Biodegradation **11:** 331–340, 2000.

Transformation of 2,4,6-trichlorophenol by the white rot fungi *Panus tigrinus* and *Coriolus versicolor*

A.A. Leontievsky[1]*, N.M. Myasoedova[1], B.P. Baskunov[1], C.S. Evans[2] & L.A. Golovleva[1]
[1] *Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142292 Pushchino Moscow region, Russia;* [2] *Fungal Biotechnology Group, University of Westminster, London W1M 8JS, U.K.*
(author for correspondence; e-mail: leont@ibpm.serpukhov.su)*

Accepted 19 September 2000

Key words: Coriolus versicolor, *Panus tigrinus*, laccase, Mn-peroxidase, 2,4,6-trichlorophenol, white rot fungi

Abstract

The toxicity of thirteen isomers of mono-, di-, tri- and pentachlorophenols was tested in potato-dextrose agar cultures of the white rot fungi *Panus tigrinus* and *Coriolus versicolor*. 2,4,6-Trichlorophenol (2,4,6-TCP) was chosen for further study of its toxicity and transformation in liquid cultures of these fungi. Two schemes of 2,4,6-TCP addition were tested to minimize its toxic effect to fungal cultures: stepwise addition from the moment of inoculation and single addition after five days of growth. In both cases the ligninolytic enzyme systems of both fungi were found to be responsible for 2,4,6-TCP transformation. 2,6-Dichloro-1,4-hydroquinol and 2,6-dichloro-1,4-benzoquinone were found as products of primary oxidation of 2,4,6-TCP by intact fungal cultures and purified ligninolytic enzymes, Mn-peroxidases and laccases of both fungi. However, primary attack of 2,4,6-TCP in *P. tigrinus* culture was conducted mainly by Mn-peroxidase, while in *C. versicolor* it was catalyzed predominantly by laccase, suggesting a different mode of regulation of these enzymes in the two fungi.

Abbreviations: ABTS – 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), CP – Chlorophenol, DCP – Dichlorophenol, DMF – Dimethylformamide, LiP – Lignin peroxidase, MnP – Manganese peroxidase, MS – Mass-spectrometry, PAGE – Polyacrylamide gel electrophoresis, PCP – Pentachlorophenol, PDA – Potato-dextrose agar, TLC – Thin layer chromatography, TCP – Trichlorophenol.

Introduction

Ligninolytic wood-rotting fungi are capable of degrading a wide range of recalcitrant compounds. It is of interest to show their ability to detoxify persistent pollutants that are produced on a large scale and contaminate different environmental media. Chlorophenols, especially 2,4-di-, 2,4,5- and 2,4,6-tri-, and pentachlorophenol, are some of the most typical example of this kind of xenobiotics.

Biodegradation of chlorophenols by bacterial species is rather effective and well-known (Golovleva et al. 1994; Zaitsev et al. 1995). However bacteria are not ideal organisms for treating chlorophenol (CP) pollution in wood based substrates due to lack of available nutrients. At the same time 2,4,6-trichlorophenol and pentachlorophenol were widely used for antimicrobial wood preservation.

Different approaches have been used to study fungal biodegradation of chlorinated phenols: intact cultures of fungi (Mileski et al. 1988; Valli & Gold 1991; Alleman et al. 1992; Reddy et al. 1998), separation of mycelium and culture liquid (Armenante et al. 1994), application of free and immobilized enzymes (Shuttleworth & Bollag 1986; Ruggiero et al. 1989; Roy-Arcand & Archibald 1991; Iimura et al. 1996; Ruttimann-Johnson & Lamar 1996). These studies revealed that lignin-degrading enzyme systems are usually responsible for the chlorophenol transformation. The ease of dechlorination decreases with the number of substituted chlorines in the aromatic ring, with trichlorophenols (TCP) and penta-

chlorophenol (PCP) being more resistant than mono- and dichlorophenols (DCP). The process of dechlorination is a first stage in the degradation of chlorophenols, a few products of degradation have been identified also with polymer formation noted as a common endpoint (Roy-Arcand & Archibald 1991; Ruttimann-Johnson & Lamar 1996; Ullah et al. 1999). The most extensive and diverse research was conducted with the white rot fungus *Phanerochaete chrysosporium*, chosen because it has been the most studied model of ligninolytic fungi. Comprehensive pathways of degradation of 2,4-di-, 2,4,5- and 2,4,6-trichlorophenols have been elucidated for this fungus (Valli & Gold 1991; Joshi & Gold 1993; Reddy et al. 1998). *P. chrysosporium* is a thermophilic fungus which has limited use in temperate climates for bioremediation in situ. Temperate fungal species offer more advantages, provided they have extracellular enzymes capable of chlorophenol transformation.

In the present report we have studied the toxic effects of several chlorophenols as well as transformation of 2,4,6-TCP using the temperate lignin-degrading fungi *Panus tigrinus* and *Coriolus versicolor*. *P. tigrinus* produces Mn-peroxidase (MnP) and laccase as its extracellular ligninolytic enzymes and does not produce lignin peroxidase (LiP) (Leontievsky et al. 1990). Different strains of *C. versicolor* are known as LiP, MnP and laccase producers (Hatakka 1994), however LiP is not essential for realization of some biotechnological processes aimed at degradation of persistent compounds (Archibald 1992; Paice et al. 1993).

Methods

Chemicals

2-, and 3-Chlorophenols (CP), 2,4-, 2,5-, 2,6-, 3,4- and 3,5-dichlorophenols (DCP) were obtained from Merck. 4-CP, 2,3-DCP, 2,3,4-, 2,4,5- and 2,4,6-trichlorophenols (TCP) and pentachlorophenol (PCP) were obtained from Aldrich.

Microorganisms and methods of cultivation

White-rot fungus *P. tigrinusr* 8/18, isolated from decayed wood in Douzhanbe (Tadjikistan), and *C. versicolor* VKM F-116, obtained from All-Russian Collection of Microorganisms (VKM), were routinely stored on malt agar slopes at 40 °C.

The fungi were grown at 29 °C on potato-dextrose agar (PDA) plates under stationary conditions or in liquid media with agitation at 200 rpm.

