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Laccases in Pollution Control

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ABSTRACT

Environmental pollution with hazardous wastes containing recalcitrant synthetic chemicals (xenobiotics) has become one of the major ecological problems. Unlike the naturally-occurring organic compounds that are readily degraded upon introduction into the environment, xenobiotics are extremely resistant to biodegradation by native microorganisms. Additionally, the implementation of more and more stringent environmental regulations on hazardous wastes has impelled the search for innovative and environmentally-friendly treatment technologies to complement or substitute the conventional ones. Thus, a great deal of research has recently been focused on investigating the potential arising from the use of enzymes that have been isolated from their parent organisms to catalyse the transformation of targeted pollutants. Among such enzymes, laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are outstanding, since they have the following properties: low substrate specificity, they do not need the addition or synthesis of a cofactor, as their cosubstrate – oxygen – is usually present in their environment, most laccases are extracellular which facilitates the purification procedures, they generally exhibit a considerable level of stability in the extracellular environment and the inducible expression of laccases in most fungal species also contributes to their easy applicability in biotechnological processes. All this makes laccase enzymes very useful for their application in bioremediation of polluted sites. The present paper reviews the potential application of laccases in pollution control.

Keywords: bioremediation, biotechnology, environment, enzymes, wastewater, xenobiotics
Abbreviations: **ABTS**, 2-2′-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid); **AHA**, acetohydroxamic acid; **AOX**, adsorbable organic halogens; **BPA**, bisphenol A; **CLECs**, cross-linked enzyme crystals; **COD**, chemical oxygen demand; **CRT**, cellular retention time; **2,4-DCP**, 2,4-dichlorophenol; **EDCs**, endocrine disrupting chemicals; **EPR**, electron paramagnetic resonance; **EDTA**, ethylenediamine tetraacetic acid; **HAs**, humic acids; ***N*-HBT**, hydroxybenzotriazole; **HPLC**, high performance liquid chromatography; **HRP**, horseradish peroxidase; **HRT**, hydraulic retention time; **LMS**, laccase mediator system; **NHE**, Nerst hydrogen electrode; **NP**, nonylphenol; **OMW**, olive mill wastewater; **PAHs**, Polycyclic aromatic hydrocarbons; **PEG**, polyethylene glycol; **PCBs**, polychlorinated biphenyls; **PCE**, perchloroethylene; **PCP**, pentachlorophenol; **RBBR**, Remazol Brilliant Blue R; **SSF**, solid-state fermentation; **TCS**, triclosan; **TNT**, 2,4,6-trinitrotoluene

CONTENTS

INTRODUCTION

The stringent legislations concerning the release of wastewater to water bodies have increased the search for efficient and green oxidation technologies to treat industrial wastewater. Processes based on enzymes appear very promising. Thus, laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) have been subject of intensive research in the last decades because they have the following properties: wide substrate specificity, 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, making the purification procedures very easy, they generally exhibit a considerable level of stability in the extracellular environment and the inducible expression of laccases in most fungal species also contributes to their easy applicability in biotechnological processes.

Laccases are multicopper proteins belonging to the family of blue-oxidase enzymes, which can oxidise a great variety of aromatic compounds with the concomitant reduction of oxygen to water. The copper centres of laccases are classified in three groups according to their spectroscopic properties: T1, blue copper displaying an absorption band at 605 nm, detectable by electron paramagnetic resonance (EPR); T2, normal copper with no absorption band in the UV-Vis region, detectable in EPR spectra; T3, coupled binuclear copper center with an absorption band at 330 nm, not detectable in EPR (Klyachko *et al*. 1992; Solomon *et al*.

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Invited Review

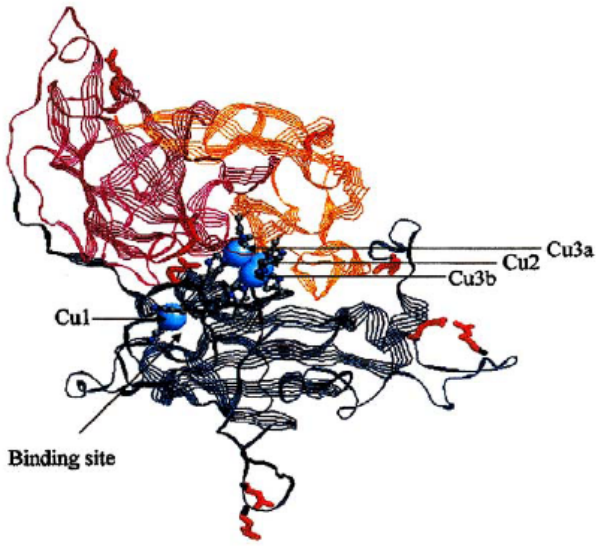

Fig. 1 Three dimensional structure of laccase from *Rhus vernicifera*. There are three cuprodoxin-like domains: the T1 site (Cu1) belongs to domain 3 and the T2/T3 site (Cu2, Cu3a and Cu3b) is the interface between the two other domains. The model also indicates the putative binding sites for glutaraldheyde. (Figure re-printed from Durante *et al.* (2004) *Journal of Molecular Catalysis B: Enzymatic* **27**, 191-206, with kind permission of Elsevier Ltd.).

1996). Laccases usually contain four copper ions: one T1, one T2 and two T3 copper centres (**Fig. 1**). The T2 and T3 copper centres form a trinuclear copper cluster site, which is involved in the binding of oxygen during its reduction to water. The T1 copper centre is involved in the oxidation of the reducing substrate and the generated electrons are then transferred back to the T2 and T3 copper centres (Call and Mücke 1997; **Fig. 2**). One of the key characteristics of laccases is the standard redox potential of the T1 site, which was found to vary between 430 and 790 mV vs NHE (Reinhammar 1972; Xu *et al*. 1996; Klonowska *et al*. 2002; Shleev *et al*. 2004a, 2005). Reinhammar (1972) reported that the redox potential of the laccase from *Polyporus versicolor* at T1 site was 785 mV whereas the redox potential of the laccase from *Rhus vernicifera* at T1 site was 434 mV. Also, Xu *et al*. (1996) showed that significant differences in the redox potential of the T1 site existed among fungal laccases. On the contrary, Shleev *et al*. (2004a) determined that the standard redox potentials of the laccases from *Trametes hirsuta* 56, *Trametes ochracea* 92-78, *Cerrena maxima* and *Coriolopsis fulvocinerea* at T1 site were very similar (780, 790, 750 and 780 mV, respectively). Klonowska *et al*. (2002) reported that the basidiomycete C30 produced simultaneously both a low and a high redox potential laccase. More recently, Shleev *et al*. (2005) suggested that the redox potentials of the T2 copper sites in many multicopper oxidases might have a formal potential value close to 400 mV vs NHE.

Although a few laccases have been isolated from plant sources, e.g., lacquer (*R. vernicifera)*, sycamore (*Ficus sycomorus*) and tobacco (*Nicotiana tabacum* L.), most known laccases are fungal in origin (e.g. white-rot fungi) and are extracellular enzymes (Schneider *et al*. 1999; Antorini *et al*. 2002). Also, it has been reported that laccases are widespread in bacteria like *Escherichia coli*, *Pseudomonas syringae*, *Xanthomonas campestris* and *Pseudomonas putida* (Alexandre and Zhulin 2000).

