Appl Microbiol Biotechnol (2000) 53: 230–234

ORIGINAL PAPER

M. A. Ullah · C. T. Bedford · C. S. Evans

Reactions of pentachlorophenol with laccase from *Coriolus versicolor*

Received: 7 June 1999 / Received revision: 18 August 1999 / Accepted: 2 September 1999

Abstract Laccase, purified from *Coriolus versicolor*, removed pentachlorophenol (PCP) from solution at pH 5, depending on initial PCP concentration and amount of laccase. With 100 units of laccase, 100% of 25 μg ml^{-1} PCP and 60% of 200 μg ml^{-1} PCP were removed respectively over 72 h. No free chloride was released in the reaction. In reaction with 100 μg PCP, products were primarily polymers (about 80,000 MW) with only 2–3 pg of *o*- and *p*-chloranils formed. Polymers were stable to acid hydrolysis and no release of PCP, or other low-molecular-weight products, was detected over several weeks. Laccase has a potential use in the biotreatment of aqueous effluents containing PCP, with polymerised products being removed from solution due to their high molecular weight.

Introduction

Since the development of pesticides, large quantities of man-made organic compounds have been introduced into the environment at concentrations that cause ecologically undesirable effects. Many of these compounds, such as pentachlorophenol (PCP), are highly resistant to biotic and abiotic degradation and as a result remain in the environment at toxic levels. PCP has been listed as a priority pollutant by the US EPA and by the European Commission, which have banned its use in these countries (McAllister et al. 1996).

The ability of white-rot fungi to degrade PCP has been demonstrated as an effective treatment system for aqueous effluents (Alleman et al. 1995). The precise mechanisms by which such fungi detoxify PCP are still unclear, with the roles of individual enzymes in the ligninolytic complex only partially characterised. Laccase, one of the lignin-degrading enzymes, reacts with a wide range of phenolic compounds (Roy-Arcand and Archibald 1991; Thurston 1994).

Laccase is a polyphenol oxidase produced prolifically as an extracellular enzyme by many species of white-rot fungi including *Coriolus versicolor* (Evans 1985; Fahraeus and Reinhammar 1967). Several forms have been isolated, with types I and II being blue copper-containing proteins [originally described as laccase A and B (Fahraeus and Reinhammar 1967; Thurston 1994]. They are products of liquid cultures, whereas type III is a colourless protein produced predominantly during cultivation on solid substrates such as wood chips (Iimura et al. 1995). Laccase isozymes identified from *Panus tigrinus*, *C. versicolor*, *Phlebia radiata* and *P. tremellosa* gave typical blue laccases from liquid cultures, but yellow laccases from solid state cultures (Leontievsky et al. 1997). Formation of yellow laccases is proposed to be a result of the enzyme protein binding the lignin-derived molecules (Leontievsky et al. 1997). Early work by Konishi and Inoue (1972) showed that laccase reacted with PCP oxidatively to produce the benzoquinones, *p*-and *o*-chloranils, while more recently it was concluded that laccase did not play an integral role in PCP mineralisation when both laccase and PCP were added to growing and autoclaved fungal cultures (Ricotta et al. 1996). Excess laccase could result in the coupling of reaction products claimed not to be susceptible to ring cleavage (Ricotta et al. 1996).

Degradation of chlorinated phenols by laccase has been particularly effective for the less chlorinated compounds such as di-, tri- and tetra-chlorinated phenols. Roy-Arcand and Archibald (1991), investigating the chloride release of a mixture of chlorinated phenols by laccase, found that the transformation efficiency decreased with increasing numbers of chlorines. PCP was the most recalcitrant of the compounds, releasing no chloride. In mixtures of chlorophenols, degradation of individual compounds was enhanced, while with crude

M. A. Ullah · C. T. Bedford · C. S. Evans (✉)
Fungal Biotechnology Group, School of Biosciences,
University of Westminster, London W1M 8JS, UK
Tel.: +44-171-9115128
Fax: +44-171-9115087

enzyme preparations the removal of chlorophenols has been correlated with laccase activity in the extracts (Kadhim et al. 1999). This study investigates the reactions of a purified laccase, isolated from *C. versicolor*, with PCP.

Materials and methods

Chemicals

All chemicals were reagent grade unless otherwise specified. PCP was obtained from Sigma (Poole, Dorset, UK) and solvents of HPLC grade from BDH (Poole, Dorset, UK).

