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ORIGINAL PAPER

B. C. Okeke · A. Paterson · J. E. Smith
I. A. Watson-Craik

Comparative biotransformation of pentachlorophenol in soils by solid substrate cultures of *Lentinula edodes*

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Abstract Sterilised and non-sterilised soils contaminated with pentachlorophenol (PCP) were inoculated with solid substrate cultures of *Lentinula edodes* LE2 ("shiitake" mushroom) to simulate monoculture bioremediation treatments and treatments in which the fungus competes with natural microflora. With monocultures of *L. edodes*, rates of PCP depletion were rapid for the initial 4 weeks and, although thereafter the rate decreased, 99% biotransformation was obtained in 10 weeks. In mixed culture, PCP biotransformation by *L. edodes* was markedly slower and only 42% of the PCP was depleted after 10 weeks. Maximal rates of PCP transformation, biomass (ergosterol) accumulation and oxidative enzymes (phenol oxidase and manganese-peroxidase) production were observed after 2 weeks of incubation. In monocultures, phenol oxidase activity was 195.5 U g^{-1} and Mn-peroxidase 138.4 U g^{-1}. In mixed cultures, fungal enzyme activities were markedly lower: 70.33 U g^{-1} for phenol oxidase and 85.0 g^{-1} for Mn-peroxidase. Analyses of soil metabolites after 10 weeks revealed that monocultures of *L. edodes* had eliminated both PCP and pentachloroanisole. Pentachloroanisole, however, was detected in soils with the mixed microflora. Both dechlorination and mineralisation of the xenobiotic compound were effected by *L. edodes* LE2.

B. C. Okeke (✉)[1] · A. Paterson · J. E. Smith
I. A. Watson-Craik
Department of Bioscience and Biotechnology,
University of Strathclyde, 204 George Street,
Glasgow, G1 1XW, UK

Present address:
[1] International Centre for Genetic Engineering and Biotechnology, Area Science Park,
Padriciano 99, I-34012 Trieste, Italy
Fax: +39 40 226555, Tel: +39 40 375 7317
e-mail: okeke@icgeb.trieste.it

Introduction

Pentachlorophenol (PCP) has been widely used as a pesticide, particularly as a wood preservative (Larsen et al. 1991) and is currently found in significant concentrations in certain soils, natural water courses and in the atmosphere. This xenobiotic compound is an inhibitor of oxidative phosphorylation and is toxic to all forms of life (Apajalahti and Salkinoja-Salonen 1984). PCP is also an attractive model for studying biotransformations of chlorinated aromatic compounds in soils because of its highly stable aromatic ring system and high halogen content. Development of such processes can be used in the design of bioremediation technology that is both cost-effective and compatible with concerns for the environment. Filamentous fungi have been reported to metabolise a range of xenobiotic compounds such as PCP (Bumpus et al. 1985; Mileski et al. 1988), polychlorinated biphenyls (Bumpus and Aust 1987; Sasek et al. 1993), trinitrotoluene (Fernando et al. 1990), trichlorophenoxyacetic acid and other non-chlorinated aromatics (Yadav and Reddy 1993). Decolourisation of pulp bleachery effluents has also been achieved with basidiomycetes such as *Phanerochaete chrysosporium* (Eaton et al. 1982), *Trametes versicolor* (Archibald et al. 1990), *Stagonospora gigaspora* (Bergbauer et al. 1992), *Lentinula edodes* and other ligninolytic fungi (Esposito et al. 1991). As the carbon skeletons of many recalcitrant environmental pollutants are similar to those found in lignins (Bumpus et al. 1985), xenobiotic breakdown by ligninolytic fungi is thought to be related to oxidative enzymes secreted by fungi to effect lignin depolymerisation (Bumpus and Aust 1987; Mileski et al. 1988). Exploitation of the ligninolytic properties of basidiomycetes is becoming increasingly important in developing bioremediation processes (Roy-Arcand and Archibald 1991; Valli and Gold 1991; Palmieri et al. 1993; Okeke et al. 1994b). In such studies *P. chrysosporium* is often the organism of choice as it has been well characterised during analyses of mechanisms of