Laccases have found several applications in bioremediation. Thus, laccases have been used for the treatment of phenolic effluents, polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Böhmer *et al*. 1998; D'Annibale *et al*. 2000; Ullah *et al*. 2000) as well as for the decolouration of textile dyes (Kandelbauer and Gübitz 2005). The recent interest in laccases is, in part, a consequence of the finding that laccases can also oxidise non-phenolic compounds in the presence of certain com-

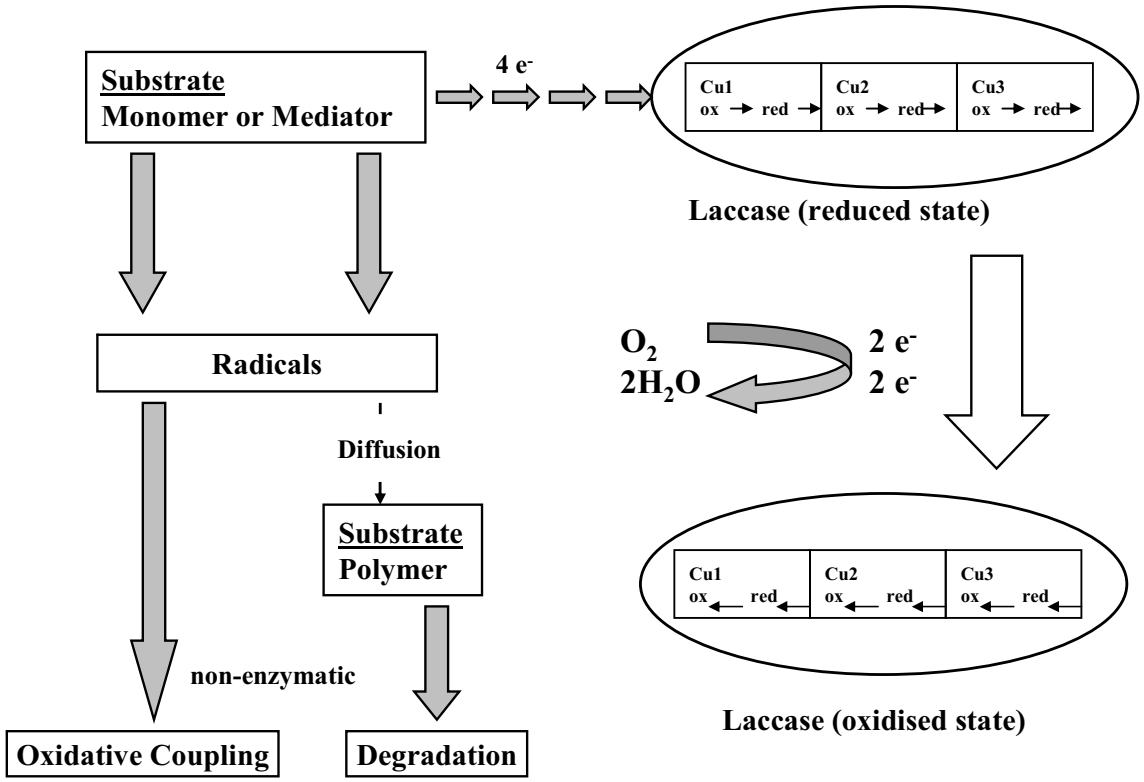

Fig. 2 Catalytic reactions of fungal laccases: The enzyme oxidises the substrate molecules with type 1 copper by four step-wise transfers of one electron. The reoxidation of laccase is brought about by the diamagnetic type 3 copper pair, which transfers four electrons in two-electron steps to O_2. The oxidation of monomers creates reactive radicals that can undergo non-enzymatic coupling reactions. Degradation of polymers is catalysed by low molecular mass substances. After activation of these mediator molecules by laccase, they diffuse from the active enzyme site to susceptible structures of the polymers. (Adapted from Claus 2003).

Fig. 3 The oxidation cycle of a laccase/mediator system towards non-phenolic substrates. (Figure re-printed from Astolfi *et al.* (2005) *New Journal of Chemistry*, **29**, 1308-1317, with kind permission of the Royal Society of Chemistry).

pounds, which act as redox mediators, (Bourbonnais and Paice 1990; Eggert *et al*. 1996). Redox mediators are low-molecular weight compounds that are easily oxidised by laccases producing, in some cases, very unstable and reactive cationic radicals, which can oxidise more complex substrates before returning to their original state (**Fig. 3**). The electrons taken by laccases are finally transferred back to oxygen to form water (McGuirl and Dooley 1999; Wong and Yu 1999). Typical mediators are 2-2′-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) (Cantarella *et al*. 2003), *N*-hydroxybenzotriazole (HBT) (Kleen *et al*. 2003) and violuric acid (Li *et al*. 1999). The laccase mediator system (LMS) has yet to be applied on a large scale due to the cost of synthetic mediators (for example, 1 g of ABTS costs 33.30 euros; 100 g of HBT 85.90 euros; 10 g of violuric acid 40.20 euros; https://www.sigmaaldrich.com/catalog/) and the lack of studies that guarantee the absence of toxic effects of these compounds or their derivatives. 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. Thus, in the presence of the above-mentioned mediators a decolouration percentage of about 80% in 5 min for the triarylmethane-type dye Aniline Blue and higher than 80% in 5 min for the diazo dye Reactive Black 5 was obtained. Laccase alone was not able to decolourise the latter.

LACCASE APPLICATIONS IN POLLUTION CONTROL

Detection of toxic compounds

Biosensors can make ideal sensing systems to monitor the effects of pollution on the environment due to their biological base, ability to operate in complex matrices, short response time and small size (Dennison and Turner 1995). The determination of phenol and its derivative compounds is of environmental importance, since these species are released into the environment by a large number of industries, e.g. the manufacture of plastics, dyes, drugs, antioxidants and wastewater from pulp and paper production (Canofeni *et al*. 1994; Yaropolov*et al*. 1995; Svitel *et al*. 1998; Nistor *et al*. 1999; Freire *et al*. 2000).

Phenols are also breakdown products from natural organic compounds such as humic substances, lignins and tannins. Certain phenols and related aromatic compounds are highly toxic, carcinogenic and allergenic and due to their toxic effects, their determination and removal in the environment are of great importance.

Most phenols exhibit different toxicities and their determination is very important for evaluating the total toxicity of an environmental sample. In general, phenolic compounds are subjected to chromatographic separation before detection. However, the separation takes time and often requires pre-concentration. In addition, the equipment is expensive and is not generally portable. A device which permits the detection of phenols in aqueous solutions at a concentration in the low micro molar range with minimal sample preparation will be useful (Vianello *et al*. 2004).

The maximum amount of phenols in wastewater allowed by the European Community is lower than 1 mg/L in European Community countries (European Community "Urban Water Directive" 91/271/EC), of 0.1 mg/L in Brazil (Secretaria de Saúde e do Medio Ambiente, State Law 5/89), of 1 mg/L in Japan (Water Pollution Control Law and Tochigi Prefecture ordinances)), of 0.5 mg/L in U.S. (Ersöz *et al*. 2003) and, therefore, the detection and monitoring of phenols in wastewater is not an easy task. Apart from the classical method of Folin-Ciocalteau, various methods based on spectrophotometry (Bosch *et al*. 1987), enzyme assay (Mosca *et al*. 2000), HPLC (Ong *et al*. 1989; Zhao and Lee 2001), gas chromatography (Bartak *et al*. 2000) and gas chromatography-mass spectrometry (Angerosa *et al*. 1995; Tasioula-Margari and Okogeri 2001) have been proposed to determine phenols in aqueous solutions. Although some of these methods are characterised by high sensitivity, they are relatively cumbersome and often need derivatisation and pre-concentration steps. To overcome these drawbacks, biosensors using laccase as a detection element have been developed to detect phenols in effluents (Yaropolov *et al*. 1995; Freire *et al*. 2002; Kulys and Vidziunaite 2003). **Table 1** shows the development of laccase-based biosensors for the detection of environmental pollutants (mainly phenols) in polluted sites in recent years. Thus, Marko-Varga *et al*. (1995) developed a biosensor in which tyrosinase, laccase and peroxidase were implemented in amperometric electrodes, allowing the screening of complex real environmental samples for phenolic compounds with an accurate detection of down to sub-µg/L levels. Freire *et al*. (2001) showed that a biosensor in which laccase was immobilised using glutaraldehyde and carbodiimide exhibited an excellent stability and maintained the laccase activity over 2 months. Freire *et al*. (2002) developed a new system for amperometric determination of phenolic compounds in paper mill effluents. The method was based on a flow system, a dialysis sampler and a laccase-based biosensor. The biosensor allowed its application for direct measurements in complex media with no sample pre-treatment and showed an excellent long-term stability allowing measurements for more than 3 months. In addition, this laccase-based biosensor showed selective measurements of micromolar concentration of phenol, *p*-chlorophenol, guaiacol and chloroguaiacol. Also, Kulys and Vidziunaite (2003) developed graphite

Table 1 Application of laccases for the detection of toxic compounds.