PDA cultures were inoculated with a fragment of mycelium (4 mm diameter) placed in the center of the plate. Inoculum for liquid media for both fungi were grown for seven days on soya-glycerol medium. The medium contained (g l^{-1}): NH_4NO_3, 0.2; KH_2PO_4, 0.2; K_2HPO_4, 0.02; $MnSO_4 \cdot 7H_2O$, 0.1; peptone, 0.5; soya powder, 0.5; glycerol, 2.0 ml l^{-1}. For inoculation, mycelium was washed twice with sterilized distilled water and homogenized by shaking with porcelain beads. Cultivation was carried out in 750-ml flasks with 100 ml of mineral medium, containing "low" or "high" concentration of nitrogen sources, equal to 2.4 and 24.0 mM of ammonium nitrogen (Kirk et al. 1978). As a carbon source, 1% glucose (*C. versicolor*) or 1% maltose (*P. tigrinus*) were used. Submerged cultures grown for purification of extracellular enzymes contained additionally 2.0 mM 3-methylbenzyl alcohol (*P. tigrinus*) or 0.2 mM tannic acid (*C. versicolor*) as inducers.

2-, 3-, 4-CP, 2,3-, 2,4-, 2,5-, 2,6-, 3,4-, 3,5-DCP, 2,3,4-, 2,4,5-, 2,4,6-TCP and PCP were added to PDA cultures (to a final concentration 5–200 ppm). 2,4,6-TCP was added to liquid media as a dimethylformamide (DMF) solution (100 μl). To control flasks 100 μl of DMF were added. 2,4,6-TCP was added to liquid media stepwise (3, 5, 7-, 5, 10, 10-, or 10, 20, 20 ppm at 0, 24 and 48 hours of cultivation to a final concentration of 15, 25 or 50 ppm) or as a single addition of 15, 25 or 50 ppm of 2,4,6-TCP on the fifth day of cultivation.

The toxicity of chlorophenols in PDA cultures was evaluated by measurements of diameters of colonies in comparison with control plates with no chlorophenols added. Control cultures of *P. tigrinus* colonized the surface of the plates in 84 hours, and control cultures of *C. versicolor* in 60 hours. In liquid cultures the effect of 2,4,6-TCP was estimated by comparison of dry biomass weight and extracellular enzyme activities (Mn-peroxidase and laccase) compared with control variants without of 2,4,6-TCP.

Enzyme assays

Laccase activity was measured by the rate of oxidation of 0.2 mM 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in 20 mM Na-acetate buffer, pH 5.0; the increase of absorbance at 436 nm was

detected. Mn-peroxidase activity was detected by the rate of NADH oxidation (Asada et al. 1986).

Enzyme purification

Mn-peroxidases and laccases of *P. tigrinus* and *C. versicolor* were purified as described earlier (Leontievsky et al. 1990) but with some modifications. Culture liquids of submerged cultures were separated from mycelium by centrifugation and filtration through the paper filters. The filtrate was applied to a 2.6 × 40-cm column with DEAE-Spheron 1000 (La Chema, Czechia) equilibrated with buffer A (20 mM Na-acetate buffer, pH 5.0 for enzymes of *P. tigrinus* or 5.5 for enzymes of *C. versicolor*). The enzymes were eluted by a linear gradient of 0–0.5 M NaCl in buffer A at flow rate 120 ml/hour. Combined active fractions were concentrated by ultrafiltration using a PM-10 membrane (Amicon, The Netherlands). The concentrated preparations (2 ml) were applied to gel-filtration column HiPrep 16/10 Sephacry S100 (Pharmacia Biotech, Sweden) equilibrated with 0.1 M NaCl in buffer A and eluted with the same buffer at flow rate 12 ml/hour. Active fractions, containing MnP and laccase, separated on the gel-filtration step, were combined, dialized against buffer A and applied to a Mono-Q HR 5/5 anion-exchange column (Pharmacia Biotech) equilibrated with buffer A. The enzymes were eluted with linear gradient of 0–0.5 M NaCl in buffer A at flow rate 60 ml/hour. Combined active fractions were dialized against deionized water and used as the purified MnP or laccase preparations.

Purified enzymes were electrophoretically homogeneous. SDS-PAGE was performed in 12% gel. Protein standards (Pharmacia Biotech) were (kDa): phosphorilase b, 94; bovine serum albumin, 67; ovalbumine, 43; caronic anhydrase, 30; soybean trypsin inhibitor, 20.1; α-lactalbumin, 14.4.

Enzyme reactions

Laccase reaction mixture (3 ml) contained 20 μg of enzyme and 1, 4 or 6 mM 2,4,6-TCP in 20 mM Na-acetate buffer, pH 5.0. MnP reaction mixture (3 ml) contained 20 μg of enzyme, 0.5 or 1.0 mM 2,4,6-TCP, 0.4 mM H_2O_2 and 0.1 mM $MnSO_4$ in 20 mM Na-lactate-succinate buffer, pH 4.5. Reaction vials were incubated 18–24 hours at 30 °C with agitation.

Isolation and identification of 2,4,6-TCP oxidation products

For studying the dynamics of 2,4,6-TCP bioconversion and identification of products of its oxidation, culture liquid was separated from mycelium by centrifugation and filtration, acidified to pH 2.0 by 0.1 M sulfuric acid, and extracted by ethylacetate three times. The extracts were combined, dried over anhydrous sodium sulfate and evaporated on a rotary evaporator at 40 °C. The residues were dissolved in a small volume of methanol and analyzed by HPLC, thin-layer chromatography (TLC) and mass-spectrometry (MS).

For determination of the amount of 2,4,6-TCP adsorbed to fungal mycelium, the mycelium was separated from the culture liquid, washed with distilled water three times, suspended in 50 ml of water, homogenized by blender MPW-302 (Mechanika Precyzyjna, Poland) and then acidified and the mycelial extract collected.

Quantitative analysis of 2,4,6-TCP was conducted using HPLC system (LKB, Sweden) with a ODS-2 column (4.6 × 250 mm) at 50 °C. Elution was carried out isocratically at a flow rate of 0.8 ml l^{-1} with 65% of methanol in 5 mM KH_2PO_4, pH 2.0. Detection was at 280 nm using a UV detector.

Qualitative analysis of 2,4,6-TCP and its metabolites was performed by TLC on Silufol UV-254 plates (Kavalier, Czechia) developed with benzene-dioxane-acetic acid (90 : 10 : 2). After processing of the plates, metabolites were detected under UV light, with diazotized benzidine (as a reagent on aromatic hydroxy-groups) and silver nitrate solution in acetone (as a reagent for chlorine groups). Diazotized benzidine spray preparation: 0.5 g of benzidine (Reakhim, Russia) dissolve in 1.4 ml of concentrated HCl, hold 15 minutes at room temperature, add distilled water to 100 ml volume (reagent A); 10% water solution of $NaNO_2$ (reagent B); reagents A and B mixed in a ratio 1 : 1 prior using; once prepared the mixture is active for 3–4 hours at room temperature. Siver nitrate spray preparation: 1 ml of 10% water solution of $AgNO_3$ mixed with 10 ml of 2-phenoxyethanol, 190 ml of acetone and one drop of 30% H_2O_2; the mixture is active for 2–3 weeks. Cl-containing compounds could be seen on the treated TLC plates after 15 minutes holding under UV light. The compounds with corresponding Rf values were eluted from the plates with methanol. These methanol solutions were used for rechromatography on TLC plates to check purity and/or for MS-analysis.

