Meshko *et al.* 2001, Nur *et al.* 2005, Yuan *et al.* 2007, Wang and Ariyanto 2007, Alpat *et al.* 2008).

Low-cost adsorbent (LCA) materials

LCA are normally natural materials, wastes/by-products of industries or synthetically prepared materials that can be used as adsorbents and generally have little or no price in the industrial chain. Such materials can be a great advantage for less industrialized/developing countries which are facing big water problems. Recently, Gupta and Suhas (2009) suggested a protocol based on the numerous studies for the development, utilization and application of LCA generally adopted by researchers. Also, they reviewed the advantages and disadvantages of adsorbents, favorable conditions for particular adsorbate–adsorbent systems, and adsorption capacities of various LCA and commercial activated carbons.

Membrane filtration

Filtration technology has been used for drinking water and wastewater treatment applications. In general terms, filtration includes microfiltration, ultrafiltration, nanofiltration and reverses osmosis processes. These processes have been investigated for the color removal; however, the microfiltration is not very used for wastewater treatment due to the large pore size (Cheremisinoff 2002, Avlonitis *et al.* 2008). The ultrafiltration and nanofiltration are effective for the removal of all classes of dyestuffs, but the dye molecules frequently cause clogging of the membrane pores, limiting the use of these separation systems for textile effluent treatment (Marmagne and Coste 1996, Cheremisinoff, 2002). Filtration systems main drawbacks are the high working pressures and energy consumption, the high cost of the membranes and their relatively short life.

On the other hand, reverse osmosis has been proved to be an effective discoloration and desalting process against a diverse range of dye wastewater, producing a water similar to the pure H_2O (Marcucci *et al.* 2001, Al-Bastaki 2004, Sostar-Turk *et al.* 2005).

Ion exchange

This technology has not been used in extend for dye wastewater treatment due to the limited range of dyes that can be removed (Slokar and Le Marechal, 1998). The main drawback of this technology is the high cost but it does not present losses in the adsorption capacity after regeneration.

6.3.2   Chemical methods

Coagulation/flocculation with the addition of agents

One of the most robust ways to remove color from dye wastewater is the chemical treatment with a coagulating/flocculating agent (Shi *et al.* 2007, Zhou *et al.* 2008). The process involves the addition of agents to the wastewater such as aluminum (Al^{3+}), calcium (Ca^{2+}) or ferric (Fe^{3+}) ions. Normally, this process is economically feasible and presents satisfactory results in the removal of disperse, sulfur and vat dyes; however, results with azo, reactive, acid and especially basic dyes are not generally good. The main drawbacks of this process are the production of sludge in large quantities and the removal of dyes is pH dependent (Kace and Linford 1975, Lee *et al.* 2006).

Advanced oxidation processes

Advanced oxidation processes are used to degrade the dyes partially or completely. They are among the most commonly used methods for discoloration since they require low quantities of reagents and have short reaction times.

Fenton's Reagent

H_2O_2 is a very pale blue liquid, slightly more viscous than water, with strong oxidizing properties. A solution of hydrogen peroxide and an iron catalyst, known as Fenton's reagent, is also used to oxidize dye wastewater (Meric *et al.* 2003, Wang 2008) and is stronger than hydrogen peroxide. It is generally very effective in the discoloration of both soluble and insoluble dyes (acid, reactive, direct, metal complex dyes) (Pak and Chang 1999). The main drawbacks are its narrow pH range of operation (< 3.5) (Cheng *et al.* 2004), the sludge generation and the long reaction time.

Chlorine

Chlorine is a strong oxidizing agent and may also be applied as calcium hypochlorite and sodium hypochlorite. It is extensively used for the removal of color. Reactive, acid direct and metal dyes are discolored readily by hypochlorite; however, water insoluble disperse and vat dyes are resistant to be discolored with this technology (Namboodri *et al.* 1994a,b).

The use of chlorine gas is a low-cost process for dye wastewater discoloration, though; it can present side reactions, producing organochlorine compounds.

Ozonation

Ozonation is a technology firstly used in the 70's and is carried out by ozone generated from oxygen. This technology has been found to be very effective, but expensive, for the discoloration of dye wastewaters (Perkins *et al.* 1996, Soares *et al.* 2006). It is suggested that ozonation can be used to completely remove color and COD to an extent, which is sufficient for water reuse. The main drawbacks of this technology are the high cost of the process and the production in-situ of the ozone.

Electrochemical

Electrochemical methodology is also used to remove color from dye wastewater. (Lin and Peng 1994, Gupta *et al.* 2007). The discoloration is either achieved by electro oxidation with non-soluble anodes or by electro-coagulation using consumable materials. The electro-degradation of dyes has been successfully carried out by using different anode materials, such as, iron, conducting polymers and boron doped diamond electrode (Dogan and Turkdemir 2005, Faouzi *et al.* 2007, Oliveira *et al.* 2007). This technology is not only effective for the discoloration of soluble and insoluble dyes, but also in the reduction of the COD. The main drawbacks are the high electrical cost, the sludge production and also the pollution generated from chlorinated organic products and heavy metals due to indirect oxidation.

Photochemical oxidation

Photochemical

This method degrades dye molecules by ultraviolet (UV) treatment in the presence of an oxidant. Degradation is caused by the production of high concentration of hydroxyl radicals. UV light is used to activate oxidants, such as H_2O_2, and the rate of dye removal is influenced by the intensity of the UV radiation, pH, dye structure and wastewater composition (Slokar and Le Marechal 1998).

Photo-Fenton

The combination of Fenton's reaction and UV light is called photo-Fenton reaction. This technique has showed to enhance the efficiency of the Fenton's process and is also effective in the dye wastewater treatment (Muruganandham and Swaminathan 2004, Bandala *et al.* 2008).

Table 8 summarizes the disadvantages of the physical and chemical methods for dye removal (Husain 2006).

Table 8. Physical and chemical methods for dye removal and their specific disadvantages (extracted from Husain 2006)

Type of treatment	Disadvantages
Physical	
Activated carbon	Very expensive, excessive sludge generation
Silica gel	Side reactions prevent commercial application
Low cost adsorbents	Requires long retention time, Specific surface area for adsorption
Membrane filtration	Concentrated sludge production
Reverse osmosis	Highly expensive, slow process
Ion exchange	Not effective for all dyes
Chemical	
Coagulation	High sludge production
Fenton's reagent	Excessive sludge generation
Chlorine	Release of aromatic amines
Ozonation	Short half-life (20 min)
Electrochemical destruction	High cost of electricity, poor color removal
Photochemical	Formation of by-products, production of more toxic compounds, poor color removal, quite slow process

6.3.3 Biological Methods

Biological treatment, either aerobic (in the presence of oxygen) or anaerobic (in the absence of oxygen), is generally considered to be the most effective means of removing the bulk of pollutants from wastewater. Different species have been used for the treatment of various dye effluents. The major advantage of this technique is the low running costs.

Bacteria and fungi are the two groups of microorganisms that have been widely studied in the treatment of dye wastewater. The enzymes secreted by aerobic bacteria can break down the organic compounds, thus, the isolation of aerobic bacterial strains capable of degrading different dyes has been carried out for more than two decades (Rai *et al.* 2005). Also, fungal strains and their enzymes have been studied in detail by various authors (Sani and Banerjee 1999, Fu and Viraraghavan 2001, Pazarlioglu *et al.* 2005). However, it is important to remark that different factors like concentration of pollutants, dyestuff concentration, initial pH and temperature of the effluent affect the discoloration process.

White-rot fungi have been also widely used for dye removal. This color removal process can take place by adsorption of dyestuff on fungal mycelia, by real degradation or by a combination of both. Nevertheless, there are some important drawbacks in the use of white-rot fungi. The addition of reagents for having appropriate growth conditions is necessary, since dye degradation is attributed to secondary metabolic pathways. Also, the production of the enzymes involved in the dye degradation is not constant with time and is influenced by inhibitors that can be present in the effluents. For overcoming these problems, the use of the enzymes involved in the dye degradation, instead of the whole fungal cultures, is getting more attention.

The potential advantages of using enzymes instead of fungal cultures are mainly associated to the following factors: shorter treatment period, operation of high and low concentrations of substrates, absence of delays associated with the lag phase of biomass, reduction in sludge volume and ease of process control (López *et al.* 2002, Akhtar and Husain 2006). In addition, it would eliminate the need for ligninolytic enzyme production, which depends on the metabolic state of the cultures, which in turn is affected by physiological conditions

such as pH and variation in effluent composition. Also, enzyme-based treatment alone might be sufficient especially when the enzymes transform toxic compounds to less harmful products. In such cases, complete degradation of the contaminants might not be necessary. However, there are several practical limitations in the use of free enzymes such as the high cost associated with production, isolation and purification of enzymes and the short lifetimes of enzymes. The former can be overcome producing the enzyme in SSF with agro-byproducts as support-substrates and applying the enzyme in crude form (Roriz *et al.* 2009, Sun *et al.* 2009). As for the latter, enzyme immobilization has shown to improve enzyme stability (Khani *et al.* 2006, Kulshrestha and Husain 2006, Michniewicz *et al.* 2008). In addition, enzyme immobilization allows enzyme reutilization and continuous operation in bioreactors which is very important for an industrial application of the enzyme. However, the idea of enzyme reutilization implies that the stability of the enzyme has to be high enough to allow its reuse. Therefore, the immobilized enzyme has to be highly stable to develop a suitable process. Thus, although there are hundreds of immobilization protocols, the design of new ones that improve enzyme properties is still a challenging goal.

Recently, Husain (2006) has reviewed the potential applications of the oxidoreductive enzymes in the discoloration of textile and other synthetic dyes.

6.3.3.1 Dye treatment using free and immobilized laccase

The use of free laccase for dye removal has been studied, commonly used in combination with mediators. In recent years, the immobilization of laccase has also been studied for the treatment of dyes or dye wastewater. The use of immobilized laccase, instead of the free enzyme or the living microorganism, is gaining interest due to the possibility of the sequential use of the immobilized enzyme and at the same time avoids the formation of sludge or biomass. Additionally, free enzymes are not always useful for biotechnological applications because of their low stability. Table 9 shows some of the most recent results in dye removal with free and immobilized laccase.

Table 9. Recent results in dye removal with free and immobilized laccase at flask scale

Dye	Laccase source	Support[a]	Disc. %	time	Reference
Acid Black 1	*T. trogii*		95	24 h	Ciullini *et al.* 2008
Acid Black 194	*T. trogii*		88.7	24 h	Ciullini *et al.* 2008
	T. trogii		77	-	Ciullini *et al.* 2008
Acid Black 210	*B. subtilis (CotA-laccase)*		35	24 h	Pereira *et al.* 2009
Acid Blue 158	*T. trogii*		99.3	24 h	Ciullini *et al.* 2008
Acid Blue 324	*T. trogii*		75	24 h	Ciullini *et al.* 2008
Acid Blue 62	*B. subtilis (CotA-laccase)*		98	7	Pereira *et al.* 2009
Acid Blue 80	*T. trogii*		98.6	24 h	Ciullini *et al.* 2008
Acid Fuchsin	*T. versicolor*		28	48 h	Casas *et al.* 2009
Acid Green 16	*T. versicolor*		90	48 h	Casas *et al.* 2009
Acid Orange 52	*T. modesta*		> 50	2 h	Tauber *et al.* 2008
	T. versicolor		63	6 h	Yamak *et al.* 2009
Acid Red 186	*T. trogii*		96.5	24 h	Ciullini *et al.* 2008
Acid Red 266	*B. subtilis (CotA-laccase)*		70	24 h	Pereira *et al.* 2009
Acid Red 299	*B. subtilis (CotA-laccase)*		2	24 h	Pereira *et al.* 2009
Acid Red 374	*T. trogii*		100	24 h	Ciullini *et al.* 2008
Acid Red 42	*T. trogii*		93.4	24 h	Ciullini *et al.* 2008
Acid Yellow 129	*T. trogii*		27.5	24 h	Ciullini *et al.* 2008
Acid Yellow 194	*B. subtilis (CotA-laccase)*		33	24 h	Pereira *et al.* 2009
Acid Yellow 49	*B. subtilis (CotA-laccase)*		3	24 h	Pereira *et al.* 2009
	T. trogii		23.3	24 h	Ciullini *et al.* 2008
Alizarin	*B. subtilis (CotA-laccase)*		75	24 h	Pereira *et al.* 2009
Basic Fuchsin	*T. versicolor*		< 20	48 h	Casas *et al.* 2009
Brilliant Green 1	*T. versicolor*		100	48 h	Casas *et al.* 2009
$C_{16}H_{13}N_3O_7S_2$	*P. cinnabarinus*		10	120 min	Rotkova *et al.* 2009
Direct Black 22	*T. trogii*		31.3	24 h	Ciullini *et al.* 2008
Direct Black 38	*B. subtilis (CotA-laccase)*		43	24 h	Pereira *et al.* 2009
Direct Blue 1	*B. subtilis (CotA-laccase)*		90	24 h	Pereira *et al.* 2009
Direct Blue 6	*P. desmolyticum*		100	16 h	Kalme *et al.* 2009
Direct Blue 71	*T. modesta*		> 50	2 h	Tauber *et al.* 2008
	T. trogii		21.5	24 h	Ciullini *et al.* 2008
Direct Red 243	*T. trogii*		90.4	24 h	Ciullini *et al.* 2008
Direct Red 80	*B. subtilis (CotA-laccase)*		8	24 h	Pereira *et al.* 2009

Dye	Source	%	Time	Reference
Direct Red R	*B. subtilis (CotA-laccase)*	60	24 h	Pereira *et al.* 2009
Direct Yellow 106	*B. subtilis (CotA-laccase)*	10	24 h	Pereira *et al.* 2009
Disperse Blue 1	*B. subtilis (CotA-laccase)*	27	24 h	Pereira *et al.* 2009
Disperse Yellow 3	*B. subtilis (CotA-laccase)*	8	24 h	Pereira *et al.* 2009
MG	*T. versicolor*	100	48 h	Casas *et al.* 2009
RB5	*B. subtilis (CotA-laccase)*	85	24 h	Pereira *et al.* 2009
	DeniLite IIS from genetically modified Aspergillus	89		Cristovao *et al.* 2009
Reactive Blue 114	*DeniLite Base from genetically modified Aspergillus*	25	24 h	Tavares *et al.* 2009
Reactive Blue 19	*B. subtilis (CotA-laccase)*	63	24 h	Pereira *et al.* 2009
Reactive Blue 222	*B. subtilis (CotA-laccase)*	18	24 h	Pereira *et al.* 2009
Reactive Blue 69	*T. trogii*	91.1	24 h	Ciullini *et al.* 2008
	T. trogii	95	-	Ciullini *et al.* 2008
Reactive Green 19A	*P. desmolyticum*	100	12 h	Kalme *et al.* 2009
Reactive Orange 107	*T. modesta*	0	70 h	Tauber *et al.* 2008
Reactive Orange 16	*T. modesta*	0	70 h	Tauber *et al.* 2008
Reactive Red 141	*P desmolyticum*	100	16 h	Kalme *et al.* 2009
Reactive Red 195	*B. subtilis (CotA-laccase)*	15	24 h	Pereira *et al.* 2009
Reactive Red 239	*DeniLite Base from genetically modified Aspergillus*	70	24 h	Tavares *et al.* 2009
	DeniLite IIS from genetically modified Aspergillus	78		Cristovao *et al.* 2009
Reactive Red 272	*T. trogii*	15.9	24 h	Ciullini *et al.* 2008
Reactive Red 4	*B. subtilis (CotA-laccase)*	40	24 h	Pereira *et al.* 2009
Reactive Yellow 145	*B. subtilis (CotA-laccase)*	16	24 h	Pereira *et al.* 2009
Reactive Yellow 15	*DeniLite Base from genetically modified Aspergillus*	84	24 h	Tavares *et al.* 2009
	DeniLite IIS from genetically modified Aspergillus	76		Cristovao *et al.* 2009
Reactive Yellow 39	*T. trogii*	0	24 h	Ciullini *et al.* 2008

Reactive Yellow 81	*B. subtilis (CotA-laccase)*		0	24 h	Pereira *et al.* 2009
Remazol Black B	*Ganoderma lucidum*		94	1 h	Murugesan *et al.* 2009
RBBR	*G. lucidum*		35	1 h	Murugesan *et al.* 2009
Sudan Orange G	*B. subtilis (CotA-laccase)*		83	24 h	Pereira *et al.* 2009
Acid Orange 52	*T. versicolor*	Semi-interpenetrating polymer P(AAm-NIPA)/Alg [b,d]	66	6 h	Yamak *et al.* 2009
	T. versicolor	Semi-interpenetrating polymer P(AAm)/Alg [c,d]	48	6 h	Yamak *et al.* 2009
	T. versicolor	Semi-interpenetrating polymer P(AAm-NIPA) [b]	50	6 h	Yamak *et al.* 2009
Reactive Red 120	*R. vernificera*	Poly(GMA/EGDMA) [e]	91	10 h	Arica *et al.* 2009

[a] In case of immobilized laccase

[b] P(AAm-NIPA): poly(acrylamide-N-isopropylacrylamide)

[c] P(AAm): poly(-acrylamide)

[d] Alg: alginate

[e] Poly(GMA/EGDMA): poly(glycidyl methacrylate/ethyleneglycol dimetacrylate)

7 Outline of this thesis

Most of the previous studies reported about ligninolytic enzymes focused on *P. chrysosporium*, *P. ostreatus* and *T. versicolor*. Lately there has been an increasing interest in studying the ligninolytic enzymes of white-rot fungi searching for better lignin-degrading systems. In this work, *Trametes pubescens* was selected for the production of laccase for its further use in the discoloration of dyes in free and immobilized form.

7.1 Hypothesis

"The use of immobilized laccase produced by *T. pubescens* is useful for the treatment of (simulated) textile effluents in lab-scale reactors".

7.2 Objectives

The cultivation of *T. pubescens* was carried out under both SmF and SSF conditions, and in some cases using different agricultural waste materials. Different variables were taken into consideration during cultivation, like carbon sources, organic nitrogen sources and inducers

of laccase activity. Once an efficient culture medium was obtained for the laccase production, cultivation was scaled up to lab-scale reactors.

Crude laccase was used to perform the removal of different dyes in order to establish the capacity of the laccase produced for this type of application. Subsequently, crude laccase was immobilized for the treatment of dyes and simulated textile effluents. Thus, the main objectives of this work can be summarized as follows:

1. Laccase production: cultivation of *T. pubescens* under SmF and SSF conditions, selecting the best culture medium and fermentation technique to scale up the process to lab-scale reactors.
2. Dye removal: use of the crude laccase for the removal of different dyes.
3. Enzyme immobilization: find an effective immobilization method for dye removal.
4. Dye degradation pathway: study the probable degradation pathway of one of the dyes used in the dye removal experiments.
5. Lab-scale reactor: simulated textile effluent treatment with immobilized laccase in lab-scale reactors.

7.3 Structure of the thesis document

The work presented in this thesis has been organized into separate articles that have been published or submitted. In the first article (Chapter 2), the adsorbing capacity of sunflower shells and mandarin peelings was evaluated for the removal of the diazo dye RB5. These materials were selected due to their potential use as a carbon source and/or as a support for *T. pubescens* cultivation.

The cultivation of *T. pubescens* under SmF was investigated in the second article (Chapter 3). Three different carbon sources (i.e. glucose, glycerol and mandarin peelings) were evaluated for the production of laccase under static conditions at flask scale. Also, the effect of different inducers on laccase activity was studied in the basal medium.

The next three publications (Chapters 4-6) remark and evaluate the potential of SSF for laccase production. In this sense, banana skin and sunflower seed shells were assessed as natural substrate/supports. Different inducers were added to the culture medium at different

cultivation stages. Subsequently, the culture medium that led to the highest activity was used for the production of laccase in lab-scale tray bioreactors. Additionally, the dye discoloration ability of the crude laccase was tested.

The immobilization of laccase was studied in the last three publications (Chapters 7-9) where laccase was covalently attached to aluminum oxide pellets. The use of polyelectrolyte multilayers as coating for the protection of the immobilized laccase was evaluated. Different experiments were carried out for the degradation of different dyes and a simulated textile effluent. The degradation pathway of the anthraquinonic dye RBBR was proposed in Chapter 8, whereas the treatment of a simulated textile effluent based on the diazo dye RB5 was studied in different configurations of lab-scale reactors in Chapter 9.

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CHAPTER 2. Sunflower seed shells: a novel and effective low-cost adsorbent for the removal of the diazo dye Reactive Black 5 from aqueous solutions

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Sunflower seed shells: A novel and effective low-cost adsorbent for the removal of the diazo dye Reactive Black 5 from aqueous solutions

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Abstract

In this paper, the potential of two low-cost adsorbents such as sunflower seed shells (SS) and mandarin peelings (MP) in the removal of the synthetic anionic dye Reactive Black 5 (RB5) from aqueous solutions was investigated. SS led to a percentage of dye removal higher than MP (85% and 71% after 210 min, respectively, for an initial RB5 concentration of $50 \, \text{mg L}^{-1}$ and an initial pH of 2.0).

The rate of adsorption followed a pseudo-second-order kinetic model and the intra-particle diffusion was found to be the rate-controlling stage. In addition, the equilibrium data fitted well both the Freundlich and multilayer adsorption isotherm equations indicating the heterogeneity of the adsorbent surface. This was also corroborated by the SEM photographs. On the whole, the results in this study indicated that SS were very attractive materials for removing anionic dyes from dyed effluents.
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Keywords: Adsorption isotherms; Adsorption kinetics; Intra-particle diffusion; Mandarin peelings; Pseudo-second-order kinetics; Reactive Black 5; Sunflower seed shells

1. Introduction

Azo dyes are synthetic organic compounds widely used in textile dyeing, paper printing and other industrial processes such as the manufacture of pharmaceutical drugs, toys and foods including candies. This chemical class of dyes, which is characterised by the presence of at least one azo bond (–N=N–) bearing aromatic rings, dominates the world-wide market of dyestuffs with a share of about 70% [1]. These dyes are highly recalcitrant to conventional wastewater treatment processes. In fact, as much as 90% of reactive azo dyes could remain unaffected after activated sludge treatment [2]. Therefore, alternative methods should be implemented for effective treatment of dyed effluents.

There are five main methods used for the treatment of dye-containing effluents: adsorption, oxidation–ozonation, biological treatment, coagulation–flocculation and membrane process [3]. Adsorption process is noted to be superior to other removal techniques because it is more economical, simpler [4] and it is capable to efficiently treat dyes in a more concentrated form [5].

Activated carbon [6,7] is the most popular and widely used dye adsorbent but it suffers from several drawbacks such as its high price of both manufacturing and regeneration and it is ineffective against disperse and vat dyes. This has impelled the search for cheaper substitutes like the solid wastes generated from the agricultural industry. The accumulation and concentration of pollutants from aqueous solutions by the use of biological materials is named bio-adsorption. The bio-adsorbents are often much more selective than traditional ion-exchange resins and commercial activated carbons and can reduce dye concentration to ppb levels.

The aim of this study was to investigate the potential of two wastes of the agricultural industry such as mandarin peelings (MP) and sunflower seed shells (SS) as bio-adsorbents in the removal of the synthetic diazo dye Reactive Black 5 (RB5) from aqueous solutions. To the best of our knowledge SS have not been used as dye adsorbents before this study.

2. Materials and methods

2.1. Adsorbents

Mandarin (*Citrus reticulata*) peelings (MP) were obtained from a local market, cut into small pieces, washed to remove

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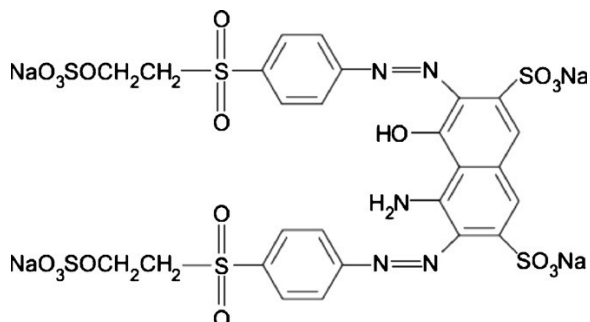*E-mail address:* susana.rodriguez@urv.cat (S.R. Couto).

Fig. 1. Molecular structure of the dye Reactive Black 5 (RB5).

any adhering dirt, air dried at room temperature for 36 h and milled (particle sized about 0.4 mm). The chemical composition of the MP was 32–34% soluble carbohydrates, 12–14% cellulose, 11–14% reducing substances, 9–11% lignin and 1.1–1.3% total nitrogen [8]. MP had a mean pore area of 231.73 μm^2.

Sunflower (*Helianthus annuus*) seed shells (SS) were obtained from a local market, washed to remove any adhering dirt and air dried at room temperature for 36 h. The chemical composition of the SS was 31.9% cellulose, 29.3% lignin and 27.2% pentosan [9]. SS showed a mean surface area of 1.55 cm^2 and a mean pore area of 244.98 μm^2.

2.2. Reagents and solutions

The anionic diazo dye Reactive Black 5 (CI 20505) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and was used without any further purification. The chemical structure of this dye is shown in Fig. 1. Distilled water was used as a solvent. The solutions contained different dye concentrations and pH was adjusted to 2.0 and 4.0 by adding a small amount of 2 M H_2SO_4.

2.3. Adsorption procedure

The adsorption processes were conducted at room temperature by mixing 4 g of milled MP or 4 g of SS in 250 mL-Erlenmeyer flasks containing 100 mL of aqueous solutions at different concentrations of the dye RB5 at pH 2.0 and 4.0 and 100 rpm (Orbital shaker, Ovan, Lovango SL, Spain), according to the experiment. The changes in absorbance were determined spectrophotometrically (Perkin-Elmer, CA, USA) at certain time intervals during the adsorption processes at a wavelength of 597 nm, which corresponds to the maximum adsorption peak of RB5, or measuring the area under the plot from 400 to 750 nm. Decolouration was expressed in terms of percentage. The experiments were performed three times, the experimental error being around 4% (mean value)

2.4. Microscopic examination

Original and dyed SS samples were sputter coated with gold and examined with a Jeol 6400 scanning electron microscope (SEM) at 15 kV, belonging to SRCiT (Scientific and Technical Services) of the Rovira i Virgili University (Tarragona, Spain).

3. Results and discussion

3.1. Effect of the adsorbent on dye removal

As shown in Fig. 2 the adsorption rate of MP was faster than that of SS for the first stages of the process; however SS led to a higher percentage of RB5 removal for 50 min onwards. Thus, in 210 min (equilibrium time) SS showed an adsorption percentage of 85% and MP of 71%. In addition, the SS-based process showed the following advantages over the MP-based one: the resulting aqueous solution was nearly colourless and SS milling was not necessary. This would reduce the cost of the overall process. Therefore, SS were selected for performing the subsequent experiments.

3.2. Effect of initial dye concentration and initial pH on dye adsorption

Adsorption of RB5 from aqueous solutions onto SS was measured at given contact times for three different initial dye concentrations (15, 30 and 50 mg L^{-1}) at an adsorbent dose of 4 g. The effect of the initial dye concentration on the rate of adsorption is shown in Fig. 3A. The percentage of dye adsorbed increased as the initial dye concentration decreased for the first 30 min. Afterwards, the dye adsorption was higher for 30 and 50 mg L^{-1} of initial dye concentration. The time taken to reach equilibrium was equal for all the initial dye concentrations used, which was 210 min. This finding is supported by the study carried out by Poots et al. [10], who reported that the initial concentration of dyes had only a small influence on the time of contact necessary to reach equilibrium in the adsorption study of Telon Blue by peat.