Organism and culture conditions

C. versicolor (FPRL-28A) was originally obtained from IMI (now CABI; Egham, Surrey, UK). Stock cultures were maintained on 3% malt-2% agar slants grown at 26 °C, stored at 4 °C, and plate cultures were prepared on the same medium, incubated for 7 days at 26 °C. Medium for liquid cultures for isolating laccase from *C. versicolor* was a glucose-amino acid-salts medium as previously described (Evans 1985; Fahraeus and Reinhammar 1967), shaken at 25 °C for 7 days, inoculated with mycelial plugs (4 mm diameter) cut from the growing edge of malt/agar plate cultures. Addition of 2,5-xylidine (2×10^{-4} M in the growth medium) as inducer of laccase was made 18 h before harvesting.

Isolation of laccase

Isolation and purification of laccase was as previously described (Evans 1985; Fahraeus and Reinhammar 1967). Activity was identified in blue laccase type II and blue laccase type I (Fahraeus and Reinhammar 1967). Each of the two protein fractions gave a single band on SDS-PAGE of 62,000 Da molecular weight (MW).

Measurement of laccase activity

Laccase activity was measured by the oxidation of catechol (10 mM) in 100 mM sodium acetate buffer, pH 5.0, by an increase in absorbance at 440 nm or by oxidation of syringaldazine (0.01%) in 60 mM sodium acetate buffer (pH 5.0) by increase in absorbance at 560 nm (Harkin and Obst 1973). One unit of laccase activity was defined as that which caused a change in absorbance of 1.0 min^{-1} ml^{-1} at 25 °C.

Reaction of laccase with PCP

PCP stock solutions were dissolved in ethanol. Assays for the reaction of laccase with PCP were carried out in 100 mM sodium acetate buffer, pH 5.0, replacing catechol by PCP, with a final ethanol concentration of 10–33% to maintain full dissolution of PCP at concentrations of 25–200 μg ml^{-1}.

Reaction of PCP with laccase was investigated at 4–55 °C, over a pH range of 3.0–7.0, at a substrate concentration of 25–200 μg ml^{-1} with 12–200 units enzyme activity. All reactions were performed statically, in the dark at 26 °C, contained in a final volume of 0.9 ml. PCP concentrations were monitored at intervals throughout the time for reaction. All reactions were performed in triplicate on at least two separate occasions. Controls for all experiments included boiled enzyme and no enzyme.

Analysis of PCP

PCP was measured by HPLC using a reversed phase column (Hichrom) 4.6 × 250 mm, packed with R-Sil C18 (10 μm) with a mobile phase of acetonitrile:water:acetic acid (75:25:0.125). Detection of PCP was at 254 nm with a retention time of 6.9 min. Residual PCP (after reaction with enzymes) was identified by retention time in comparison to an authentic PCP standard (in 100% ethanol) and co-elution with added PCP. Calibration plots of peak areas of PCP standards were linear in the range 20–175 μg ml^{-1}.

Analysis of reaction products by gel exclusion chromatography

Gel filtration of the crude reaction mixture of enzymes with PCP was used to determine the relative MW of the products of reaction. The reaction mixtures were fractionated on a column of Sephadex G75 (with MW cut-off at 80,000), with a column volume of 100 ml (1.5 × 50 cm). Components were identified on line by their absorption at 254 nm. Each peak was collected and concentrated to a minimum volume by vacuum rotary evaporation (Buchi Rotavapor, Switzerland). Calibration of columns used PCP, decachlorobiphenyl, and dextran to correlate elution volumes with MW.

Chloride ion determination was based on the method of Iwasaki et al. (1956) with calibrations in the range 1–100 ppm Cl^-.

Mass spectrometry

Products from enzyme-PCP reactions were extracted with 33% methanol, washed three times with doubly distilled ether and the water layer discarded. The ether layer was retained in a sealed flask, kept at 0 °C overnight to freeze out residual water. To the ether extract free of water, 0.1 ml of 100 ng ml^{-1} 2,4-dibromoanisole was added as an internal standard. After evaporation to near dryness, 0.2 ml of residual ether was used for GC-MS analysis. For GC-MS, a column of BPX5 (50 m × 0.32 mm; SGE, Milton Keynes, UK) was used with the oven programme at 60 °C for 1 min rising by 5 °C min^{-1} to 250 °C; injection temperature held at 250 °C splitless for 30 s, then split at a ratio of 15:1. The MS temperature was 170 °C, emission current was 70 eV equivalent to 50 μA, with selective ion monitoring.