Identification of pure compounds isolated from TLC plates was made with a Finnigan Mat 8430 mass-spectrometer (Germany) operated at on ionization energy of 70 eV with direct evaporation of sample. Sensitivity of the method was not less than 1 μg.

Results

PDA plate cultures

Chlorophenolic compounds are toxic to fungi, reducing hyphal extension depending on their concentration. In static PDA plate cultures, the diameter of hyphal growth of *P. tigrinus* and *C. versicolor* was measured in the presence of increasing concentrations of mono-, di-, tri- and pentachlorophenols up to 200 ppm (Figure 1). The toxic effect of chlorophenols increased with the number of chlorine substituents. In general, both of the tested fungi were affected by the chlorophenols to a similar degree with a few exceptions, where *C. versicolor* was more resistant to 2-CP and PCP and much more sensitive to 3,5-DCP, than *P. tigrinus*. The chlorophenols that produced most inhibition of hyphal growth were 2,3,4-TCP, 2,4,5-TCP, 2,4,6-TCP and PCP at the lowest concentrations of 15–50 ppm. Comparison of the most toxic chlorophenols showed that PCP completely inhibited hyphal growth of *P. tigrinus* and *C. versicolor* at different concentrations, of 25 and 75 ppm respectively. All TCP molecules inhibited hyphal growth completely at 50 ppm, but 2,4,6-TCP was marginally less toxic at 15–25 ppm than 2,3,4- and 2,4,5-substituted compounds.

Liquid media cultures

In liquid cultures the dynamics of biomass growth of both fungi were dependent on the nitrogen content of the growth media: low nitrogen medium restricted any increase of biomass weight after the fifth day of cultivation (Figure 2).

To minimize the toxic effect of 2,4,6-TCP two different schemes for its addition to liquid cultures of fungi were tested: stepwise addition starting from the point of inoculation and single addition at fifth day of growth when cultures had reached sufficient weight of biomass.

Final concentrations of 2,4,6-TCP for the stepwise addition (15 and 25 ppm for *C. versicolor*, and 25 and 50 ppm for *P. tigrinus*), were chosen based on results of experiments with PDA-cultures, where there was relatively stronger resistance to this compound by

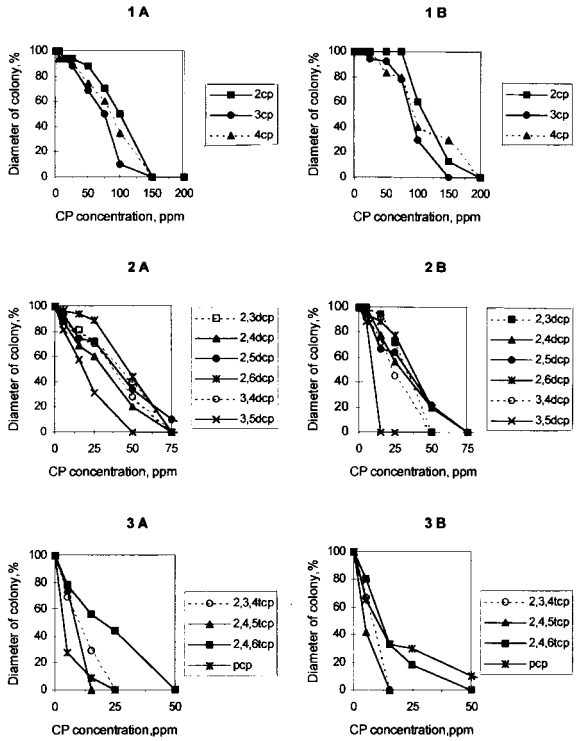

Figure 1. The effect of increasing concentration of chlorophenols on hyphal growth of PDA cultures of *P. tigrinus* (A) and *C. versicolor* (B). 1, mono-, 2, di-, 3, tri- and pentachlorophenols.

P. tigrinus (Figure 1). The experiments with liquid fungal cultures resulted in a similar effect: 25 ppm of 2,4,6-TCP almost completely inhibited growth of *C. versicolor* while the *P. tigrinus* culture had some increase of its biomass. On the other hand, marginally toxic concentrations of 2,4,6-TCP (15 ppm for *C. versicolor* and 25 ppm for *P. tigrinus*), which still permitted some biomass growth, negated the differences between low- and high- nitrogen cultures (Figure 2). In both cases general biomass weight reached after 2 weeks growth was significantly less than that in control cultures with low nitrogen media without addition of 2,4,6-TCP.

When 2,4,6-TCP (50 ppm) was added on the fifth day after inoculation of the liquid cultures, differences between low and high nitrogen cultures disappeared, its effect was less toxic, so cultures of both *P. tigrinus* and *C. versicolor* after two weeks growth, reached the same amount of biomass as low nitrogen control cultures (Figure 3).

During submerged cultivation of *P. tigrinus* and *C. versicolor* in liquid media without addition of 2,4,6-TCP, both fungi produced Mn-peroxidase and laccase