lignin biodegradation. We have previously shown, however, that its optimum temperature and water activity for PCP biotransformation (Okeke et al. 1996) are not those found in temperate soils typical in Central and Northern Europe. In contrast, the "shiitake" mushroom *L. edodes* appears to remain active at lower temperatures (Okeke et al. 1996) and biomass is now readily available following cropping in horticulture. As such biomass in sawdust is currently a waste it was important to determine whether this could form an important product that could be incorporated into soils where the organism could be able to compete with the indigenous microflora.

Although *L. edodes* has been shown to biotransform PCP in monoculture (Okeke et al. 1993, 1994b), the efficiency and extent of transformations in mixed culture in non-sterile soil were not known. In this study, PCP biotransformation by *L. edodes* biomass in mono and mixed cultures was related to the development of fungal biomass, quantified as ergosterol and oxidative activities in sterilised and non-sterilised soils.

Materials and methods

Chemicals

Pentachlorophenol (99% pure) and [U-^{14}C]pentachlorophenol (9.3 Ci mol^{-1}, 22 200 cpm µl^{-1}), were purchased from Sigma Chemical Company. Ergosterol (95% pure), 3,3′,5,5′-tetramethylbenzidine (97% pure) and 2,4,6-tribromophenol (99% pure) were obtained from Aldrich Chemical Company. Other chemicals were of analytical grade and purchased from commercial sources.

Fungal strain and biotransformation conditions

L. edodes strain LE2, obtained from Prof. K. K. Tan, Everbloom Biotechnology, Singapore, was maintained on malt extract/agar slants at 4 °C. Fungal biomass was prepared by solid substrate fermentation (Okeke et al. 1996). Soil and culture conditions were as described previously (Okeke et al. 1996) except that the initial moisture was adjusted to 31% with 2 ml sterile nutrient-nitrogen-sufficient mineral salts solution (10 g glucose, 0.48 g NH_4NO_3 0.2 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 0.1 mg thiamine/HCl, 100 ml distilled water, and supplemented with 1 ml mineral elements solution: 3 g $MgSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot H_2O$, 1.0 g NaCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $CoSO_4$, 0.082 g $CaCl_2$, 0.01 g $ZnSO_4$, 0.01 g $CuSO_4 \cdot 5H_2O$, and 1000 ml distilled water) (Kirk et al. 1978). Following incubation, residual PCP was extracted and quantified by reverse-phase HPLC (Okeke et al. 1996). Biotransformation (%) was calculated relative to residual PCP in control soils.

Biomass determination

Fungal biomass was quantified as ergosterol according to the method of Seitz et al. (1979) with modification (Okeke et al. 1994a). Saponification was carried out in a water bath (75 °C, 45 min) as described by Dewey et al. (1992).

Enzymatic activities

Enzymes were extracted by steeping soil culture in distilled water (1:1, w/v) for 90 min at 4 °C and removing the soil by centrifugation (13 000 *g*, 4 °C), recording the pH. Phenol oxidase (laccase) activity was quantified by the method of Babasaki and Ohmasa (1991), modified as described previously (Okeke et al. 1994a). Mn-peroxidase activity was assayed in a reaction mixture of 1 mM 3,3′,5,5′-tetramethylbenzidine in 25 mM sodium acetate, pH 4.0 (2 ml), 100 mM $MnCl_2 \cdot 4H_20$ (100 µl), enzyme solution (100 µl), and 54 mM H_2O_2 (24 µl) at 25 °C for 10 min. In each case, one unit of enzyme activity was defined as 0.01 unit change in A_{650} min^{-1} (Okeke et al. 1994a). The specific activity (U mg^{-1} ergosterol) was calculated.