Results

The reactions of laccase I and II with PCP were investigated. The solubility of PCP in water is low (0.014 mg l^{-1} at 25 °C), so ethanol–water was used as solvent. The effect of ethanol on laccase activity showed that increasing concentrations of ethanol in the assay mixture with catechol reduced the activity of laccase I linearly (Kazandjian et al. 1986; Tome et al. 1978). Similar inhibition of activity of laccase II was observed. To solubilise >200 μg ml^{-1} PCP in the reaction mixture, >33% ethanol was required. With catechol as substrate, 33% ethanol reduced laccase activity to approximately half that in the absence of ethanol, but sufficient activity was retained to allow the reaction with PCP to be studied under these conditions.

Figure 1 shows the reaction of laccase I with PCP in the range of 25–200 μg ml^{-1}. At 25 μg ml^{-1}, PCP was completely removed from solution over 48 h, while 65–45% of 50–200 μg ml^{-1} PCP were removed respectively over 72 h. As similar rates of reaction were achieved with laccase II (data not shown), all further studies were carried out with laccase I because more of this isomer was available.

The effect of temperature on the extent of PCP removal by laccase showed that the reaction was optimal

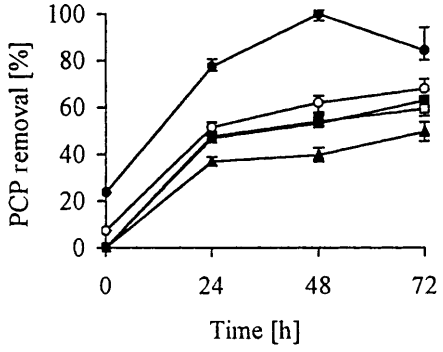

Fig. 1 Removal of PCP at various concentrations by laccase I: ● 25 μg ml^{-1}, ○ 50 μg ml^{-1}, □ 100 μg ml^{-1}, ■ 150 μg ml^{-1}, ▲ 200 μg ml^{-1}

at 25 °C, with a range of 10–45 °C (Fig. 2). Minimal activity was still observed at 5 and 55 °C. The pH optimum for the PCP reaction with laccase was pH 5.0 (Fig. 3), though activity over a broad pH range showed that 5% activity was retained at pH 3.5–6.5. This pH profile is similar to that obtained using catechol or syringaldazine as substrates.

Reaction of 100 μg ml^{-1} PCP with laccase was proportional to the amount of laccase between 12 and 100 units (Fig. 4). In these experiments for consistency with previous data, PCP was solubilised in a final ethanol concentration of 33% but, when this was reduced to 10%, total removal of 100 μg ml^{-1} PCP was achieved in 24 h, compared to only 60% removal in 33% ethanol. At lower ethanol concentrations, PCP was insoluble and gave a cloudy suspension, but laccase was still able to react with PCP in suspension (Kazandjian et al. 1986; Tome et al. 1978). The reaction of PCP with 100 units of laccase over 24 h in the presence of 33% ethanol showed Michaelis-Menton kinetics in the substrate range of 25–200 μg ml^{-1} (0.09–0.75 mM), with a K_m value of 3 mM and a V_{max} of 0.07 mM min^{-1}. These data confirm the very low specificity and low turnover rate of laccase with PCP, compared with substances such as catechol, dihydroxyphenylalanine and syringaldizine which have K_m values of 6.7 mM, 15.6 mM and 12.3 mM respectively.

The products of reaction of laccase (100 units) incubated with 100 μg ml^{-1} PCP for 24 h were analysed

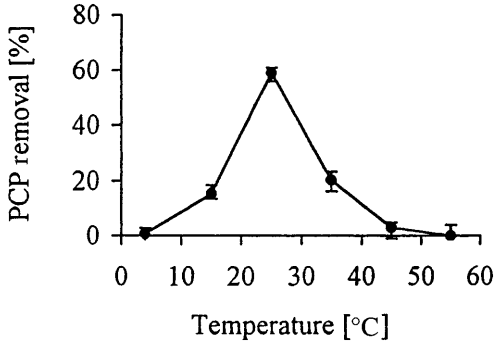

Fig. 2 The effect of temperature on the activity of laccase I with catechol as substrate