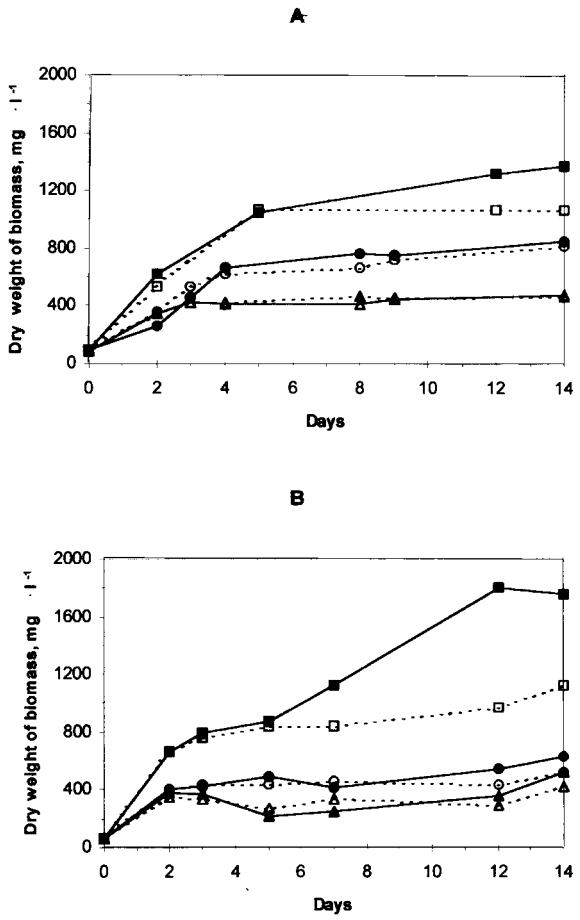

Figure 2. Dynamics of biomass growth in the liquid cultures of *P. tigrinus* (A) and *C. versicolor* (B) with stepwise addition of 2,4,6-TCP. Solid lines with black symbols – high nitrogen cultures, dashed lines with open symbols – low nitrogen cultures. Concentration of 2,4,6-TCP in *P. tigrinus* cultures (A): 0 (■), 25 (●) and 50 (♦) ppm. Concentration of 2,4,6-TCP in *C. versicolor* cultures (B): 0 (■), 15 (●) and 25 (♦) ppm.

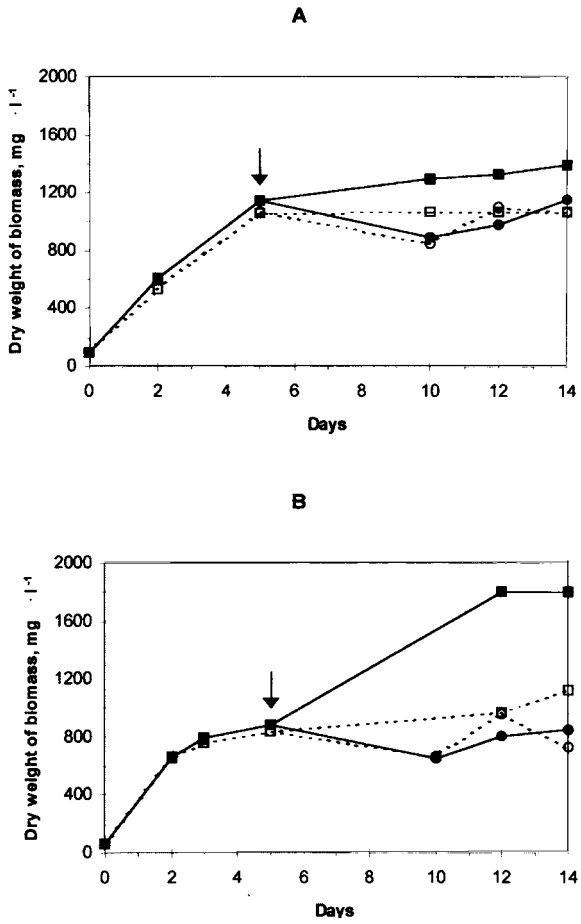

Figure 3. Dynamics of biomass growth in the liquid cultures of *P. tigrinus* (A) and *C. versicolor* (B) with addition of 2,4,6-TCP at fifth day of cultivation. Solid lines with black symbols – high nitrogen cultures, dashed lines with open symbols – low nitrogen cultures; 2,4,6-TCP concentration: 0 (■) and 50 (●) ppm. The arrows mark the moment of 2,4,6-TCP addition.

(Figure 4). No lignin peroxidase activity was found in the culture liquid of these fungi. Low nitrogen concentration in culture liquids of both *P. tigrinus* and *C. versicolor* increased Mn-peroxidase (MnP) production by a factor of two, in comparison with high nitrogen media. Laccase production by *C. versicolor* was not dependent on nitrogen concentration, while *P. tigrinus* showed increased laccase production in high nitrogen medium.

Stepwise addition of 25 ppm 2,4,6-TCP to *P. tigrinus* cultures reduced MnP activity and delayed laccase production up to the sixth day of cultivation compared with an increase in its activity measured in both low and high nitrogen media (Figure 4a). Both of these enzyme activities were hardly detected at 50 ppm of toxicant (data not shown), with a substantial decrease of MnP and laccase activities. Stepwise addition of 15 ppm of 2,4,6-TCP to *C. versicolor* cultures completely inhibited MnP production and only changes in laccase activity could be detected (Figure 4b). No enzyme activity was found in *C. versicolor* cultures with 25 ppm of 2,4,6-TCP (data not shown).

The addition of 2,4,6-TCP on the fifth day of growth of *P. tigrinus* cultures almost completely suppressed extracellular laccase activity but stimulated a reciprocal increase of MnP activity. When 2,4,6-TCP was added under the same conditions to culture of *C. versicolor*, its effect on the enzyme composition in the culture liquid was opposite to that of *P. tigrinus*. The dynamics of laccase activity was not significantly