Solution pH affects both aqueous chemistry and surface binding-sites of the adsorbents. The effect of initial pH on adsorption percentages of RB5 was studied at pHs 2 and 4. As shown in Fig. 3A and B, the dye removal ratio was minimal at the initial pH 4, especially for initial RB5 concentrations of 30 and 50 mg L^{-1}. This could be attributed to the adsorption
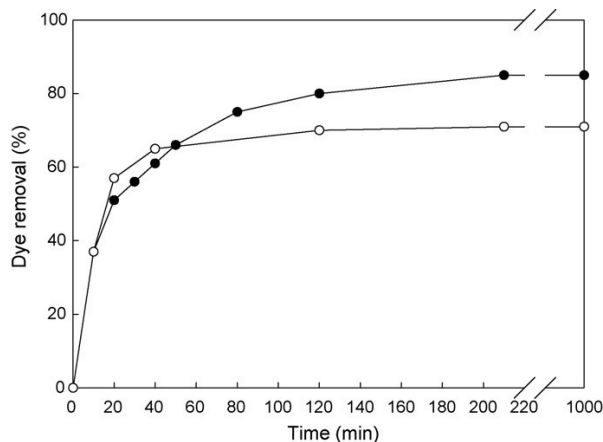

Fig. 2. Percentage of RB5 removal for SS (●) and MP (○) (pH 2; C_0, 50 mg L^{-1}).
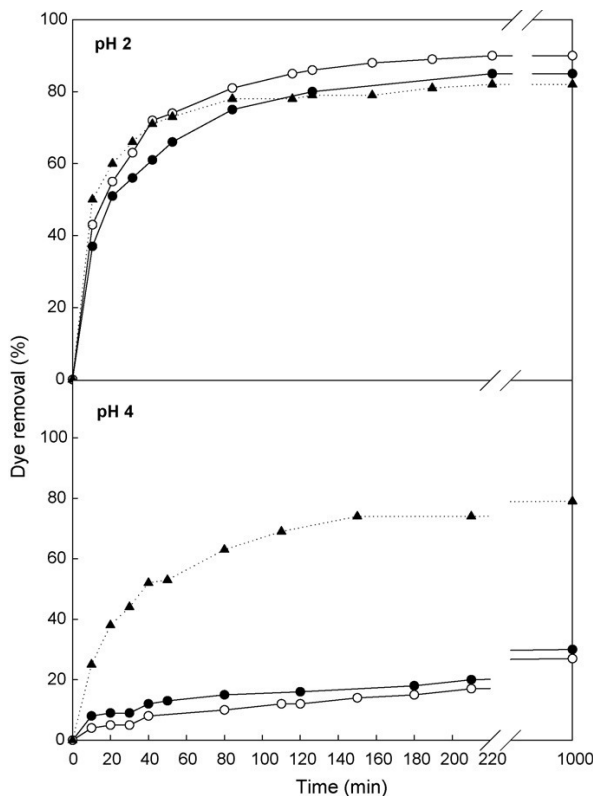
Fig. 3. Percentage of RB5 removal for SS at different pHs: (A) pH 2 and (B) pH 4 and at different initial RB5 concentrations: (●) 50 mg L^{-1}; (○) 30 mg L^{-1}; (▲) 15 mg L^{-1}.

Fig. 4. Pseudo-second-order adsorption kinetics of RB5 on SS at pH 2 and different initial RB5 concentrations: (●) 50 mg L^{-1}; (○) 30 mg L^{-1}; (▲) 15 mg L^{-1}.

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e}$$

where k_2 is the pseudo-second-order rate constant (g mg^{-1} min^{-1}); q_e the quantity of dye adsorbed at equilibrium (mg g^{-1}); q_t the quantity of dye adsorbed at time t (mg g^{-1}) and t is the time (min).

As shown in Fig. 4 the data fitted well with the second order kinetics model ($R^2 > 0.999$). Also, the calculated q_e values agree very well with the experimental data (Table 1). Similar kinetic results were reported in the biosorption of Reactive Black 5 by powdered active carbon (PAC) and fly ash [13].

3.4. Intra-particle diffusion study

The intra-particle diffusion model [14] was applied to describe the dye adsorption. Assuming that the rate is controlled by pore and intra-particle diffusion, in a non-flow-agitated system, the amount adsorbed (q_t) is proportional to the square root of time ($t^{1/2}$), as per the relationship given by Weber and Moris [14].

$$q_t = k_p t^{1/2}$$

where q_t (mg g^{-1}) is the adsorbate uptake at time t (min); k_p (mg g^{-1} min$^{-1/2}$) is the intra-particle diffusion rate constant.

The plot of q_t *versus* $t^{1/2}$ may present multi linearity [15], which indicates that two or more steps occur in the adsorption processes. The first sharper portion is the external surface

of dye molecules onto SS was driven by the electrostatic attraction between adsorbed H$^+$ groups and the anionic dye. Since the SS are negatively charged, the repulsion between the anionic dye and the negatively charged SS surface will be enhanced with an increase in pH, thereby decreasing the binding strength.

Our results are in agreement with those reported by Sivaraj et al. [11], who found that the removal of Acid Violet 17 by orange peels was maximal at pH 2. More recently, Gong et al. [12] also reported that the removal of three anionic dyes by powdered peanut hull was maximal at pH 2.

According to the above results pH 2 was selected for performing the subsequent experiments.

3.3. Kinetics of the adsorption process

Fig. 4A illustrates the adsorption kinetics of RB5. The removal rate of RB5 was very fast during the initial stages of the adsorption processes, especially for an initial dye concentration of 30 mg L^{-1}. However, the adsorption equilibrium was reached at 210 min for all the three concentrations tested. The kinetic data in Fig. 4A were treated with a pseudo-second-order rate equation. The second-order kinetic model [13,14] is expressed as:
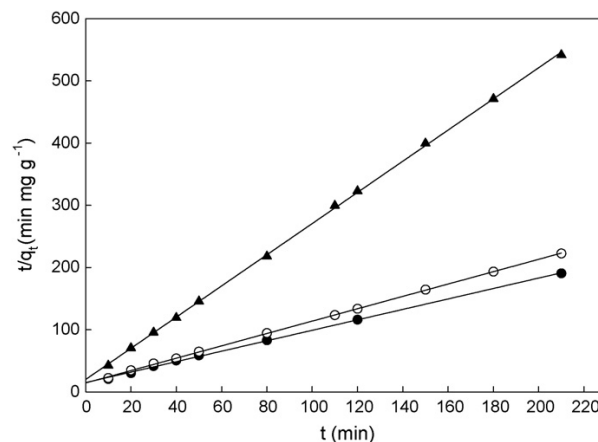
Table 1
Pseudo-second-order adsorption rate constants and calculated and experimental q_e values for different initial RB5 concentrations at pH 2

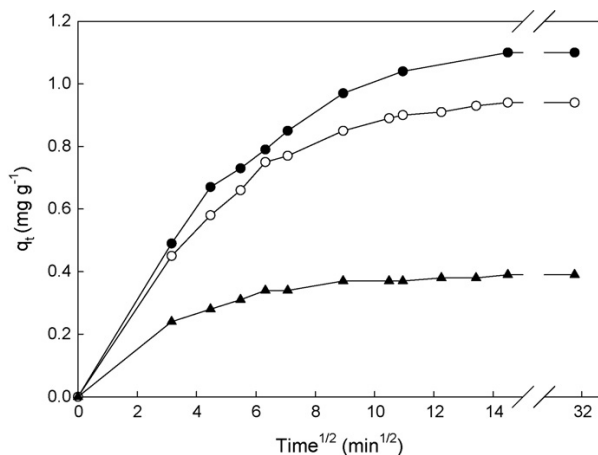| RB5 (mg L^{-1})	K_2 (g mg^{-1} min^{-1})	q_e calc. (mg g^{-1})	q_e exp. (mg g^{-1})	R^2
15	0.314	0.40	0.39	0.9998
30	0.047	1.19	0.94	0.9991
50	0.069	1.01	1.10	0.9998

Fig. 5. Intra-particle diffusion plot for RB5 adsorption by SS at pH 2 and different initial RB5 concentrations: (●) 50 mg L^{-1}; (○) 30 mg L^{-1}; (▲) 15 mg L^{-1}.

adsorption or instantaneous adsorption stage. The second portion is the gradual adsorption stage, where the intra-particle diffusion is rate-controlled. The third portion is the final equilibrium stage, where the intra-particle diffusion starts to slow down due to the extremely low solute concentration in solution [16]. Fig. 5 shows the plot of q_t *versus* $t^{1/2}$. The slope of the line in each stage is written as the rate parameter $k_{p,i}$ ($i = 1$–3). The corresponding rate parameters are listed in Table 2.

Fig. 5 indicates that two linear stages are involved with a rapid diffusion rate in the initial stage at higher RB5 concentrations. The lines pass through the origin, indicating that intra-particle diffusion is the rate-controlling step of the adsorption process. So, predominantly, adsorption of RB5 took place rapidly by external mass transfer followed by intra-particle diffusion. The value of the rate constants for intra-particle transport ($k_{p,1}$, $k_{p,2}$ and $k_{p,3}$) increased with the increase in the initial RB5 concentration (Table 2).

3.5. Adsorption isotherms

The equilibrium data were fitted into Freundlich and multilayer adsorption equations to determine the correlation between the isotherm models and experimental data.

3.5.1. Freundlich isotherm

The Freundlich isotherm is an empirical model that considers heterogeneous adsorptive energies on the adsorbent surface. It

Table 2
Intra-particle diffusion rate constants for different initial RB5 concentrations at pH 2

RB5 (mg L^{-1})	$k_{p,1}$ (g mg^{-1} min$^{-1/2}$)	$k_{p,2}$ (g mg^{-1} min$^{-1/2}$)	$k_{p,3}$ (g mg^{-1} min$^{-1/2}$)
15	0.082	0.0146	0.0039
30	0.1267	0.0515	0.0164
50	0.1416	0.0687	0.024

Table 3
Freundlich constants and calculated and experimental q_e values for different initial RB5 concentrations at pH 2

RB5 (mg L^{-1})	q_e calc. (mg g^{-1})	q_e exp. (mg g^{-1})	k_F	n	R^2
10	0.17030	0.1875	0.0376	0.6061	0.9553
20	0.4826	0.3825			
30	0.6444	0.6100			
40	0.7826	0.8425			
50	0.9532	1.0725			

is expressed by the following equation:

$$q_e = k_F C_e^{1/n}$$

where q_e (mg g^{-1}) is the amount of dye adsorbed at equilibrium, C_e (mg L^{-1}) the dye concentration at equilibrium and k_F and n are the Freundlich constants for the system, which are indicators of adsorption capacity and intensity, respectively [17].

To determine the constants k_F and n the linear form of the equation is used:

$$\ln q_e = \ln k_F + \frac{1}{n} \ln C_e$$

The plot of $\ln q_e$ *versus* $\ln C_e$ is employed to generate the intercept K_F and the slope $1/n$. The values of K_F, n, the experimental and the calculated q_e (mg g^{-1}) values and the linear regression correlation (R^2) for Freundlich are given in Table 3. The value of $1/n > 1$ indicates that saturation was not attained [18]. The fit of the data to the Freundlich equation indicates the heterogeneity of the adsorbent surface.

3.5.2. Multilayer adsorption isotherm

The multilayer adsorption isotherm model described by Wang et al. [19] was also used to describe the experimental adsorption data. The model is expressed by the following equation:

$$q_e = \frac{Q_m K_1 C_e}{(1 - K_2 C_e)[1 + (K_1 - K_2)C_e]}$$

where q_e (mg g^{-1}) is the amount of dye adsorbed at equilibrium, Q_m (mg g^{-1}) the maximum monolayer adsorption capacity, C_e (mg L^{-1}) the dye concentration at equilibrium, K_1 the equilibrium constant for the first layer adsorption and K_2 is the equilibrium constant for multilayer adsorption.

Writing the above equation in another way and regrouping terms:

$$\frac{C_e}{q_e} = \frac{C_e^2(K_2^2 - K_1 K_2) + C_e(K_1 - 2K_2) + 1}{Q_m K_1}$$

Plotting C_e/q_e *versus* C_e and adjusting to a second-order polynomial equation Q_m, K_1 and K_2 are obtained. The values of Q_m, K_1, K_2, the experimental and the calculated q_e (mg g^{-1}) values and the linear regression correlation (R^2) are given in Table 4. The calculated q_e values agree quite well with the experimental ones. It can be seen that the multilayer adsorption constant is much lower than that of the first layer.

Table 4
Multilayer model constants and calculated and experimental q_e values for different initial RB5 concentrations at pH 2

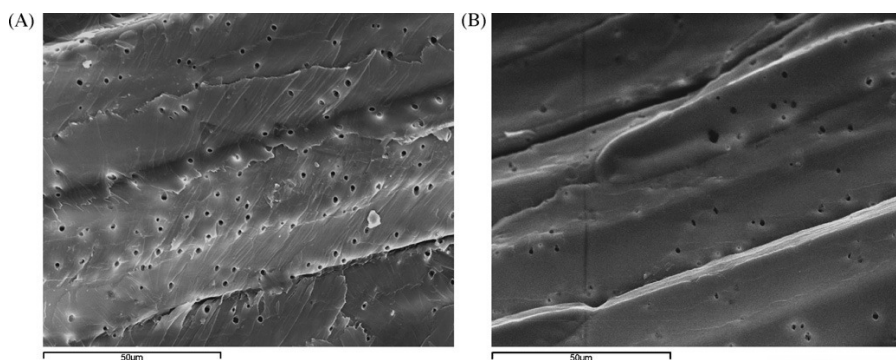| RB5 (mg L^{-1})	q_e calc. (mg g^{-1})	q_e exp. (mg g^{-1})	Q_m (mg g^{-1})	k_1 (L mg^{-1})	k_2 (L mg^{-1})	R^2
10	0.1852	0.1875	0.873	0.8977	0.00688	0.9425
20	0.4133	0.3825				
30	0.5743	0.6100				
40	0.7693	0.8425				
50	1.1676	1.0725				

Fig. 6. SEM photographs of the SS: (A) original and (B) at the end of the adsorption process.

3.6. SEM photographs

Fig. 6A and B show the SEM photographs of the original and the dyed SS, respectively. SS has heterogeneous surface and macro-pores as seen from its SEM photograph. After RB5 adsorption, SS presents a smoother surface because of the packing of the dye what makes surface irregularities less sharp.

4. Conclusions

Sunflower seeds shells, which are discarded as waste material from the food industry, are promising bio-adsorbents for the removal of dyes from aqueous solutions. Hence, in the present study their potential to adsorb an anionic dye in extremely acidic conditions (pH 2) was shown. The dyed SS have potential to be used as substrates in solid state fermentation (SSF). Thus, degradation of the adsorbed dye takes place whilst simultaneously enriching the protein content of the substrate by the presence of fungal biomass. The fermented mass can be utilised as a fertiliser or a soil conditioner. Another alternative is to burn the dyed SS to generate power. The utilisation of this waste would contribute to the so-called green biotechnology.

Acknowledgements

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CHAPTER 3. Mandarin peelings: the best carbon source to produce laccase by static cultures of *Trametes pubescens*

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Mandarin peelings: The best carbon source to produce laccase by static cultures of *Trametes pubescens*

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Abstract

In the present study, we investigated the effect of different carbon sources (glucose, glycerol and ground mandarin peelings) on laccase production by *Trametes pubescens* grown on stainless steel sponges under static conditions. The cultures with ground mandarin peelings gave the highest laccase activities, showing values of about $100 \, \text{U} \, \text{l}^{-1}$. This is a very interesting result, since mandarin peelings are common agricultural wastes in some regions such as Mediterranean and Asiatic countries. Therefore, their reutilisation, besides reducing medium cost, also helps to solve the pollution problems caused by their disposal.

Also, we studied the effect of supplementing the culture medium with different potential laccase-inducing compounds (ABTS, Tween 20, soya oil, Malaquite Green, Cu^{2+}, tannic acid) on laccase production. Soya oil was the best inducer of laccase activities, attaining values 4-fold higher than those obtained in the reference cultures.
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Keywords: Agricultural wastes; Culture medium optimisation; Inducers; Static cultures; Stainless steel sponges; Submerged fermentation

1. Introduction

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are particularly abundant in white-rot fungi, which are the only living organisms able to degrade the whole wood components (Kirk and Fenn, 1982). Accordingly, this type of fungi has been the subject of extensive research as potential laccase producers. In particular, the genus *Trametes* is assumed to be one of the most efficient lignin degraders. Among them, *Trametes pubescens* has been described as a promising laccase producer (Galhaup and Haltrich, 2001).

Laccases catalyse the oxidation of a great variety of phenolic and inorganic compounds (Bourbonnais and Paice, 1997), which make them very useful for biotechnological purposes. However, the application of these oxidative enzymes to biotechnological processes requires the production of high amounts of enzyme at low cost. Therefore,

research in this area is oriented towards the search for new efficient production systems.

A good strategy to increase the productivity of the laccase fermentation process is the optimisation of the fermentation medium and then enhancing laccase activity by using inducers. In particular, the selection of an appropriate carbon source is crucial in the development of an efficient and economic process. Thus, the aim of this study was to evaluate the significance of various carbon sources (glucose, glycerol and ground mandarin peelings) for laccase production by *T. pubescens* grown on stainless steel sponges under static conditions. The effect of different laccase-inducing compounds on laccase activity was also assessed.

2. Materials and methods

2.1. Microorganism

T. pubescens (CBS 696.94) was maintained on malt extract agar (MEA) plates at 4 °C and sub-cultured every three months.

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2.2. Support

Stainless steel sponge (Mapa Spontex Ibérica SA, Spain) was used as an immobilisation support. The sponges were pre-treated by boiling for 10 min and washing thoroughly three times with distilled water. Thereafter, they were dried overnight at room temperature. Prior to use, the supports were autoclaved at 121 °C for 20 min.

2.3. Culture conditions

The composition of the basal medium was prepared according to Rodríguez Couto et al. (2006). To investigate the effect of the carbon source on laccase production by *T. pubescens*, three carbon sources were tested: glucose (10 g l^{-1}), glycerol (10 g l^{-1}) and ground mandarin (*Citrus reticulata*) peelings (30 g l^{-1}). The chemical composition of the mandarin peelings is 1.1–1.3% total nitrogen, 11–14% reducing substances, 32–34% soluble carbohydrates, 12–14% cellulose and 9–11% lignin (Stajić et al., 2006).

The effect of different laccase-inducing compounds on laccase production was determined in the cultures with glucose. Six potential inducers of the laccase activity were assayed: 2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) (1 mM), Tween 20 (1 g l^{-1}), soya oil (1% v/v), Malaquite Green (10 μM), Cu^{2+} (1 and 2 mM) and tannic acid (50 and 100 μM). They were added at the time of inoculation. A reference culture with no inducer addition was also done for comparison.

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 ml) containing a stainless steel sponge (weight: 4.0 g; diameter: 4.5 cm) and 100 ml of culture medium. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter, 7.0 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculum. The Erlenmeyer flasks were incubated statically under an air atmosphere at 30 °C and in complete darkness (Fig. 1).
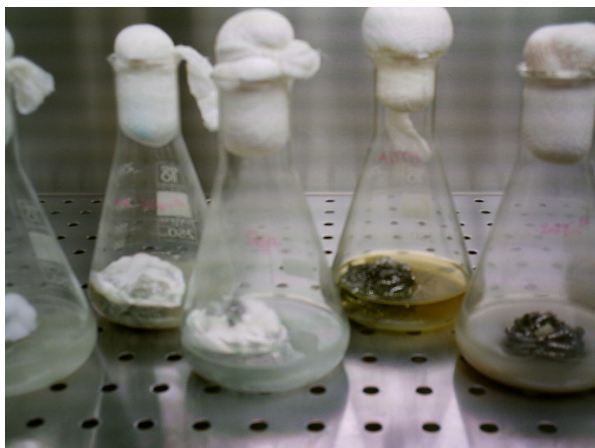

Fig. 1. Photograph of static cultures of *T. pubescens* immobilised on stainless steel sponge.

2.4. Analytical determinations

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. (1990) with ABTS as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min. The activities were expressed in U l^{-1}.

2.5. Microscopic examination

Samples of stainless steel sponge were fixed with 6% glutaraldehyde in phosphate buffer 0.1 M for 4 h. Afterwards, the samples were washed twice with phosphate buffer 0.1 mM for 15 min at 4 °C. Then, the samples were post-fixed with osmium tetroxide 0.1 M at 4 °C. Following fixation, the samples were washed twice with phosphate buffer 0.1 mM for 15 min. After that, the samples were dehydrated through an ethanol-amylacetate series to pure amylacetate and critical point dried using CO_2 as the transition liquid. The dried samples were mounted on aluminum stubs, sputter coated with gold (20 nm) and examined with a Jeol 6400 scanning electron microscope (SEM) at 15 kV, belonging to SRCiT (Scientific and Technical Services) of the Rovira i Virgili University (Tarragona, Spain).

3. Results and discussion

Laccase was produced by static cultures of *T. pubescens* immobilised on stainless steel sponge. Stainless steel sponges were selected, from the different immobilisation materials described in the literature, to perform this study due to several reasons: this material has shown to be very suitable for laccase production by the white-rot fungus *Trametes hirsuta* (Rodríguez Couto et al., 2004), its loose structure offers a large area for attachment and growth of the mycelium together with appropriate oxygen and nutrients diffusion and it is made of an inert material, which means that it is not susceptible to degradation during the fermentation process.

3.1. Effect of the carbon source on laccase production

Three carbon sources were tested: glucose, glycerol and ground mandarin peelings. In the cultures with glucose laccase first appeared on the 10th day (22 U l^{-1}), and then, it increased reaching a maximum value of 51 U l^{-1} on the 15th day of cultivation (Fig. 2). Afterwards, laccase activity decreased until the end of cultivation.

In the cultures with glycerol, laccase production began on the 5th day (15 U l^{-1}) and from here onwards it increased attaining a maximum value of 43 U l^{-1} on the 8th day. Then, it decreased and from the 14th day onwards it increased again peaking on the 16th day (89 U l^{-1}) (Fig. 2). The laccase activities obtained are about 1.5-fold higher than those attained in the cultures with glucose. These results are in disagreement with those found by Hou et al. (2004) in static cultures of *Pleurotus ostreatus*,
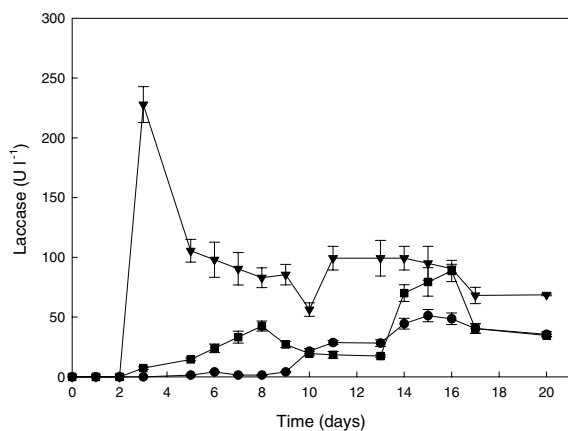
Fig. 2. Laccase production by static cultures of *T. pubescens* immobilised on stainless steel sponge using different carbon sources: (▼) mandarin peelings; (●) glucose; (■) glycerol.
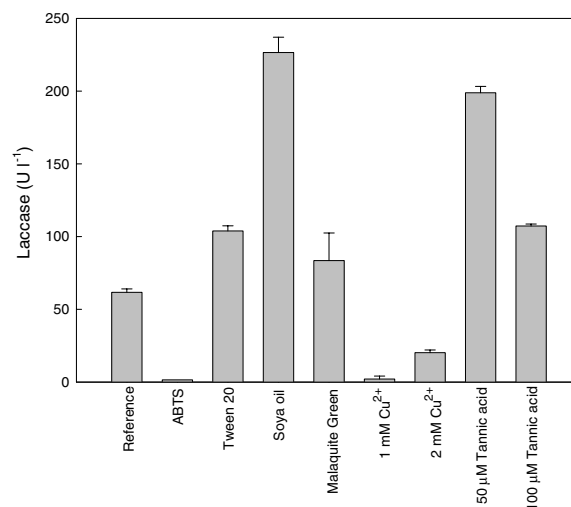

Fig. 3. Maximum laccase activities obtained in static cultures of *T. pubescens* immobilised on stainless steel sponge supplemented with different inducers.

in which glucose and glycerol led to similar laccase activities. Undoubtedly, laccase production is dependent on the microbial taxa employed.

As regards the cultures with ground mandarin peelings, laccase production started on the 3rd day ($228 \, U \, l^{-1}$), which coincided with its maximum value, then it abruptly decreased and from the 5th day onwards it was maintained at values around $100 \, U \, l^{-1}$ (Fig. 2). These values are higher than those obtained in the cultures with both glucose and glycerol (12% and 96% higher, respectively).

The highest activities produced in the cultures with ground mandarin peelings are likely due to their content in water-soluble aromatic compounds (flavones and flavonoids) capable of inducing or stimulating the biosynthesis of ligninolytic enzymes. Also, the content in cellulose of mandarin peelings acted as an activator of laccase activity (Srinivasan et al., 1995).

Our results are in agreement with those recently reported by Stajić et al. (2006), Elisashvili et al. (2006) and Mikiashvili et al. (2005). In addition, this result is very interesting, since mandarin peelings are common agricul-

tural wastes in several countries (Spain, Italy, China, Morocco, Turkey, Japan and EEUU) and their reutilisation would help to solve the pollution problems caused by their disposal. Moreover, the use of such wastes supposes a great advantage from the economical point of view, since it highly reduces the cost of the fermentation medium. Therefore the utilisation of such wastes besides to promote high laccase activity levels, contributes to diminish the production costs.

3.2. Effect of different inducers on laccase production

Extracellular laccases are constitutively produced in small amounts. However, their production can be greatly stimulated by the presence of a wide variety of inducing substances (Bollag and Leonowicz, 1984). Hence, in order to enhance laccase activities the addition of different inducers to cultures of *T. pubescens* immobilised on stainless steel sponge was investigated. Among the different laccase
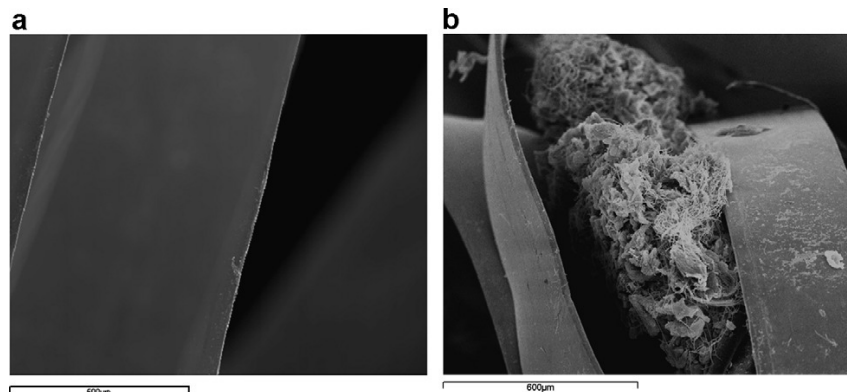

Fig. 4. SEM microphotographs of stainless steel sponge: (a) without fungus; (b) with fungus.

inducers found in the literature ABTS,Tween 20, soya oil, Malaquite Green, Cu^{2+} and tannic acid were selected to perform the present study. As shown in Fig. 3 soya oil-supplemented cultures led to the highest activities followed by 50 μM tannic acid-supplemented ones. These values are 4-fold and 3-fold higher, respectively, than that obtained by the reference cultures (Fig. 3). These results are very interesting since the above-mentioned compounds are non-toxic contrary to those commonly used to stimulate laccase synthesis (Revankar and Lele, 2006). It was observed that the addition of copper to the culture medium highly inhibited the fungal growth. This indicates that *T. pubescens* is extremely sensitive to this metal at the concentrations used here.

3.3. Microscopic examination

The most important characteristics that influence adhesive behaviour of filamentous fungi to the support are hydrophobicity and surface charge. Fig. 4 shows SEM microphotographs of stainless steel sponge without (a) and with fungus (b). It can be observed that the fungus grew well attached to the stainless steel fibres of the sponge. This is due to the high hydrophobicity of the stainless steel sponge (Sinde and Carballo, 2000), which eases the attachment of the fungus to the carrier (Kotrba et al., 2002). In addition, the loose structure of the sponges allows a suitable diffusion of oxygen and nutrients into the cultures.