Analysis of products of PCP biotransformation

High-resolution gas chromatography/mass spectrometry (HRGC-MS) analysis of PCP metabolites was carried out as described by Okeke et al. (1994b). Ionic chloride was quantified using mercuric thiocyanate (Schering Agrochemicals 1987). A chloride standard was prepared by diluting 2.8 ml 0.1 M HCl to 100 ml using deionised water. Soil culture was extracted with deionised water (1:1 w/v). The reaction mixture comprised a 0.5-ml sample, 2 ml 1 M nitric acid, 0.5 ml ammonium ferric sulphate solution (0.25 M in 9 M nitric acid), 0.5 ml mercuric thiocyanate solution (4 g l^{-1} ethyl alcohol) and 1.5 ml deionised water. The absorbance of the resulting solution at 456 nm was determined, using a Shimadzu Spectronic UV-120-02, after 10 min. Percentage dechlorination efficiency was calculated on the basis of chloride as PCP at 200 µg g^{-1} soil being equivalent to 133.13 µg chloride g^{-1} soil.

In studies of true PCP mineralisation by *L. edodes*, soils were contaminated using a mixture of [^{12}C] and [^{14}C]PCP. A stock solution was prepared by mixing 4.8 ml [^{14}C]PCP (20.8 mg ml^{-1} in toluene) with 0.2 ml (4.49×10^6 dpm) of the [^{14}C]PCP in toluene. Sterile soil (50 g in a 250-ml conical flask) was contaminated with 500 µl PCP solution to 200 mg kg (4.49×10^5 dpm). The flasks, with soil adjusted to 31% moisture content, were each fitted with rubber stoppers through which a capillary air inlet and non-capillary air outlet tube were inserted. Each culture flask capillary glass tube was connected via a PVC rubber tubing to a 2-l flask containing 250 ml sterile water, fitted with a rubber tubing through which seven glass tubes (one at the centre and six at the periphery) were inserted. The central glass tubing was connected to an air pump via a PVC rubber tubing fitted with an air filter. Air outlet tubing from each flask was connected to a 250-ml flask containing an ethanolamine trap and cultures were flushed 5 days in a week for 3 h, using sterile air. Aliquots (1 ml) from the ethanolamine trap was mixed with 10 ml liquid scintillation cocktail (Safefluor S, Lumac LSC, BV) and $^{14}CO_2$ was analysed in a Canberra Packard (1500 Tri-Carb) liquid scintillation counter.

Statistical validation of data

Experiments were carried out in triplicate. Means and standard deviations (SD) of replicates were calculated. Correlation analyses between variables were performed and a Mann-Whitney non-parametric test was used to confirm significance of factor effect, at 5% error.

Results

Biotransformation of PCP in sterilised soil by monocultures of *L. edodes*

The kinetics of PCP biotransformation by monocultures of *L. edodes* demonstrates that PCP was depleted rapidly within the initial 4 weeks of incubation; afterwards the rate decreased (Fig. 1A). A 99% depletion (0.73 mg kg^{-1}) was achieved in a 10-week period. Phenol oxidase and Mn-peroxidase activities rose rapidly in the cultures