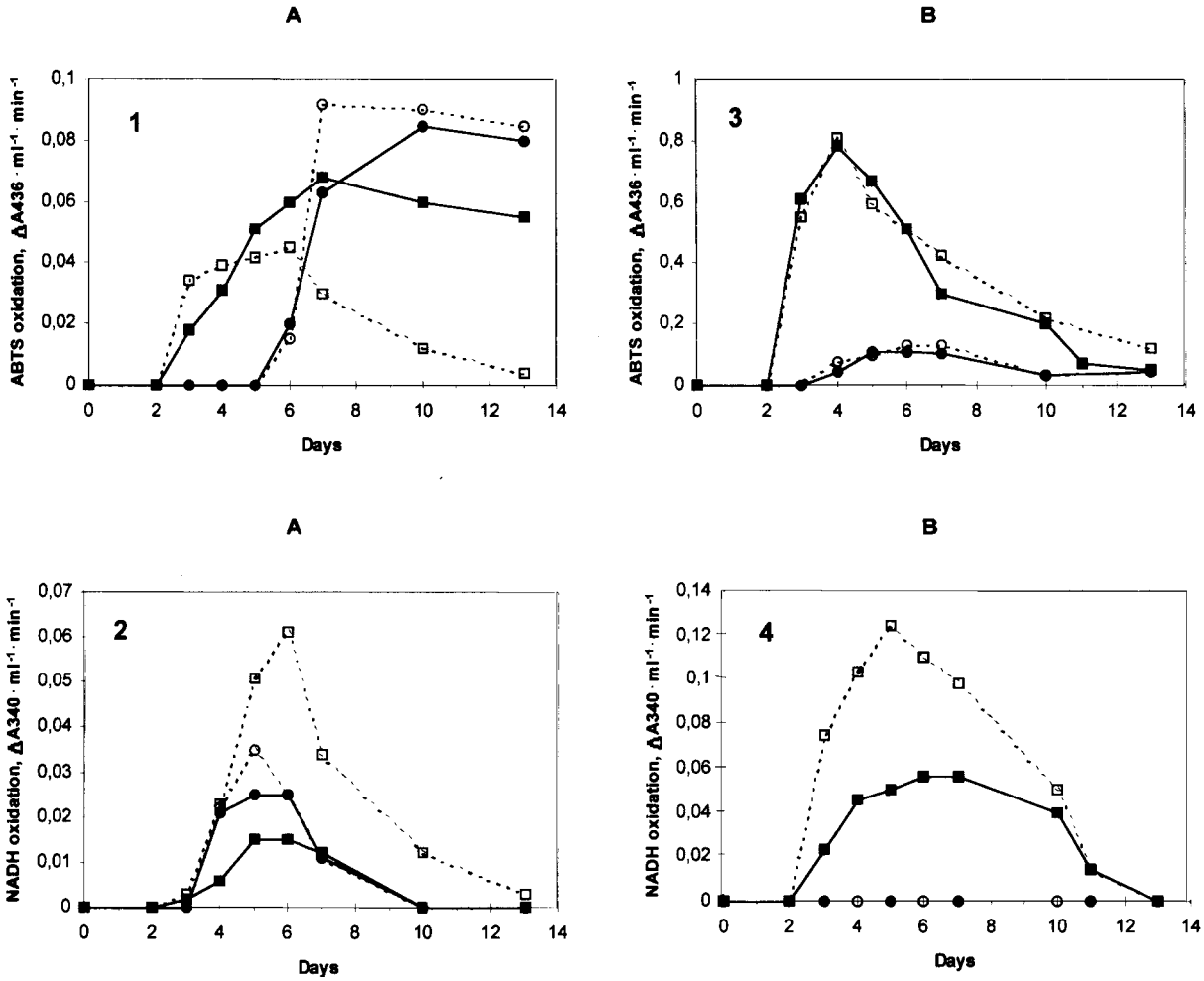

Figure 4. Dynamics of laccase (1, 3) and MnP (2, 4) activities in the liquid cultures of *P. tigrinus* (A) and *C. versicolor* (B) with stepwise addition of 2,4,6-TCP. Designations are as in the Figure 2.

changed, but MnP activity declined drastically (Figure 5).

Dynamics of 2,4,6-TCP transformation

Quantitative analysis of the concentration of 2,4,6-TCP in triplicate liquid cultures of *P. tigrinus* and *C. versicolor* revealed that this compound was adsorbed by the fungal mycelium: $9 \pm 1\%$ of TCP was adsorbed in low nitrogen cultures and $14 \pm 1\%$ – in high nitrogen cultures. These data were not dependent on culture age nor on the total concentration of 2,4,6-TCP in the culture. Further data concerning the chlorophenol concentration was calculated as a sum of "free" TCP in culture liquid and adsorbed TCP.

In the liquid fungal cultures with stepwise addition of 2,4,6-TCP (up to 25 ppm for *P. tigrinus* and 15 ppm for *C. versicolor*) only a residual amount of TCP was found in low nitrogen cultures by HPLC analysis on the sixth day of cultivation. In the high nitrogen cultures, from 10 to 20% of this chlorophenol was still detected. These data were confirmed by recording the absorption spectra of the culture liquid (data not shown). In both cultures with higher concentration of 2,4,6-TCP (50 ppm, *P. tigrinus* and 25 ppm *C. versicolor*) up to 20% of 2,4,6-TCP could be detected after two weeks of cultivation independently from the nitrogen concentration.

When 50 ppm of 2,4,6-TCP was added to cultures on the fifth day of growth, 90% had been removed from the cultures, for both *P. tigrinus* and *C. versicolor*. In low nitrogen cultures the rate of removal