4. Conclusions

The results obtained in this paper clearly showed the enormous potential of ground mandarin peelings as a carbon source for the production of laccase by static cultures of *T. pubescens*. Furthermore, the use of mandarin peelings as a carbon source besides to promote high laccase activities makes the process more economical.

On the other hand, the addition of soya oil to glucose cultures of *T. pubescens* greatly improved laccase activities.

In view of these encouraging results more studies in order to determine the optimal operation conditions (C/N ratio, optimal concentration of inducers, time point of inducer addition, synergistic effects, etc.) are underway in our laboratory.

Acknowledgements

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CHAPTER 4. Potential of solid-state fermentation for laccase production

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Potential of solid-state fermentation for laccase production

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Solid-state fermentation (SSF) processes involve the growth of microorganisms (typically fungi) on a solid material in the absence or near absence of free-flowing water. The wide range of solid materials used in SSF can be classified into two great categories: inert (synthetic materials) and non-inert (organic materials). The former only acts as an attachment place for the fungus, whereas the latter also functions as a source of nutrients, due to which it is called support-substrate. Utilisation of agro-industrial residues as support-substrates in SSF processes provides an alternative avenue and value-addition to these otherwise under- or non-utilised residues. SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi, since they reproduce the natural living conditions of such fungi. In the present chapter the production of laccase enzyme by white-rot fungi under SSF is described.

Keywords: solid-state fermentation; laccase; agro-wastes; filamentous fungi; bioprocesses

1. Introduction

Enzyme production is an increasing field of Biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the use of the solid-state fermentation (SSF) technique to produce several enzymes. SSF is known from ancient times in Asian countries thus, SSF is used, for example, in the production of koji and sake. However, in western countries SSF was nearly ignored after 1940. This was due to fact that SmF had become a model technology for production of any compound by fermentation as a result of the development of penicillin. Table 1 gives a brief summary of the historical evolution of SSF [1]. This technique reproduces the natural microbiological processes like composting and ensiling. This natural process can be utilised in industrial applications in a controlled way to produce a desired product. In addition, it presents several advantages over the traditionally employed SmF (Table 2) [2].

A direct comparison between SSF and SmF cultivation techniques is difficult to make because the two processes are quite different. Studies on fungal enzyme production in SSF have shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes with a higher temperature or pH stability. Also, the fermentation time is shorter and the degradation of the produced enzymes by undesirable proteases is minimised [3]. Castilho et al (2000) [4] performed a comparative economic analysis of SSF and SmF processes for the production of lipases by the ascomycete *Penicillium restrictum*. They found that for a plant producing 100 m^3 lipase concentrate per year, the process based on SmF needed a total capital investment 78% higher than the one based on SSF and its product had a unitary cost 68% higher than the product market price. In addition, Viniegra-González et al. (2003) [5] compared the productivity of three fungal enzymes, invertase, pectinase and tannase, using SSF and SmF techniques. They reported that the higher titres found in SSF were due to SSF cultivation works as a fed-batch culture with fast oxygenation but slow sugar supply and, in addition, SSF has the added advantage of being a static process without mechanical energy expenditures. Moreover, recently Roy et al. (2006) [6] reported that SSF was a better treatment method for rubber biodegradation than SmF.

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Despite the numerous both processing and biological advantages that SSF offers over SmF [3], there are few designs available in the literature for bioreactors operating in SSF conditions. This is principally due to several problems encountered in the control of different parameters such as pH, temperature, aeration and oxygen transfer, moisture and agitation [7]. SSF lacks the robust control mechanisms that are usually associated with SmF. Control of the environment within the bioreactors is also difficult to achieve, particularly temperature and moisture. Several authors have reported remarkable results in terms of yield or quality when cultivating microorganisms on solid substrates at laboratory-scale bioreactors. However, when these processes are reproduced at larger scales results are mostly disappointing. Scaling-up of SSF processes is difficult and unreliable [8-12]. This can be attributed to the radically different growing conditions microorganisms find in a large-scale reactor. At lab-scale, conditions such as temperature, water activity and pH can easily be maintained homogeneous and constant at optimum levels throughout the fermentation processes [10–12]. In contrast, bed heterogeneity in large-scale SSF bioreactors is inevitable as simulations with a distributed parameter model [13] and experiments with a pilot-scale SSF bioreactor [14] have shown. This is due to the low heat and mass transfer rates characteristic of the solid porous bed and that stirring is limited to avoid damaging the microorganisms [8, 10–12, 15]. Effective control strategies have shown to reduce time and space variability in growing conditions in large-scale SSF bioreactors [16, 17]. However, process complexity and the lack of reliable and affordable instrumentation make designing control strategies difficult. Computer simulation, on the other hand, is an alternative way to test new automatic control designs, since even hundreds of simulation runs are fast and relatively inexpensive. Nevertheless, only simulations with complex models provide credible results. Recently, Fernández-Fernández and Pérez-Correa (2007) [18] developed a complex model for a packed-bed solid-state bioreactor, which will be useful in the design of effective control systems for intermittently mixed SSF bioreactors. Also, Sahir et al. (2007) [19] developed a mathematical model for a packed-bed solid-state bioreactor utilising the *N*-tanks in series approach which reduced computational complexities, thus, facilitating the design of packed-bed SSF bioreactors.

Table 1. History and development of SSF [1]

Period	Development
2,600 BC*	Bread making by Egyptians
BC in Asia (recorded history 1,000 BP**)	Cheese making by *Penicillium roqueforti*
2,500 BP	Fish fermentation/preservation with sugar, starch, salts, etc Koji process
7th Century	Koji process from China to Japan by Buddhist priests
18th Century	Vinegar from pomace Gallic acid used in tanning, printing, etc
1860-1900	Sewage treatment
1900-1920	Fungal enzymes (mainly amylases), kojic acid
1920-1940	Fungal enzymes, gluconic acid, rotary drum fermenter, citric acid
1940-1950	Fantastic development in fermentation industry. Penicillin production by SSF and SmF
1950-1960	Steroid transformation by fungal cultures
1960-1980	Production of mycotoxins, protein enriched feed
1980-present	Various other products like alcohol, gibberellic acid

*BC, before Christ
**BP, before present

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Table 2. Advantages and disadvantages of SSF over SmF (extracted from Pérez-Guerra et al., 2003 [2])

Advantages	Disadvantages
Similar or higher yields than those obtained in the corresponding submerged cultures	Only microorganisms that can grow at low moisture levels can be used
The low availability of water reduces the possibilities of contamination by bacteria and yeast. This allows working in aseptic conditions in some cases	Usually the substrates require pre-treatment (size reduction by grinding, rasping or chopping, homogenisation, physical, chemical or enzymatic hydrolysis, cooking or vapour treatment)
Similar environment conditions to those of the natural habitats for fungi which constitute the main group of microorganisms used in SSF	Biomass determination is very difficult
Higher levels of aeration, especially adequate in those processes demanding an intensive oxidative metabolism	The solid nature of the substrate causes problems in the monitoring of the process parameters (pH, moisture content, and substrate, oxygen and biomass concentration)
The inoculation with spores (in those processes that involve fungi) facilitates their uniform dispersion through the medium	Agitation may be very difficult. For this reason static conditions are preferred
Culture media are often quite simple. The substrate usually provides all the nutrients necessary for growth	Frequent need of high inoculum volumes
Simple design reactors with few spatial requirements can be used due to the concentrated nature of the substrates	Many important basic scientific and engineering aspects are yet poor characterised. Information about the design and operation of reactors on a large scale is scarce
Low energetic requirements (in some cases autoclaving or vapour treatment, mechanical agitation and aeration are not necessary)	Possibility of contamination by undesirable fungi
Small volumes of polluting effluents. Fewer requirements of dissolvents are necessary for product extraction due to its high concentration	The removal of metabolic heat generated during growth may be very difficult
The low moisture availability may favour the production of specific compounds that may not be produced or may be poorly produced in SmF	Extracts containing products obtained by leaching of fermented solids are often of viscous nature
In some cases, the products obtained have slightly different properties (e.g. more thermotolerance) when produced in SSF in comparison to SmF	Mass transfer limited to diffusion
Due to the concentrated nature of the substrate, smaller reactors in SSF with respect to SmF can be used to hold the same amounts of substrate	In some SSF, aeration can be difficult due to the high solid concentration
	Spores have longer lag times due to the need for germination
	Cultivation times are longer than in SmF

2. General aspects of SSF

SSF is a microbial process occurring mostly on the surface of solid materials, which can absorb or contain water, in the presence or absence of soluble nutrients [20]. SSF comprises two very different modes [21]. In the first one, a divided and humidified solid (organic material) acts as both support and nutrient source and the process essentially occurs in the absence of free water [22-24] (Fig. 1). In the second mode, a nutritionally inert solid (synthetic material), which exclusively acts as a support, is

soaked in a nutrient solution (Fig. 2). This model is less used, but it reports some advantages. The use of a defined liquid medium and an inert support with a homogenous physical structure improves controlling and monitoring the process and the reproducibility of fermentations. However, the use of inert supports presents economical disadvantages [25].

Fig. 1. Solid-state cultures of the white-rot fungus *T. pubescens* grown on different natural supports: mandarin peelings (left), banana skins (center) and wheat bran flakes (right)

Fig. 2. The white-rot fungus *T. hirsuta* grown on cubes of nylon sponge (inert support) under SSF conditions

In both cases, the success of the process is directly related to the physical characteristics of the support (particle size, shape, porosity, consistency), which favour both gas and nutrient diffusion and the attachment of the microorganisms [26]. Generally, smaller substrate particles provide a larger surface area for microbial colonisation but if they are too small may result in substrate agglomeration as well as poor growth. In contrast, larger particles provide better aeration but a limited surface for microbial colonisation. Therefore, a compromised particle size must be selected for each particular process [27]. Availability and cost are also criteria of great importance. In the case of the organic materials the chemical composition also plays an important role [28]. Thus, Rodríguez Couto et al. (2003) [29] studied the lignin peroxidase (LiP) production by the white-rot fungus *Phanerochaete chrysosporium* under solid-state conditions utilising organic materials with different lignin content. They found that the materials with higher lignin content led to the higher LiP activities. This stimulating effect of lignin was also observed for laccase production by the white-rot fungus *Trametes hirsuta* [30]. More recently, Osma et al. (2007) [31] reported that the white-rot fungus *Trametes pubescens* grown on banana skin produced laccase with high ability to decolourise synthetic dyes. Also, Rodríguez Couto (2007) [32] found that laccase produced by *T. hirsuta* grown on paper cuttings was able to decolourise synthetic dyes at alkaline pHs. This illustrates the enormous importance of selecting a suitable support-substrate for each particular purpose.

SSF processes have shown to be particularly suitable for the production of enzymes by filamentous fungi [33, 34], since they reproduce the natural living conditions of such fungi [27] due to which they may be more capable of producing certain enzymes with high productivity in comparison to SmF. Fig. 3

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shows a photograph of the white-rot fungi *T. pubescens* and *T. hirsuta* as grow in nature. In addition, the morphology of filamentous fungi allow them colonising and penetrating the solid support-substrates in search for nutrients. Fig. 4 shows scanning electron microscopy (SEM) microphotographs of banana skin with and without fungus. It can be observed that the fungus grew well attached to the skin. The application of agro-industrial residues in SSF bio-processes not only provides an alternative substrate but reduces the pollution problems caused by their accumulation.

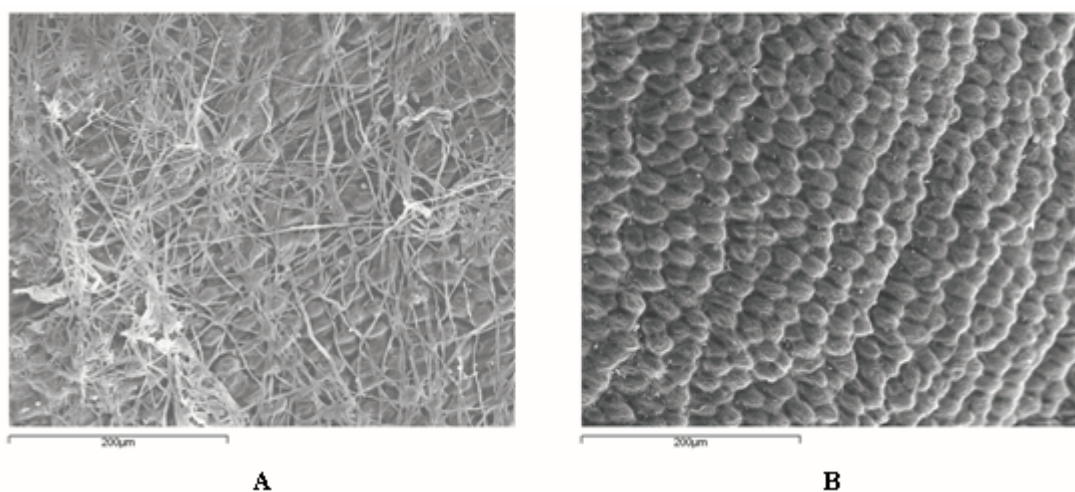**Fig. 3.** The white-rot fungi *T. pubescens* (A) and *T. hirsuta* (B) as grow in nature

Fig 4. SEM micropotographs of banana skin: A (with fungus); B (without fungus) (extracted from Osma et al., (2007) [31])

3. Example of SSF application: laccase production

White-rot fungi are the only organisms able to degrade the whole wood components [35] due to the secretion of an extracellular ligninolytic complex during their secondary metabolism in response to nutrient limitation. The main components of this ligninolytic complex consist of a family of peroxidases named lignin peroxidases (LiPs) and manganese-dependent peroxidases (MnPs) and a family of multicopper oxidases named laccases.

To study the ligninolytic ability of white-rot fungi the oxidation of model compounds such as 2,2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) and the polymeric dye Poly R-478 on low-nutrient agar plates is performed. Positive ligninolytic fungi turned ABTS from light green to dark green and Poly R-478 from purple to yellow. Fig. 5 and Fig. 6 show the ligninolytic ability of *T. pubescens* grown on ABTS and PolyR-478 agar plates, respectively.
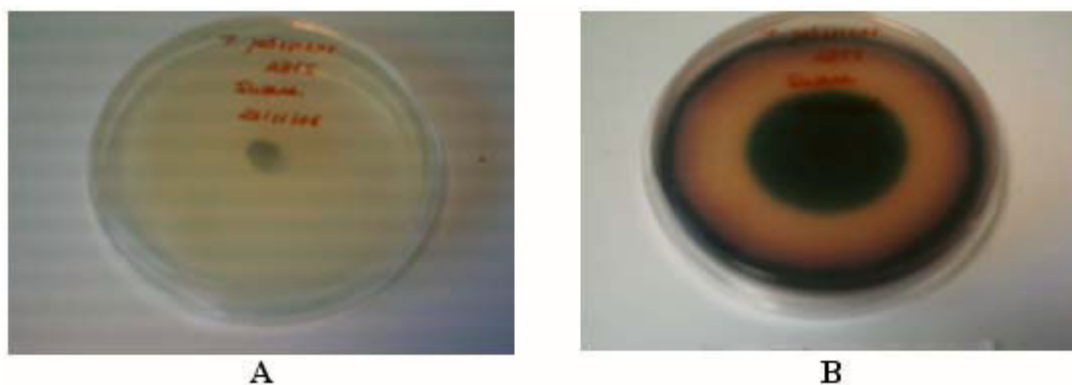

Fig. 5. ABTS oxidation by *T. pubescens* on agar plates, A: day 0, B: day 8
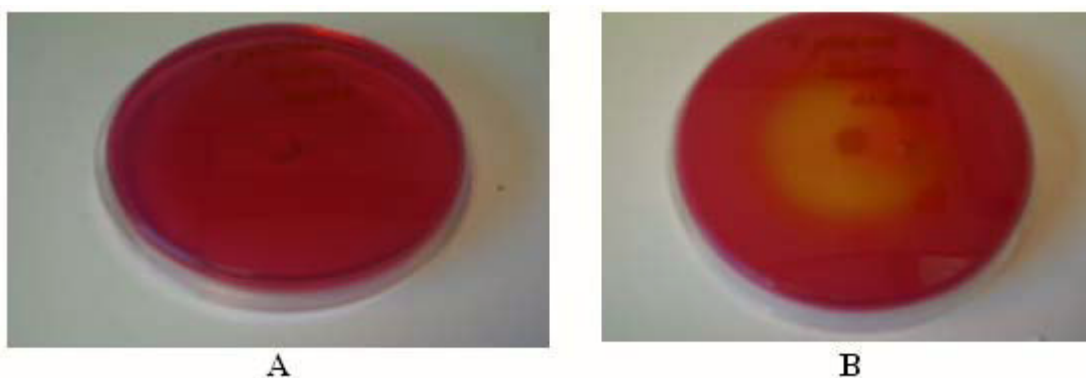

Fig. 6. Poly R-478 oxidation by *T. pubescens* on agar plates, A: day 0, B: day 11

Laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are particularly abundant in white-rot fungi. Laccases catalyse the oxidation of both phenolic and non-phenolic compounds [36] and are able to mineralise a wide range of synthetic dyes [37-40]. Fig. 7 shows a typical laccase-catalysed reaction where a diphenol is oxidised to form a free radical, which can further undergo a second enzymatic catalysis to form a quinone,
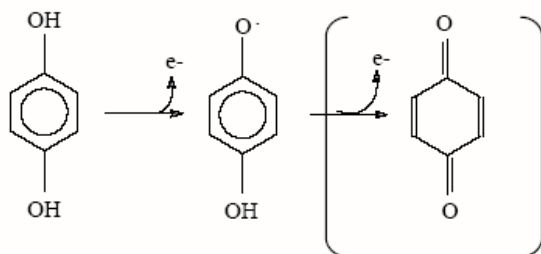

Fig. 7. Typical laccase-catalysed reaction for a diphenol (extracted from Tavares (2006) [41])

Laccases have been subject of intensive research in the last decades because they have the following properties: broad substrate specificity [42], do not need the addition or synthesis of a low molecular weight cofactor, as their cosubstrate – oxygen – is usually present in their environment, most laccases are extracellular enzymes, making the purification procedures very easy and they generally exhibit a considerable level of stability in the extracellular environment. Such characteristics make laccases very suitable for their application to several bioprocesses such as biopulping, biobleaching and the treatment of industrial wastewater.

The application of laccases to the above-mentioned processes requires the production of large amounts of enzyme at low cost. Therefore, research in this area is oriented towards the search for efficient production systems. A good strategy for this purpose is the production of laccase by SSF using agro-industrial wastes as a support-substrate. The food, agricultural and forestry industries produce large volumes of wastes annually world-wide which cause a serious disposal problem. In addition, the reutilisation of biological wastes shows a great interest, since due to legislation and environmental reasons the industry is more and more forced to find an alternative use(s) for its residual matter. Most of such wastes are rich in soluble carbohydrates and also contain inducers of laccase synthesis, ensuring an efficient production of laccase [43-46]. Furthermore, agro-wastes have shown to produce higher laccase activities than inert supports for the same fungal strain and culture conditions [47-50]. Table 3 reports the laccase production by several white-rot fungi grown on different natural supports under solid-state conditions.

Table 3. - Laccase production by different white-rot fungi grown on different natural supports under SSF conditions

Support	Microorganism	Reference
Sugarbeet bagasse	*Trametes versicolor*	[51]
Wheat straw	*Phlebia radiata*	[52]
Ballico seed	*Botryosphaeria sp.*	[53]
Corn stalks	*Lentinus edodes* strain CS-495	[54]
Straw	*Pleurotus sp.*	[55]
Cotton wastes	*Pleurotus ostreatus, Pleurotus cystidiosus, Pleurotus pulmonarius, Pholiota nameko*	[56]
Barley bran	*P. chrysoporium*	[57]
Cotton stalks	*P. chrysosporium, Funalia trogii*	[58]
Corncob	*P. chrysoporium*	[59]
Sawdust, grapewine cuttings	*Coriolus hirsutus, Daedaleopsis confragosa, Marasmius allaceus, P. chrysosporium*	[60]
Wheat straw	*P. ostreatus*	[61]
Corncob	*P. chrysosporium, P. radiata*	[62]
Wheat bran, wheat straw	*P. pulmonarius*	[63]
Neem hull, wheat bran, sugarcane bagasse	*P. ostreatus, P. chrysosporium*	[64]
Wheat straw, barley straw, wood shavings, barley bran	*T. versicolor*	[47]
Barley bran, apple peelings, orange peelings, potato peelings	*T. hirsuta*	[48]
Canola roots	*Cyathus olla*	[65]

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Eucalyptus grandis	*Ceriporiopsis subvermispora*	[66]
Grape seeds, barley bran	*T. hirsuta*	[30]
Wheat straw	*Fomes sclerodermeus*	[67]
Banana waste	*P. ostreatus, Pleurotus sajor-caju*	[43]
Barley bran	*T. versicolor*	[68]
Corncob	*P. pulmonaris*	[69]
Barley bran	*T. hirsuta, T. versicolor*	[49]
Chestnut shell, barley bran	*Coriolopsis rigida*	[70]
Coconut flesh	*T. hirsuta*	[61]
Kiwi fruit	*T. hirsuta*	[45]
Wheat bran flakes	*T. pubescens*	[72]
Groundnut seeds Groundnut shells	*T. hirsuta*	[73]
Grape seeds	*T. hirsuta*	[50]
Rubberwood sawdust, oil palm frond parenchyma tissue, sago hampas	*Pycnoporus sanguineus*	[74]
Banana skin	*T. pubescens*	[31]
Paper cuttings	*T. hirsuta*	[32]
Orange peelings	*T. hirsuta*	[75]

Given the potential applications of laccases and the need for the development of economical methods for improving laccase production from fungi with an overall aim of reducing the cost of the industrial processes, the use of SSF, especially using agro-wastes as a support-substrate, is an appalling alternative. However, there are few designs available in the literature for bioreactors operating in solid-state conditions, especially at a large-scale due to the reasons stated in the introduction section. Moreover, the use of agro-wastes as a support presents additional problems, which are not found operating with inert supports, such as support degradation and/or support accretion may occur during the fermentation process. This would cause mass and oxygen restrictions into the reactor bed hampering its proper performance. Therefore, advances in the design of SSF bioreactors are needed for the industrial explotation of SSF.

4. Conclusions

SSF is a very promising cultivation technique for the production of industrially-relevant enzymes such as laccases, especially utilising agro-wastes as support-substrates. The scarcity of bioreactor designs to perform solid-state processes together with the advantages offered by such processes promote the necessity of developing new bioreactor configurations or modifying the designs that already exist. These bioreactor designs should be able to operate in continuous mode with high enzyme productivity for prolonged periods of time as well as permit the scale-up of the process.

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CHAPTER 5. Banana skin; a novel waste for laccase production by *Trametes pubescens* under solid-state conditions

J.F. Osma, J.L. Toca Herrera and S. Rodríguez Couto. Banana skin; a novel waste for laccase production by *Trametes pubescens* under solid-state conditions. Application to synthetic dye decolouration. *Dyes & Pigments* 2007, 75: 32-37.
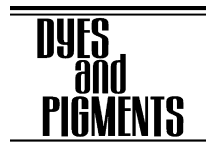
Banana skin: A novel waste for laccase production by *Trametes pubescens* under solid-state conditions. Application to synthetic dye decolouration

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Abstract

In this paper, we investigated the potential of banana skin as a support-substrate for the production of extracellular laccase by the white-rot fungus *Trametes pubescens* CBS 696.94. Laccase showed a maximum activity of 1570 U/l. In addition, we assessed the degrading ability of the extracellular liquid obtained. For this, we performed the *in vitro* decolouration of two structurally different dyes such as the anthraquinonic dye Remazol Brilliant Blue R (RBBR) and the triphenylmethane dye Methyl Green (MG). The former was decolourised about 57% in 4 h, whereas the latter presented a lower decolouration rate (40.9% in 4 h). Interestingly, RBBR decolouration was considerably higher than that attained by a commercial laccase (23.2% in 4 h), whereas MG decolouration (46% in 4 h) was very similar for both laccases. This shows the high potential of *T. pubescens* laccase for synthetic dye decolouration, especially for anthraquinonic dyes.
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Keywords: Banana skin; Laccase; Methyl Green; Remazol Brilliant Blue R; Solid-state fermentation; *Trametes pubescens*

1. Introduction

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) have been the subject of continuous study since the end of the 19th century. The genus *Trametes*, which belongs to the white-rot fungi, is assumed to be one of the main producers. Among them, *Trametes pubescens* has been described as a promising laccase producer [1]. The biotechnological importance of this enzyme lies in its ability to oxidise both phenolic and non-phenolic lignin-related compounds [2,3] as well as highly recalcitrant environmental pollutants [4,5].

Solid-state fermentation (SSF) is defined as any fermentation process occurring in absence or near absence of free liquid, using an inert substrate or a natural substrate as a solid support [6]. The former only functions as an attachment place for the microorganism, whereas the latter also acts as a carbon source, which considerably reduces the production costs [7]. SSF is advantageous in obtaining concentrated metabolites and subsequent purification procedures are economical [8]. In SSF, the microorganisms grow under conditions close to their natural habitat. This may allow them to produce certain enzymes and metabolites, which usually would not be produced or would only be produced at a low yield in submerged cultivation [6].

The selection of an adequate support for performing SSF is essential, since the success of the process depends on it. The most important factors to take into account are particle size, porosity and chemical composition. In addition to this, availability and cost are also criteria of great importance. In recent years, there has been an increasing trend towards the utilisation of organic wastes such as residues from the agricultural, forestry and alimentary industries as raw materials to produce value-added products by SSF technique [9]. The use of such wastes, besides providing alternative substrates, helps to solve

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environmental problems, which are caused by their disposal. Furthermore, most of these wastes contain lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more economical. All these make them very suitable as raw materials for the production of secondary metabolites of industrial significance by microorganisms. In particular, the present study focuses on laccase production by *T. pubescens*.

Banana skin has been selected to perform the present study due to its high content in carbohydrates, which due to their organic nature are easily metabolised by microorganisms, and it has the physical integrity to serve as a supporting material. In addition, its content in ascorbic acid exerts an inhibitory effect against bacteria [10]. Moreover, the banana processing industry generates a huge amount of solid wastes, which are dumped in landfills, rivers, oceans and unregulated dumping grounds. Therefore, their reutilisation would help to diminish the pollution problems caused by their disposal.

The goal of the present paper was to investigate the potential of banana skin as a support-substrate for the production of laccase by *T. pubescens* under SSF conditions, since the utilisation of such a support would mean an important reduction in production costs. It is very interesting to find new ways of producing laccase with higher activities at lower cost due to the enormous potential that this enzyme offers for the development of efficient biotechnology processes (biopulping, biobleaching, treatment of wastewater, etc.). To the best of our knowledge, this is the first report on laccase production using banana skin as a support-substrate.

2. Materials and methods

2.1. Microorganism

T. pubescens (CBS 696.94) was maintained on malt extract agar (MEA) plates at 4 °C and sub-cultured every three months.

2.2. Waste material

Chopped banana (*Musa cavendishii*) skins (particle size 7.5 mm × 7.5 mm), purchased at a local market, were used as support-substrates for laccase production by *T. pubescens* under SSF conditions. Table 1 shows the composition of the banana skin.

Banana skins were pre-treated as follows: they were first soaked for an hour in 30 ml of KOH 83.17 mM (10 g of fresh support) to neutralise organic acids [11]. Then, they were thoroughly washed with distilled water and dried at moderate temperature. Prior to use, the skins were autoclaved at 121 °C for 20 min.