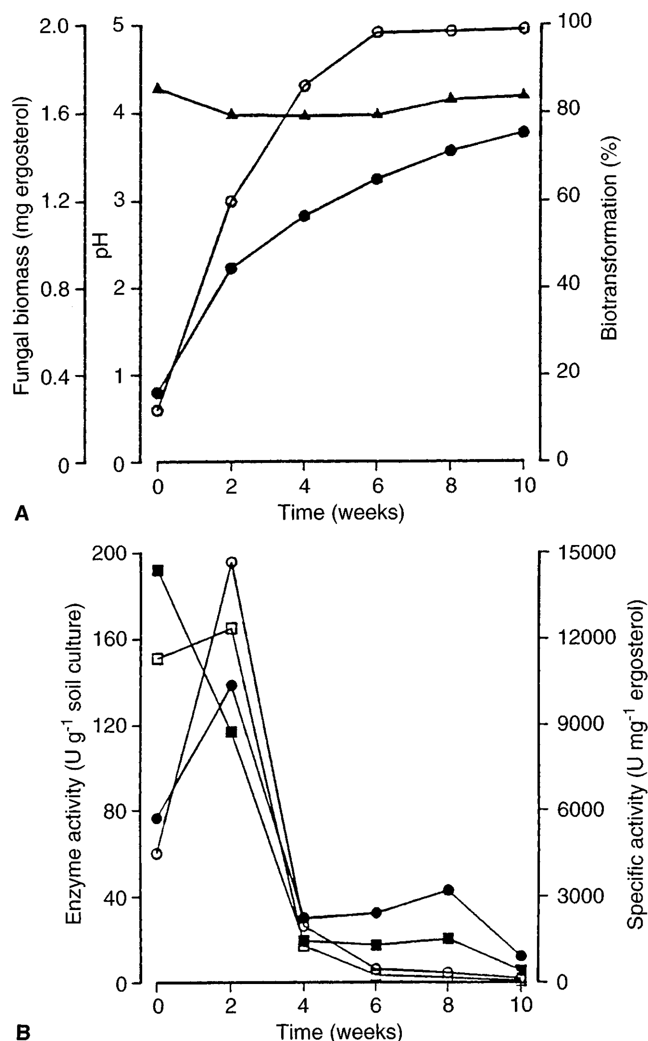

Fig. 1 A Fungal biomass, pH and depletion of pentachlorophenol (PCP) in sterile soil inoculated with a monoculture of *Lentinula edodes* and incubated at 25 °C for 10 weeks: PCP depletion (○), biomass as ergosterol (●), pH (Δ). Pooled SD values: PCP depletion 2.0%, biomass 0.08 mg, pH 0.1. PCP depletion (percentage biotransformation) was calculated relative to residual PCP in controls (Table 1). **B** Activities of oxidative enzymes secreted during growth of monocultures of *L. edodes* in sterile soil at 25 °C for 10 weeks: phenol oxidase (○), specific activity (□); Mn-peroxidase (●), specific activity (■). Pooled SD values: phenol oxidase 3.20 U g^{-1} Mn-peroxidase 3.50 U g^{-1}

reaching 195.46 ± 7.10 U g^{-1} and 138.40 ± 6.70 U g^{-1} after 2 weeks respectively (Fig. 1B). The highest rate of PCP transformation was reached after 2 weeks of incubation and was also maximal for enzyme secretion (Fig. 1B) and ergosterol accumulation (Fig. 1A). Enzymatic activities in relation to ergosterol concentration have similar profiles (Fig. 1B). PCP disappearance and biomass (ergosterol) accumulation were strongly correlated ($r = 0.988$, $P < 0.05$). During the growth of *L. edodes*, there was a decrease in pH of the soil culture in the initial 2 weeks (Fig. 1A). Recoverable PCP decreased from 139.00 mg kg^{-1} (69.50% recovery) to 79.46 mg kg^{-1} (39.73% recovery) in control soils and from 122.35 mg kg^{-1} (61.20% recovery) to 0.73 mg kg^{-1} (0.37% recovery) in treated soils during the 10-week incubation period (Table 1).

Table 1 Pentachlorophenol (*PCP*) recovery in sterilised soil culture of *Lentinula edodes* LE2 over a 10-week incubation period at 25 °C. Values are means of triplicate experiments ± SD. Values in parenthesis represent percentage recovery relative to initial PCP concentration (200 mg kg^{-1})

Time (weeks)	Residual PCP (mg kg^{-1})	
	Control	Treated
0	139.00 ± 7.66 (69.50)	122.35 ± 6.33 (61.20)
2	104.24 ± 3.10 (52.12)	41.81 ± 2.58 (20.91)
4	96.14 ± 3.00 (48.07)	13.22 ± 0.37 (6.61)
6	81.82 ± 2.77 (40.91)	1.29 ± 0.15 (0.65)
8	80.66 ± 1.56 (40.33)	1.10 ± 0.10 (0.55)
10	79.46 ± 1.82 (39.73)	0.73 ± 0.06 (0.37)