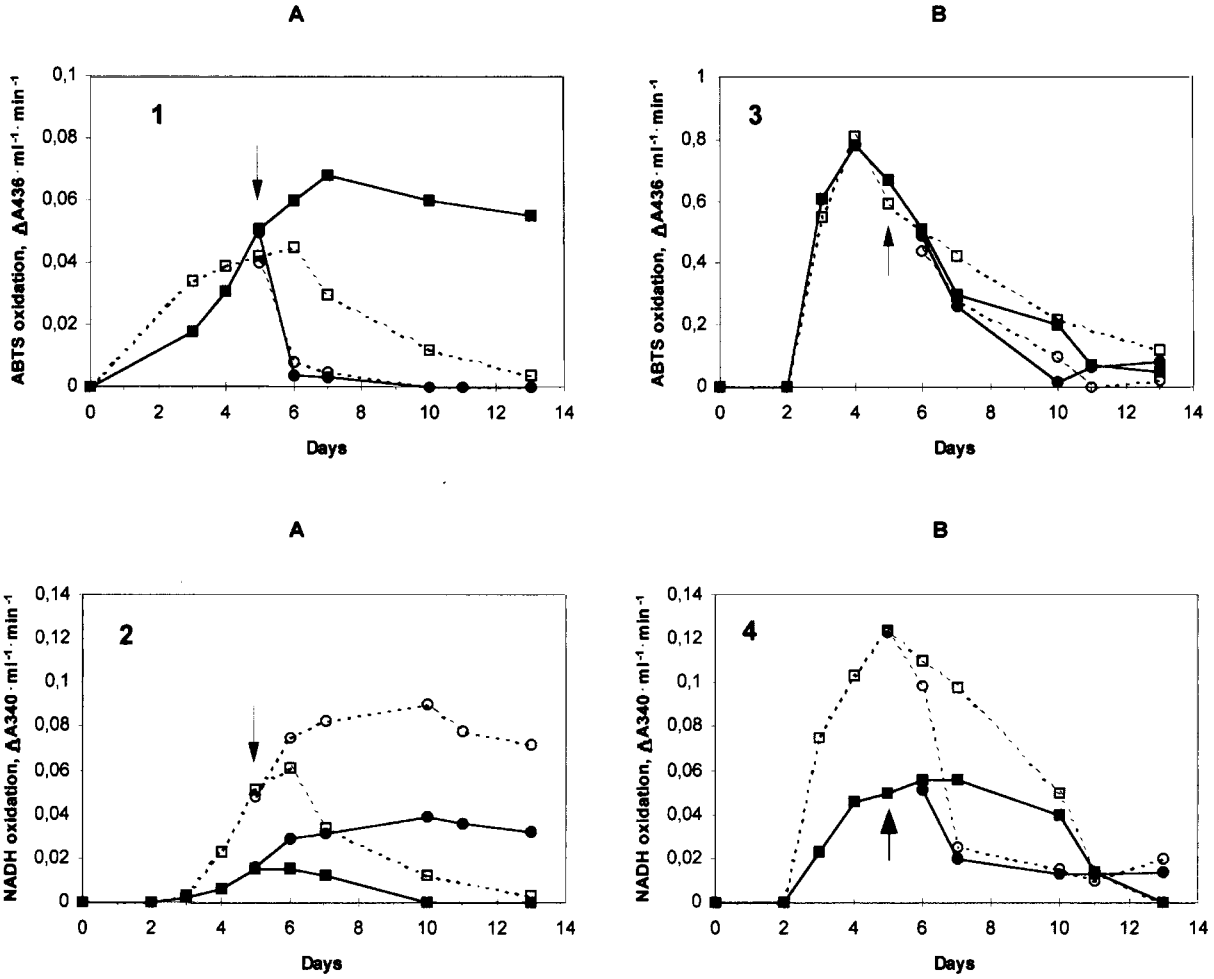

Figure 5. Dynamics of laccase and MnP activity in the liquid cultures of *P. tigrinus* (A) and *C. versicolor* (B) with addition of 2,4,6-TCP at fifth day of cultivation. Designations are as in the Figure 2.

was more rapid than in high nitrogen cultures for the first 12–48 hours (Figure 6).

As the main products of 2,4,6-TCP (I) degradation by both fungal cultures 2,6-dichloro-1,4-benzoquinone (II), 2,6-dichloro-1,4-hydroquinol (III) (Figures 7, 8) and unidentified products with M+ 360 (IV) were found. A trace amount of 2,6-dichloro-4-methoxyphenol (V) and 2,6-dichloro-4-methoxy-1,3-dihydroxybenzene (VII) were also detected (data not shown) (Figure 8). The formation of two chlorine-containing derivatives demonstrated that the fungi effected dechlorination of 2,4,6-TCP in the *para*-position. In the case of stepwise addition of TCP, this set of products was detected after 3 day of cultivation in both low and high nitrogen cultures. When 2,4,6-TCP was added at day 5 to the fungal cultures, the products were found already after 3 (low

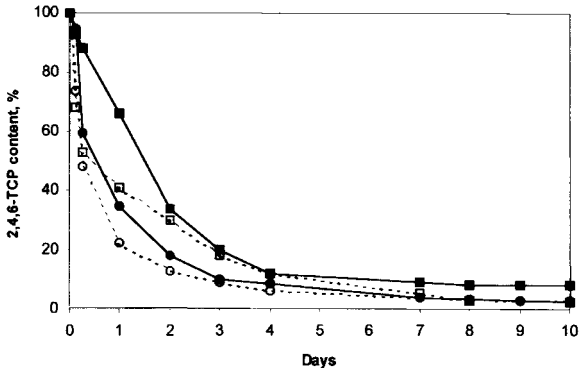

Figure 6. Dynamics of the rate of 2,4,6-TCP transformation. 50 ppm of 2,4,6-TCP was added at fifth day of cultivation to low and high nitrogen cultures of *P. tigrinus* (■) and *C. versicolor* (●). Solid lines with black symbols – high nitrogen cultures, dashed lines with open symbols – low nitrogen cultures.