2.3. Culture conditions

The composition of the culture medium was prepared according to Rodríguez Couto et al. [12] except that glucose was at a concentration of 2 g/l. The cultures were performed

Table 1
Chemical composition (%dry matter) of the banana skin [10]

Compound (g per 100 g)	
Dry matter	14.08
Crude protein	7.87
Crude fat	11.60
Crude fibre	7.68
Total ash	13.44
Carbohydrates	59.51
Moisture	78.4
Mineral and ascorbic acid content (mg per 100 g)	
Calcium	7
Sodium	34
Phosphorus	40
Potassium	44
Iron	0.93
Magnesium	26
Sulphur	12
Ascorbic acid	18

in cotton-plugged Erlenmeyer flasks (250 ml) containing 7 g of chopped banana skins and 20 ml of culture medium. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter, 7 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculums. The Erlenmeyer flasks were incubated statically under an air atmosphere at 30 °C and in complete darkness.

2.4. Analytical determinations

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [13] with ABTS (2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate]) as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol ABTS per minute. The activities were expressed in U/l.
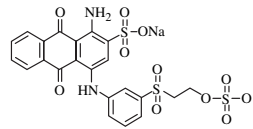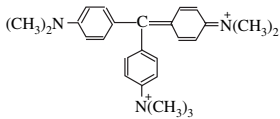
2.5. Decolouration studies

The dyes used were Remazol Brilliant Blue R (RBBR), purchased from Sigma Aldrich (St. Louis, MO, USA) and Methyl Green (MG), purchased from Merck (Germany). The characteristics of the dyes are summarised in Table 2. Stock solutions (0.1% w/v in water) were stored in the dark at room temperature.

Culture broth from banana skin cultures of *T. pubescens*, collected on day 14, and a commercial laccase (EC 1.10.3.2), supplied from Novo Nordisk (Denmark), were used for dye decolouration experiments.

The reaction was carried out directly in the spectrophotometer cuvette and the reaction mixture (final volume 1.5 ml) consisted of an aqueous solution of dye and extracellular liquid or commercial laccase (300 U/l, final concentration) in succinic buffer (pH 4.5). Dye concentrations were selected in order to obtain around 1.3 absorbance units at the maximum wavelength in the visible spectrum (0.133 g/l for RBBR and 0.033 g/l for MG, final concentration). All the reactions were incubated at room temperature, in static conditions and in complete darkness.

Table 2
Characteristics of the dyes employed

Dye	Class	λ_{max} (nm)	CI number	CI name	Structure
Remazol Brilliant Blue R	Anthraquinonic	595	61 200	Reactive Blue 19	
Methyl Green	Triphenylmethane	630	42 585	Basic Blue 20	

CI = colour index.

The residual dye concentration was measured spectrophoto-metrically and was associated with the decrease in the absorbance at the peak of maximum visible wavelength (595 nm for RBBR and 630 nm for MG). Dye decolouration was expressed in terms of percentage. A control test containing the same amount of a heat-denatured laccase was performed in parallel. The assays were done twice, the experimental error being below 10%.

2.6. Microscopic examination

Banana skin samples were fixed with 6% glutaraldehyde in phosphate buffer 0.1 M for 4 h. Afterwards, the samples were washed twice with phosphate buffer 0.1 mM for 15 min at 4 °C. Then, the samples were post-fixed with osmium tetroxide 0.1 M at 4 °C. Following fixation, the samples were washed twice with phosphate buffer 0.1 mM for 15 min. After that the samples were dehydrated through an ethanol–amyla-cetate series to pure amylacetate and critical point dried using CO_2 as the transition liquid. The dried samples were mounted on aluminum stubs, sputter coated with gold (20 nm) and examined with a Jeol 6400 scanning electron microscope (SEM) at 15 kV, belonging to SRCiT (Scientific and Technical Services) of the Rovira i Virgili University (Tarragona, Spain).

2.7. Mathematical analysis of the SEM images

SEM images were analysed using MATLAB 6.5 (MathWorks Inc., Natick, MA). The relief of the SEM images was obtained by a cross-section analysis of the gray-scale information. Discrete Fourier Transformation (DFT) was applied to the cross lines of SEM images in order to obtain the frequency information according to the following equation:

$$X(k) = \sum_{n=1}^{N} x(n) e^{(2j\pi(k-1)(n-1)/N)}, \quad 1 \leq k \leq \frac{N}{2} \quad (1)$$

where

N is the number of pixels of one line of the SEM images
j is the imaginary unit

$x(n)$ is the normalised gray-scale value of each nth-pixel
$X(k)$ is the kth armonic component of the signal

3. Results and discussion

3.1. Laccase production

As shown in Fig. 1, laccase production began on the 3rd day (63 U/l) and, then, it sharply increased up to a maximum activity of nearly 1600 U/l at the end of the cultivation. No other ligninolytic activities were detected in the extracellular culture. This is very interesting for the subsequent application of this enzymatic complex to the biotechnological processes, since the purification stage would be more economical.

3.2. Microscopic examination

The most important characteristics that influence adhesive behaviour of filamentous fungi to the support are hydrophobicity and surface charge. Fig. 2 shows SEM microphotographs of banana skin with (A) and without (B) fungus. It can be
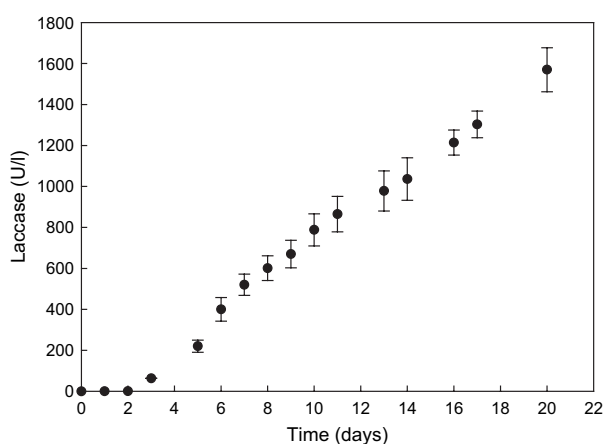

Fig. 1. Laccase production by solid-state cultures of *T. pubescens* grown on banana skins.
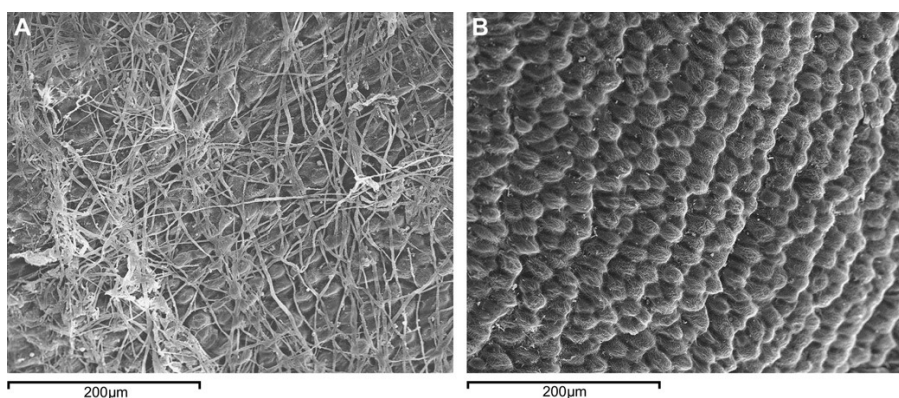
Fig. 2. SEM microphotographs of banana skin: (A) with fungus; (B) without fungus.

observed that the fungus grew well attached to the banana skin. This is due to the high hydrophobicity of the banana skin, which eases the attachment of the fungus to the carrier [14]. Therefore, banana skin is very suitable as an attachment place for filamentous fungi. This together with its high content in carbohydrates (Table 1) makes banana skin very suitable as a support-substrate to perform solid-state processes.

SEM images were analysed by software techniques in order to obtain information about the position of the fungus on the banana skin cells. Dotted line in Fig. 3 represents the cross-section of the banana skin cells, a cyclic shape can be appreciated and represents the position of each cell. Higher values correspond to the upper part of the cells; meanwhile, lower values correspond to the space between cells. Solid line represents the cross-section of the banana skin with fungus on it. More frequent peaks can be noticed as an indicator of the tubular structure of the fungus. Also, big covered areas show places where the fungus agglomerates. The growth of the fungus is not regular and does not cover symmetrically the surface of the cells; it is concentrated in different places where
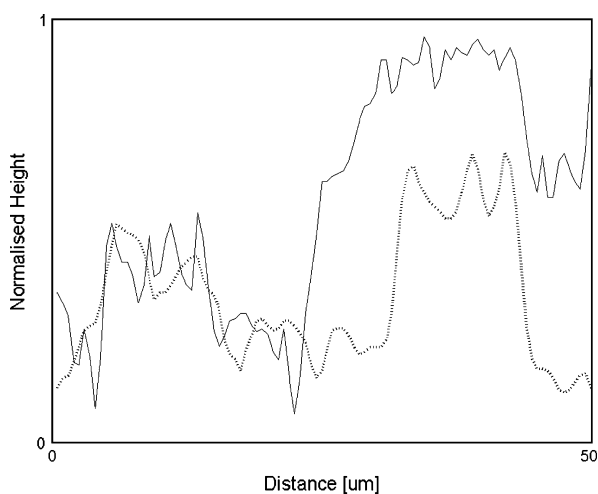
carbon source is surely more accessible. It also presents tubular structures that make clusters and grow irregularly, not following a specific parameter.

Fig. 4 presents a Fourier frequency analysis of the same SEM images, where low frequency peaks correspond to the size of the cells and high frequency peaks correspond to the tubular structure of the fungus and the roughness of the cells. Fourier analysis shows the mean size of the banana skin cells through the vertical and the horizontal axes (17 and 25 μm, respectively).

3.3. Decolouration studies

The ability of white-rot fungi to decolourise synthetic dyes has been widely studied, particularly with *Phanerochaete chrysosporium* and *Trametes versicolor* [15]. In the present study, we assessed the ability of the extracellular fluid from

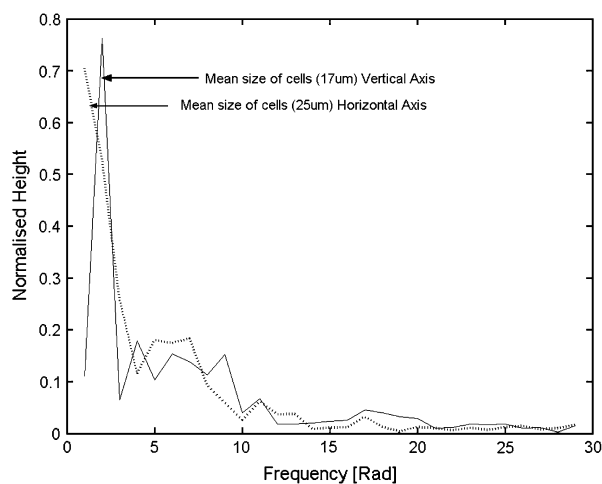Fig. 3. Cross-sections of banana skin with (solid line) and without fungus (dotted line).

Fig. 4. Fourier frequency analysis of the cross-section of the banana skin with fungus. Vertical axis analysis (solid line) and horizontal axis analysis (dotted line). The two peaks, 25 and 17 μm, correspond to the second and third armonics, respectively, of the Discrete Fourier Transformation (DFT) with $N = 50$ (1 pixel = 1 μm) extracted from information in Fig. 3 (cross-section of banana skin with fungus). The DFT equations used can be found in the following link: http://astronomy.swin.edu.au/~pbourke/other/dft/.

T. pubescens, a little studied white-rot fungus, to decolourise two structurally different synthetic dyes (RBBR and MG). The decolourisation of type model dyes is a simple method to assess the aromatic degrading capability of ligninolytic enzymes [16].

As it can be seen in Fig. 5, the decolouration rate obtained was very different in each case. Thus, RBBR showed a degree of decolouration about 57% after 4 h of treatment. However, from here onwards decolouration proceeded very slowly and it reached a value of 84.5% in 21 h. This could be due to either enzyme inhibition by some products generated in the decolouration process or substrate inhibition. These results differ from those found by Soares et al. [17], who reported that the addition of a redox mediator was necessary for RBBR decolouration by a laccase from a genetically modified *Aspergillus* microorganism. The discrepancy between our results and those from Soares [17] could be due to the difference in fungal species from which the laccase was obtained. Also, the redox potential of laccases varies depending on the laccase source [18], which could also dictate the need of a redox mediator for the decolouration of a particular dye to occur.

MG was decolourised about 41% in 4 h reaching a decolouration of 96.4% after 21 h. So, the decolouration rate was slower than that of RBBR but the final decolouration was higher (Fig. 5). It has been reported that highly substituted

triphenylmethane dyes required longer time to be decolourised or could only be decolourised to a certain extent [19].

When a commercial laccase was used, RBBR decolouration was significantly lower than that attained by laccase from banana skin cultures of *T. pubescens*. On the other hand, MG decolouration was very similar for both laccases (Fig. 5).

It was observed that from 24 h of incubation onwards decolouration did not increase (data not shown). This could be due to enzyme inhibition by some products generated in the decolouration process.

Since equal doses (300 U/l) of laccases were used in the decolouration process, the difference in the decolouration efficiency of the two laccases was most likely due to the difference in laccase isoenzymes produced by the different strains as well as due to the difference in specificities to different dyes of diverse structures [20]. In addition, as commented above, it could also be due to the difference in the redox potential of laccases from different microorganisms.

The fact that *T. pubescens* was able to decolourise the dye RBBR with no mediator addition is very interesting, since this dye is frequently used as a starting material in the production of polymeric dyes. Also, it represents an important class of often toxic and recalcitrant organopollutants [21].

4. Conclusions

The results clearly showed the enormous potential of banana skin as a support-substrate for the production of laccase by *T. pubescens* under solid-state conditions. In addition, the laccase produced presented a highly decolourising ability, especially for anthraquinonic dyes. This makes laccase from this fungus very attractive for further investigations as well as for its application to different biotechnology areas. More studies in order to optimise the culture conditions and the decolouration process are underway in our laboratory.

Acknowledgements

Fig. 5. Profile of dye decolouration attained: (●) extracellular liquid from banana skin cultures of *T. pubescens*; (○) commercial laccase.

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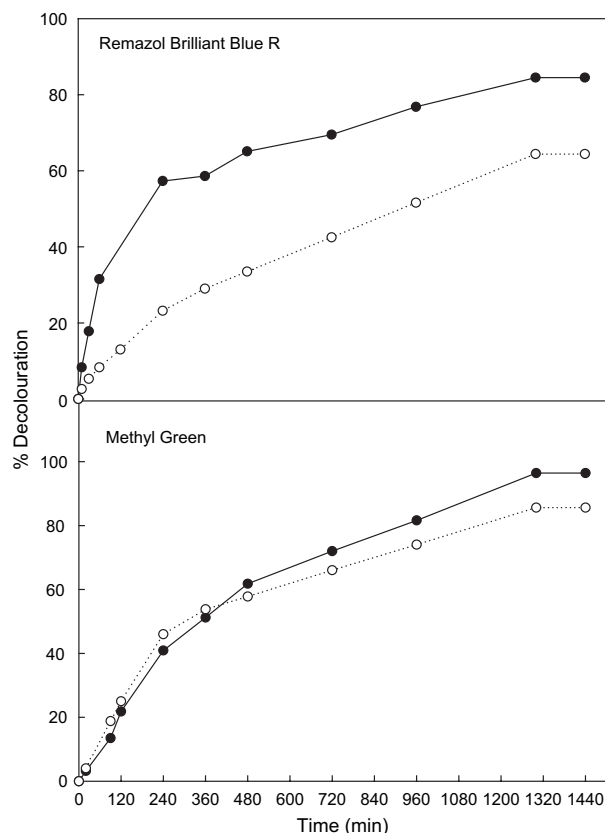
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CHAPTER 6. Removal of synthetic dyes by an eco-friendly strategy

S. Rodríguez Couto, J. F. Osma and J. L. Toca-Herrera. Removal of synthetic dyes by an eco-friendly strategy. *Engineering in Life Sciences* 2009

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Research Article

Removal of synthetic dyes by an eco-friendly strategy

In this paper an eco-friendly strategy for the removal of synthetic dyes from aqueous solutions was proposed. Thus, in a first step the dyes were adsorbed onto sunflower seed shells (SS) by using a batch technique. Subsequently, in a second step, these dyed SS were used as support-substrates to produce laccase by the white-rot fungus *Trametes pubescens* under semi-solid-state conditions. The effect of inducer addition on laccase production was studied. The optimum conditions (addition of both 0.5 mM Cu^{+2} and 50 µM tannic acid on the 3rd day of cultivation) led to a maximum laccase activity of 30272 U/L. Further, the system was efficiently scaled-up to laboratory bioreactors producing a maximum activity of 40172 U/L together with a total decolouration of the adsorbed dye. In addition, SDS-PAGE showed that laccases were the only enzymes present in the extracellular fluid. Therefore, apart from dye removal this approach allowed the production of high titres of laccase, which was obtained almost pure (only needed to be ultra-filtrated).

Keywords: Dye removal / Laccase / Sunflower seed shells

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1 Introduction

Approximately 10,000 different dyes and pigments are produced annually worldwide and used extensively in the dye and printing industries. This has resulted in the generation of large volumes of highly polluted wastewater. Apart from the aesthetic deterioration of the natural water bodies, dyes also cause harm to the flora and fauna in the natural environment [1, 2]. Therefore, wastewater-containing dyes must be treated prior to their discharge into the environment.

The usual treatment methods for dye-containing wastewater include combinations of biological treatment, chemical coagulation and activated carbon adsorption [3]. The latter is proven to be very effective in treating dye-containing wastewater but it suffers from several drawbacks such as its high price of both manufacturing and regeneration and it is ineffective against disperse and vat dyes. This has impelled the search for cheaper substitutes such as the solid wastes generated from the agricultural and food industry. The accumulation and concentration of pollutants from aqueous solutions by the use of biological materials is named bio-adsorption. The bio-adsorbents are often much more selective than traditional ion-exchange resins and commercial activated carbons and can reduce dye concentration to ppb levels. A second step should be the degradation of the adsorbed dyes.

White-rot fungi are the only micro-organisms able to degrade the whole wood components as a result of their non-specific extracellular ligninolytic enzyme system. The main components of their ligninolytic system are lignin peroxidases (LiP, EC 1.11.1.14), manganese peroxidases (MnP, EC 1.11.1.13) and laccases (EC 1.10.3.2). The latter have been subject of recent research due to (i) laccases present a better thermostability than LiPs and MnPs, (ii) laccases only require the presence of oxygen from air but neither manganese nor hydrogen peroxide and (iii) in the presence of appropriate substrates (redox mediators), which act as electron shuttles, laccases can also oxidise non-phenolic compounds [4, 5].

The aim of the present paper was the removal of synthetic dyes by means of an eco-friendly strategy involving two steps: a bio-adsorption process followed by a fungal treatment.

2 Material and methods

2.1 Microorganism

Trametes pubescens MB 89 (CBS 696.94; Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was obtained

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from the Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences (Vienna, Austria) and was maintained on malt extract agar (MEA) plates at 4°C and sub-cultured every three months.

2.2 Support-substrate

Sunflower (*Helianthus annuus*) seeds were obtained from a local market and the shells were collected after normal human consumption of the seeds. The sunflower seed shells (SS) were washed to remove any adhering dirt and air dried at room temperature for 36 hours. The chemical composition of the SS according to Demirbas [6] is 17% lignin, 48.4% cellulose and 34.6% hemicellulose.

2.3 Adsorption of dyes

The dyes used were Remazol Brilliant Blue R (RBBR), Congo Red (CR), Reactive Black 5 (RB5), Lanaset Grey (LG), purchased from Sigma Aldrich (St. Louis, MO, USA) and Methyl Green (MG) (Merk, Germany). Stock solutions (0.1% w/v) were stored in the dark at room temperature.

The adsorption processes of the dyes by the SS were conducted as indicated in a previous paper [7]. They were performed with a single dye (250 mg/L final concentration) and with a mixture containing the five dyes (RBBR, CR, RB5, LG and MG, 200 mg/L each).

2.4 Culture conditions

The composition of the culture medium was as follows: 10 g/L glucose, 20 g/L yeast extract, 0.9 g/L $(NH_4)_2SO_4$, 2 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ $7H_2O$, 0.1 g/L $CaCl_2$ $2H_2O$, 0.5 g/L KCl and 0.5 g/L thiamine in citrate-phosphate buffer (pH 4.5). The medium was sterilised at 121°C for 20 min. After cooling the thiamine, previously sterilised by filtration (0.22 µm), was added to the medium. Five potential inducers of laccase activity were tested: soy oil (1 and 2% v/v), coconut oil (1 and 2% v/v), Cu^{+2} (0.1, 0.3 and 0.5 mM), 2,5-xylidine (1 mM) and tannic acid (25 and 50 µM). They were added, under sterilised conditions, at different time points of the cultures (at the time of inoculation, tropophase and idiophase). A reference culture with no inducer addition was also performed for comparison.

The cultures were performed in cotton-plugged Erlenmeyer flasks (250 mL) containing 1.5 g of dyed SS (3.7–4.9 mg of dye adsorbed per gram of dry matter) and 20 mL of culture medium. Inoculation was carried out directly in the Erlenmeyer flasks. Three agar plugs (diameter, 7 mm), from an actively growing fungus on MEA, per Erlenmeyer were used as inoculum. The Erlenmeyer flasks were incubated statically under an air atmosphere at 30°C and in complete darkness. A culture with non-dyed SS was also performed for comparison.

2.5 Bioreactor configuration and culture conditions

A tray bioreactor configuration was used: it consisted of a plastic flat tray with an area of 20 cm × 15.7 cm and a height of 7 cm, where the dyed SS were placed forming a layer of about 0.5 cm of thickness (11.25 g dyed SS/150 mL medium). The bioreactor was sterilised by autoclaving at 121°C for 20 min.

Fig. 1 shows a scheme of the tray bioreactor used. Inoculation was carried out directly in the bioreactor with 22 agar plugs (7 mm diameter), from an actively growing fungus on MEA. The bioreactor was kept at 30°C, with passive aeration and in complete darkness. The culture medium composition was that determined as optimum in the experiments at flask scale.

2.6 Analytical determinations

Laccase activity was spectrophotometrically determined as described by Niku-Paavola et al. [8] with 2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS) as a substrate. One activity unit (U) was defined as the amount of enzyme that oxidised 1 µmol of ABTS per min. The activities were expressed in U/L.

Manganese-dependent peroxidase activity was spectrophotometrically assayed at 468 nm by the method of Kuwahara et al. [9]. The reaction was started by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 µmol of 2,6-dimethoxyphenol oxidised per minute and the activities were expressed in U/L.

Lignin peroxidase activity was spectrophotometrically determined at 310 nm according to Tien and Kirk (1984) [10]. The reaction was starting by adding 0.4 mM H_2O_2. One activity unit (U) was defined as 1 µmol of veratryl alcohol oxidised in 1 minute and the activities were reported as U/L.

Protease activity was determined according to Ginther [11], using a solution of azocasein (Sigma) at pH 4.5 as a substrate and 30°C during the whole reaction. One activity unit (U) was defined as the amount of enzyme that produced an increase of 0.1 absorbance units for a reaction hour per mL of sample and the activities were expressed in U/mL.
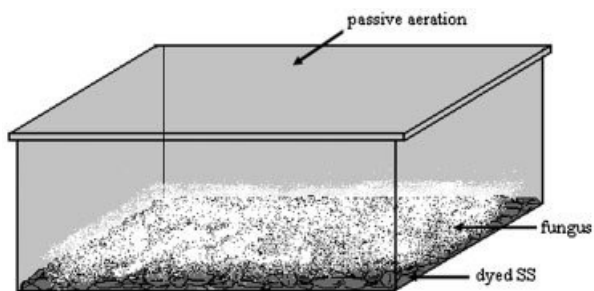

Figure 1. Scheme of the tray bioreactor used

2.7 Biomass estimation

Since mycelium was bound to the SS, it was firstly necessary to determine the amount of support degraded by the action of the fungal enzymes. In order to determine this variable, 25% of the SS were labelled using small holes in different distributions to make possible the monitoring during the cultivation process. After being dyed, all SS were dried and weighed to determine the original dry weight (w_0). After cultivation, the complex support-mycelium was collected and dried until constant weight (w_c). Afterwards, labelled SS without fungus were separated and weighed for calculating the amount of degraded support. The amount of degraded support (D_s) corresponded to the mean difference between original and final dry weight of labelled SS without fungus and was determined from:

$$D_S = \frac{1}{N} \sum_{n=1}^{N} (w_0 - w_f)_n \qquad (1)$$

where w_0 and w_f were the original and the final dry weight of each labelled SS (n), respectively [g] and N the total labelled SS used in the test.

The ratio between D_S and the original dry weight of the labelled SS without fungus corresponded to the mean amount of degraded SS (RD_S) as indicated in the following equation:

$$RD_S = \frac{D_S}{\sum_{n=1}^{N} (w_0)_n} \qquad (2)$$

On the other hand, fungal biomass (FB) was calculated as the difference between w_c, w_0 plus RD_S multiplied by w_0 as given in

$$FB = w_c - w_0 \cdot (1 - RD_S) \qquad (3)$$

Fungal biomass production correlated very well with the dry weight loss of this system, therefore, higher losses of dry weight (%) were assumed as higher biomass production [12].

2.8 Decolouration of the adsorbed dyes

To calculate the decolourised dye, the dyed SS were placed in a desorption medium that consisted of ethanol:water (2:3) and heated to 80°C for 24 h. The concentration of dye in the desorption medium at time 0 and after 12 days of fungal cultivation was spectrophotometrically measured from 400 to 700 nm and calculated measuring the area under the plot. Dye decolouration was expressed in terms of percentage.

2.9 Microscopic examination

SS samples were examined with a FEI–Quanta 600 environmental scanning electron microscope (ESEM), belonging to SRCiT (Scientific and Technical Services) of the Rovira i Virgili University (Tarragona, Spain). Samples were examined at 20 kV under environmental operation conditions at 2°C, with about 1500x of magnification.

2.10 SDS polyacrylamide gel electrophoresis under non-denaturing conditions

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 10% resolving gel and a 4% stacking gel according to the method of Laemmli [13]. To detect laccase activity after SDS-PAGE, prior to gel application the samples were heated to 50°C for 20 min and not boiled. Following Coomassie Blue staining the gel was destained, rinsed with water and subsequently submerged in 10 mL succinic buffer (pH 4.5) with 1% v/v guaiacol where the laccase active bands were highlighted in dark brown.

2.11 UV-Visible absorption spectrum

The UV-Visible absorption spectrum of crude laccase (235 mg/mL in succinic buffer pH 4.5) from *T. pubescens* cultures was recorded at room temperature on a UV-Visible spectrophotometer (Hewlett Packard 8453) in a 1 cm-path length quartz cuvette.

3 Results and discussion

3.1 Adsorption of dyes onto sunflower seed shells

The adsorption of dyes onto SS was conducted as indicated in a previous paper [7]. The rate of adsorption followed a pseudo-second-order kinetic model and the intra-particle diffusion was found to be the rate-controlling stage. In addition, the equilibrium data fitted well both the Freundlich and multilayer adsorption isotherm equations [7].

3.2 Dye decolouration and laccase production

In order to solve the disposal problem of the dyed SS generated from the adsorption process, the decolouration of the adsorbed dyes by the white-rot fungus *T. pubescens* was investigated. Cultures with non-dyed SS were also performed for comparison.

Absorption spectra of desorption medium from *T. pubescens* are shown in Fig. 2. The absorbance peaks in the absorption spectrum obtained at day 0 showed the presence of dye in the desorption medium. These peaks disappeared after 12 days of cultivation indicating that the fungus decolourised the dyes adsorbed onto SS (about 94% for RBBR, 49% for CR, 90% for RB5, 69% for LG and 97% for MG).