Biotransformation of PCP in non-sterilised soil by mixed cultures

In mixed cultures, where competing microorganisms had not been eliminated by autoclaving, there was also a rapid depletion of PCP for the initial 2 weeks after which the rates decreased so that 42% disappearance (41.26 ± 3.08 mg kg^{-1}) was recorded after 10 weeks (Fig. 2A). Again fungal biomass increased rapidly in the initial 2 weeks and enzymatic activities (Fig. 2B) were maximal after 2 weeks: laccase at 70.33 U g^{-1}; Mn-peroxidase at 85.0 U g^{-1}. Specific activities of both enzymes also followed a similar pattern. There was no significant correlation between PCP depletion and biomass (ergosterol) accumulation ($r = 0.01$, $P > 0.05$) over the 10 weeks. Microbial growth in the non-sterilised soil culture resulted in increases in pH from an initial value of 4.38 to 5.53 following 10 weeks of incubation (Fig. 2A). PCP recovery decreased from 127.33 mg kg^{-1} (63.67% recovery) to 71.62 mg kg^{-1} (35.81% recovery) in control soils and from 116.82 mg kg^{-1}(58.41% recovery) to 41.26 mg kg^{-1} (20.63% recovery) in the treated soil culture (Table 2).

PCP biotransformation products

HRGC-MS, showed the prescence of biotransformation products: pentachloroanisole (mass ion, = m.i., 280.3) and tetrachloroanisole (m.i. 245.9), and tetrachlorophenol (m.i. 231.9) in the first 4 weeks in both sterilised and non-sterilised soils. Pentachloroanisole was the major biotransformation product (Fig. 3). After 10 weeks of monoculture treatment with *L. edodes* chloroanisoles