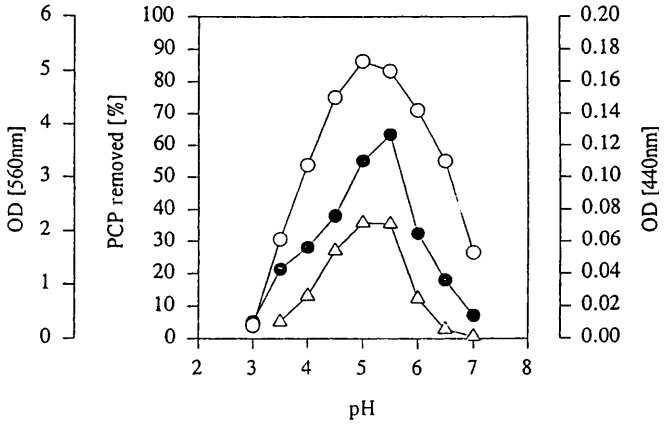

Fig. 3 The pH optimum of laccase with △ PCP, ● catechol, ○ syringaldazine, *OD* absorbance

using gel exclusion chromatography. Fractionation of the reaction mixture on a column of G75 Sephadex showed a major peak absorbing at 254 nm. This was eluted at the void volume of the column (MW above 80,000), with a further peak identified as unreacted PCP (MW 266) (Fig. 5). The identity of PCP was confirmed using HPLC by co-chromatography with authentic PCP, as both PCP and decachlorobiphenyl eluted from the G75 Sephadex column after the total effective column volume (V_t). Aromatic hydroxyl and methoxyl substituents are known to increase adsorption onto Sephadex (Ruttimann-Johnson and Lamer 1996), so elution of PCP beyond V_t was probably due to interactions of the phenolic group with the Sephadex matrix. No evidence for dimer formation was observed in the products from the reaction of laccase with PCP.

GC-MS of products did not identify any low-molecular-mass components in substantial quantities. The mass spectrum identified *o*- and *p*-chloranils (tetrachlorobenzoquinones) as trace components in replicate samples, in picogram quantities in incubations with 100 μg PCP. MS with selective ion monitoring showed abundant ions at *m/z* 248 [M], 210 [M-Cl, 2H], 175 [M-2Cl, 2H], 147 [M-2Cl, 2O] and 113 [M-3Cl, 2O].

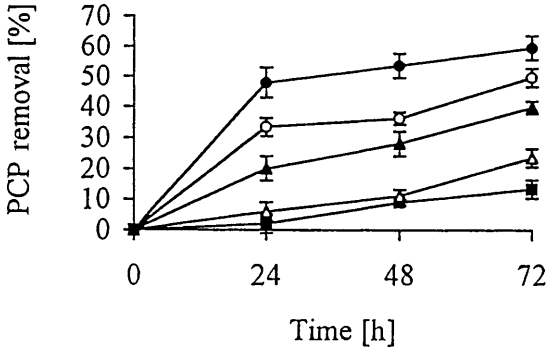

Fig. 4 Reaction of 100 μg ml^{-1} PCP with laccase I at various concentrations: ■ 12 units, △ 25 units, ▲ 50 units, ○ 75 units, ● 100 units