Application	Laccase source	Reference
Biosensors for monitoring lignin in wastewater from pulp and paper industry	*Trametes hirsuta*	Shleev *et al*. 2006
Biosensor for detection of phenols in OMW	*Rigidoporus lignosus*	Vianello *et al*. 2006
Biosensor for determination of phenols in environmental control	*Trametes versicolor*	Roy *et al*. 2005
Biosensor for determination of xenobiotics in wastewater	*T. versicolor*, *Aspergillus niger* and *Agaricus bisporus* tissues	Timur *et al*. 2004
Biosensor for detection of phenols in OMW	*R. lignosus*	Vianello *et al*. 2004
Biosensor for determination of phenolic and related compounds in wastewater	*Polyporus pinsitus*, *Myceliophthora thermophila**	Kulys and Vidziunaite 2003
Biosensor for determination of phenolic compounds in effluent samples from paper mills	*Coriolus hirsutus*	Freire *et al*. 2002
Biosensor for phenol monitoring in wastewater	*T. versicolor*	Freire *et al*. 2001
Biosensor for determination of phenolic compounds in real water samples	*C. hirsutus*	Marko-Varga *et al*. 1995

electrodes based biosensors containing recombinant laccase of *Polyporus pinsitus* and *Myceliophthora thermophila* for the determination of phenol and related compounds under steady-state and flow-through regimes.

On the other hand, Vianello *et al*. (2004) showed that a biosensor based on covalent immobilisation of laccase from *Rigidoporus lignosus* detected phenols at a sensitivity of 3 nA/μM and a detection limit of 2 μM, when 1,4-hydroquinone was used as a substrate. Although the amount of enzyme immobilised (about 140 ng laccase/cm^2) was tiny, the biosensor had a lifetime comparable with devices with much higher enzyme loads such as those above-mentioned developed by Freire *et al*. (2002) and Kulys and Vidziunaite (2003). In addition, because of the low substrate specificity of the immobilised laccase from *R*. *lignosus*, the proposed biosensor can be used to detect a large number of phenols occurring in olive mill wastewater (OMW). Timur *et al*. (2004) developed thick film electrode based biosensors containing *T. versicolor* and *Aspergillus niger* laccases and *Agaricus bisporus* tissues for determining phenolic compounds. They showed that the obtained biosensors could be used as simple, rapid and direct methods for determining xenobiotics in wastewater samples without requiring sample pretreatment. Recently, Roy *et al*. (2005) showed that cross-linked enzyme crystals (CLECs) can be used for biosensor application. Thus, they developed a biosensor containing CLECs of laccase from *T. versicolor* which was able to detect phenols at 50-1000 μmol concentration level. The CLECs of laccase has an added advantage over the soluble enzyme in the biosensor application: it has an optimum pH range of 5.5-6, which is nearer to the neutral, whereas the optimum pH range for the soluble enzyme is 3-4. This biosensor could be used to detect the quantity of catechol and catechins in tea and antioxidants like pyrogallol and ferulic acid in food and beverages and organic pollutants like 2-amino phenol in wastewater. More recently, Shleev *et al*. (2006) designed biosensors with laccase from *T. hirsuta* to monitor kraft and soluble pine lignin in wastewater from the pulp and paper industry and Vianello *et al*. (2006) presented a laccase-based biosensor that detected phenolics with a 100 nA/μM sensitivity and a detection limit of about 30 nM.

Bioremediation of industrial wastewater

The rapid expansion and technological improvement in industrial fields in the last 30 years has meant an increasing amount and complexity of toxic waste effluents. At the same time, regulatory authorities have paid more attention to environmental problems and as a consequence industrial companies are forced to treat their waste effluents before discharging them into the environment.

Wastewater from the textile industry

One of the more urgent problems facing the textile industry is the removal of colour from dyebath effluents prior to discharge them to local sewage treatment facilities. During textile processing, it is estimated that due to inefficient methods almost 15% of all dyestuff is lost to the environment (Zollinger 1987), leading to the accumulation of highly undesirable pollution load in water bodies. Wastewater from textile industries is a complex mixture of many polluting substances such as organochlorine-based pesticides, heavy metals, pigments and dyes. Its composition has been discussed in detail by O'Neill *et al*. (1999).

Currently, through new regulations, pressure is being placed on companies to reduce the amount of colour in industrial wastewater in developed and developing countries. Several industrial-scale decolouration systems are commercially available (Willmott *et al*. 1998), which include adsorption, filtration, precipitation and activated sludge systems. All of these technologies work by concentrating the dyestuffs and transferring them to a solid phase that subsequently needs disposal. Anaerobic degradation of synthetic dyes by bacteria has been reported to produce carcinogenic and/or mutagenic products (Valli *et al*. 1992).

Biodegradation using ligninolytic enzymes has been suggested as one of the most attractive alternatives for the treatment of dyes (Robinson *et al*. 2001). Among such enzymes, laccases are highly interesting for the treatment of wastewater from the textile industry due to their broad substrate specificity. Thus, laccases have been found able to decolourise a wide range of synthetic dyes (**Table 2**). However, despite of the potential of laccases for the decolouration of textile wastewater, there are very few studies involving real textile wastewater (**Table 2**). Hence, Rodríguez Couto *et al*. (2002) studied the decolouration of different synthetic dyes (Acid Fuchsine, Congo Red and Indigo Carmine) by barley bran cultures of *T. versicolor* grown under SSF (solid-state fermentation) conditions. Dye decolouration was almost complete (85-96%) after 6 days of dye incubation. Moldes *et al*. (2003) studied the decolouration of several synthetic dyes by laccase obtained from *T. hirsuta* cultures grown on grape seeds under solid-state conditions. They found that the dyes Indigo Carmine and Bromophenol Blue were totally decolourised in 24 h whereas Methyl Orange and Phenol Red were decolurised by 65% and 36% for the same period, respectively. Also, Rancaño *et al*. (2003) studied the decolouration of the synthetic dye Phenol Red by laccase from *T. versicolor* produced in an airlift reactor and found that 34% of Phenol Red was decolourised in 27 h.