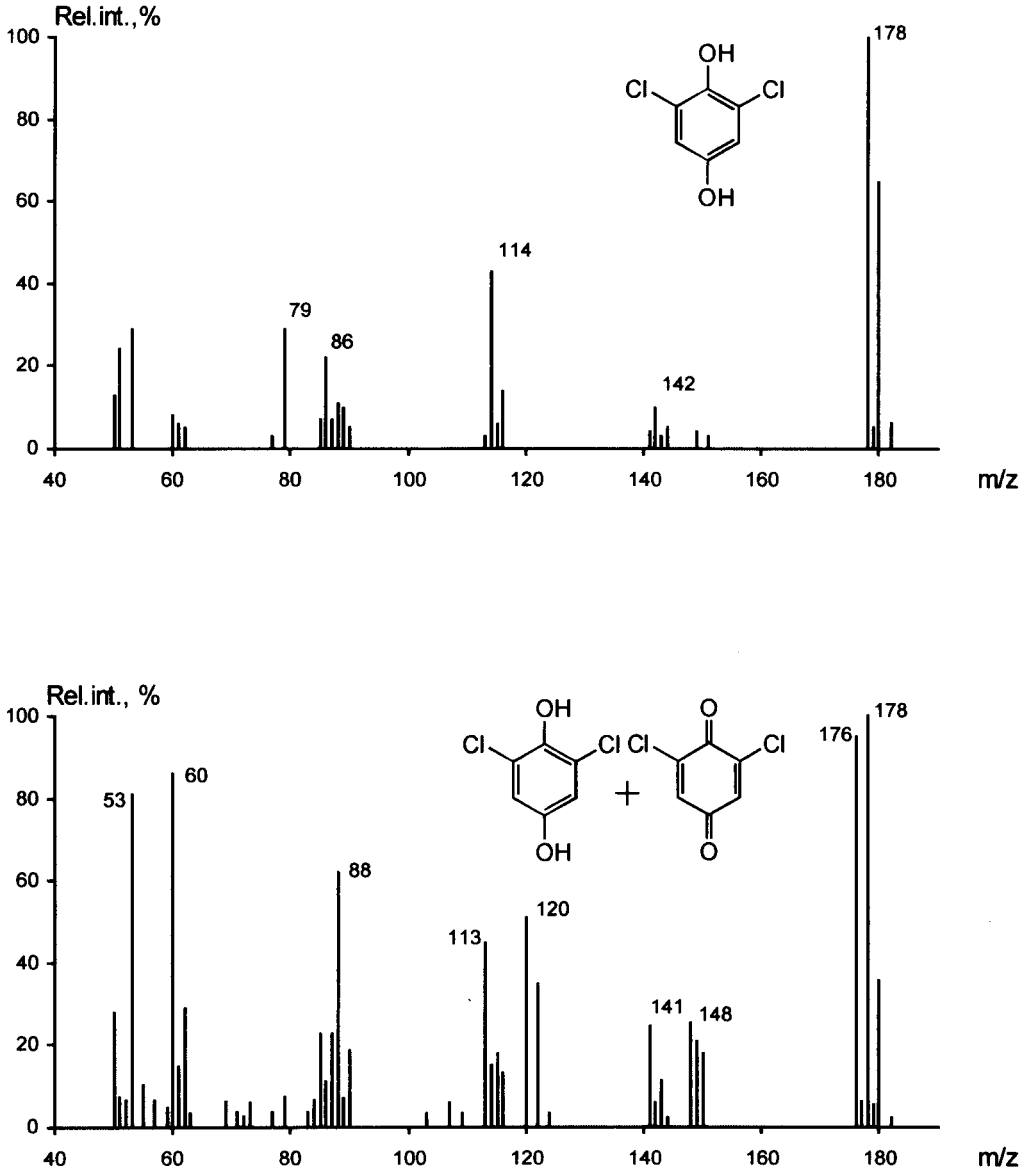

Figure 7. Mass-spectra of the complex of 2,6-dichloro-1,4-hydroquinol with 2,6-dichloro-1,4-benzoquinone and 2,6-dichloro-1,4-hydroquinol.

N) or 6 (high N) hours. Preliminary evaluations of unidentified product (IV) suggested that it might be a condensed oligomeric compound containing less than two chlorine substituents per each aromatic nucleus, as a result of further dechlorination of 2,4,6-TCP.

Purified MnPs and laccases from *P. tigrinus* and *C. versicolor* incubated with 0.5-6.0 mM 2,4,6-TCP for 18–24 hours at 30 °C oxidized 70-90% of TCP. 2,6-Dichloro-1,4-benzoquinone (II) and 2,6-dichloro-1,4-hydroquinol (III), as well as condensed oligomeric compound (IV), were found as the only products of this reaction (Figure 8).

Discussion

The results obtained demonstrate that PDA cultures of *P. tigrinus* and *C. versicolor* were affected similarly by the majority of the tested CPs. The CPs with chlorine substitution at *meta*-positions were shown to be less able to be metabolized by fungal enzymes (and probably more toxic), than CPs substituted in *ortho*- and

para-positions (Dec & Bollag 1990; Kadhim et al. 1999). We also found that 3-CP, 3,5-DCP and 2,3,4-TCP were more toxic for both *P. tigrinus* and *C. versicolor* (as well as 2,3- and 3,4-DCP for *C. versicolor*), than other mono-, di- and tri-chlorophenols. On the other hand, for cultures of *P. tigrinus* no differences in toxicity of 2,3-, 3,4- and 2,4-, 2,5- and 2,6-DCPs were found (Figure 1). The increased resistance to PCP of our strain of *C. versicolor* corresponded to data of Alleman et al. (1992), when another strain of *Trametes (Coriolus) versicolor* in the presence of PCP was the most fast growing and CP removing from six tested fungi.

The results found for PDA cultures with 2,4,6-TCP correlated with the data of liquid cultures of the fungi. Despite the similar range of toxicity of this compound to both fungi in PDA cultures (in contrast, for instance, to PCP), *P. tigrinus* was more resistant to 2,4,6-TCP in the range of concentrations 15-25 ppm. Experiments with liquid cultures with stepwise addition of TCP revealed that *P. tigrinus* was again more tolerant to the toxicant at these concentrations (Figure 2).

The comparison of stepwise addition of 2,4,6-TCP and addition at the fifth day of growth of liquid cultures of *P. tigrinus* and *C. versicolor* showed an obvious advantage of the second scheme. While 25 ppm of 2,4,6-TCP almost completely suppressed the biomass growth and expression of extracellular enzymes in the culture of *C. versicolor* with stepwise addition of the toxicant, in the case of addition of 50 ppm of this compound at the fifth day of growth, only a minimal toxic effect could be observed (Figure 2–4). These data are in accordance with results of other researchers, stressed the importance of addition of toxicants to fungal cultures after formation of mycelial mat (Milleski et al. 1988), as well as the expression of effective concentrations of toxicant as a ratio of the mass of chemicals to the mass of mycelium instead of solution concentrations (Alleman et al. 1992).

The expression of ligninolytic enzyme systems of the white rot fungi is dependent from the concentration of definite nutrients in the growth media (Kirk & Farrell 1987). According to this approach, the limited amount of nitrogen sources in the medium initiated the expression of ligninolytic enzymes, while high nitrogen medium resulted in complete or partial suppression.

Both *P. tigrinus* and *C. versicolor* showed a greater rate of 2,4,6-TCP oxidation in low nitrogen media than in high nitrogen media (Figure 6), suggesting that the lignin-degrading enzyme systems participated in this process. The absence of differences in this respect in the cases of cultures with maximal concentrations of the TCP at stepwise addition (50 ppm for *P. tigrinus* and 25 ppm for *C. versicolor*), seems to be a result of its strong toxic effect.

MnP and laccase were found as components of the ligninolytic enzyme systems of both tested fungi. In the *P. tigrinus* cultures with stepwise addition of 2,4,6-TCP, irrespective of nitrogen concentration in the growth medium, the majority of TCP disappeared by the sixth day of growth. Before this time only MnP activity could be found in the culture liquid (Figure 4a). When 2,4,6-TCP was added to *P. tigrinus* culture grown for 5 days, its rapid disappearance was detected during the first day, coinciding with the sharp suppression of laccase and activation of MnP activities (Figure 5a). This permits us to suggest that in *P. tigrinus* cultures, from the two ligninolytic enzymes being found in the culture liquid, MnP was responsible for primary attack of 2,4,6-TCP. In the cultures of *C. versicolor* with stepwise addition of toxicant, no MnP activity was found at all but only laccase activity (Figure 4b). In the cultures of *C. versicolor* with addition of 2,4,6-TCP on the fifth day of cultivation, resulting in fast removal of TCP, the activity of MnP was reduced, while laccase activity was unaffected by TCP in comparison with blank cultures without the toxicant (Figure 5b). Therefore in contrast to *P. tigrinus* cultures, in the case of *C. versicolor* laccase was responsible for initial transformation of 2,4,6-TCP, not MnP.

2,6-Dichloro-1,4-benzoquinone (II) and 2,6-dichloro-1,4-hydroquinol (III) were the primary products of 2,4,6-TCP transformation in *P. tigrinus* and *C. versicolor* cultures. When 2,4,6-TCP was oxidized by purified MnP from *P. tigrinus* and laccase from *C. versicolor*, only these two compounds (as well as oligomeric product (IV)) were found (Figure 8). The formation of 2,6-dichloro-4-methoxyphenol (V) and 2,6-dichloro-4-methoxy-1,3-dihydroxybenzene (VII) when 2,4,6-TCP was oxidized by intact fungal cultures, is probably a result of a side reaction catalyzed by cell-associated methyltransferases (Joshi & Gold 1993; Reddy et al. 1998). The existence of polymeric products which might contain less than two chlorines on the aromatic nucleus is in agreement with a pathway of degradation of 2,4,6-TCP by *P. chrysosporium*, described earlier (Reddy et al. 1998).