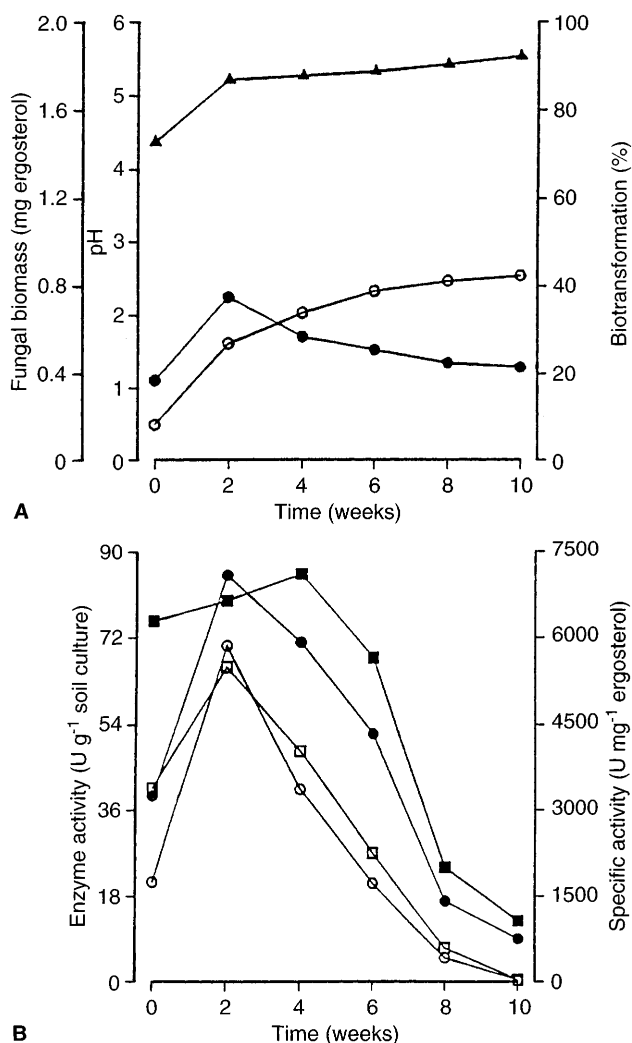

Fig. 2 A Fungal biomass, pH and depletion of PCP in natural soil inoculated with *L. edodes* and growing in mixed culture at 25 °C for 10 weeks: PCP depletion (○), biomass as ergosterol (●), pH (Δ). Pooled SD values: PCP depletion 2.0%, biomass 0.08 mg, pH 0.1. PCP depletion (percentage biotransformation) was calculated relative to residual PCP in controls (Table 2). **B** Activities of oxidative enzymes secreted during growth of *L. edodes* in mixed culture soil at 25 °C for 10 weeks: phenol oxidase (○), specific activity (□); Mn-peroxidase (●), specific activity (■). Pooled SD values: phenol oxidase, 2.20 U g^{-1}, Mn-peroxidase 3.20 U g^{-1}

were absent from the soils. Pentachloroanisole, however, persisted in soils treated with the organism in the presence of the natural soil microflora (mixed culture). Ionic chloride was detected in both natural and sterilised soils (Table 1). Dechlorination efficiency was significantly ($P < 0.05$) greater with monoculture of *L. edodes* (29.50%) than in mixed culture (22.40%). A certain amount in dechlorination was also detected in sterile control soils (3.63%), but in the non-sterile control soil there was a significant activity (10.30%). Total degradation of PCP by *L. edodes* was confirmed by quantification of mineralization using [^{14}C]PCP. Evolution of $^{14}CO_2$ was rapid in the initial 3 weeks (3.69×10^4 dpm, 8.2% initial ^{14}C) but thereafter the rate

Table 2 Pentachlorophenol recovery in non-sterilised soil culture of *L. edodes* LE2 over a 10-week incubation period at 25 °C Values are means of triplicate experiments ± SD. Values in parenthesis represent percentage recovery relative to initial PCP concentration (200 mg kg^{-1})

Time (weeks)	Residual PCP (mg kg^{-1})	
	Control	Treated
0	127.33 ± 6.57 (63.67)	116.82 ± 7.00 (58.41)
2	95.64 ± 9.60 (47.82)	69.82 ± 4.80 (34.91)
4	83.10 ± 8.33 (41.55)	54.87 ± 6.20 (27.44)
6	76.31 ± 9.38 (38.16)	46.63 ± 4.01 (23.32)
8	73.46 ± 8.12 (36.73)	43.20 ± 3.51 (21.60)
10	71.62 ± 5.38 (35.81)	41.26 ± 3.08 (20.63)

decreased. A cumulative total of 5.91×10^4 dpm (13.2% initial ^{14}C) was recovered as $^{14}CO_2$ after 10 weeks of incubation. A degree of mineralization was observed in sterile control soils: 0.63×10^3 dpm in week 1 (0.14%); 1.14×10^3 dpm by week 10 (0.25%) but not significant ($P > 0.05$).

Discussion

L. edodes is currently produced in large quantities in horticulture, generating spent biomass in sawdust that could be readily utilised for bioremediation of pesticide-contaminated soils. We have demonstrated that *L. edodes* biomass in sawdust can effect depletion of PCP in both the presence and absence of the natural soil microflora. Both in monoculture and when competing against indigenous soil microorganisms, initial PCP depletion was rapid and, during this time, secretion of both phenol oxidase (laccases) and Mn-peroxidase was maximal. After 4 weeks, the rate of PCP depletion had slowed, possibly because of a reduction in enzymatic activities. This observation supports the hypothesis that the enzymes secreted by *L. edodes* to effect oxidative attack on lignins are principal agents effecting PCP biotransformation (Okeke et al. 1994b). Xenobiotics in natural environments are normally found as mixtures so that the non-specificity of the ligninolytic enzymes is attractive, since these enzymes effect oxidation of both a wide variety of substances and mixtures of chemicals (Aust 1990; Bewley 1992).