Novotny *et al*. (2004b) reported that *Irpex lacteus* decolourised a broad spectrum of chemically different synthetic dyes at a concentration of 200 mg/L in stationary liquid cultures. Decolouration levels after two weeks were 60-100%. Also, *I. lacteus* immobilised on pinewood cubes decolourised 100% of Remazol Brilliant Blue R (RBBR) (150 mg/L) within six days. It also efficiently decolourised textile industry effluents containing colour mixtures Drimarene Blue, Drimarene Red, Remazol Green and Acid Black, achieving decolouration percentages of 100%, 80%, 45% and 35%, respectively, within 3-5 days. Rodríguez Couto *et al*. (2004a) found that laccase produced by *T. hirsuta* immobilised on stainless steel sponges in an immersion bioreactor was able to decolourise the leather dyes Luganil Green (16.2% in 2 h) and Sella Solid Red (40% in 2 h). In addition, Rodríguez Couto *et al*. (2004b) reported the decolouration of several synthetic dyes by laccase from *T. hirsuta* produced in solid-sate cultures of barley bran. High decolourisation percentages in short incubation times were achieved for Bromophenol Blue, Indigo Carmine and Methyl Orange, whereas Poly R-478 presented much more resistance to degradation. Moreover, Rodríguez Couto *et al*. (2004c) reported that the textile dye Indigo Carmine was almost totally degraded in 3 days by *T. hirsuta* immobilised on stainless steel sponge grown in a fixed-bed bioreactor, while Lanaset Marine was degraded in two successive batches, reaching in the first batch a decolouration percentage of about 82% in 15 h and in the second one 71% in 28 h. Gómez *et al*. (2005) found that laccase from barley bran cultures of the white-rot fungus *Coriolopsis rigida* decolourised the synthetic dyes Indigo Carmine (100% in 4 h), Methyl Green (90% in 24 h) and Methyl Orange (80% after 24 h). Rodríguez Couto and Sanromán (2005) studied the decolouration ability of the white-rot fungus *T. hirsuta* grown on coconut flesh under SSF conditions. For this, the decolouration of the textile dye Lissamine Green B *in vivo* and *in vitro* was performed. The former showed a decolouration percentage higher than 96% in 2.5 h whereas the latter led to a decolouration percentage between 42% and 66% in 12 h depending on the culture age.

Held *et al*. (2005) showed for the first time that spore-bound laccases, which are stable at high temperatures and pH values, could be used for the decolouration of the common textile dyes Mordant Black 9, Mordant Brown 96/ Mordant Brown 15 and Acid Blue 74. The dyes were decolourised within 90 min of incubation time. In addition, the decolourised solutions were successfully used in re-dyeing. Kamida *et al*. (2005) reported the decolouration of a textile

Table 2 Application of laccases to bioremediation

Application	Laccase source	Reference
Decolouration of a textile dye	*Trametes versicolor*	Blánquez *et al*. 2007
Degradation of NP, BPA and TCS	*Coriolopsis polyzona*	Cabana *et al*. 2007
Degradation of BPA	*T. versicolor*	Diano *et al*. 2007
Decolouration of textile dyes	*Ischnoderma resinosum*	Kokol *et al*. 2007
Treatment of effluent from Kraft bleaching process	*T. versicolor*	Minussi *et al.* (2007)
Decolouration of Reactive Black 5	*Pleurotus sajor-caju*	Murugesan *et al*. 2007a
Decolouration of reactive dyes	*Ganoderma lucidum*	Murugesan *et al*. 2007b
Decolouration of synthetic dyes	*Trametes pubescens*	Osma *et al*. 2007
Decolouration of an industrial effluent containing a mixture of dyes	*Ganoderma* sp. WR-1	Revankar and Lele 2007
Degradation of phenols in OMW	*T. versicolor*	Ryan *et al*. 2007
Decolouration of synthetic dyes	*Collybia dryophila*, *Mycena inclinata*, *Stropharia rugosoannulata*, *Pleurotus ostreatus* and *T. versicolor*	Baldrian and Šnajr 2006
Decolouration of a textile dye	*T. versicolor*	Blánquez *et al*. 2006; Romero *et al*. 2006
Decolouration of textile dyes	*Phanerochaete chrysosporium*, *T. versicolor*	Böhmer *et al*. 2006
Degradation of PCE	*T. versicolor*	Marco-Urrea *et al*. 2006
Decolouration of an anthraquinoic dye	*Trametes trogii*	Mechichi *et al*. 2006
Decolouration of textile dyes	*Cyathus bulleri*	Mishra and Bisaria 2006
Remediation of OMW	*P. ostreatus*	Olivieri *et al*. 2006
Degradation of different PAHs	*P. ostreatus*	Pozdnyakova *et al*. 2006
Decolouration of synthetic dyes	*Trametes hirsuta*	Rodríguez Couto *et al*. 2006
Decolouration of synthetic dyes	*T. hirsuta*	Rodríguez Couto and Sanromán 2006
Decolouration of synthetic dyes	*T. hirsuta*	Rodríguez Couto and Sanromán 2007
Decolouration of textile azo dyes	*Trichophyton rubrum*	Yesiladal *et al*. 2006
Decolouration of textile dyes	*T. trogii*	Zouari-Mechichi *et al*. 2006
Decolouration of synthetic dyes	*Coriolopsis rigida*	Gómez *et al*. 2005
Decolouration of phenolic dyes	*Bacillus SF*	Held *et al*. 2005
Degradation of a textile effluent	*P. sajor-caju*	Kamida *et al*. 2005
Degradation of phenolics	*Trametes* sp.	Michizoe *et al*. 2005
Degradation of BPA	Novozymes	Modaressi *et al*. 2005
Decolouration of an anthraquinoic dye	*P. ostreatus*	Palmieri *et al*. 2005a, 2005b
Decolouration of textile dyes	*T. versicolor*	Ramsay *et al*. 2005
Degradation of lindane	*P. ostreatus*	Rigas *et al*. 2005
Decolouration of Lissamine Green B	*T. hirsuta*	Rodríguez Couto and Sanromán 2005
Degradation of phenols, colour and organic load in OMW	*Panus tigrinus*	D'Annibale *et al*. 2004
Decolouration and dephenolisation of OMW	*P. chrysosporium* and basidyomicete Euc-1	Dias *et al*. 2004
Degradation of PAHs	*T. versicolor*	Dodor *et al*. 2004
Detoxification of wastewater polluted with aromatic compounds	*Rhus vernicifera*	Durante *et al*. 2004
Degradation of xenobiotics	*Marasmius quercophilus*	Farnet *et al*. 2004
Degradation of methoxyclor	*T. versicolor*	Hirai *et al*. 2004
Degradation of PCBs	*T. versicolor* and *P. ostreatus*	Keum and Li 2004
Degradation of hydroxylated compounds	*R. vernicifera*	Möder *et al*. 2004
Degradation of synthetic dyes	*Irpex lacteus*	Novotny *et al*. 2004a
Degradation of PAHs, PCBs and synthetic dyes	*T. versicolor*, *Coriolopsis polyzona*, *P. ostreatus* and *I. lacteus*	Novotny *et al*. 2004b
Degradation of PAHs	*Cladosporium sphaerospermum*	Potin *et al*. 2004
Degradation of 2,4-dichorophenol and benzo(a)pyrene	*Pleurotus eryngii*, *P. ostreatus*, *Pleurotus pulmonarius* and *P. sajorcaju*	Rodríguez *et al*. 2004
Decolouration of leather dyes	*T. hirsuta*	Rodríguez Couto *et al*. 2004a
Decolouration of synthetic dyes	*T. hirsuta*	Rodríguez Couto *et al*. 2004b
Decolouration of textile dyes	*T. hirsuta*	Rodríguez Couto *et al*. 2004c
Oxidation of BPA and NP	a fungus isolated from soil (family *Chaetomiaceae*)	Saito *et al*. 2004
Biotransformation of humic acids from soil	*P. tigrinus*	Zavarzina *et al*. 2004
Phenolic removal from OMW	*P. ostreatus*	Aggelis *et al*. 2003
Dephenolisation of OMW	*Lentinula edodes*	Casa *et al*. 2003
Degradation of nitrobenzene and anthracene	*T. trogii*	Levin *et al*. 2003
Decolouration of synthetic dyes	*T. hirsuta*	Moldes *et al*. 2003
Decolouration of Phenol Red	*T. versicolor*	Rancaño *et al*. 2003
Decolouration of industrial effluents	*P. ostreatus*	Rodríguez *et al*. 2003
Transformation of chlorophenols	*T. versicolor*	Sedarati *et al*. 2003
Treatment of NP	*Trametes* sp.	Tanaka *et al*. 2003
Treatment of 2,4-DP-polluted soil	*Trametes villosa*	Ahn *et al*. 2002
Oxidation of PAHs	*C. hirsutus*	Cho *et al*. 2002
Removal of phenolics in OMW	*P. ostreatus*	Fountoulakis *et al*. 2002
Degradation of lignin from olive pomace	*P. chrysosporium*, *Oxysporus* sp., *Schizophyllum commune*, *Hyphoderma* sp. and *Ganoderma* sp.	Haddadin *et al*. 2002
Decolouration of synthetic dyes	*T. versicolor*	Rodríguez Couto *et al*. 2002
Phenolic removal in OMW	*Pleurotus* spp.	Tsioulpas *et al*. 2002
Degradation of BPA	*T. villosa*	Fukuda *et al*. 2001

Table 2 (Cont.)