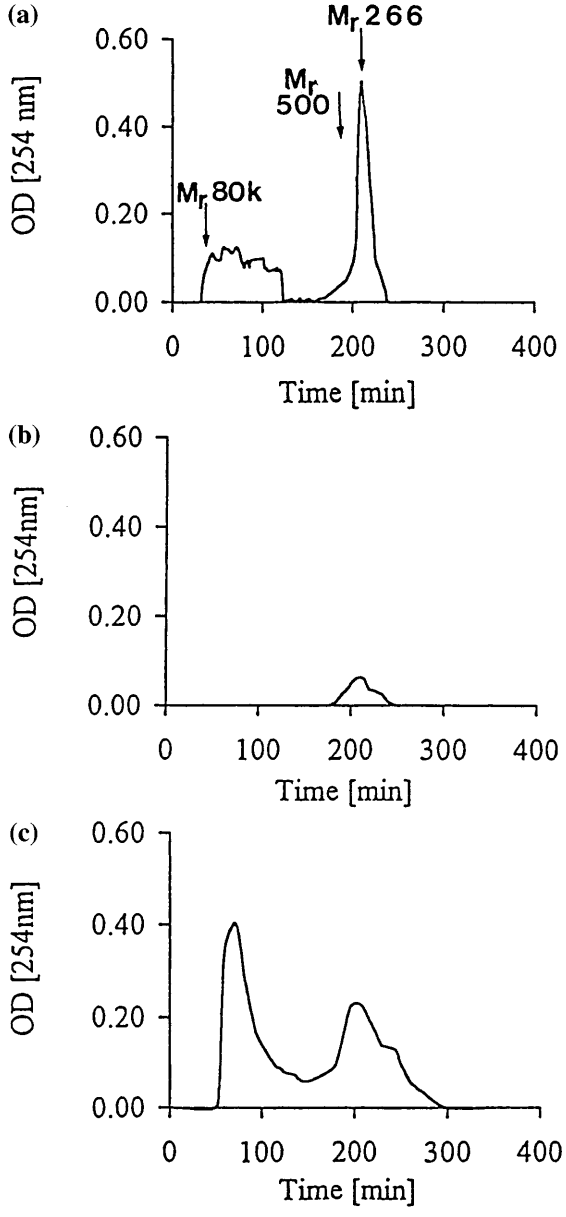

Fig. 5 Gel filtration on G-75 Sephadex of reaction products from **a** laccase with PCP, **b** MnP with PCP, and **c** laccase and MnP with PCP

No free chloride was released from the action of laccase with PCP, although the sensitivity of the assay was set at 2 ppm chloride. Because the major product formed was a high MW polymer and no chloride was released during the reaction, the stability of the polymer was examined. Acid hydrolysis did not release PCP from the polymer, nor did any PCP leach from the polymer in aqueous solution at pH 5 over at least 30 days.

Discussion

An increasing range of white-rot fungi is now being investigated for bioremediation purposes (Brandt et al. 1997; Lamar and Dietrich 1990; Lin et al. 1990; Mileski et al. 1988; Pfender et al. 1997). It is therefore important that the specific enzymic reactions involved with remediation of individual pollutants are characterised. For the removal of PCP, reaction with laccase as a predominant enzyme in many white-rot species such as *C. versicolor* and *Pleurotus ostreatus* has been described here. In this reaction, PCP is a substrate for laccase, similar to the substrates catechol and syringaldazine with respect to pH optimum and temperature of reaction. The reaction is dependent on both PCP and laccase concentrations. The K_m value of 3 mM indicated low specificity of the enzyme for the substrate. Reaction of PCP with laccase must be solely with the phenolic group as no detectable free chloride was released during reaction, which confirms the observation of Roy-Arcand and Archibald (1991). PCP has been reported to be the most recalcitrant of the chlorophenolic compounds, as all other chlorophenols release some free chloride after reaction with peroxidases or laccase (Roy-Arcand and Archibald 1991). Because free chloride was released only when PCP was present in mixtures of chlorophenols, our data confirm that in the reaction of PCP alone with laccase, measurable amounts of chloride are not released.

The number of chloro substituents on the phenolic ring affects the optimum pH for peroxidases (Dec and Bollag 1990), with decreases from pH 8.3 for tetrachlorophenol, to pH 6.5 for dichlorophenol, to pH 6.1 for trichlorophenol and to pH 5.4 for pentachlorophenol. Substituents in the *meta* position reduced the reactivity of the chlorophenols in studies with crude enzyme extracts washed from wheat grain cultures of *C. versicolor* (Kadhim et al. 1999).

It is important that products of reaction in bioremediation systems are less toxic than the pollutant. The primary product of the PCP reaction with laccase was found to be a high MW polymer, which appeared stable under aqueous conditions in alkali and in acid. Trace quantities of highly toxic tetrachlorobenzoquinones were detected, but at less than 0.1 ppm compared with the original amount of PCP (100 μg). Polymerisation provides effective removal of PCP, yielding a stable compound. In aqueous effluents, polymerisation of PCP may offer a means to collect and remove the polymer, so permitting complete removal of PCP from solution. Recalcitrant organochlorine compounds have been degraded in fixed film bioreactors (Alleman et al. 1995; Salkinoja-Salonen et al. 1983). Stability of laccase can be improved if used in an immobilised state (Leonowicz et al. 1988).

Fungi producing large quantities of laccase may offer a preferable enzyme system for the removal of PCP, compared with high peroxidase-producing organisms. Characterisation of the reaction between laccase and PCP highlights the potential for using laccase-producing fungi in bioremediation of aqueous effluents, where polymerised products could be readily removed. Searches for fungi which produce more laccase than *C. versicolor*, through rapid screening of laccase activity and removal

of PCP, will permit the identification of the most useful organisms for successful bioremediation of PCP.

Acknowledgements We thank the University of Westminster for a fee-waiver scholarship for M.U., and Dr R.A. Rastall, Reading University, for MS analysis.