It is of interest, that purified laccase from *P. tigrinus* and MnP from *C. versicolor* catalyzed the same reaction with 2,4,6-TCP, as MnP from *P. tigrinus*

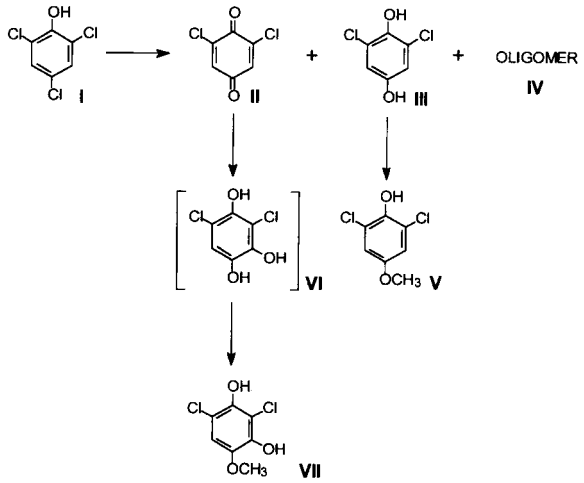

Figure 8. Scheme of primary reaction of 2,4,6-TCP oxidation by ligninolytic enzymes of *P. tigrinus* and *C. versicolor*. I, 2,4,6-TCP, II, 2,6-dichloro-1,4-benzoquinone, III, 2,6-Dichloro-1,4-hydroquinol, IV, oligomeric condensed product, V, 2,6-dichloro-4-methoxyphenol, VI, 3,5-dichloro-1,2,4-trihydroxybenzene, VII, 2,6-dichloro-4-methoxy-1,3-dihydroxybenzene.

and laccase from *C. versicolor* which seem to be preferentially responsible for primary oxidation of this compound in the intact fungal cultures. It may reflect the fact that the different roles of MnP and laccase for 2,4,6-TCP oxidation in the cultures of *P. tigrinus* and *C. versicolor* resulted from a different mode of regulation of expression of these enzymes in the two fungi, rather than their different properties.

Acknowledgments

This work was supported by NATO Science for the Peace Program, Grant No. SfP 972294 and the Russian Foundation of Basical Research, Grants Nos. 99-04-48185 and 98-04-48197.

References

Alleman BC, Logan B & Gibertson RL (1992) Toxicity of pentachlorophenol to six species of white rot fungi as a function of chemical dose. Appl. Environ. Microbiol. 58: 4048–4050

Archibald F (1992) Lignin peroxidase activity is not important in biological bleaching and delignification of unbleached Kraft pulp by *Trametes versicolor*. Appl. Environ. Microbiol. 58: 3101–3109

Armenante PM, Pal N & Lewandowski G (1994) Role of mycelium and extracellular protein in the biodegradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 60: 1711–1718

Asada Y, Miyabe M, Kikkava M. & Kuwachara M (1986) Oxidation of NADH by peroxidase of a lignin-degrading basidiomycete *Phanerochaete chrysosporium* and its involvement in the degradation of lignin model compound. Agric. Biol. Chem. 50: 525–529

Golovleva LA, Zaborina OE, Pertsova RN & Baskunov BP (1994) Degradation of polychlorinated phenols by *Streptomyces rochei* 303. Biodegradation. 2: 201–208

Dec J & Bollag JM (1990) Detoxification of substituted phenols by oxidoreductase enzymes through polymerization reactions. Arch. Environ. Contam. Toxicol. 19: 543–550

Hatakka A (1994) Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol. Rev. 13: 125–135

Iimura Y, Hartikainen P & Tatsumi K (1996) Dechlorination of tetrachloroguaiacol by laccase of white-rot basidiomycete *Coriolus versicolor*. Appl. Microbiol. Biotechnol. 45: 434–439

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

Kirk TK & Farrell RL (1987) Enzymatic "combustion": the microbial degradation of lignin. Ann. Rev. Microbiol. 41: 465-505

Kirk TK, Schultz E, Connors WJ, Lorens LF & Zeikus JG (1978) Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*. Arch. Microbiol. 117: 227–285

Leontievsky AA, Myasoedova NM, Maltseva OV, Termkhitarova NG, Krupyanko VI & Golovleva LA (1990) Mn-dependent peroxidase and oxidase of *Panus tigrinus* 8/18: purification and properties. Biochemistry (Engl. Transl. Biokhimiya) 55: 1375–1380

Mileski GJ, Bumpus JA, Jurek MA. & Aust, SD (1988) Biodegradation of pentachlorophenol by the white rot fungus *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 54: 2885–2880.

Joshi DK & Gold MH (1993) Degradation of 2,4,5-trichlorophenol by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. 59: 1779–1785

Paice MG, Reid ID, Bourbonnais R, Archibald FS & Jurasek L (1993) Manganese peroxidase, produced by *Trametes versicolor* during pulp bleaching, demethylates and delignifies Kraft pulp. Appl. Environ. Microbiol. 59: 260–265

Reddy GVB, Gelpke MDS & Gold MH (1998) Degradation of 2,4,6-trichlorophenol by *Phanerochaete chrysosporium*: involvement of reductive dechlorination. J. Bacteriol. 180: 5159–5164

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

Ruggiero P, Sarkar JM & Bollag J-M (1989) Detoxification of 2,4-dichlorophenol by a laccase immobilized on soil or clay. Soil Science 147: 361–370

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

Shuttleworth KL & Bollag JM (1986) Soluble and immobilized laccase as catalysts for the transformation of substituted phenols. Enzyme Microb. Technol. 8: 171–177

Ullah MA, Bedford CT & Evans CS (2000) Reaction of pentachlorophenol with laccase from *Coriolus versicolor*. Appl. Microbiol. Biotechnol. 53: 230–234

Valli K & Gold M (1991) Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. J. Bacteriol. 173: 345–352

Zaitsev GM, Uotila JS, Tsitko IV, Lobanok AG & Salkinoja-Salonen MS (1995) Utilization of halogenated benzenes, phenols and benzoates by Rhodococcus opacus GM-14. Appl. Environ. Microbiol. 61: 4191–4201