Application	Laccase source	Reference
Degradation of NP, octylphenol, BPA and ethynylestradiol	*Trametes* sp.	Tanaka *et al*. 2001
Degradtion of BPA	*T. villosa*	Uchida *et al*. 2001
Phenolic removal in OMW	*L. edodes*	D'Annibale *et al*. 2000
Degradation of phenolic pollutants	*P. ostreatus*	Hublick and Shinner 2000
Biodegradation of phenols	*Pyricularia oryzae*	Lante *et al*. 2000
Degradation of PAHS	*T. versicolor*	Majcherczyk and Johannes 2000
bioremediation of chlorophenols in aqueous effluents	*Coriolus versicolor*	Ullah *et al*. 2000
Treatment of OMW	*L. edodes*	D'Annibale *et al*. 1999
Degradation of aromatic xenobiotics	*Cerrena unicolor*	Gianfreda *et al*. 1998
Oxidation of PAHs	*T. versicolor*	Majcherczyk *et al*. 1998

effluent containing Indigo from a textile factory located at Americana (São Paulo, Brasil) by the edible fungus *Pleurotus sajor-caju*. Palmieri *et al*. (2005a) investigated the decolouration of the recalcitrant dye RBBR by the fungus basidiomycete *Pleurotus ostreatus*. They found that when *P. ostreatus* grew in liquid media supplemented with veratryl alcohol, it completely decolourised the dye RBBR in 3 days and, in addition, its toxicity was reduced by 95%. Also, Palmieri *et al*. (2005b) reported the decolouration of the synthetic dye RBBR by laccase from *P. ostreatus* immobilised by entrapment in copper alginate beads. Operating under optimal conditions a maximum dye decolouration of 70% was obtained even after 20 cycles. In addition, Ramsay *et al*. (2005) reported that *T. versicolor* immobilised into alginate beads decolourised the dyes Amaranth, Reactive Black 5, Reactive Blue 19 and Direct Black 22 and mixtures of these dyes in a stirred-tank reactor.

Blánquez *et al*. (2006) reported the long-term continuous decolouration of the textile dye Grey Lanaset G (150 mg/L) in an air-pulsed bed bioreactor with retained pellets of the white-rot fungus *T. versicolor*. For a maximum cellular retention time (CRT) of 40 days, a colour reduction of 90% was obtained. In order to carry out a long-term continuous treatment, they performed a strategy of purge and biomass renovation and found that with a CRT of 21 days carrying out partial biomass renovations every 7 days and with a hydraulic retention time (HRT) of 2 days, decolourisation percentages higher than 80% were obtained. Böhmer *et al*. (2006) reported the advantages of adapting the temporary immersion RITA®-System (R*écipient à* I*mmersion* T*emporaire* A*utomatique*) as a bioreactor for laccase production by white-rot fungi and its application to decolouration of the textile dyes Levafix Blue and Remazol Brilliant Red. A successful series of four batch-decolouration processes was performed, which allowed dye decolouration over a long period.

Kokol *et al*. (2007) showed that the culture liquid produced by the white-rot fungus *Ischnoderma resinosum* in combination with redox mediators was able to decolourise synthetic dyebaths containing inorganic salts and the metal chelator ethylenediamine tetraacetic acid (EDTA). Mechichi *et al*. (2006) found that culture filtrates of *Trametes trogiii* induced with Cu^{+2} as well as a purified laccase from the same organism decolourised the dye RBBR. The purified laccase decolourised the dye efficiently at a concentration of 100 mg/L in the presence of only 0.2 U/mL of enzyme. Mishra and Bisaria (2006) investigated the decolouration of a number of recalcitrant reactive azo and acid dyes using the culture filtrate and purified laccase from the fungus *Cyathus bulleri*. They observed that both the decolouration rate and the decolouration percentage were considerably increased by the addition of ABTS.

Rodríguez Couto *et al*. (2006) studied the decolouration of different synthetic dyes by the extracellular liquid of *T. hirsuta* grown on grape seeds under SSF conditions in a tray bioreactor operating with grape seeds as a support. They found that the dyes Bromophenol Blue, Indigo Carmine, Methyl Green, Malachite Green and Methyl Orange were decolourised higher than 80% in 20-24 h. Also, Rodríguez Couto and Sanromán (2006) showed the ability of the extracellular liquid from *T. hirsuta* cultures grown on groundnut seeds under SSF conditions to decolourise the dyes Nickel (II) phthalocyanine, Lissamine Green B and Acid Black 48 (higher than 30% in 24 h).

Romero *et al*. (2006) showed that using an air-pulsed bioreactor with *T. versicolor* under laccase production conditions to decolourise the dye Grey Lanaset G was better than using the laccase enzyme, because possible product inhibition was avoided. In addition, they also showed that the dye degradation could be improved by using an appropriate dye pulse strategy. More recently, Blánquez *et al*. (2007) established the operational conditions for the continuous treatment process of the metal complex dye Grey Lanaset G (150 mg/L), in a fluidized-bed bioreactor using air pulses with retained pellets of the white rot fungus *T. versicolor*. Decolourisation was highly efficient (>80%) for the different HRTs tested ranging from 18 to 120 h, and the dye removal rates ranged from 6.73 to 1.16 mg/L/h. However, no direct relationship between decolourisation and extracellular laccase activity was found and high laccase activities were not necessary to obtain high decolourisation percentages.

Yesiladah *et al*. (2006) studied the potential of the wood-degrading fungus, *Trichophyton rubrum* LSK-27, for effective decolouration of textile azo dyes. Within two days of dye addition, the fungus was able to decolourise 83% of Remazol Tiefschwarz, 86% of Remazol Blue RR and 80% of Supranol Turquoise GGL in liquid cultures. The reactive dyes, Remazol Tiefschwarz and Remazol Blue, were removed by fungal biodegradation, while the acid dye Supranol Turquoise GGL was mainly accomplished by bioadsorption. Also, Zouari-Mechichi *et al*. (2006) found that crude laccase as well as purified laccase from *T. trogii* were able to decolourise dyes from the textile industry.

Baldrian and Šnajr (2006) compared ligninolytic enzyme production and synthetic dye decolouration ability of litter-decomposing basidiomycete fungi and white-rot fungi. They found that litter-decomposing fungi represent a promising alternative to white-rot fungi with respect to dye decolouration.

Murugesan *et al*. (2007a) showed that the presence of HBT was essential for the decolouration of the dye Reactive Black 5 by a purified laccase from the white-rot fungus *P. sajor-caju*. Murugesan *et al*. (2007b) showed the dye decolourising potential of the crude laccase from the white rot fungus *Ganoderma lucidum* for recalcitrant textile dyes such as RBBR. Osma *et al*. (2007) investigated the potential of the extracellular liquid from banana skin cultures of the white-rot fungus *Trametes pubescens* for dye decolouration. The dye RBBR was decolourised about 57% in 4 h and the dye Methyl Green 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.

Revankar and Lele (2007) investigated the decolouration of recalcitrant dyes by the white-rot fungus *Ganoderma* sp. They found a maximum decolouration of 96% for the dye Amaranth (100 mg/L) in 8 h in an optimised medium.