Fig. 3 shows ESEM photographs of the original SS (Fig. 3A), dyed SS (Fig. 3B) and dyed SS after fungal treatment (Fig. 3C). SS has a heterogeneous surface and micro-pores (about 1.5 μm) as seen from its ESEM photograph. After dye adsorption, SS presented a smoother surface and smaller micro-pores (about 1 μm) because of the packing of the dye what made surface irregularities less sharp. After fungal treatment dyed SS were almost totally colonised by the fungus and micro-pores were fully covered. Furthermore, the amount of
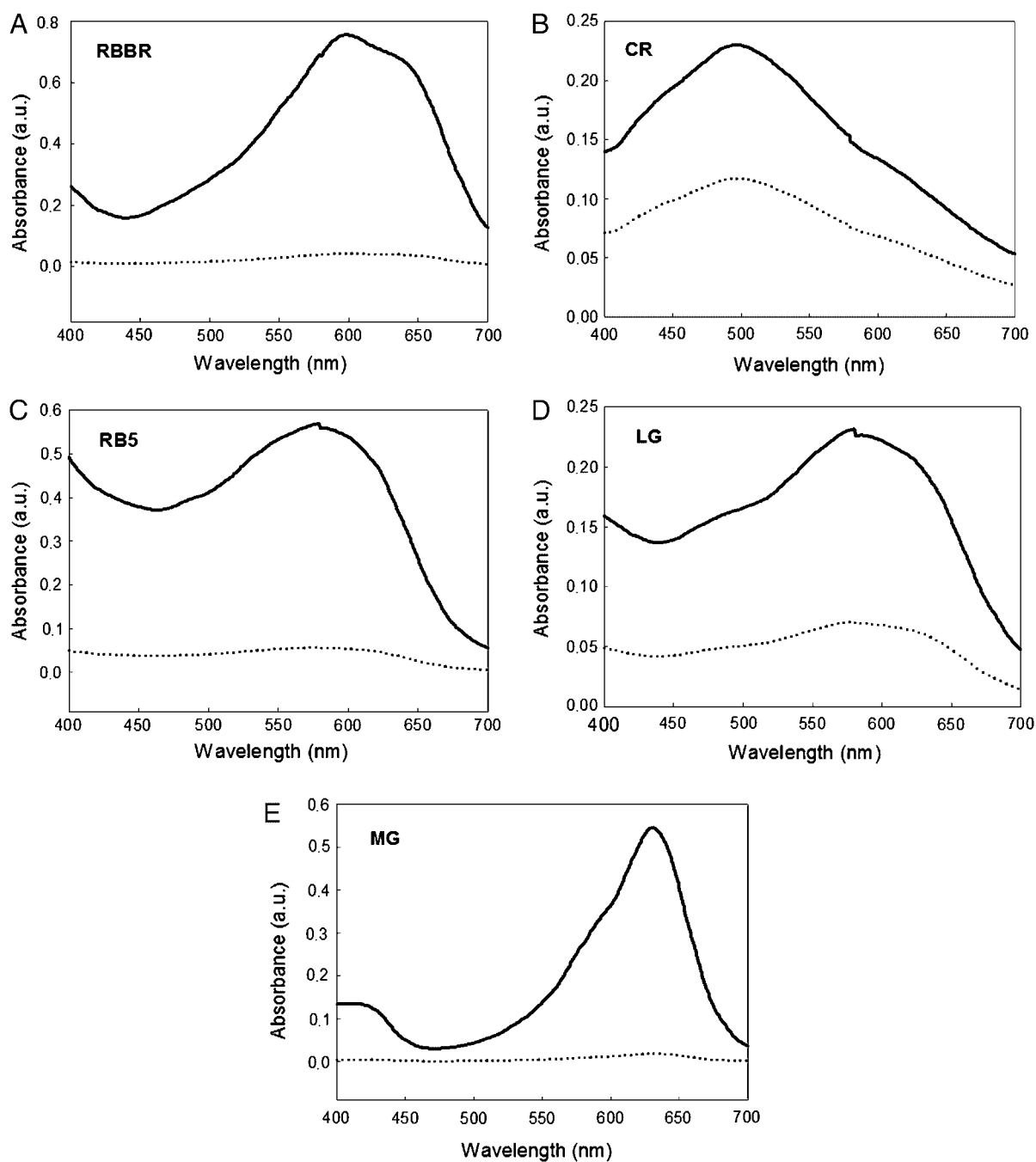
Figure 2. Absorption spectra of desorption media from *T. pubescens* cultures on SS. Time zero (continuous line) and after 12 cultivation days (dotted line)

SS degraded by fungal enzymes after 12 days of cultivation was estimated by comparing the initial and ending dry weight of the SS. The mean amount of degraded SS (RD$_S$) was determined to be 0.024 degraded grams per original gram of support.

As shown in Fig. 4, laccase production began between the 2nd and the 3rd cultivation day (around 120 U/L) and, then, it sharply increased up to a maximum activity of nearly 7000 U/L at the end of the cultivation. Although the laccase activities

obtained with the non-dyed SS were higher than those attained with the dyed ones (Fig. 4), the latter led to activities high enough to consider this material suitable as a support-substrate for laccase production under semi-solid-state conditions. The effect of using shells dyed with a single dye (RBBR or RB5) or shells dyed with a mixture of dyes (RBBR, RB5, CR, LG and MG) on laccase production was minimal. In addition, the laccase activities obtained were about 147-fold higher than that obtained in submerged cultures using stainless
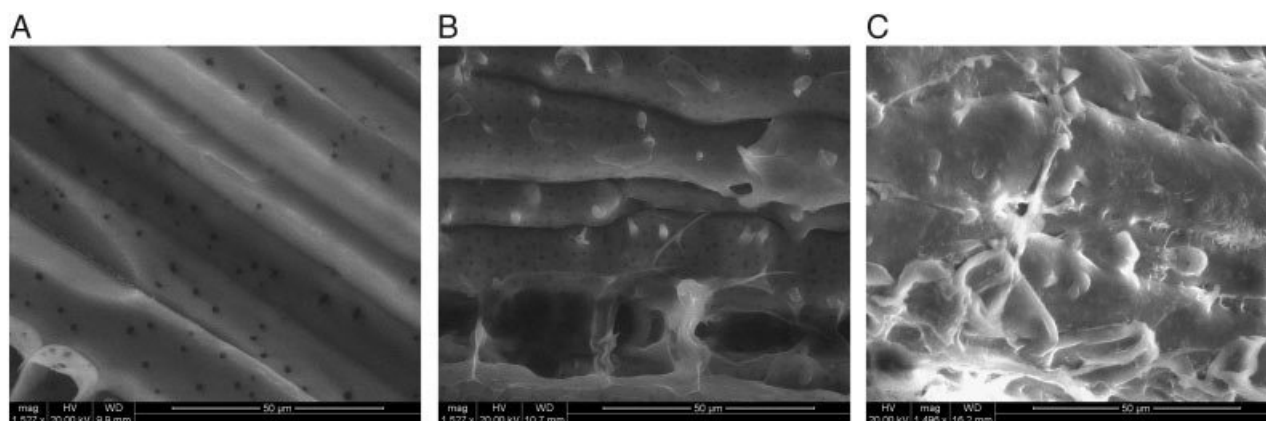
Figure 3. ESEM photographs of SS. A: original; B: dyed at time zero; C: dyed after fungal cultivation. Scale bar: 50 μm
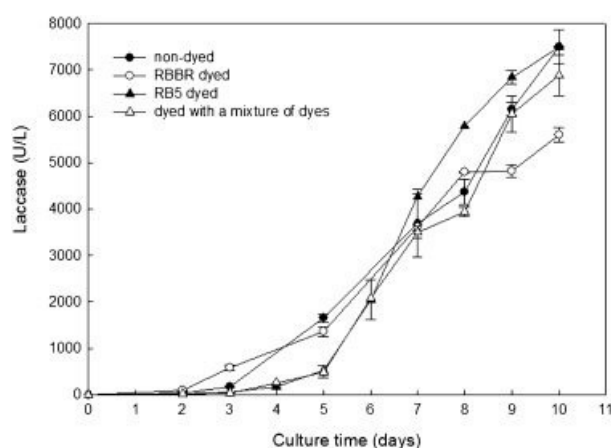

Figure 4. Laccase production by *T. pubescens* grown under semi-solid-state conditions: (—●—) non-dyed SS; (—○—) RBBR-dyed SS; (—▲—) RB5-dyed SS; (—△—) SS dyed with a mixture of dyes. Laccase activities obtained with non-dyed SS are higher than those attained with the dyed ones. It should be pointed out that laccase activities are ca. 147-fold higher than those obtained in submerged cultivation

Table 1. Maximum laccase activity obtained by *T. pubescens* under different cultivation conditions

Support	Type of cultivation	Maximum laccase activity (U/L)	Reference
Stainless steel sponge	SmC*	51	[14]
Banana skin	SSC**	1570	[15]
Wheat bran	SSC	2437	[16]
Sunflower seed shells	SSC	7495	This study
Dyed sunflower seed shells	SSC	5603	This study

*SmC: submerged cultivation
**SSC: semi-solid-state cultivation

steel sponges as supports (Table 1). Also, they were considerably higher than those attained under semi-solid-state conditions using other agro-wastes as support-substrates such as banana skin and wheat bran (Table 1).

In view of the above results further experiments in order to enhance laccase production using dyed SS as support-substrates were performed. For this SS dyed with RB5 were selected due to this dye is widely used in the textile industry.

3.3   Effect of inducer addition on laccase production

It is known that the addition of inducers into the culture medium considerably influences the production of laccase [17]. Thus, we studied the addition of five potential laccase-inducing substances into the culture medium such as Cu^{+2}, 2,5-xylidine, soy oil, coconut oil and tannic acid. The inducer concentration and the time point of the inducer addition (at the time of inoculation, tropophase and idiophase) were also investigated. As shown in Fig. 5 the highest laccase activity (25773 U/L) was obtained by adding 0.5 mM Cu^{+2} to the culture medium on the 3rd day of cultivation. This activity is more than 3-fold higher than that obtained in cultures with no inducer addition. There was not a linear correlation between biomass and laccase production, so the influence of inducer compounds on laccase production was not due to increased biomass production (Fig. 5). Other authors have also reported the positive effect of Cu^{+2} as an inducer of laccase activity in fungi belonging to the *Trametes* genus [18–21]. Experiments with purified laccase showed that Cu^{+2} not only induces laccase by the expression of laccase genes, but it also positively affects the activity and stability of the enzyme [22].

3.4   Joint effect of inducer addition on laccase production

In view of the above results the joint effect of supplementing the medium with Cu^{+2} *plus* xylidine and Cu^{+2} *plus* tannic acid was
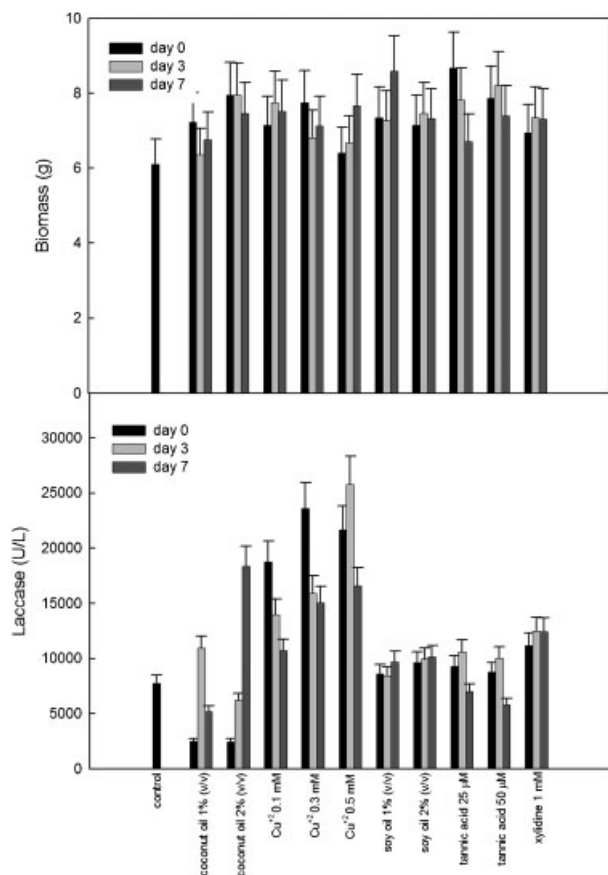
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Figure 5. Effect of different inducers on laccase and biomass production by *T. pubescens* grown under semi-solid-state conditions using RB5-dyed SS as support-substrates. The highest laccase activity is obtained for the medium supplemented with 0.5 mM Cu^{+2} which is at least 3-fold higher than those obtained in cultures with no inducer addition. These results show that there is no linear correlation between biomass and laccase production. Duplicate experiments were run for comparison and samples were analysed twice. The values in the figures correspond to mean values with a standard deviation lower than 15%

Figure 6. Joint effect of Cu^{+2} *plus* tannic acid and Cu^{+2} *plus* xylidine added at the 3rd cultivation day on laccase production by *T. pubescens* grown under semi-solid-state conditions using RB5-dyed SS as support-substrates: control (—▲—); 0.5 mM Cu^{+2}+ 1 mM xylidine (—●—);0.5 mM Cu^{+2}+50 µM tannic acid (—○—)

Figure 7. Protease production by *T. pubescens* grown under semi-solid-state conditions using RB5-dyed SS as support-substrates: control cultures (—●—); copper-induced cultures (---○---)

investigated. Thus, the experiments were performed with 0.5 mM Cu^{+2} *plus* 1 mM xylidine and with 0.5 mM Cu^{+2} *plus* 50 µM tannic acid added on the 3rd day of cultivation. As can be observed in Fig. 6 the joint addition of Cu^{+2} (0.5 mM) and tannic acid (50 µM) increased the laccase activity by 4-fold in relation to the reference cultures (Fig. 5). However, this increase was not acute (17%) in relation to the addition of only 0.5 mM Cu^{+2} (Fig. 5). The fact that Cu^{+2} acted synergistically with tannic acid to increase laccase activity is very interesting, since tannic acid is a non-toxic compound whereas xylidine is a toxic one.

Fig. 7 shows the protease production in cultures with and without inducers (Cu^{+2} and tannic acid). Protease production was lower in the induced cultures, which was likely the reason of the higher laccase activities produced in such cultures. This is in agreement with a paper by Palmieri et al. [23] in which was stated that the positive effect of copper on laccase stability may be due to inhibition by Cu^{+2} of an extracellular protease.
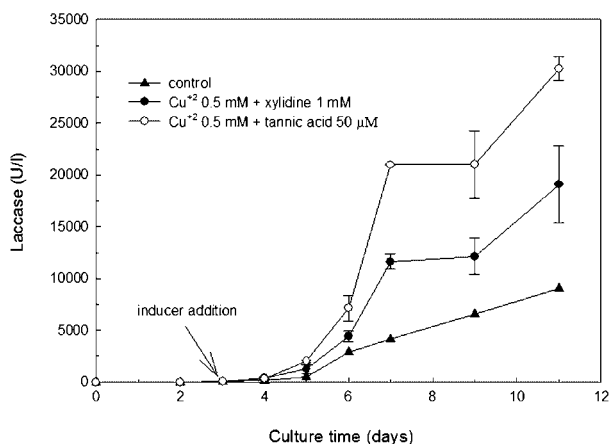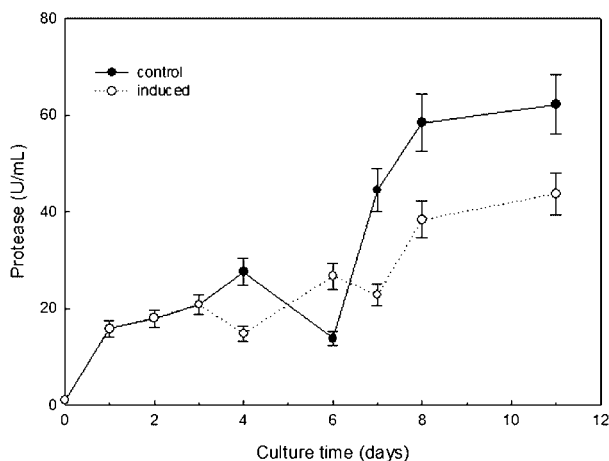
3.5 Dye decolouration and laccase production at reactor scale

The above process was scaled-up to laboratory reactors. The static tray bioreactor, also known as koji bioreactor, was the reactor configuration utilised since it is the generally used bioreactor for solid-state cultures. Tray bioreactors are very simple in design, with no forced aeration or mixing for the solid support-substrate. The design of tray bioreactors has remained almost unchanged over the last decades. Tray bioreactors are extensively used for the production of fermented oriental foods and enzymes.

The results obtained are shown in Fig. 8. They were quite similar to those attained at flask scale which shows the potential of this process for its implementation at industrial scale.
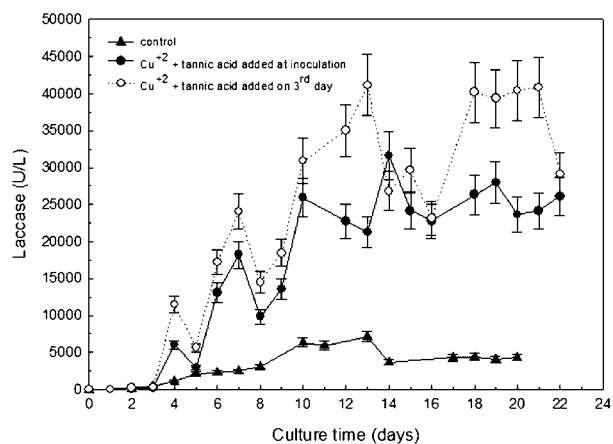
Figure 8. Laccase production by *T. pubescens* grown on RB5-dyed SS in a tray bioreactor under semi-solid-state conditions: control (—▲—); 0.5 mM Cu^{+2}+50 µM tannic acid added at the inoculation (—●—); 0.5 mM Cu^{+2}+50 µM tannic acid added on the 3rd cultivation day (—○—)

Figure 9. Guaiacol stained SDS-PAGE gel. Lane 1: molecular weight standards (kDa); lane 2: extracellular crude from cultures without inducers; lane 3: extracellular crude from cultures with inducers (Cu^{+2} and tannic acid added on the 3rd cultivation day)

3.6 Electrophoresis of the extracellular crude obtained

Samples showing the highest laccase activities from the cultures without inducers and supplemented with 0.5 mM Cu^{+2} and 50 µM tannic acid on the 3rd cultivation day were collected, centrifuged and ultra-filtrated in 20 mL-Vivaspin tubes (Sartorius AG, Göttingen, Germany) with a membrane cut-off of 10 kDa. The resulting crude showed a specific laccase activity of 40 U/mg. From the activity staining it was clear that laccase enzymes were the main ligninolytic enzymes produced in both cultures with molecular weights of around 83 kDa and 50 kDa (Fig. 9).

3.7 UV-visible absorption spectrum of the extracellular crude obtained

The UV-visible absorption spectrum of the crude obtained showed two peaks at 280 and 620 nm and a shoulder at 330 nm (Fig. 10). The peak at 620 nm is typical for the type I Cu (II), which is responsible for the deep blue colour of the enzyme [24] and the shoulder at 330 nm indicated the presence of the type III binuclear Cu (II) pair [25]. The spectral characteristics of laccase from *T. pubescens* were similar to that observed for other fungal laccases [26, 27].

4 Conclusions

Due to its low cost and high adsorption capacity (around 4 mg/g), SS was found to be a promising material for dye removal from wastewater. Subsequently, the dye adsorbed onto SS was decolourised by the white-rot fungus *T. pubescens*, which, in addition to this, produced high titres of laccase enzymes. Further, the system was successfully scaled-up to
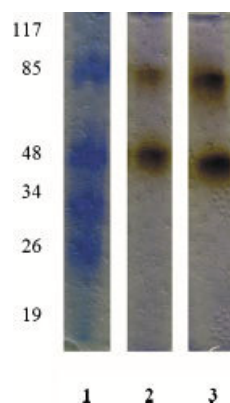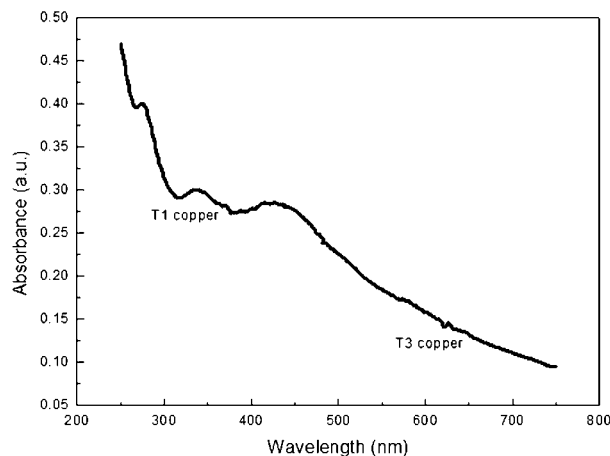

Figure 10. UV-Visible absorption spectrum of crude laccase (235 mg/mL in succinic buffer pH 4.5) from induced cultures of *T. pubescens*

laboratory bioreactors. However, several aspects related to improving the bioreactor design, optimising more process parameters and greater automation are needed for the industrial exploitation of this process.

Acknowledgements

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CHAPTER 7. Coating of immobilised laccase for stability enhancement: a novel approach

Coating of immobilised laccase for stability enhancement: A novel approach

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Abstract

We report on the coating of laccase immobilised on Al_2O_3 pellets by the sequential adsorption of oppositely charged polyelectrolytes for stability enhancement. The activity of the coated laccase was not only preserved but also increased in relation to the non-coated laccase. The decolouration of two synthetic dyes of different structure and ionisation, i.e. Methyl Green (MG) and Remazol Brilliant Blue R (RBBR) by the coated and the non-coated laccase was assessed. Both laccases led to similar decolouration levels but desorption from the pellets of the non-coated laccase was detected. This phenomenon did not occur in the coated laccase which showed the protective effect exerted by the layers.

On the other hand, both the coated and the non-coated laccase retained its laccase activity after more than 28 cycles of ABTS reaction. It was observed that the retention of laccase activity increased with the number of layers. Also, laccase activity of both the coated and the non-coated laccase was retained after 5 days of RBBR decolouration, this effect being especially acute in the one-layer coated laccase. This would permit the reusability of laccase, which would mean a considerable economical advantage.
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Keywords: Laccase; Immobilisation; Layer by layer; Coating; Decolouration

1. Introduction

The development of methods for removing toxic compounds from the environment has increased in recent years. Biodegradation with oxidative enzymes appears a promising technology. Among the different existing oxidative enzymes, laccases (*p*-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are outstanding because they do not need any cofactor, as their co-substrate – oxygen – is usually present in their environment. Furthermore, laccases catalyse the oxidation of a great variety of phenolic and non-phenolic compounds [1], which make them very useful for biotechnological purposes.

Nevertheless, the stability and the catalytic activity of laccases considerably decrease by a wide variety of environmental conditions that characterise effluents (pH, ionic concentrations, inhibitors, etc.) and which seriously limit their industrial application. Therefore, the improvement of both laccase stability and reusability is a goal of considerable importance. So far, the immobilisation of laccases in/on water-insoluble carriers has improved their stability and lasting.

The layer by layer technique (LbL) [2] is based on the alternation of oppositely charged polyelectrolytes to build multilayers. It has widely been used to make biosensors, coatings and films [3–6] and recently to build artificial cell walls [7]. In addition, LbL has been demonstrated to be an effective means for the immobilisation of enzymes [8]. Thus, glucose oxidase [9–12], catalase [13,14] and peroxidase [13,15] have been immobilised via the LbL technique. However, as far

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as we know this technique has not been applied to laccase enzyme before this study.

In this study, we applied LbL to coat laccase previously immobilised on alumina (Al_2O_3) pellets in order to obtain a long-life biocatalyst. Alumina pellets were chosen as a support for laccase immobilisation due to their mechanical resistance at high pHs and temperatures [16].

2. Experimental

2.1. Chemicals

Poly(allylamine hydrochloride) (PAH, M_w 70 000) and poly(sodium 4-styrenesulfonate (PSS, M_w 70 000), alumina (Al_2O_3) spherical pellets (3 mm diameter), γ-aminopropyl-triethoxy silane, glutaraldehyde, 2,2′-azino-di-[3-ethyl-benzo-thiazolin-sulfonate] (ABTS), bovine serum albumin (BSA) and Remazol Brilliant Blue R (RBBR) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Methyl Green (MG) was purchased from Merck (Germany). 0.01 M polyelectrolyte solutions with a NaCl concentration of 0.5 M were prepared.

2.2. Enzyme

Trametes hirsuta laccase was produced as previously described by Almansa et al. [17].

2.3. Laccase immobilisation

Alumina (Al_2O_3) spherical pellets (3 mm diameter) were silanised with 2.0% (v/v) γ-aminopropyltriethoxy silane in acetone at 45 °C for 20 h [18]. The silanised supports were washed once with acetone and silanised again for 24 h with the above procedure. Afterwards, they were washed several times with deionised water and dried through air. The supports were treated with 2% (v/v) aqueous glutaraldehyde (50%, v/v) for 2 h at room temperature, washed again with deionised water and dried through air. One hundred and thirty grams of support were immersed in 200 mL of laccase preparation (4.7 g protein/L; 4026 U/L) for 48 h at room temperature. Afterwards, the

supports were washed several times with 0.05 M phosphate buffer (pH 7) to remove the unbounded protein and kept at 4 °C until further use. Bound laccase and bound protein were determined as a difference between the initial and residual laccase and protein concentrations, respectively (immobilisation yield 68%, 5.6 mg protein/g support).

2.4. Enzyme coating

The Al_2O_3-immobilised laccase was coated as follows: the Al_2O_3 pellets with immobilised laccase were washed three times with 0.1 M NaCl. The polymer layers were then assembled onto the immobilised laccase by the sequential deposition of PAH and PSS, since the Al_2O_3 pellets with immobilised laccase were negatively charged. Capsules with one layer (PAH), two layers (PAH + PSS), three layers (PAH + PSS + PAH) and four layers (PAH + PSS + PAH + PSS) were made. After each layer, the excess of polyelectrolyte was removed with 0.1 M NaCl.

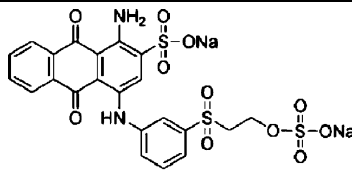### 2.5. Analytical determinations

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [19] with ABTS as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol ABTS/min. The activities were expressed in U/L.

Protein concentration was determined spectrophotometrically at 595 nm according to Bradford [20] using the reagent commercialised by Bio-Rad (Richmond, USA). BSA was used as a standard.

2.6. Decolouration experiments

The characteristics of the dyes used are summarised in Table 1. Stock solutions (0.1% (w/v) in water) were stored in the dark at room temperature. The reactions were carried out directly in the spectrophotometer cuvette and the reaction mixture (final volume 1.0 mL) consisted of an aqueous solution of dye and a Al_2O_3 pellet with immobilised laccase either coated or non-coated, according to the experiment.

Table 1
Characteristics of the dyes used

Dye	Class	λ_{max} (nm)	CI no.	CI name	Ionisation	Structure
RBBR	Anthraquinonic	595	61200	Reactive Blue 19	Anionic	
MG	Tryaryilmethane	630	42585	Basic Blue 20	Cationic	

CI: colour index.

Dye concentrations were selected in order to obtain around 1.0 absorbance unit at the maximum wavelength in the visible spectrum (0.133 g/L for RBBR and 0.033 g/L for MG, final concentrations in the cuvette). The residual dye concentrations were measured spectrophotometrically and associated with the decrease in the absorbance at the peak of maximum visible wavelength (Table 1). Dye decolouration was expressed in terms of percentage. Adsorption of the dyes to the pellets was determined by immersing the pellets in ethanol (70%, v/v) for 10 days. The amount of the dye bound to the pellets was calculated from the absorption of the supernatants.

3. Results and discussion

Several authors have showed the potential application of immobilised laccase in dyestuff treatment [21–24]. However, laccase desorption can occur, especially by changes in pH or ionic strength. To avoid laccase leaching from the support a novel approach was performed. This approach consisted in coating the immobilised laccase with polyelectrolytes according to the LbL technique.