References

Alleman BC, Logan BE, Gilbertson RI (1995) Degradation of pentachlorophenol by fixed films of white rot fungi in rotating tube bioreactors. Water Res 29: 61–67

Brandt S, Zeng A-P, Decker W-D (1997) Adsorption and desorption of pentachlorophenol on cells of *Mycobacterium chlorophenolicum* PCP-1. Biotech Bioeng 55: 480–488

Dec J, Bollag J-M (1990) Detoxification of substituted phenols by oxidoreductive enzymes through polymerisation reactions. Arch Environ Contam Toxicol 19: 543–550

Evans CS (1985) Laccase activity in lignin degradation by *C. versicolor*: *in vivo* and *in vitro* studies. FEMS Microbiol Lett 27: 339–343

Fahraeus G, Reinhammar B (1967) Large scale production and purification of laccase from the fungus *Polyporus versicolor* and some properties of laccase. Acta Chem Scand 21: 2367–2378

Harkin M, Obst JR (1973) Syringaldazine an effective reagent for detecting laccase and peroxidase in fungi. Experientia 29: 381–508

Iimura Y, Katayama Y, Kawai S, Morohoshi N (1995) Degradation and solubilization of C13 and C14 side chain labelled synthetic lignin (dehydrogenative polymerizate) by laccase III of *Coriolus versicolor*. Biosci Biotech Biochem 59: 903–905

Iwasaki I, Utsumi S, Hagino K, Ozawa T (1956) A new spectrometric method for the determination of small amounts of chloride using the mercuric thiocyanate method. Bull Chem Soc Jpn 29: 860–864

Kadhim H, Graham C, Barratt P, Evans CS, Rastall RA (1999) Removal of phenolic compounds in water from *C. versicolor* grown on wheat bran. Enzyme Microbiol Technol 24: 303–307

Kazandjian RZ, Dordick JS, Klibanov AM (1986) Enzyme analyses in organic solvents. Biotech Bioeng 28: 417–421

Konishi K, Inoue Y (1972) Detoxification mechanism of pentachlorophenol by laccase of *Coriolus versicolor*. Mokuzai Gakkaishi 18: 463–469

Lamar RT, Dietrich DM (1990) In situ depletion of pentachlorophenol from contaminated soil by *Phanerochaete* spp. Appl Environ Microbiol 56: 3093–3100

Leonowicz A, Sarkar JM, Bollag J (1988) Improvement in stability of an immobilised fungal laccase. Appl Microbiol Biotech 29: 129–135

Leontievsky AA, Vares T, Lankinen P, Shergill JK, Pozdnyakova NN, Myasoedova N, Kalkkinen N, Golovleva LA, Cammack R, Thurston CF, Hatakka A (1997) Blue and yellow laccases of ligninolytic fungi. FEMS Microbiol Lett 156: 9–14

Lin J-E, Wang HY, Hickey RF (1990) Degradation kinetics of pentachlorophenol by *Phanerochaete chrysosporium*. Biotech Bioeng 35: 1125–1134

McAllister KA, Lee H, Trevors JT (1996) Microbial degradation of pentachlorophenol. Biodegradation 7: 1–40

Mileski GJ, Bumpus JA, Jurek MA, Aust SD (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. Appl Environ Microbiol 54: 2885–2889

Pfender WF, Maggard SP, Gander LK, Watrud LS (1997) Comparison of three bioremediation agents for mineralisation and transformation of pentachlorophenol in soil. Bull Environ Contam Toxicol 59: 230–237

Ricotta A, Unz RF, Bollag J-M (1996) Role of laccase in the degradation of pentachlorophenol. Bull Environ Contam Toxicol 57: 560–567

Roy-Arcand L, Archibald FS (1991) Direct dechlorination of chlorophenolic compounds by laccases from *Trametes* (*Coriolus*) *versicolor*. Enzyme Microb Technol 13: 194–203

Ruttimann-Johnson C, Lamar RT (1996) Polymerisation of pentachlorophenol and ferulic acid by fungal extracellular lignin-degrading enzymes. Appl Environ Microbiol 62: 3890–3893

Salkinoja-Salonen M, Hakulinen R, Valo R, Apajalahti J (1983) Biodegradation of recalcitrant organochlorine compounds in fixed film reactors. Water Sci Technol 15: 309–319

Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140: 19–26

Tome D, Nicholas J, Drapron R (1978) Influence of water activity on the reaction catalysed by polyphenoloxidase (E.C.1.14.18.1) from mushrooms in organic liquid media. Lebensm Wiss Technol 11: 38–41