In addition, *Ganoderma* sp decolourised the dyes Reactive Orange 16, Cibacron Brilliant Red 3B-A, Acid Red 106, Orange II and RBBR. Moreover, complete decolouration of an industrial effluent containing a mixture of reactive dyes was achieved in 12 days.

Rodríguez Couto and Sanromán (2007) investigated the effect of the redox mediator violuric acid on the decolouration of the two recalcitrant acid dyes Acid Red 97 and Acid Green 26 by crude laccase from *T. hirsuta*. The LMS led to a higher extent of decolouration in shorter times than that obtained without mediator addition, especially for the dye Acid Red 97 which was decolourised by 90% in only 3 min.

From the exposed above, it can be concluded that individual dye structures influence the decolourisation extent obtained by laccase, indicating the specificity of laccase towards different dye structures. In addition, laccase produced for different organisms and/or under different culture conditions has different decolouring abilities.

Wastewater from the pulp and paper industry

Chlorine is an effective and widely used bleaching agent for chemically produced wood pulps. However, chlorination followed by alkaline extraction results in large volumes of effluents containing substantial levels of adsorbable organic halogens (AOX), primarily in the form of chlorophenols, chloroguaiacols, chloroaliphatics, chlorocatechols, chlorosyringols and large polymerised chloroaromatics. The presence of such compounds causes a great environmental impact. The treatment of such effluent streams involving activated sludge frequently faces serious problems to control the activity of wild microorganisms due to their biodiversity and unpredictability. Laccases appear as very promising enzymes to treat this type of effluents due to their potential to degrade both highly toxic phenolic compounds and lignin (Mansur *et al*. 1998; Gianfreda and Rao 2004). In addition, laccases are also able to oxidise the non-phenolic subunits of lignin by the addition of redox mediators (Bourbonnais and Paice 1992).

Minussi *et al*. (2007) studied for the first time the treatment of E_1 effluent (first-stage of basic extraction of Kraft bleaching process using *Eucalyptus grandis* woods) by laccase and N-OH mediator system. They found a phenol reduction around 23% in the presence of 100 U laccase and in the absence of mediators and observed that the presence of HBT did not increase phenol reduction. However, acetohydroxamic acid (AHA) at a concentration of 0.34 mM, which was not degraded by laccase (50 U), acted very efficiently on E1 effluent reducing 70% and 73% of the total phenol and total organic carbon, respectively, in 3 h. At the same conditions 50 U of laccase in the absence of AHA reduced only 15% of the total phenols after 3 h.

Wastewater from the food industry

Some fractions of beer-factory wastewater represent an important environmental concern due to their high content of polyphenols (mainly tannins) and dark-brown colour. Yagüe *et al*. (2000) studied the ability of *Coriolopsis gallica*, a white-rot fungus producer of laccase, to degrade this high-tannin-containing wastewater. They found a reduction in polyphenol pyrolysis product content with the incubation time. Thus, they found a decrease of 22.4% for phenol, 60.8% for guaiacol, 57.6% for 4-methylguaiacol and 31.6% for 4-vinylguaiacol at day 12 of incubation in a medium containing 20% (v/v) of a beer-factory effluent.

The disposal of vinasse, the major effluent from the ethanol industry, represents a considerable environmental problem. This black liquid that is produced at a rate 10 to 15 times greater than the ethanol itself is a mixture of water and organic and inorganic compounds. These compounds remain after different steps involving the sugar cane production and processing. Rodríguez *et al*. (2003) studied the decolouration of vinasse effluents from a destillery factory by submerged cultures of *Pleurotus* spp. They found a decreased in chemical oxigen demand (COD) (38% after 10 days) and colour (39% after 10 days) of such effluents after fungal treatment.

OMW is a characteristic by-product of olive oil production and a major environmental problem in the Mediterranean area, where is produced in quantities higher than 30 million m^3 per year, constituting an important phenolic waste. Its phenolic compounds are responsible for its black colour and its toxic properties in ecosystems (Pérez *et al*. 1992; Martirani *et al*. 1996). The main risks associated with the release of OMW in the environment are due to its high organic load and to the significant presence of phenolic components (Moreno *et al*. 1987; Sayadi *et al*. 2000), the concentration of which may easily reach 5-10 g/L, depending on cultivar, harvesting season and extraction process (D'Annibale *et al*. 2004). Several authors have shown that laccase is able to remove OMW phenolics (**Table 2**). Thus, Gianfreda *et al*. (1998) showed that laccase from *Cerrena unicolor* was able to oxidise different phenolic substances usually present in OMW with oxidation percentages ranging from 60 to 100% after 24 h of laccase incubation. D'Annibale *et al*. (1999) reported the decolouration and detoxification of OMW with a laccase from *Lentinus edodes* immobilised on chitosan. Subsequently (D'Annibale *et al*. 2000), they found that the same laccase immobilised on Eupergit® C led to a significant reduction in total phenols of an OMW. Also, Fountoulakis *et al*. (2002) investigated the capability of *P. ostreatus* to degrade phenols of OMW in different conditions. Thus, the degradation of phenols reached up to 78.3% for the sterilised and 50% diluted OMW and 66.7% and 64.7% for the thermally processed OMW, with and without dilution, respectively.

Haddadin *et al*. (2002) studied the delignification of alkaline pretreated pommace from olive oil processing by several wood-decaying fungi and found an evident relationship between ligninase and laccase activity and the extent of lignin degradation. Tsioulpas *et al*. (2002) showed the ability of several *Pleurotus* spp. strains to grow in OMW without any addition of nutrients and to remove a significant part (69-76%) of the phenolic compounds present as well as to produce high laccase activity. However, it was found that the remaining phenolics and/or some of the oxidation products of the laccase reaction in the treated OMW were more toxic than the original phenolic compounds. On the contrary, Casa *et al*. (2003) evaluated the potential of a laccase-based treatment for removing OMW phytotoxicity and found that the treatment with laccase resulted in a 65% and 86% reduction in total phenols and ortho-diphenols, respectively, due to their polymerisation.

Aggelis *et al*. (2003) reported that *P. ostreatus* was able to reduce the phenolic content and toxicity of sterilised OMW in bioreactor cultures. However, high OMW dilutions should be used, and/or additional treatment should be applied before using the OMW in the environment, e.g. as water for irrigation. Also, D'Annibale *et al*. (2004) assessed the potential of the white-rot fungus *Panus tigrinus* in removing organic load, colour and toxic phenols from OMW. They observed a delay in removal of colour, organic load and phenol by the fungus at an initial soluble COD of 85000 mg/L which was associated with a delayed onset of laccase and manganese-dependent peroxidase production. However, *P. tigrinus* removed the above-mentioned components promptly and efficiently when grown on OMW with an initial soluble COD content of 43000 mg/L. Dias *et al*. (2004) reported that the basidiomycete Euc-1, a laccase producing strain, removed 90% of phenols (initial concentration 800 mg/L), 73% of colour (initial A_{465}=4.4) and 45% of COD in batch cultures containing OMW. Since partial phenol removal occurred before the detection of laccase activity, no plausible correlation could be established between them. In contrast, decolouration occurred only after the detection of laccase activity and coincided with its production over time.

Olivieri *et al*. (2006) showed that *P. ostreatus* effectively grew on raw OMW at polyphenol concentrations as

large as 1.4 g/L and exhibited a remarkable ability to catalyse polyphenol bioconversion. Thus, bioconversion of polyphenols was as large as 70% over 4-7 incubation days increasing to 95% over the same time period when added nutrients were supplied. No appreciable decolouration took place along with remediation. The process was satisfactorily scaled to an internal loop airlift bioreactor.