In order to check the effect of coating on laccase activity, the laccase activity of the coated and the non-coated laccase was measured. It was found that laccase activity of the coated laccase was higher than that of the non-coated one. Thus, it was about 1.6-fold higher for the one-layer coated laccase, about twofold for the two- and four-layer coated laccase and almost threefold for the three-layer coated laccase (Fig. 1). The decrease of activity of the four-layer coated laccase in relation to the three-layer coated one was likely due to this number of layers acts as a barrier between laccase and substrate. So, the laccase activity was not only preserved but also increased. This might be due to the polyelectrolyte layers protect laccase from potential denaturising agents present in the solution. This is in agreement with the results reported by Wang and Caruso [14]. They found that coated catalase retained 98% of its activity after 2 h of protease incubation whereas the non-coated one retained only 20% after 1 h.
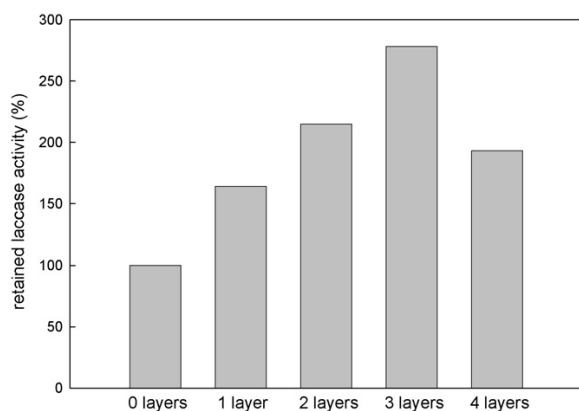

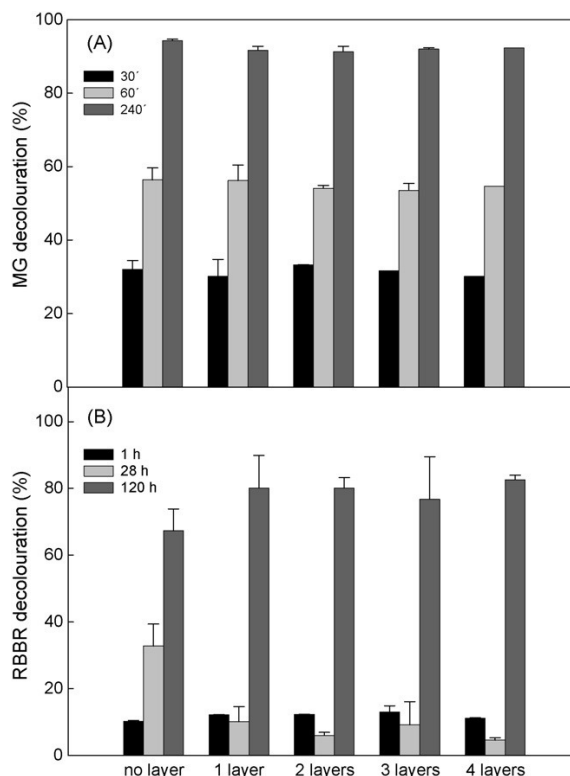Fig. 1. Laccase activity of the non-coated and the coated (one, two, three and four layers) laccase.

Fig. 2. MG (A) and RBBR (B) decolouration obtained by the non-coated and the coated laccase (one, two, three and four layers) at different times.

The decolouration of two different charged dyes by the coated and the non-coated laccase was studied. As shown in Fig. 2 the decolouration rate of MG (cationic) was much higher than that of RBBR (anionic) (higher than 91% in 240 min and higher than 76% in 120 h, respectively). It can also be seen that the non-coated and the coated laccase led to similar decolouration levels. However, laccase desorption from the pellets of the non-coated laccase was detected. This is presumably due to some laccase was immobilised by weak bounds, so the dye could remove it. This phenomenon did not occur in the coated laccase which showed the protective effect exerted by the layers. It was observed that decolouration was due to two processes: adsorption of the dye on the support and dye degradation due to laccase action. It was determined that dye adsorption on the pellets was very low (less than 5%), so dye decolouration was mainly due to laccase action.

Both the non-coated and the coated laccase were allowed to react with ABTS for several successive batch reactions (each batch lasted 10 h). It was found that both laccases retained their activity after 28 cycles, although in different proportions. Thus, the non-coated laccase retained 14% of its activity, meanwhile the coated laccase retained 25%, 32%, 54% and 66% for the one, two, three and four layers, respectively. Therefore, it can be seen that the layers preserved the laccase activity. The percentage of retained activity was calculated with respect to the initial activity for each case.
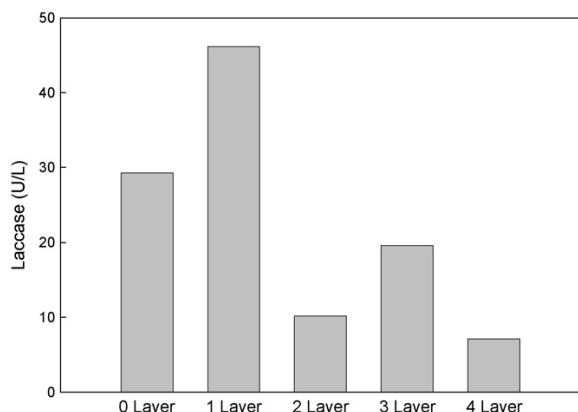
Fig. 3. Laccase activity after 30 min of ABTS reaction of the non-coated and the coated (one, two, three and four layers) laccase after 5 days of RBBR decolouration.

On the other hand, we measured the laccase activity of the coated and the non-coated laccase after 5 days of RBBR decolouration. The ordinary ABTS assay could not be performed because the absorbance did not increase linearly at the beginning of the reaction, presumably due to interferences between the RBBR adsorbed on the pellets and the ABTS. The absorbance became linearly after 10 min of ABTS reaction (saturation of RBBR on pellets), so the reaction was monitored for 30 min. Fig. 3 shows the laccase activities obtained after 30 min of ABTS reaction for each case. It can be observed that the one-layer laccase led to the highest activity followed by the non-coated one. Also, the laccase with an even number of layers showed higher absorbance than those with an odd number. This is likely related to the charge, the thickness and the permeability of the whole layers.

The difference in the ABTS activity of both experiments can be related with the fact that the adsorbed RBBR could behave as extra-layers decreasing the porosity of the coating, and thus, acting as a barrier decreasing the ABTS activity. However, this is a very complex system in which at least four different interactions took place (numbered in Fig. 4). The first one was related to the laccase molecules that were not covalently bond and were trapped by weak interactions with other laccase molecules. When a substrate was present in the solution, the physical movement of the laccase molecules. (in order to interact and create the complex laccase–substrate) could remove the non-covalent bond laccase molecules. This could be the reason why laccase desorption of the non-coated laccase was detected. The second interaction was due to the difference in size between the laccase and the dyes (the laccase molecular weight is around 100-fold higher than the dye molecular weight) due to which some cross linkers remained free letting some dyes covalently bond to the support. This could explain why some adsorbed dye did not get out from the pellets after washing them with 70% (v/v) ethanol for 10 days. Also, in the presence of dye molecules in the solution a third interaction may occur, since some dye molecules could be trapped in between the layers, reducing the porosity of the coating and, thus, incrementing the barrier effect. This would explain why the ABTS activity of the non-coated laccase was higher than the two-, three- and four-layer coated laccase. However, this effect did not affect the one-layer coated laccase in the same way since the dye had fewer chances to get trapped in just one layer than in multiple ones. On the whole, the coating of immobilised laccase *via* LbL seems to be an effective method for protecting laccase of adverse environmental conditions without compromising its activity, thus, overcoming the problem of enzyme desorption often encountered with direct immobilisation of enzymes on solid supports. In addition, it allowed laccase reutilisation. Therefore, the system presented in the current paper supposes a step forward in the development of an efficient method for applying laccase-based treatments at an industrial scale. More studies in order to investigate the effect of different
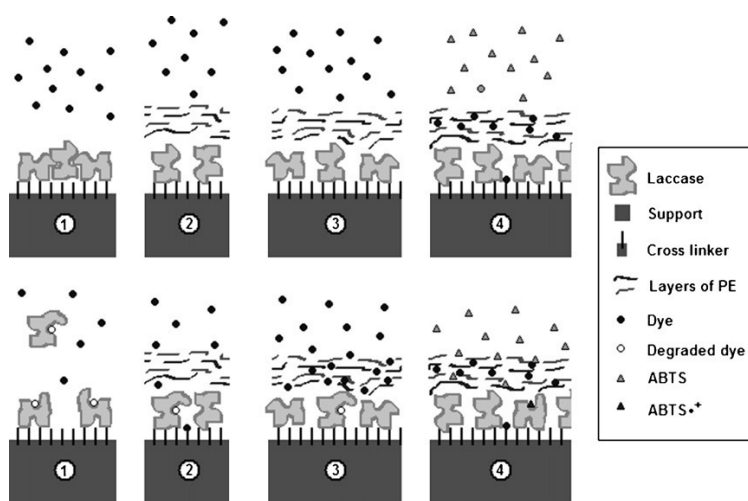

Fig. 4. Different interactions in the laccase immobilisation procedure: (1) desorption of non-coated laccase; (2) adsorption of dye molecules by covalent bonds; (3) entrapment of dye molecules between layers; (4) ABTS degradation with RBBR trapped between layers which reduced the porosity of the coating.

environmental conditions (pH, ionic strength, the presence of denaturising agents, inhibitors, etc.) on the coated laccase as well as the permeability properties of the wall capsule are undergoing in our laboratory.

Acknowledgements

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CHAPTER 8. Degradation pathway of Remazol Brilliant Blue R by immobilised laccase

J.F. Osma, J.L. Toca Herrera and S. Rodríguez Couto. Degradation pathway of Remazol Brilliant Blue R by immobilised laccase. Submitted to *Water Research*.

Degradation pathway of Remazol Brilliant Blue R by immobilised laccase

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Abstract

This study deals with the biodegradation products obtained from the Remazol Brilliant Blue R (RBBR) after its treatment by immobilised laccase from the white-rot fungus Trametes pubescens. 44% of dye decolouration was achieved within 42 h. RBBR degradation products were investigated using ultraviolet-visible (UV-vis) spectrum scan and High Performance Liquid Chromatography/Mass Spectrometry (LC-MS) analysis. Two compounds were identified as the degradation intermediates (m/z 304.29 and m/z 342.24) and other two as the final degradation products (m/z 343.29 and m/z 207.16). No backward polymerisation of the degradation products resulting in recurrent colouration was observed after laccase treatment of RBBR. It was also found that the decolouration of RBBR by immobilised laccase resulted in a considerable decrease of phytotoxicity.

Keywords: Remazol Brilliant Blue R; Biodegradation; Laccase; Immobilisation; Trametes pubescens

1. Introduction

About 50,000 tons of dyes are discharged into the environment world-wide every year (Lewis, 1999) causing a serious environmental impact in the ecosystems. In addition, government legislation is becoming stricter regarding the removal of dyes from industrial effluents, especially in the more developed countries (O'Neill et al., 1999). Therefore, removal of dyes from effluents prior to their discharge into the environment is an issue of considerable importance. Currently, dye-containing effluents are mainly treated by physical and chemical methods. However, these methods show several drawbacks such as sludge generation, high cost and use of high amounts of chemicals (Nigam et al., 2000). This has impelled the search for alternative methods such as the use of oxidative enzymes produced by white-rot fungi, which are so far the best-known dye-decolourising microorganisms. Among the different oxidative enzymes secreted by white-rot fungi, laccases (p-diphenol:dioxygen oxidoreductases; EC 1.10.3.2) are outstanding because they only need molecular oxygen (air) as a co-substrate and catalyse the oxidation of a great variety of phenolic and non-phenolic compounds (Bourbonnais et al., 1997). Laccases are multi-copper proteins and usually contain four copper

ions of two different types: one type I Cu+2, whose redox potential determines the substrates to be oxidised and other three Cu+2 ions transferring electrons to O2.

Immobilisation of laccase is a proven approach of increasing its stability and allowing its reuse. From the different approaches found in the literature to immobilise laccases (Durán et al., 2002 and references therein; Arica et al., 2008; Georgieva et al., 2008; Russo et al., 2008; Teerapatsakul et al., 2008; Wang et al., 2008; Rekuc et al., 2009; Zhang et al., 2009) attachment by covalent bond onto alumina pellets was selected to perform the present study, since it was shown to be a very suitable method for laccase immobilisation retaining high activities (Rodríguez Couto et al., 2007). In addition, laccases from *Trametes hirsuta*, *Sclerotium rolfsii*, *Trametes villosa*, *Trametes modesta* and *Trametes pubescens* were successfully immobilised onto alumina pellets and efficiently decolourised several synthetic dyes (Abadulla et al., 2000; Ryan et al., 2003; Zille et al., 2003; Kandelbauer et al., 2004; Rodríguez Couto et al., 2007).

Anthraquinone dyes constitute the second most important class of textile dyes, after azo dyes (Baughman and Weber, 1994). They have a wide range of colours in almost the whole visible spectrum, but they are most commonly used for violet, blue and green colours (Christie, 2001; Fontenot et al., 2003). Among them, Remazol Brilliant Blue R (RBBR) is an industrially important dye that is frequently used as a starting material in the production of polymeric dyes. RBBR is an anthracene derivative and represents an important class of toxic and recalcitrant organopollutants. There are several reports on decolouration of RBBR by laccases (Soares et al. 2001; Peralta-Zamora et al. 2003; Mechichi et al., 2006; Mohorcic et al. 2006; Susla et al. 2007; Kunnameni et al.,

2008;Russo et al., 2008; Hu et al., 2009; Rotková et al., 2009) but none has considered its degradation pathway. The identification of the metabolites produced during biodegradation as well as toxicity studies must be done to ensure the safety of the laccase-treated effluents. However, so far only a few studies regarding the metabolic pathway of azo dye degradation by ligninolytic enzymes have been performed (Martins et al., 2002; Martins et al., 2003; Lopez et al., 2004; Svobodová et al., 2007; Pereira et al., 2009). The aim of the present work was to determine the biodegradation products resulting from the laccase treatment of the anthraquinone-type dye RBBR. Also, the toxicity of the degraded RBBR was assessed.

2. Materials and methods

2.1. Laccase production and crude laccase preparation

Laccase was produced by cultivation of the white-rot fungus *Trametes pubescens* (MB 89; Institute of Applied Microbiology, University of Agricultural Science, Vienna, Austria), under semi-solid-state fermentation conditions using dyed sunflower seed shells as support-substrates as described in Rodríguez Couto et al. (2009). Culture broth was collected at the maximum laccase activity (day 10), filtered, clarified by centrifugation at 8000xg for 15 min, frozen, defrosted and, then, filtered to remove the precipitated polysaccharides. The resulting clear filtrate was concentrated on an Amicon membrane with a molecular cut-off of 10 kDa. The experiments were performed with this concentrated clear filtrate (40 U/mg specific laccase activity).

2.2. Laccase immobilisation

Crude laccase was immobilised onto alumina (Al2O3) spherical pellets (3 mm diameter) as

indicated in Rodríguez Couto et al. (2007). The immobilisation yield was 73% (0.18 mg protein/g support; 7.4 U/g support).

2.3. Analytical Techniques

Laccase activity was spectrophotometrically determined as described by Niku-Paavola et al. (1990) with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 µmol ABTS/min. The activities were expressed in U/L.
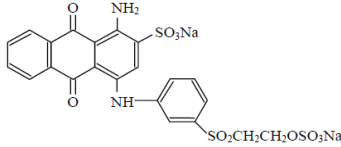
Protein concentration was spectrophotometrically determined at 595 nm according to Bradford (1976) using the reagent commercialised by Bio-Rad (Richmond, USA). Bovine serum albumin (BSA) was used as a standard.

2.4. Dye decolouration experiments

The recalcitrant dye Remazol Brilliant Blue R (CI Reactive Blue 19; 1-amino-9,10-dihydro-9,10-dioxo-4-[(3-{[2-(sulfooxy)ethyl]sulfonyl}phenyl)amino] 2-anthracenesulfonic acid), purchased from Sigma–Aldrich (St. Louis, MO, USA), was selected to perform the present study as a model of anthraquinone dyes. The general characteristics of RBBR are indicated in Table 1. A stock solution (0.1% w/v in water) was stored in the dark at room temperature. The reactions were carried out directly in the spectrophotometer cuvette and the reaction mixture (final volume 1.0 mL) consisted of an aqueous solution of dye and a Al_2O_3 pellet with immobilised laccase (500 U/L, final concentration). RBBR concentration was selected in order to obtain around 0.6 absorbance unit at the maximum wavelength in the visible spectrum (133 g/L, final concentration in the cuvette). The residual

RBBR concentration was spectrophotometrically measured from 400 to 700 nm and calculated by measuring the area under the plot. This approach takes into account the conversion of the dye molecules to other compounds absorbing at different wavelengths and then, the ratio of the area under the visible spectrum is always equal or lower than the ratio of the absorbances at the peak. Dye decolouration was expressed in terms of percentage. Adsorption of the dye to the pellets was determined by immersing the pellets in ethanol (70%, v/v) for 10 days at 50ºC. The amount of the dye bound to the pellets was calculated from the absorption of the supernatants. A control test containing the same amount of a heat-denatured laccase was also performed in parallel.

Table 1. General characteristics of the dye Remazol Brilliant Blue R (RBBR)

Properties	Remazol Brilliant Blue R
C.I. name	Reactive Blue 19
C.I. number	61200
Class	anthraquinone
Apparent colour	Blue
Molecular weight	626.54 g/mol
Molecular formula	$C_{22}H_{16}N_2Na_2O_{11}S_3$
Chemical structure	
Maximum absorption wavelength	592 nm
Water solubility	10 g/L

2.5. LC-MS analysis

RBBR and its final degradation mixture were analysed using a LC 1200 system from Agilent Technologies (Santa Clara, CA, USA) and an Eclipse XDB C-18 column (Agilent

Technologies), with a particle size of 5 µm, 4.5 mm i.d. and 150 mm in length. All samples were passed through a 0.20 µm filter before injection into the LC-MS system. Optimum separation was achieved with a binary mobile phase at a flow-rate of 0.5 mL/min. The mobile phase was composed by solvent A: acetonitrile; and solvent B: 30 mM acetic acid/ammonium acetate buffer at pH 4.5. The gradient elution programme was: 0–0.5 min, 5% A, 0.5–15 min, 5–20% A; 15–18 min, 20–60% A; 18–24 min, 60% A; 24–27 min, return to initial conditions and 8 min post-run delay according to Barceló-Barrachina et al. (2004). The volume of sample injected was 20 µL. The LC system was coupled to a MS 6210 time-of-flight (TOF) mass spectrometer (Agilent Technologies) equipped with a Z-spray ESI source working in positive mode. Optimal ionisation source working parameters were 3.5 kV of capillary voltage, 350ºC of drying gas temperature, 12 L/min of drying gas flow and a nebuliser pressure of 60 psi. Continuum mode TOF mass spectra were recorded using a fragmentor voltage of 150 V and a skimmer voltage of 65 V. Complete system control and data evaluations were done with Mass-hunter workstation software - Qualitative Analysis - v. B.02.00 (Agilent Technologies).

2.6. Phytotoxicity studies

The toxicity of the original and the degraded dye was assessed by measuring the phytotoxicity effect of water solutions (1:6) on seeds germination of ryegrass (Lolium perenne) according to Zucconi et al. (1985). 4 replicates of 10 seeds were used for each test. After 5 days of incubation in the dark, the seed germination percentage and root length of seeds immersed in the dye solutions as well as in deionised water were determined. The values obtained for the deionised water were used as a control. The germination index (GI) was calculated as

follows: GI = GPxLa/Lc, where GP is the number of germinated seeds expressed as a percentage of control values, La is the average value of root length in the dye solutions and Lc is the average value of root length in the control.

2.7. Statistical analysis

Analytical determinations and biological tests were carried out by triplicate and means with standard deviation are given.

3. Results and discussion

3.1. Degradation pathway of RBBR by immobilised laccase
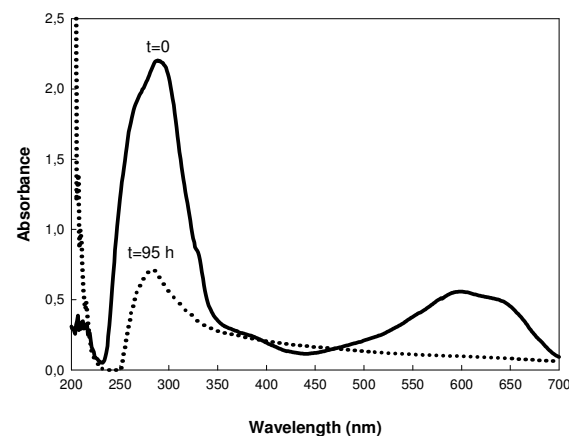
Figure 1. UV–vis spectrum of Remazol Brilliant Blue R solution (133 mg/L initial) before (solid line) and after (dotted line) degradation by immobilised laccase from T. pubescens. Data were obtained from three replicates, standard deviation values were below 5%

Decolouration of RBBR by immobilised laccase was accompanied by changes in its absorption spectrum (Fig. 1). The visible peak (600 nm) disappeared at the end of the experiment (95 h) indicating total decolouration, which implies the cleavage of the chromophore group.
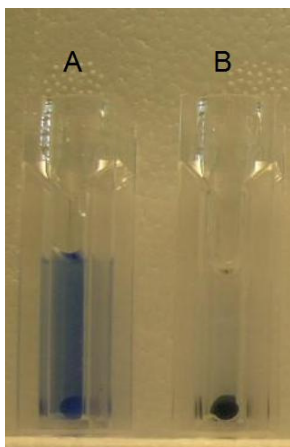
Figure 2. Photograph of Remazol Brilliant Blue R solution (133 mg/L initial concentration) before (A) and after (B) degradation by immobilised laccase from T. pubescens. Data were obtained from three replicates, standard deviation values were below 5%
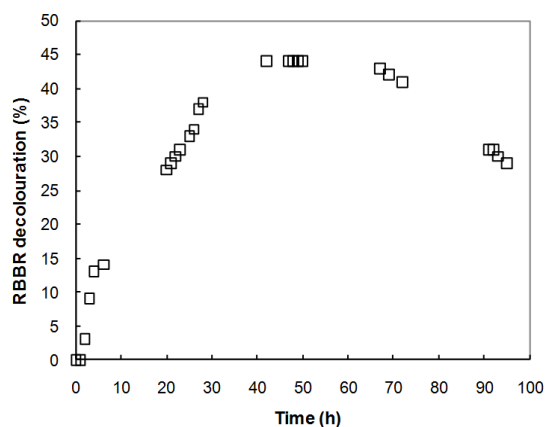
Figure 3.- Remazol Brilliant Blue R (133 mg/L initial concentration) decolouration along time. Data were obtained from three replicates, standard deviation values were below 5%
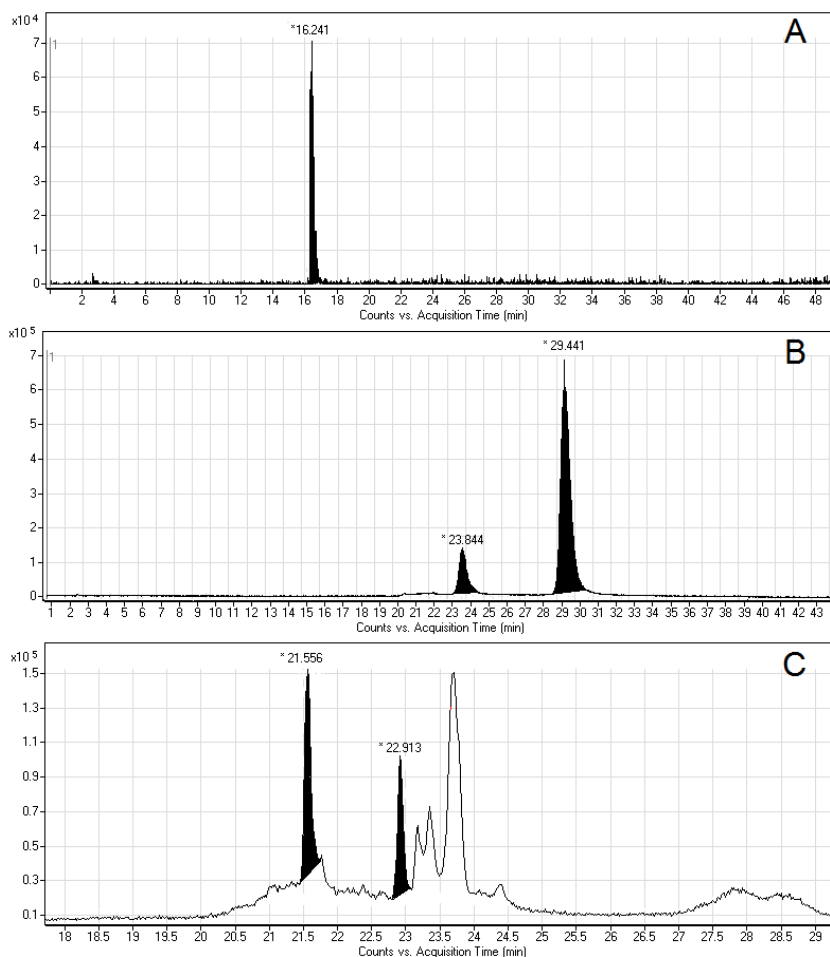
Figure 4.- Chromatograms of the Remazol Brilliant Blue R (133 mg/L) degradation by immobilised laccase from T. pubescens: (A) 0 h; (B) 2 h; (C) 95 h

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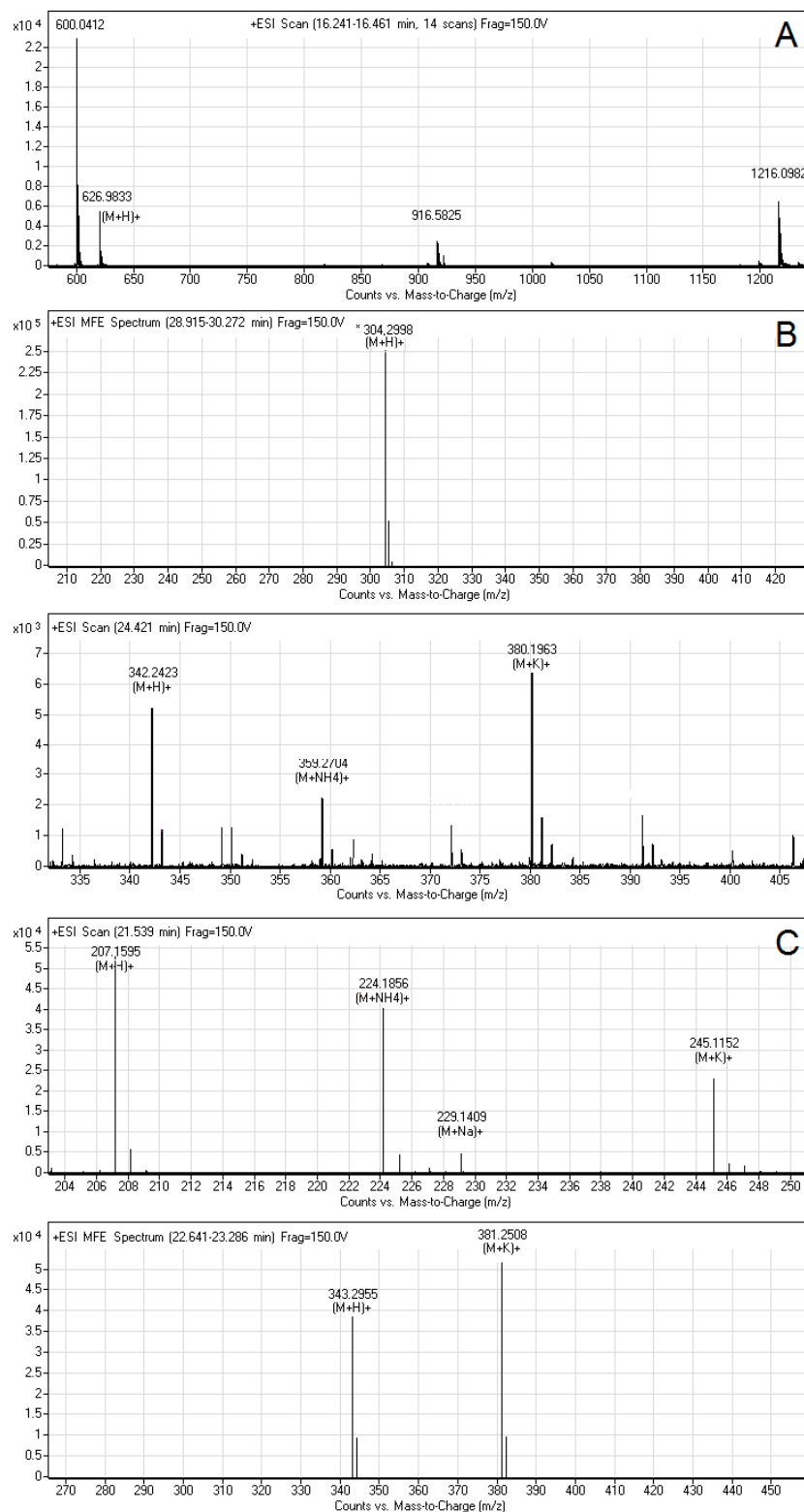
Figure 5.- Mass spectra of the Remazol Brilliant Blue R (RBBR) degradation products by immobilised laccase from T. pubescens at different time intervals: (A) 0 h; (B); 2 h; (C) 95 h

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Fig. 2 shows a photograph of the dye solution before and after the enzymatic treatment, where the significant colour removal can be observed. Fig. 3 shows the decolouration of the RBBR in terms of percentage, where maximum decolouration (44 %) was obtained in 42 h. Then, this value was kept for 25 h and from 67 h onwards it decreased likely due to the formation of coloured and/or cloudy sub-products. In the UV spectrum, the peak at 289 nm considerably decreased but it did not disappeared indicating that partial mineralisation took place (Fig. 1). It was determined that dye adsorption on the pellets was less than 5%, so dye decolouration was mainly due to laccase action. Experiments with denaturised laccase also showed a low adsorption of the dye on the pellet (about 5%), similar to the one observed by the pellets with active laccase.