Loss of PCP from the sterilised control soil was attributed to binding of the xenobiotic compound to soil organic matter (Berry and Boyd 1985; Dec and Bollag 1988). Edgehill and Fin (1983) observed about 25%–30% loss of PCP in sterile controls after 12 days of incubation. A similar reason would account for PCP depletion in the non-sterilised soil controls and additional losses are

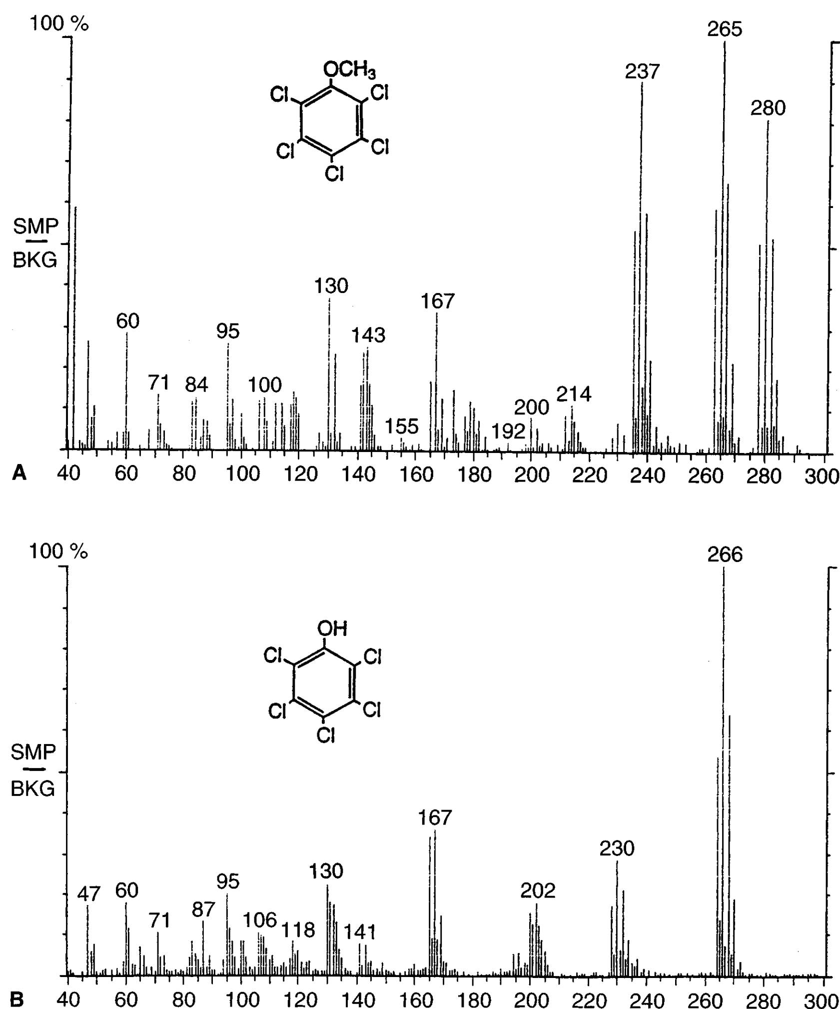

Fig. 3 Mass spectra of pentachloroanisole (**A**), and pentachlorophenol (**B**)

possibly due to the metabolic activities of the indigenous soil microbial flora.

Accumulation of fungal biomass paralleled PCP depletion when *L. edodes* was growing in monocultures but not when it was competing with the natural soil microflora. The rate and final extent of PCP depletion were markedly reduced in the presence of indigenous soil microbes. This is a problem central to fungal bioremediation of soils as the indigenous bacteria are able to grow faster, utilising available nutrients more rapidly and antagonising the exogenous fungus. Bacteria isolated from polluted and agricultural soils have been shown to inhibit growth of *P. chrysosporium* on agar medium (Radtke et al. 1994). One pseudomonad isolate, capable of producing phenazines, exhibited a marked inhibitory effect on *P. chrysosporium* while a second, producing a fluorescent siderophore did not (Radtke et al. 1994). Field soil contaminated with PCP, however,