Wastewater containing EDCs (xenoestrogens)

There are increasing concerns about potential adverse effects on human health and environment resulting from the disposal of numerous chemicals that otherwise improve human life and economic activities. Household chemicals, pharmaceuticals and other consumables as well as biogenic hormones are released into the environment after passing through wastewater treatment processes, which are not designed to remove them. Such substances are potential endocrine disrupting chemicals (EDCs) (xenoestrogens). Among them, nonylphenol (NP) (4-nonylphenol), bisphenol A (BPA) (2,2-bis(4-hydroxyphenol)propane) and triclosan (TCS) (5-chloro-2(2,4-dichlorophenoxy) phenol) are the most frequently detected in downstream effluents of intense urbanization (Kolpin *et al*. 2002; Boyd *et al*. 2004).

The presence of the above-mentioned compounds in the aquatic environment is of special concern since they tend to bioacumulate causing a serious health and environmental problem. Laccases appear as a promising alternative to remove these xenobiotics from the aquatic environment (**Table 2**). In addition, recently Cabana *et al*. (2007) showed that enzymatic treatment with laccase of NP, BPA and TCS removed their estrogenic activity.

Hublick and Shinner (2000) showed that a laccase from *P. ostreatus* immobilised on Eupergit® C allowed the continuous elimination of 2,6-dimethoxyphenol. Interestingly, the precipitates resulting from oxidative coupling of such a compound were found to be insoluble at conditions predominating in industrial wastewater which would make possible their further filtration. Lante *et al*. (2000) found that a commercial laccase immobilised on a spiral-wound asymmetric polyethersulphone membrane was able to oxidise a wide range of phenols including chlorophenols, cresols and methoxyphenols. Their results confirmed that the type and/or the position of the substituent group affected the level of oxidation. Ullah *et al*. (2000) showed that laccase from *C. versicolor* grown in both wheat husk and wheat bran removed 100% of 2,4-dichlorophenol (2,4-DCP) (50 mg/L) in 5 h and 75-80% of pentachlorophenol (PCP) (50 mg/L) in 24 h at flask scale. In addition, *C. versicolor* immobilised on wheat bran pellets was added to chlorophenol solutions in 200-4000-mL bioreactors resulting in a removal of chlorophenols higher than 90% in 100 min. Also, Sedarati *et al*. (2003) showed that *T. versicolor* immobilised on nylon mesh in a 2-L bioreactor removed PCP and 2,4-DCP more efficiently than free cultures. They found that 85% of 2,4-DCP (2000 mg/L) and 70% of PCP (3400 mg/L) were transformed by enzymes (laccase and manganese-peroxidase) after 1020 h of treatment. Moreover, Durante *et al*. (2004) showed that non-isothermal bioreactors with laccase from *R. vernicifera* immobilised on a nylon membrane were a promising tool for the detoxification of wastewater polluted with phenolic compounds.

Fukuda *et al*. (2001) found that BPA was rapidly degraded by a laccase, which was extracted and purified from DeniLite, a Novozymes' product (Novozymes A/S, Denmark), leading to two kinds of compounds one of which was identified as 4-isopropylphenol. Hirai *et al*. (2004) found that the pesticide methoxychlor was converted into methoxychlor olefin and 4,4′-dimethoxybenzophenone by laccase-HBT treatment. Keum and Li (2004a) tested commercial laccases from *T. versicolor* and *P. ostreatus* to degrade hydroxy PCBs and found that laccase from *T. versicolor* degraded hydroxy PCBs more rapidly than that from *P. ostreatus*. They also found that degradation rate constants decreased with increase of chlorination and no degradation was observed with tetra-, penta- and hexa-chloro hydroxy PCBs in non-mediated reactions.

Möder *et al*. (2004) studied the degradation of selected hydroxylated aromatic compounds (3,4-dimethylphenols, 4-ethylphenol, 2-hydroxy-1,2,3,4-tetrahydronaphthalene, 2-hydroxy-decahydronaphthalene and 4-hydroxy-biphenyl) from water samples by microporous polypropylene hollow fiber membranes impregnated with horseradish peroxidase (HRP) and laccase. It was found that, with the exception of 2-hydroxydecahydronaphthalene, all substrates were efficiently degraded (50-100% within 48 h). Interestingly, laccase exhibited more unselective degradation results than HRP. Saito *et al*. (2004) found that a purified laccase from a fungus (family *Chaetomiaceae*) rapidly oxidised the EDCs BPA and NP in the absence of mediators and, in addition, their estrogenic activities were completely removed in 24 h.

Michizoe *et al*. (2005) showed that the combination of surfactant-laccase complexes and reverse micelles created an homogeneous organic solvent system for biocatalysis, which would lead to efficient degradation of environmental pollutants at higher concentrations than in aqueous degradation systems. In particular, they found that the oxidation of BPA led to two products: 4-isopropylphenol and 4-isopropenylphenol, indicating the oxidative degradation of the bis-phenolic structure of BPA. They also found that the surfactant-laccase complex turned out to handle other environmental pollutants, chlorophenols, by the simultaneous addition of water and a redox mediator into the reaction medium using reverse micelles. Also, Modaressi *et al*. (2005) treated synthetic wastewater containing BPA with laccase enzyme. Optimisation of pH, laccase concentration, polyethylene glycol (PEG) as an additive allowed the conversion and precipitation of BPA (95%) over 3 h of reaction period. In addition, PEG reduced enzyme inactivation. Thus, they found that in the absence of PEG the precipitate formed inactivated laccase while the precipitate formed in the presence of PEG protected laccase.

Rigas *et al*. (2005) studied the degradation of lindane in liquid-agitated cultures of a commercial strain of *P. ostreatus*. Under optimal conditions, the maximum biodegradation of lindane, expressed as the extent of biodegradation relative to initial lindane mass and to final biomass, was found equal to 25.8 mg/g/g (degraded lindane/initial lindane/biomass), in 12.45 days.

Cabana *et al*. (2007) investigated the degradation of the EDCs NP (5 mg/L), BPA (5 mg/L) and TCS (5 mg/L) by laccase from the white rot fungus *Coriolopsis polyzona*. After a 4-h treatment NP and BPA were totally removed whereas TCS was removed by 65%. The addition of ABTS significantly increased the efficiency of the laccase treatment. In addition, it was shown that the laccase treatment produced high molecular weight metabolites through a radical polymerisation mechanism of NP, BPA and TCS.

Diano *et al*. (2007) showed the useful application of the technology of non-isothermal reactors with immobilised laccase in processes of bioremediation of water polluted by phenol compounds, in particular certain EDCs such as BPA.

Ryan *et al*. (2007) studied the bioremediation of a phenolic wastewater from a coal gasification plant located in Australia by *T. versicolor* at flask scale. They found that under optimised conditions 0.125 g phenol/g biomass and 0.231 g o-cresol/g biomass were removed from solution per day.

Soil bioremediation

For the treatment of recalcitrant compounds in soils, bioremediation has been receiving much attention in last decades (Crawford 1996).

Bollag and co-workers (Bollag *et al*. 1982; Dec and Bollag 1990; Hatcher *et al*. 1993; Tatsumi *et al*. 1994a, 1994b) have repeatedly demonstrated the ability of laccases to detoxify different xenobiotics by cross-linking them to various humic constituencies. Irreversible binding of these pollutants by laccases has been shown to prevent further

spread of the contaminants through soil or leaching into underground water. Thus, laccase was able to mediate the coupling of reduced 2,4,6-trinitrotoluene (TNT) metabolites to an organic soil matrix, which resulted in detoxification of the munition residue (Durán and Esposito 2000). Also, Nyanhongo *et al*. (2006) showed that laccase isoenzymes were involved in immobilisation of TNT degradation products.