LC-MS analyses were carried out to investigate the intermediates and the final degradation products of RBBR after laccase treatment. As shown in Fig.4, dye sample collected at 0 h showed a major peak with a retention time of 16.24 min (m/z 600.04 (z=2), 626.98, 916.58 (z=2) and 1216.09 (z=2)). The presence of different molecules at the same retention time suggested the formation of monomers, dimmers (m/z 600.04), trimers (m/z 916.58) and tetramers (m/z 1216.09) of the dye when ionised in the mass detector. Two intermediates were identified after two hours of treatment (m/z 304.30 and m/z 342.24). However, no decolouration was observed during this interval of time which indicated that the chromophore was not broken. After laccase treatment (95 h) the above peaks disappeared and another two peaks with retention times of 21.55 min (m/z 207.16) and 22.91 (m/z 343.29) appeared (Fig. 5). As a result a pathway for RBBR degradation by laccase was proposed (Fig. 6).

Figure 6.- Proposed pathway for Remazol Brilliant Blue R degradation by immobilised laccase from T. pubescens

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3.2. Phytotoxicity study

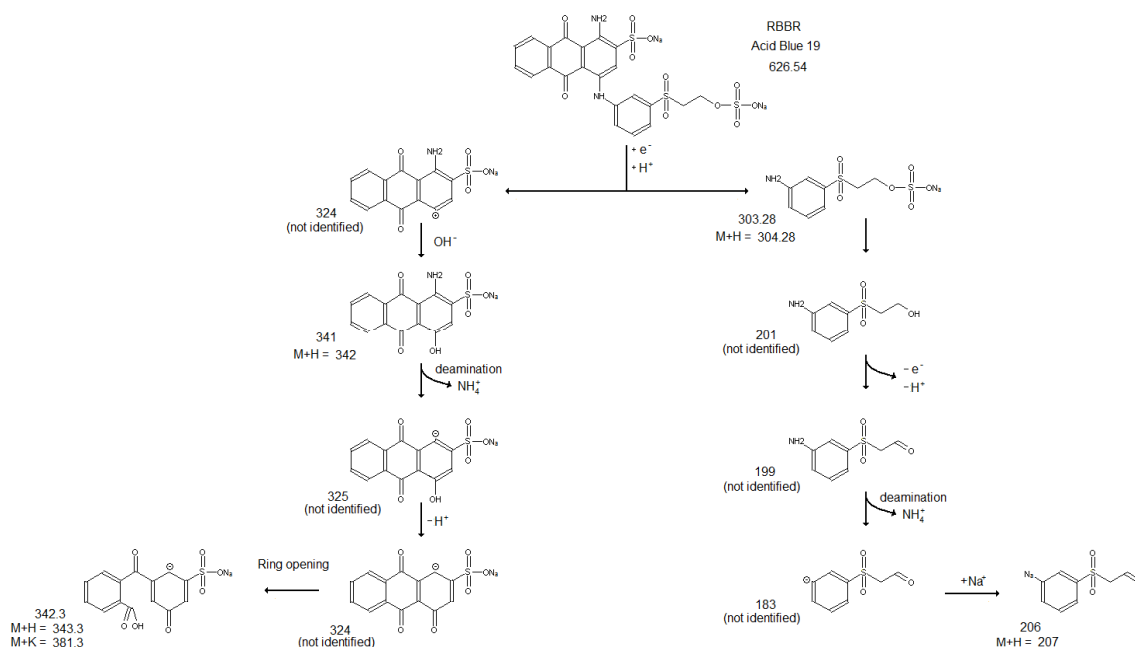Untreated dyeing effluents may cause serious environmental problems and health hazards. They are being discharged into water bodies and this water can be used for agriculture. Thus, it is of concern to assess the phytotoxicity of the dye before and after degradation. For this, the relative sensitivity of ryegrass seeds towards the dye RBBR and its degradation products was studied. The mean of root elongation of ryegrass was 32±5.1 mm of 40 seeds in distilled water as a control with 100% germination. The root elongation and seed germination were 9.1±2.5 mm and 93% when seeds were treated with RBBR and 22.2±5.1 mm and 100% when seeds were treated with RBBR degradation products, respectively. This means that seeds grown in the presence of RBBR presented a GI of 26% while those grown in the presence of RBBR degradation products presented a GI of 69%. According to Zucconi et al. (1985), values for the GI lower than 50 % mean high phytotoxicity, values between 50 and 80 % mean moderate phytotoxicity and values over 80 % indicate that the material presents no phytotoxicity. Therefore, the degradation products of RBBR are still toxic for ryegrass although much less than the original dye. Fig. 7 shows the root length of ryegrass seeds after 3 incubation days in water (Fig. 7A), RBBR (Fig. 7B) and RBBR degradation products (Fig. 7C).

4. Conclusions

In this paper we contribute to the knowledge of dye degradation mechanisms used by oxidative enzymes, suggesting a pathway for RBBR degradation by laccase enzyme. Using LC-MS analysis we were able to detect and further identify two dye intermediate metabolites and two final degradation products. It is noteworthy to point that no re-colouration was observed during RBBR degradation by laccases, making the application of these enzymes very suitable

for the degradation of anthraquinone dye derivatives. In addition, the phytotoxicity of the resulting products of RBBR degradation by laccase was considerably reduced.

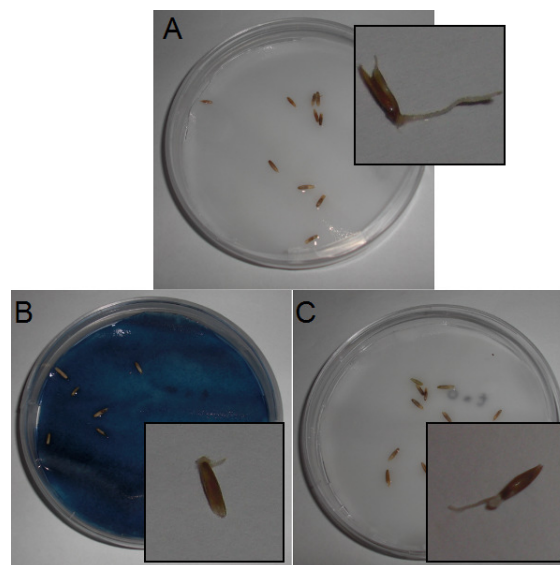*Figure 7.- Photograph of ryegrass seeds showing the root length after 3 incubation days in: (A) water (control); (B) RBBR (133 mg/L); (C) RBBR degradation products*

Acknowledgements

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CHAPTER 9. Biodegradation of a simulated textile effluent by immobilised-coated laccase in laboratory-scale reactors

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Biodegradation of a simulated textile effluent by immobilised-coated laccase in laboratory-scale reactors

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Abstract

Laccase from Trametes pubescens was immobilised on alumina pellets and coated with polyelectrolytes. It was shown that this approach enhanced both laccase stability and reusability. Further, the immobilised coated laccase was applied to the decolouration of a simulated textile effluent in laboratory-scale reactors. The simulated textile effluent was based on the recalcitrant diazo dye Reactive Black 5 (0.5 g/L). It was found that decolouration was due to two processes: dye adsorption onto the immobilisation support and coating and dye degradation by the laccase enzyme. The adsorption process represented less than 10% of colour removal for all cases, so decolouration was mainly due to laccase action. Decolouration was performed in both batch and continuous mode obtaining a complete decolouration of the effluent after 30-36 h for the former and 48 h for the latter without the addition of redox mediators. In addition, the decolourised effluent showed lower phytotoxicity than the original one. These encouraging results make the process suitable for its potential implementation at industrial scale.

Keywords: Remazol Brilliant Blue R; Biodegradation; Laccase; Immobilisation; Trametes pubescens

1. Introduction

Wastewater from textile industries constitutes a serious environmental concern in large parts of the world. During processing, 2-50% of the initial used dyes is not fixed on the textile fibres and finally is lost in the process water [1]. Besides of the aesthetic deterioration of the natural water bodies, many synthetic dyes are toxic, mutagenic and carcinogenic [2]. Therefore, dye-containing wastewater must be treated prior to its discharge into the environment. The current existing methods for removal of dyes from effluents have several drawbacks such as high cost, formation of sludge, low efficiency and do not apply to a great variety of dyes. Bioremediation with oxidative enzymes is seen as a very promising alternative. Among them, enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a particularly promising enzyme for dye removal. Laccases are multicopper oxidases

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that are able to oxidise a wide variety of xenobiotics compounds such as synthetic dyes, chlorinated phenolics, and polycyclic aromatic hydrocarbons. In addition, they can be produced in large amounts [3] and can be applied in crude form [4, 5].

The crucial step in dye degradation is the cleavage of the chromophore rendering dye fragments more susceptible to biodegradation by less specialised organisms. Since few enzymes are involved in the cleavage of chromophores, it can be advantageous to design reactors that are based directly on those enzymes omitting the organisms. Nevertheless, the stability and the catalytic activity of free enzymes considerably decrease by a wide variety of environmental conditions that characterise effluents (pH, ionic concentrations, inhibitors, etc.) and which seriously limit their industrial application. Immobilisation is the most used stabilisation method which, in addition, allows enzyme reutilisation. Laccases have been immobilised on different supports for the removal of several pollutants including dyes [6-10]. However, although a few studies at laboratory-scale with immobilised laccase have been performed [11-14], most studies have used reactors with small volumes [15-23], which does not apply to the large wastewater volumes generated by the textile industry. More studies at laboratory-scale are needed for the further scale-up of the process.

Previous studies by our research group [24] have shown the suitability of the coating approach to enhance the stability of the covalent-immobilised laccase and to decolourise different synthetic dyes. In the present work, that approach have been used to immobilise the laccase obtained from *Trametes pubescens* cultivation for its subsequent application to the treatment of a simulated textile effluent based on the dye Reactive Black 5 (RB5) in

laboratory-scale reactors operating both in batch and continuous mode. In addition, the phytotoxicity of the degraded effluent was assessed.

2. Experimental

2.1. Chemicals

Poly(allylamine hydrochloride) (PAH, Mw 70000) and poly(sodium 4-styrenesulfonate (PSS, Mw 70000), alumina (Al_2O_3) spherical pellets (3 mm diameter), γ-aminopropyltriethoxy silane, glutaraldehyde, 2,2'-azino-di-[3-ethyl-benzo-thiazolin-sulphonate] (ABTS), bovine serum albumin (BSA) and Reactive Black 5 (RB5) were purchased from Sigma Aldrich (St. Louis, MO, USA). 0.01 M poyelectrolyte solutions with a NaCl concentration of 0.5 M were prepared.

2.2. Laccase production and crude laccase preparation

Laccase was produced by cultivation of the white-rot fungus *Trametes pubescens* (MB 89; Austrian Centre of Biological Resources and Applied Mycology, University of Natural Resources and Applied Life Sciences, Vienna, Austria), under semi-solid-state fermentation conditions using dyed sunflower seed shells as support-substrates as described in Rodríguez Couto et al. [25]. Culture broth was collected at the maximum laccase activity (day 10), filtered, clarified by centrifugation at 8000xg for 15 min, frozen, defrosted and, then, filtered to remove the precipitated polysaccharides. The resulting clear filtrate was concentrated on an Amicon membrane with a molecular cut-off of 10 kDa. The experiments were performed with this concentrated clear filtrate (14.4 U/mg specific activity).

2.3. Analytical determinations

Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [26] with ABTS as a substrate. One activity unit was defined as the amount of enzyme that oxidised 1 μmol of ABTS per min. The activities were expressed in U/L.

Protein concentration was determined spectrophotometrically at 595 nm according to Bradford [27] using the reagent commercialised by Bio-Rad (Richmond, USA). BSA was used as a standard.

2.4. Laccase immobilisation and coating

Crude laccase was immobilised onto alumina (Al_2O_3) spherical pellets (3 mm diameter) as indicated in Rodríguez Couto et al. [24]. Bound laccase and bound protein were determined as a difference between the initial and residual laccase and protein concentrations, respectively (immobilisation yield 70%, 0.14 mg protein/g support and 2 U/g support). Then, the Al_2O_3-immobilised laccase was coated by the sequential deposition of PAH and PSS as indicated in Rodríguez Couto et al. [24].

2.5. pH and temperature stability of free, immobilised non-coated and coated laccase

The pH stability of free, immobilised non-coated and coated laccase was determined in the pH range 2–7 by incubating the enzyme in respective buffer for 30 min and then measuring the residual laccase activity at each corresponding pH with ABTS.

The effect of temperature on laccase stability was determined by incubating free, immobilised non-coated and coated laccase in 25 mM succinic buffer pH 4.5 for 30 min in the temperature range 20–80ºC and then the residual laccase activity was determined with the ABTS assay method.

2.6. Reusability of the immobilised non-coated and coated laccase

The reusability of the immobilised non-coated and coated laccase was investigated in a batch experiment using 4.67 mM ABTS as a substrate. The enzyme was incubated with ABTS for 10 h (1 cycle). At the end of each oxidation cycle, the immobilised laccase (0.70 U) and the immobilised-coated laccase (0.70 U) were washed three times with desionised distilled water and the procedure was repeated with a fresh aliquot of substrate. The activity determined for the first time was considered as the control (100%) for the calculation of the remaining percent activity after each cycle.

2.7. Inhibiting effect of sodium azide on free, immobilised non-coated and coated laccase

The activity of free, immobilised non-coated and coated laccase against the well-known laccase inhibitor sodium azide (NaN_3) was tested. For this, the enzymes were pre-incubated with the inhibitor for 15 min in 25 mM succinic buffer (pH 4.5) and then the residual laccase activity was determined with the ABTS assay method. A control experiment with heat-denatured laccase was also performed in parallel.

2.8. Decolouration of a simulated textile effluent

The composition of the simulated textile effluent was based on instructions of the manufacturer Bezema AG (Montlingen, Switzerland) for reactive dyes [28]. It consisted of 0.5 g/L RB5, 30 g/L NaCl, 5g/L Na_2CO_3 and 1.5 mL/L of 32.5% (w/v) NaOH in deionised distilled water. The pH was adjusted to 4.5 with HCl. The residual dye concentration was spectrophotometrically measured from 400 to 700 nm and calculated by measuring the area under the plot. This approach takes into account

the conversion of the dye molecules to other compounds absorbing at different wavelengths and then, the ratio of the area under the visible spectrum is always equal or lower than the ratio of the absorbances at the peak. Dye decolouration was expressed in terms of percentage. Adsorption of the dye to the support was determined by immersing the pellets in ethanol (70% v/v) for 10 days and heating the pellets at 70ºC for 2 h before the measurement. The amount of the dye bound to the pellets was calculated from the absorption of the supernatants. A control experiment with heat-denatured laccase was also performed in parallel.

2.9. Bioreactor configurations and operation conditions

Fluidised-bed bioreactor (FBBR): it consisted of a glass column with dimensions of 20 cm height and 4.5 cm in internal diameter (working volume of 200 mL). Air flow (0.5 vvm) was supplied to the bioreactor in a continuous way (Fig. 1).

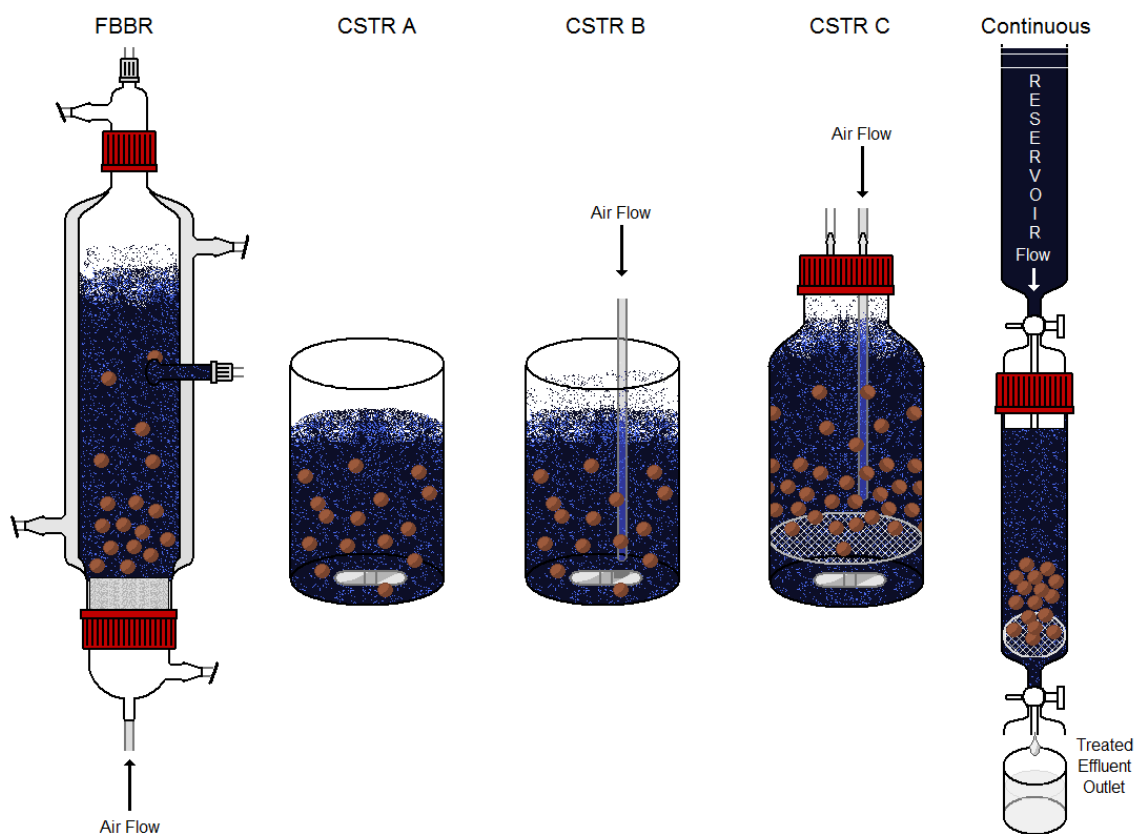*Figure 1. Scheme of the laboratory-scale reactors used*

Continuous-stirred tank reactors (CSTRs): they consisted of glass vessels (7.0 cm in external diameter; working volume 200 mL) magnetically stirred (200 rpm). Different approaches were considered:

CSTR A: it consisted of a 250 mL-glass beaker of 9.5 cm height (Fig. 1).

CSTR B: it was identical to CSTR A but with air flow (0.5 vvm) (Fig. 1).

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CSTR C: it consisted of a 250 mL-glass flask of 13.8 cm height with an air flow of 0.5 vvm and a stainless steel grid to avoid the breaking of the immobilisation support by the magnetic stirrer (Fig. 1).

For the continuous operation a tubular configuration (working volume 100 mL) was used (Fig 1). The effluent was supplied in continuous down-flow mode at a hydraulic retention time (HRT) of 33h without recycling and under passive aeration conditions.

All the bioreactors contained immobilised laccase coated with 3 layers, the ratio biocatalyst/effluent being ¼. The bioreactors were maintained at room temperature. Samples from the bioreactors were collected at convenient times, centrifuged (5000×g, 10 min), filtered (0.2 µm) and analysed.
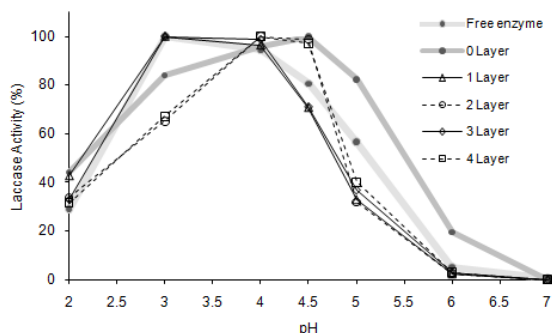

Figure 2. pH profiles of the free, immobilised non-coated and coated laccase. Data were obtained from three replicates, standard deviation values were less than 5%.

2.9. Phytotoxicity studies

The toxicity of the original and the degraded effluent was assessed by measuring the phytotoxicity effect of water solutions (1:6) on seeds germination of ryegrass (*Lolium perenne*) [29]. 4 replicates of 10 seeds were used for each test. After 5 days of incubation in the dark, the seed germination percentage and root length of seeds immersed in the dye solutions as well as in deionised distilled water were determined. The values obtained for the deionised distilled water were used as the control. The germination index (GI) was calculated as follows: GI = GP×La/Lc, where GP is the number of germinated seeds expressed as a percentage of control values, La is the average value of root length in the dye solutions and Lc is the average value of root length in the control.

2.10. Statistical analysis

Analytical determinations and biological tests were carried out by triplicate and means with standard deviation are given.

3. Results and discussion

The potential of alumina (Al_2O_3) pellets for the immobilisation of laccases from *Trametes hirsuta*, *Sclerotium rolfsii*, *Trametes villosa* and *Trametes modesta* and their further use for the decolouration of several synthetic dyes have been reported by several researchers [11, 30-32]. In addition, our research group has shown that the subsequent coating of the Al_2O_3-immobilised laccase with polyelectrolyte layers increased laccase stability [24].

3.1. pH and temperature stability of free, immobilised non-coated and coated laccase

In order to check the effect of the coating on laccase, the laccase activity of the free, immobilised non-coated and coated laccase was measured after incubation at different pH values (Fig. 2). It was found that laccase activity of the immobilised laccase coated with an even number of layers was maximum at pH values ranging from 4.0 to 4.5, whereas the immobilised laccase coated with an odd number of layers showed maximum activity at pH values ranging from 3.0 to 4.0. This might be due to the formation of a microenvironment in the vicinity of the last layer which shifts the pH operation range of the coated enzyme. From pH

6 onwards laccase activity considerably decreased.

The above results shown that the coating did not protect laccase from the inhibiting effect of the ion OH⁻. This is due to polyelectrolyte multilayers are permeable to small mobile molecules present in the solution [33].
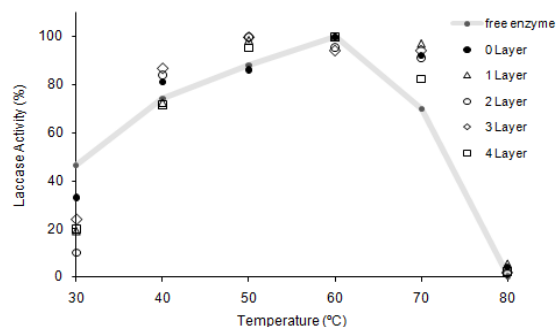
Figure 3. Temperature profiles of the free, immobilised non-coated and coated laccase. Data were obtained from three replicates, standard deviation values were less than 5%.

The thermal stability of the free, immoblised non-coated and coated laccase was studied in the range from 30 to 80ºC (Fig. 3). In all cases, the immobilised laccases showed higher stability at temperatures higher than 60ºC than the free one. However, the free laccase presented higher stability at 30ºC. This can be likely due to immobilisation limits the conformational flexibility at high temperatures. This is in agreement with the results reported by Rekuc et al [22] for laccase from *Cerrena unicolor* immobilised on mesostructured cellular foams.

3.2. Reusability of the immobilised and immobilised-coated laccase

It was found that both laccases retained their activity after 30 cycles of ABTS reaction although in different proportions (Fig. 4). Thus the non-coated laccase retained 21% of its activity, whereas the 1 layer-coated laccase

retained 36%, the 2 layer-coated laccase 42%, the 3 layer-coated laccase 53% and the 4 layer-coated laccase 58%. The half life of the non-coated laccase was shown at 15 cycles, whereas for the 1 and 2 layer-coated laccase was around 25 cycles and for the 3 and 4 layer-coated laccase at more than 30 cycles. Therefore, it can be seen that the layers increased laccase reusability . These results are in agreement with those previously found when coating crude laccase from *Trametes hirsuta* [24].
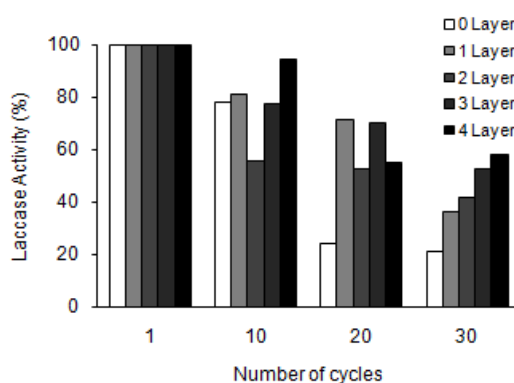
Figure 4. Laccase activity retained by the immobilised non-coated and-coated laccase after 10 h-cycles of ABTS oxidation. Standard deviation values were less than 5%.

3.4. Inhibiting effect of sodium azide on free, immobilised non-coated and coated laccase
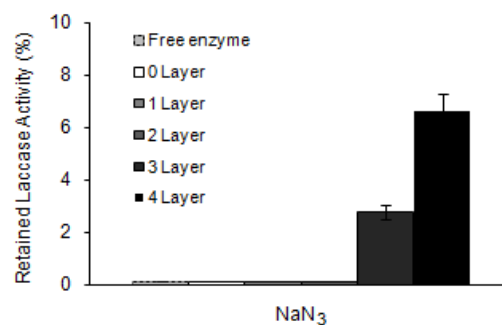
Figure 5. Laccase activity retained by the free, immobilised non-coated and-coated laccase after 15 min exposure to sodium azide (NaN3)

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In order to check the protecting effect of the coating on laccase, the laccase activity of the free, immobilised non-coated and coated laccase was measured after incubation with 30 mM NaN_3 (final concentration) for 15 min. It was found that the layers hardly protected laccase from the inhibitor (Fig. 5).This is due to polyelectrolyte multilayers are permeable to small mobile molecules present in the solution [33] as commented in the section 3.1.