Table 3 Dechlorination of PCP by mono- and mixed culture treatments of soil with *L. edodes* for 10 weeks at 25 °C
Δ Chloride or dechlorination efficiency (*DchE*) due to treatment effect

Culture	Chloride ($\mu g\ g^{-1}$)	Chloride Δ ($\mu g\ g^{-1}$)	DchE (%)	DchE Δ (%)
Natural soil (Control)	13.71 (0.60)[a]	0	10.30	0
Natural soil with *L. edodes* (mixed culture)	29.76 (0.93)[a]	+16.05	22.40	+12.06
Sterile soil (Control)	4.83 (0.20)[a]	0	3.63	0
Sterile soil with *L. edodes* (monoculture)	34.33 (1.31)[a]	+25.78	29.50	+22.16

[a] Standard deviations (n − 1) of triplicate experiments

will also contain microbes that have adapted towards metabolism of the xenobiotic, notably bacteria (Valo and Salkinoja-Salonen 1986). A further factor reducing fungal activity may be increases in pH in the mixed cultures generating suboptimal conditions for PCP biotransformation by fungi (Okeke et al. 1996).

The potential of *L. edodes* for field bioremediation could be enhanced by a number of strategies. Increasing the inoculum biomass-to-soil ratio may enhance the competitive advantage of the fungus and increase the effective surface area in the soil environment for growth of fungal mycelia and biotransformation of xenobiotic compounds. Supplementation of the soil culture system with lignocelluloses such as bark chips, straw and sawdust has been demonstrated to have similar and beneficial effects, as the polymeric lignocellulosic material is largely unavailable as a carbon source to competing soil organisms (Aust 1990). Partial sterilisation of soil through fumigation may also assist the process (Lamar and Dietrich 1990).

The presence of chloroanisoles in soils undergoing PCP biotransformation by fungal metabolism has previously been reported (Lamar et al. 1990; Lamar and Dietrich 1990; Okeke et al. 1993, 1994b). HRGC-MS analyses of metabolites after the 10-week treatment period, showed no detectable levels of pentachloroanisole in the sterilised soil culture. Pentachloroanisole was, however, detected in the non-sterilised soil culture. This is possibly due to inhibition of *Lentinula* growth and activity by competing soil organisms. Pentachloroanisole and other chloroanisoles are considered more hazardous in the environment than PCP, as such compounds have a higher lipophilicity and thus a greater tendency to bioaccumulate in animal tissues (Kennedy et al. 1990). However, chloroanisoles may degrade with prolonged treatment, as observed with the sterile soil culture, in this study. Transformation of PCP to pentachloroanisole is an important route of PCP depletion during the early stages of PCP biotransformation by *L. edodes* (Okeke et al. 1993, 1994b). Lamar et al. (1990), using *Phanerochaete chrysosporium*, also reported that pentachloroanisole accummulated initially and was degraded during a second stage beginning after 9 days of incubation.

Dechlorination of certain xenobiotics is central to their detoxification (Armenante et al. 1993; Fava et al. 1994). *L. edodes* effects significant dechlorination of PCP in both mono- and mixed soil cultures. The dechlorination of PCP by the lignin peroxidase of *P. chrysosporium* (Hammel and Tardone 1988) and other organic compounds by fungi and their extracellular enzymes has been reported (Roy-Arcand and Archibald 1991). Mixtures of laccases isolated from culture fluids of *T. versicolor* were able to effect 13.6%–17.6% dechlorination of a mixture of chlorophenolics. In this process, the release of chloride ions from single polychlorophenols was accompanied by both oxygen consumption and substrate disappearance (Roy-Arcand and Archibald 1991).

In summary, this study demonstrates that *L. edodes* degrades the xenobiotic compound PCP. Mineralisation and dechlorination are important activities. The potential for commercial application of fungal culture residues in soil bioremediation is apparent. But before this xenobiotic biotransformation potential can be realised, it is necessary to clarify how such fungi can be given a competitive advantage in soils containing an indigenous microflora, possibly by addition of lignocellulosic substrates providing surfaces to stimulate fungal growth and activity.

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