PAHs together with other xenobiotics are a major source of contamination in soil; therefore, their degradation is of great importance for the environment. The oxidation of PAHs by laccases from numerous fungi has been reported (**Table 2**). Majcherczyk *et al*. (1998) reported that laccase from *T. versicolor* was able to oxidise *in vitro* more than 14 PAHs. Thus, acenaphthylene was removed by 37%, anthracene and benzo(*α*)pyrene by 18 and 19%, respectively and an oxidation of about 10% was found for acenaphthene, fluoranthene, pyrene, benzo(*α*)anthracene, chrysene, benzo (*β*)fluoranthene, benzo(*κ*)fluoranthene and perylene after incubation for 72 h by laccase. Addition of HBT to the reaction mixture increased the oxidation of the following PAHs: acenaphthylene, acenaphthene, fluorene, anthracene, benzo(*α*)pyrene and perylene, which were almost completely removed. In addition, the oxidation of pyrene and benzo(*α*)anthracene increased from 8 and 6% without a mediator to 48 and 53%, respectively, in the presence of HBT. Also, Majcherczyk and Johannes (2000) reported the oxidation of a high molecular model compound of PAH by the LMS with the formation of two main oxidation products. Cho *et al*. (2002) studied the oxidation of five PAHs (20 µM): anthracene, benzo(*α*)pyrene, fluoranthene, phenanthrene and pyrene by laccase from *Coriolus hirsutus* in the presence of the redox mediators ABTS (1 mM) and HBT (1 mM). ABTS increased the oxidation of benzo(*α*)pyrene more than HBT but the oxidation of the other PAHs tested were the opposite. Also, it was found that the mediators used in conjunction increased the oxidation of benzo(*α*) pyrene (100% after 20 min) compared to using the mediators alone (60% in 20 min).

Tanaka *et al*. (2001) studied a laccase-based treatment for the remediation of sand contaminated with phenolic EDCs. They found that laccase from *Trametes* sp decreased the amounts of NP, octylphenol, BPA and ethynylestradiol (synthetic estrogen) adsorbed on sea sand (2 µmol/g) in a test tube with shaking. The system was efficiently scaled-up to a rotating reactor. This treatment system will be useful for the rapid remediation of soil and bottom sediments highly contaminated with EDCs in the leachates and effluents from waste landfills, industrial plants and sewage treatment works. Uchida *et al*. (2001) studied the metabolism of BPA with a highly purified laccase from the basidiomycete *Trametes villosa*. They found that the laccase reaction may contain successive BPA polymerisation, followed by either the addition of phenol to the formed oligomers or their decomposition to release 4-isopropenylphenol. Ahn *et al*. (2002) treated two soils containing 2,4-DCP with both free and immobilised laccase from *T. villosa*. In the first one both laccases removed 100% of 2,4-DCP whereas in the second one the immobilised enzyme performed better. For practical soil remediation applications the lost of enzyme activity due to immobilisation together with the higher cost of the immobilised laccase makes the free laccase a better option.

Levin *et al*. (2003) analysed the ability of the white-rot basidiomycete *T. trogii* to degrade *in vitro* concentrations of 250-500 mg/L of nitrobenzene and anthracene. They found that more than 90% of the organic pollutants added to the fungal cultures were removed within 12-24 days. Enzyme estimations indicated a high and relatively stable activity of laccase, therefore, laccase activity could be implicated in the degradation of the above-mentioned compounds. Tanaka *et al*. (2003) showed that laccase from *Trametes* sp. efficiently treated sand contaminated with NP in a rotating reactor. The estrogenic activity decreased to 1/6-1/90 after 24 h of the treatment. This treatment system will be useful in the development of a remediation system for soils and bottom sediments contaminated with phenolic EDCs.

Dodor *et al*. (2004) studied the potential of laccase from *T. versicolor* immobilised on kaolinite to oxidise anthracene and benzo[a]pyrene in a sole-substrate system in the presence of ABTS. After 24 h of incubation, immobilised laccase-ABTS system oxidised more than 80% of the initial 70 µM of PAHs present. Farnet *et al*. (2004) described the biochemical features and the ability of a new laccase isoform from a *Marasmius quercophilus* strain collected on evergreen oak litter to transform various aromatic compounds. Thus, they found that laccase from this strain was able to transform 2-chlorophenol, 2,4-DCP and 2,4,6-trichlorophenol without mediator addition.

Potin *et al*. (2004) investigated the ability of a Deuteromycete fungus, *Cladosporium sphaerospermum*, previously isolated from soil of an aged gas manufacturing plant in France, to degrade PAHs. This strain was able to degrade PAHs in non-sterile soils (average 23%), including high molecular weight PAHs, after 4 weeks of incubation. In liquid culture, *C. sphaerospermum* degraded rapidly benzo (*α*)pyrene during its early exponential phase of growth (18% after 4 days of incubation). Among different extracellular ligninolytic enzyme activities tested, only laccase activity was detected in liquid culture in the absence or in the presence of benzo(*α*)pyrene.

Rodríguez *et al*. (2004) studied the ability of *Pleurotus eryngii*, *P. ostreatus*, *Pleurotus pulmonarius* and *P. sajor-caju* to degrade phenolic and non-phenolic aromatic pollutants in liquid and SSF cultures, the ligninolytic enzymes secreted by these fungi, i.e. laccase and versatile peroxidase, participating in the degradation processes. Their results suggest that the addition of these fungi grown on agricultural wastes contaminated soils could be used in bioremediation strategies. Under these conditions the fungi could degrade the pollutants and the partial delignification of the agricultural wastes used as a substrate could exert a positive effect on the growth of other soil microorganisms including those contributing to the total degradation of recalcitrant pollutants.

Zavarzina *et al*. (2004) showed that a purified laccase from the white-rot basidiomycete *P. tigrinus* 8/18 was capable of both polymerisation and depolymerisation of humic acids (HAs) *in vitro*. The direction of transformations depended on the nature and properties of HAs. This finding clarifies and extends the role of laccase in natural processes as not only an important agent of lignin degradation but also as a biocatalyst in oxidative transformations of humic substances – the most abundant and stable form of organic carbon in soils and aquatic systems. However, due to competitive inhibition of laccase by HAs, laccase concentration and activity in soils have to be rather high.

Pozdnyakova *et al*. (2006) reported that a laccase produced by submerged cultures of *P. ostreatus* was able to degrade the following PAHs: anthracene (91%), phenanthrene (72%), fluorene (53.5%), pyrene (65.5%), fluoranthene (69.7%) and perylene (73%) only in the presence of a synthetic mediator.

Marco-Urrea *et al*. (2006) reported for the first time the aerobic degradation of perchloroethylene (PCE) by the white rot fungus *T. versicolor* to less hazardous products. Aerobic degradation rate of PCE was 0.20 and 0.28 nmol/h/mg dry weight of fungal biomass.

FUTURE PERSPECTIVES

The removal of organic pollutants by laccases is an interesting alternative for the treatment of contaminated sites. Laccases oxidise the organic compounds to usually less harmful or even innocuous products. However, one of the major limitations in developing laccase catalysis for industrial applications is the susceptibility of the enzyme to inactivation. Thus, laccases are often easily inactivated by a wide variety of environmental conditions that characterise the polluted sites (pH, salts, inhibitory molecules, extreme

temperature gradients). Therefore, the application of laccase to the decontamination of both effluents and soils requires a careful investigation of the possible interactions between laccase and the chemicals present therein. The inactivation of laccase by such chemicals is initially due to depletion of the copper ion from the active site of the enzyme. Further reactions such as complex formation and unfolding may result in irreversible denaturation of laccase (Keum and Li 2004b).

In general, immobilisation protects laccase from deactivating agents. We are currently working on new strategies to immobilise laccase (Rodríguez Couto *et al*. 2007), such as the use of Al_2O_3 pellets as an immobilisation matrix followed by the sequential adsorption of oppositely charged polyelectrolytes (layer by layer technique). We have already shown that the immobilisation of laccase improves its stability properties with time. Several questions are still open, namely, the efficieny of immobilised laccase as a function of temperature and also its reuse. Moreover, all this will considerably reduce the process cost.

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