3.5. Decolouration of a simulated textile effluent by immobilised-coated laccase in laboratory-scale reactors

Different bioreactor configurations were tested in order to find the most suitable one for the treatment of textile effluents with immobilised laccase.

3.5.1. Batch mode

The decolouration of a simulated textile effluent by the immobilised-coated laccase in different laboratory-scale reactors operated in batch mode was studied. As shown in Fig. 6 the complete decolouration of the effluent was obtained in 36 hours for all the configurations tested (Fig. 7). Dye decolouration was due to two processes: dye adsorption onto the immobilisation support and coating, which lasted 5 hours, and dye degradation by laccase enzyme. The adsorption of the dye on the support and coating was determined and was found that it represented less than 5% of the colour removal, thus dye decolouration was mainly due to laccase action. CSTR reactors without grid led to the breaking of the pellets in a short time, making the process unfeasible. There was not a significant statistic difference between the results obtained for all the reactors, a complete decolouration being obtained within 30-36 h (Fig. 6). The fluidised-bed reactor has the advantage of saving energy since magnetic stirring is not used, making this configuration

more suitable from an economical point of view.
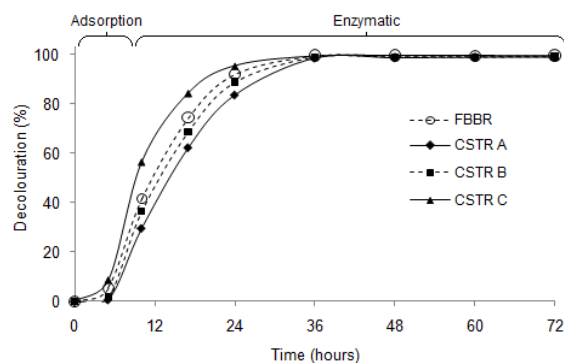
Figure 6 . Effluent decolouration (RB5 0.5 g/L) obtained in laboratory-scale bioreactors operated in batch mode. Standard deviation values were less than 5%.
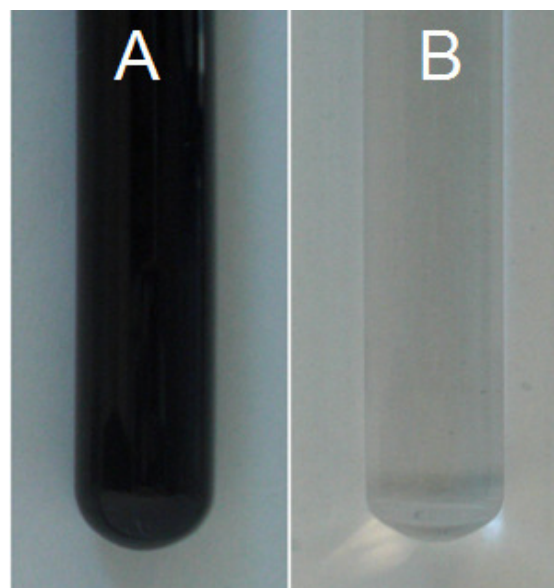
Figure 7. Photograph of the simulated textile effluent before (A) and after (B) decolouration by immobilised-coated laccase from T. pubescens

3.5.2 Continuous mode

An efficient application of effluent decolouration at industrial scale requires the performance of a continuous system technology. Thus, in this section the decolouration of a simulated textile effluent in a laboratory-scale reactor operating in continuous mode was

considered. According to the above results a tubular reactor configuration was selected to perform this experiment. The simulated textile effluent was continuously supplied to the bioreactor in a down-flow mode at an HRT of 33h. As shown in Fig. 8 complete decolouration was observed after reaching the stationary state. Similarly to the batch mode, the decolouration process also presented an initial adsorption step, which lasted 10 h. After this time, the decolouration process was only due to the catalytic action of the enzyme laccase.
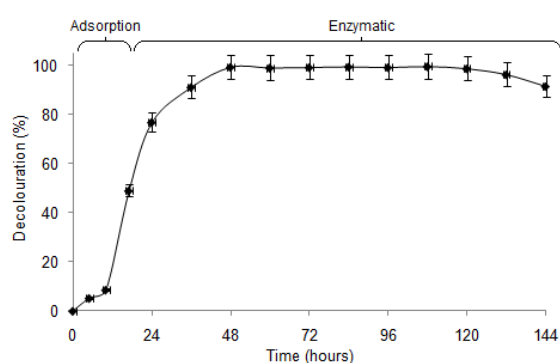
Figure 8. Effluent decolouration (RB5 0.5 g/L) obtained in a laboratory-scale bioreactor (tubular configuration) operated in continuous mode

3.4. Phytotoxicity study

Untreated dyeing effluents may cause serious environmental problems and health hazards. They are being discharged into water bodies and this water can be used for agriculture. Thus, it was of concern to assess the phytotoxicity of the effluent before and after degradation. For this, the relative sensitivity of ryegrass seeds towards the simulated textile effluent and its degradation products was studied. The mean of root elongation of ryegrass was 32±5.1 mm of 10 seeds in deionised distilled water as a control with 100% germination. The root elongation and seed germination were 26.6±1.2 mm and 100% when seeds were treated with the textile effluent and 30±3.7 mm and 100% when seeds were treated with the degraded simulated textile

effluent, respectively (Fig. 9). This means that seeds grown in the presence of the simulated textile effluent presented a GI of 83% whereas those grown in the presence of the biodegradation products of the simulated textile effluent showed a GI of 94%. According to Zucconi et al. [29], values for the GI lower than 50 % mean high phytotoxicity, values between 50 and 80 % mean moderate phytotoxicity and values over 80 % indicate that the material presents no phytotoxicity. Therefore, neither the simulated textile effluent nor its biodegradation products were toxic for ryegrass seeds. Anyway, the GI of the biodegradation products was higher than that of the original textile effluent.
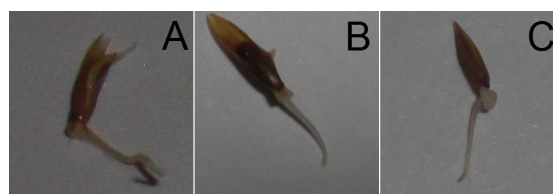
Figure 9. Photograph of ryegrass seeds showing the root length after 3 incubation days in: (A) water (control); (B) simulated textile effluent (RB5 0.5 g/L); (C) decolourised simulated textile effluent

4. Conclusions

The immobilisation procedure previously applied for *T. hirsuta* laccase was also effective for *T. pubescens* laccase. The polyelectrolyte multilayer coating enhanced laccase stability, however did not protect it from the inhibiting effect of OH^- and NaN_3. This is due to the pore size of the layers, which was originally intended for the passing of the substrate (dye) from the solution to the enzyme, also allows the passing of smaller molecules.

The decolouration of a simulated textile effluent was achieved using the coated laccase in different laboratory-scale bioreactors. Complete decolouration was obtained operating both in batch and continuous mode. In addition, the decolourised effluent showed lower

phytotoxicity than the original one. These encouraging results make the use of immobilised coated laccase suitable for its potential implementation at industrial scale.

Acknowledgements

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General Conclusions

Wastewater from textile industries constitutes a serious environmental concern in large parts of the world. During processing, 2-50% of the initial used dyes is not fixed on the textile fibers and finally is lost in the process water. Besides of the aesthetic deterioration of the natural water bodies, many synthetic dyes are toxic, mutagenic and carcinogenic. Therefore, dye-containing wastewater must be treated prior to its discharge into the environment. The current existing methods for removal of dyes from effluents have several drawbacks such as high cost, formation of sludge, low efficiency and do not apply to a great variety of dyes. Bioremediation with oxidative enzymes is seen as a very promising alternative. Among them, enzymes recycling on molecular oxygen as an electron acceptor are the most interesting ones. Thus, laccase is a particularly promising enzyme for dye removal.

The application of laccases to industrial processes requires the production of large amounts of enzyme at low cost. Therefore, our first goal in this study was the search for an efficient production system of the laccase enzyme. For this, the white-rot fungus *Trametes pubescens* was selected. *T. pubescens* was grown under both submerged (SmF) and solid-state fermentation (SSF) conditions for laccase production. Different variables affecting laccase production such as the immobilization support, the carbon source and the addition of inducers of laccase activity were assessed in order to obtain the optimal conditions for laccase production. Maximum laccase activities (30,000 U/L) were obtained operating under SSF conditions using dyed sunflower seed shells (previously evaluated as dye adsorbents) as support-substrate, yeast extract as an organic nitrogen source and Cu^{+2} and tannic acid as inducers. Subsequently, the process was scaled-up to lab-scale tray bioreactors (working volume 150 mL). Maximum laccase activities of about 40,000 U/L were attained. This activity was 822-fold higher than the one obtained operating under SmF conditions with stainless steel sponges as supports and basal medium (Annex I). Also, the cost of production of the laccase in lab-scale bioreactors is comparable to the price of commercially available laccase (Annex I). The results obtained clearly evidenced the

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benefits of SSF over SmF technology for laccase production with *T. pubescens*, probably due to the similarity of the SSF technology to the natural habitat conditions of white-rot fungi (wood). Also, the degrading ability of the laccase produced was assessed. For this, the discoloration of two synthetic dyes (RBBR and MG) was studied. It was found that the dyes were successfully removed by the action of the laccase enzyme without the addition of redox mediators.

The stability and the catalytic activity of laccases considerably decrease by a wide variety of environmental conditions that characterize effluents (pH, ionic concentrations, inhibitors, etc.) and which seriously limit their industrial application. Therefore, the improvement of both laccase stability and reusability is a goal of considerable importance. Immobilization is the most used stabilization method which, in addition, allows enzyme reutilization. Thus, the next objective of the present study was to develop an immobilization protocol for laccase enzyme and its further use in dye removal. From the different supports described in the literature, alumina pellets were chosen as a support for laccase immobilization due to their mechanical resistance at high pHs and temperatures. In addition, polyelectrolyte multilayers were applied to protect the immobilized laccase from possible inhibitors presented in dye effluents. It was found that the coating of immobilized laccase by polyelectrolyte multilayers was an effective method for protecting laccase of adverse environmental conditions without compromising its activity, thus, overcoming the problem of enzyme desorption often encountered with direct immobilization of enzymes on solid supports.

The identification of the metabolites produced during biodegradation as well as toxicity studies must be done to ensure the safety of the laccase-treated effluents. However, so far only a few studies regarding the metabolic pathway of azo dye degradation by ligninolytic enzymes have been performed. Thus, the next objective of the present study was to determine the biodegradation products resulting from the laccase treatment of the dye RBBR. For this, the degradation products of the anthraquinonic dye RBBR by immobilized laccase were investigated using ultraviolet-visible (UV-vis) spectrum scan and High Performance Liquid Chromatography/Mass Spectrometry (LC-MS) analysis. As a result, a degradation pathway for RBBR by laccase was proposed.

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In the next step of the study the immobilized-coated laccase was tested for the treatment of a simulated textile effluent, based on the diazo dye RB5, in lab-scale reactors. A complete discoloration of the effluent was observed after 36 hours in different reactor configurations operating in batch mode.

An efficient application of effluent discoloration at industrial scale requires the performance of a continuous system technology. So, continuous operation of the bioreactors was considered. For this, the tubular configuration was selected. The reactor operated for 144 h at a HRT of 33 h and a complete discoloration was obtained after 48 h. These encouraging results mean a step forward in the potential implementation of the enzymatic treatment for the treatment of dye-containing wastewater at large scale.

Conclusiones Generales

Las aguas residuales de la industria textil constituyen una seria preocupación ambiental en grandes partes del mundo. Durante el procesado, 2-50% de los tintes usados inicialmente no se fijan a las fibras textiles y se pierden en las aguas de proceso. Además del impacto visual en las aguas de lagos y ríos, muchos tintes sintéticos son tóxicos, mutágenos y carcinógenos. Por lo tanto, las aguas residuales con contenido de tintes deben ser tratadas antes de su vertido al medio ambiente. Los métodos existentes en la actualidad para la remoción de tintes de los efluentes tienen varias desventajas tales como un coste elevado, formación de lodos, bajo rendimiento y no son aplicables a una gran variedad de tintes. El tratamiento con enzimas oxidativas se considera una alternativa muy prometedora. Entre ellas, las enzimas que reciclan oxígeno molecular como aceptor de electrones son las más interesantes. Así, la lacasa es una enzima particularmente prometedora para la remoción de tintes.

La aplicación de lacasas en procesos industriales requiere la producción de grandes cantidades de enzima a bajo costo. Por lo tanto, nuestro primer objetivo en el presente estudio fue la búsqueda de un sistema de producción eficiente de la enzima lacasa. Para ello, se seleccionó el hongo de putrefacción blanca Trametes pubescens, el cual se cultivó en condiciones de fermentación sumergida (SmF) y de estado sólido (SSF). Se evaluaron diversas variables que afectan la producción de la lacasa tales como el soporte de inmovilización, la fuente de carbono y la adición de inductores de la actividad de la lacasa con el fin de obtener las condiciones óptimas para la producción de lacasa. Se obtuvieron actividades máximas de lacasa (30.000 U/L) en condiciones de SSF usando cáscaras teñidas de semillas de girasol (previamente evaluadas como adsorbentes de tintes) como soporte-substrato, extracto de levadura como fuente orgánica de nitrógeno y Cu+2 y ácido tánico como inductores. Posteriormente, el proceso se escaló a biorreactores de bandeja (volumen de trabajo 150 mL) obteniéndose actividades máximas de lacasa de alrededor de 40.000 U/L. Esta actividad fue 822 veces mayor que la obtenida en condiciones de SmF operando con esponjas de acero inoxidable como soporte y medio basal (Anexo I). Además, el coste de producción de la lacasa en biorreactores a escala laboratorio es comparable al precio de la lacasa disponible en el mercado (Anexo I). Los resultados

obtenidos evidenciaron claramente las ventajas de SSF sobre la tecnología de SmF para la producción de lacasa con el hongo T. pubescens, probablemente debido a la semejanza de la tecnología de SSF con el hábitat natural (madera) de los hongos de putrefacción blanca. También, se determinó la capacidad de degradación de la lacasa producida. Para ello, se estudió la decoloración de dos tintes sintéticos (RBBR y MG). Se encontró que ambos tintes fueron degradados efectivamente por la enzima lacasa sin la adición de mediadores redox.

La estabilidad y la actividad catalítica de la lacasa disminuyen considerablemente debido a una gran variedad de condiciones ambientales que caracterizan a los efluentes (pH, concentraciones iónicas, inhibidores, etc.) y que limitan seriamente su aplicación industrial. Por lo tanto, la mejora de la estabilidad y de la reutilización de la lacasa es un objetivo de considerable importancia. La inmovilización es el método de estabilización más utilizado el cual, además, permite la reutilización de la enzima. Así, el siguiente objetivo del presente estudio fue desarrollar un protocolo de inmovilización para la enzima lacasa y su uso posterior en la remoción de tintes. De los diversos soportes descritos en la literatura, se eligieron las esferas de alúmina como soporte para la inmovilización de la lacasa debido a su resistencia mecánica a pHs y temperaturas altas. Además, se aplicaron multicapas de polielectrolitos para proteger a la lacasa inmovilizada de los posibles inhibidores presentes en efluentes con tintes. Se encontró que el cubrimiento de la lacasa inmovilizada por multicapas de polielectrolitos era un método eficaz para proteger la lacasa de condiciones ambientales adversas sin comprometer su actividad superando, así, el problema de la desorción de la enzima encontrado frecuentemente en la inmovilización directa de enzimas en soportes sólidos.

Es necesaria la identificación de los metabolitos producidos durante la biodegradación así como la realización de estudios de toxicidad para asegurar la inocuidad de los efluentes tratados con lacasas. Sin embargo, hasta ahora solamente se han realizado algunos estudios con respecto a las rutas metabólicas de degradación de tintes azo por enzimas ligninoliticas. Así, el siguiente objetivo del presente estudio fue determinar los productos de biodegradación resultantes del tratamiento del tinte antraquinónico RBBR con lacasa. Para ello, se investigaron los productos de degradación del tinte RBBR por lacasa inmovilizada

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usando técnicas de espectrofotometría ultravioleta-visible (UV-vis) y cromatografía líquida de alto rendimiento/espectrometría de masas (HPLC-MS). Como resultado, se propuso una ruta de degradación para RBBR tratado con lacasa. En la siguiente etapa del estudio la lacasa inmovilizada-recubierta se probó para el tratamiento de un efluente textil simulado, basado en el tinte diazo RB5, en reactores de escala laboratorio. Se obtuvo una decoloración total del efluente después de 36 horas utilizando diversas configuraciones de reactores operando en modo discontinuo.

Un uso eficiente de la decoloración de efluentes a escala industrial requiere una tecnología de sistema en continuo. Así pues, se consideró la operación continua de los biorreactores. Para ello, se seleccionó la configuración tubular. El reactor funcionó durante 144 h con un TRH de 33 h y se obtuvo una decoloración total después de 48 h. Estos alentadores resultados suponen un paso adelante en la posible implementación del tratamiento enzimático de aguas residuales con tintes a gran escala.

ANNEX I. Estimation of the costs of the laccase production

For a process to be feasible at industrial scale, it is necessary to be competitive from an economical point of view. Thus, in this annex the costs of the laccase produced with *T. pubescens* are evaluated and compared with the price of the commercially available laccases.

The cost of producing laccase was based on two different items: costs of materials and operating costs. For the material costs, most of the market prices of reagents or compounds were obtained from SIGMA-Aldrich® [1]. In some special cases, such as agricultural materials, the cost was assumed to be equal to the lowest one of any commercialized product with similar characteristics [2-5]. Thus, in the case of the mandarin peelings, the price of a cooking product of fresh mandarin peelings was used, whereas for the sunflower seed shells, the price of bricks, made of sunflower hulls, for heating was used. Unfortunately, the price of banana skin could not be estimated and had to be assumed as null.

For operating costs, the energy spent by the use of the autoclave and the incubator was taken into consideration [6]. Nevertheless, the cost of manpower and equipments was not considered in any analysis, since it will depend on the automation and monitoring of the process, the experience of the worker and the volume of laccase to be produced.

The cost of producing laccase was calculated as:

$$Cost_{Lac} = \frac{\sum Price_{Mat} \cdot Quantity_{Mat} + \sum Price_{Energy} \cdot Quantity_{Energy}}{Activity_{Lac} \cdot Volume_{Lac}}$$

which basically means that the total cost of the laccase produced is the sum of all the costs of materials and operation, divided by the product of the volume and activity of the laccase produced. Table A-1 shows in detail all the calculations performed and the final cost of the laccase produced in each cultivation. Additionally, it includes the price of some

commercially available laccases. The price of basal media and the operating cost of equipments are detailed in Tables A-2 and A-3.

Table A-1. Cost of producing laccase using the different culture media and fermentation technologies described in the document and price of commercially available laccases

Type	Culture Type	Price of specific material [€/g]	Amount used [g/l]	Culture volume [l]	Total Cost of materials [€]	Days of culture until max is reached	Total hours of operation [h]	Total Cost of operation [€]	Max. laccase activity [U/l]	Amount produced [l]	Laccase Produced [U]	Cost per unit of activity [€/U]
SmF (Flask scale)	SmF glucose	0.027	10	0.1	0.576	15	360	57	51	0.08	4	14.222
	SmF glucose + ABTS	46.1	0.55	0.1	3.112	16	384	61	5	0.08	0	160.881
	SmF glucose + Tween 20	46.32	1	0.1	7.744	16	384	61	110	0.08	9	7.839
	SmF glucose + Soy oil	0.0007	0.91	0.1	7.744	16	384	61	225	0.08	18	3.832
	SmF glucose + Malaquite Green	0.04	0.005	0.1	7.744	16	384	61	80	0.08	6	10.779
	SmF glucose + 1mM Cu+2	0.02	0.16	0.1	7.744	15	360	57	7	0.08	1	116.416
	SmF glucose + 2mM Cu+2	0.02	0.32	0.1	7.745	10	240	38	25	0.08	2	23.117
	SmF glucose + 50uM Tannic Acid	0.11	0.09	0.1	7.746	10	240	38	200	0.08	16	2.890
	SmF glucose + 100uM Tannic Acid	0.11	0.17	0.1	7.747	16	384	61	105	0.08	8	8.213
	SmF glicerol	0.088	10	0.1	0.637	16	384	61	89	0.08	7	8.691
	SmF mandarin peeling	0.022	30	0.1	0.615	3	72	12	228	0.08	18	0.689
SSF (Flask scale)	SSF Banana Skin	0*	350	0.02	0.110	20	480	76	1600	0.005	8	9.565
	SSF sunflower flask	0.004	75	0.02	0.146	10	240	38	7751	0.01	78	0.498
	SSF sunflower flask + 1% v/v Coconut oil	0.012	0.91	0.02	0.146	8	192	31	10951	0.01	110	0.284
	SSF sunflower flask + 2% v/v Coconut oil	0.012	1.82	0.02	0.147	9	216	35	18382	0.01	184	0.190
	SSF sunflower flask + 0.1 mM Cu+2	0.02	0.016	0.02	0.147	10	240	38	18773	0.01	188	0.206
	SSF sunflower flask + 0.3 mM Cu+2	0.02	0.048	0.02	0.147	10	240	38	23621	0.01	236	0.164
	SSF sunflower flask + 0.5 mM Cu+2	0.02	0.08	0.02	0.147	10	240	38	25773	0.01	258	0.150

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SSF (Lab-scale Bioreactors)	SSF sunflower flask + Soy oil 1% (v/v)	0.001	0.91	0.02	0.147	9	216	35	9708	0.01	97	0.359
	SSF sunflower flask + Soy oil 2% (v/v)	0.001	1.82	0.02	0.147	10	240	38	10165	0.01	102	0.380
	SSF sunflower flask + 25 µM Tannic acid	0.11	0.09	0.02	0.147	10	240	38	10635	0.01	106	0.363
	SSF sunflower flask + 50 µM Tannic acid	0.11	0.17	0.02	0.147	10	240	38	10064	0.01	101	0.384
	SSF sunflower flask + 1 mM Xylidine	0.0738	0.121	0.02	0.147	10	240	38	12494	0.01	125	0.309
	SSF sunflower flask + Cu^{+2} + tannic acid (added 3rd day)	Combination of previous cases		0.02	0.146	11	264	42	30272	0.01	303	0.140
	SSF sunflower flask + Cu^{+2} + xylidine (added 3rd day)			0.02	0.146	11	264	42	19085	0.01	191	0.222
	SSF sunflower flask + Cu^{+2} + tannic acid (added 3rd and 7th day)	Combination of previous cases		0.02	0.147	11	264	42	22946	0.01	229	0.185
	SSF sunflower flask + Cu^{+2} + xylidine (added 3rd and 7th day)			0.02	0.146	8	192	31	26645	0.01	266	0.117
	SSF Tray reactor	0.004	75	0.15	1.094	13	312	50	7130	0.08	570	0.089
	SSF Tray bioreactor + Cu+2 + tannic acid (added 0 day)	Combination of previous cases		0.15	1.095	14	336	54	31610	0.08	2529	0.022
	SSF Tray bioreactor + Cu+2 + tannic acid (added 3rd day)			0.15	1.095	13	312	50	41135	0.08	3291	0.015
Commercial Laccase	Laccase from *Rhus vernicifera*, crude acetone powder, >50 units/mg (catechol as a substrate) (Sigma)											0.015
	Laccase from *Trametes versicolor*, BioChemika, powder, ≥0.5 units/mg (catechol as a substrate) (Sigma)											0.076

* Price of the substrate asume as null due to lack of information

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Table A-2. Detailed cost of the basal media

Basal Medium	Price [€/g]	Quantity [g/l]	Price [€/l]
$(NH_4)_2SO_4$	0.0615	0.9	0.055
KH_2PO_4	0.1815	2	0.363
$MgSO_4$	0.0281	0.5	0.014
$CaCl_2$	0.0482	0.1	0.005
KCl	0.0922	0.5	0.046
Thiamine	0.3434	0.5	0.172
C_6H8O_7	0.1292	19.1	2.466
Na_2HPO_4	0.0827	28.7	2.369
		Total Price	5.491
Yeast extract	0.0618	20	1.236
Glucose	0.027	10	0.270
		Total Price	6.997

Table A-3. Detailed operating costs of equipment

Equipment	Price [€/kW·h]	Power [kW]	Operation time [h]	Total [€]
Autoclave	0.0632 [6]	4.5	2	0.5688
Incubator	0.0632 [6]	2.5	Depending on cultivation time	0.158·Cultivation time

In general terms, the cost of producing laccase under SSF is lower than when using SmF technology. However, there is an exceptional case, when using mandarin peelings in SmF, with a final cost for the production of laccase comparable to those obtained with SSF technology. In most of the cases, the addition of inducers not only incremented the laccase activity but also lowered the cost of production. Also, the scaling up of SSF cultivation considerably lowered the final cost of laccase production (about 1.5 cents of Euro per Unit of activity). This final cost is equal or lower than the price of commercially available laccases.

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[1] Sigma Aldrich Website. http://www.sigmaaldrich.com/spain.html Prices April 2009, Region Spain.
G8270   Glucose ≥ 99.5%
G6279   Glycerol ≥ 99%, ρ=1.25 g/ml
92144   Yeast extract (Fluka)
70175   Peptone from meat, enzymatic digest (2) (Fluka)
82303   Peptone from casein, enzymatic digest (1) (Fluka)
U5378   Urea for molecular biology, >98%, powder (Sigma)
T7293   Tryptone (2) Microbiologically tested. (Fluka)

11557   2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (1) ≥99.0% (Fluka)

P1379   Tween 20 ® viscous liquid (Sigma-Aldrich)

B6756   Malaquite Green (2) Dye content ~90 % (Sigma)

209201   Copper(II) sulfate hydrate (1) 98% (Sigma-Aldrich)

403040   Tannic Acid (7) ACS reagent (Sigma-Aldrich)

D145807Xylidine 99% (Aldrich)

A4418   Ammonium sulfate (18) for molecular biology, ≥99.0% (Sigma)

P9791   Potassium phosphate monobasic (21), ≥98% (Sigma)

230391   Magnesium sulfate heptahydrate (13) ACS reagent, ≥98% (Sigma-Aldrich)

223506   Calcium chloride dihydrate (15) ACS reagent, ≥99% (Sigma-Aldrich)

P9333   Potassium chloride (23) SigmaUltra, ≥99.0% (Sigma-Aldrich)

T4625   Thiamine hydrochloride (8) reagent grade, ≥99% (Sigma-Aldrich)

251275   Citric Acid (15) ACS reagent, ≥99.5% (Sigma-Aldrich)

S7907   Sodium phosphate dibasic (18) SigmaUltra, ≥99.0% (Sigma-Aldrich)

L2157   Laccase from Rhus vernificera, crude acetone powder, >50 units/mg solid (Sigma)

38429   Laccase from Trametes versicolor, BioChemika, powder, light brown, ≥0.5 units/mg (Sigma)

38837   Laccase, Coriolus versicolor, CLEA, BioChemika, ≥0.3 units/mg (Fluka)

[2] http://www.efooddepot.com/products/Jiabao_Brand/5834/Preserved_Mandarin_Peel.html

[3] Soy oil http://www.indexmundi.com/es/precios-de-mercado/?mercancia=aceite-de-soja

[4] Sunflower shells hulls: http://www.czechdirect.ie/ecowoodpellets (20kg package - 97 dollars) = 74.7€

[5] Coconut oil (Nutiva Organic Extra Virgin Coconut Oil -- 15 oz =425g, 6.64U$=5.11€)

http://www.vitacost.com/Nutiva-Organic-Extra-Virgin-Coconut-Oil-15-oz?csrc=GPF-692752200014

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Biographical note

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Kind of participation: Poster

Conference: Internacional Symposium on Environmental Biocatalysis

Number or authors: 3

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