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RESEARCH ARTICLE

Transformation of high concentrations of chlorophenols by the white-rot basidiomycete *Trametes versicolor* immobilized on nylon mesh

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Abbreviations:
2,4-DCP: 2,4-dichlorophenol;
2,4,6-TCP: 2,4,6-trichlorophenol;
DMOP: dimethoxyphenol;
MnP: Manganese-dependent peroxidase;
PCP: pentachlorophenol;

Free-cell cultures of *Trametes versicolor* were compared with cultures immobilized on nylon mesh in a 2-litre bioreactor for transformation of pentachlorophenol (PCP) and 2,4-dichlorophenol (2,4-DCP), added at intervals to the liquid culture medium over a period of 816 hrs. Increasing amounts of PCP from 200 ppm to 2000 ppm added batchwise to cultures permitted acclimatization of the fungus to these toxic pollutants. A total addition of 2000 ppm of 2,4-DCP and 3400 ppm PCP were removed from the immobilized cultures with 85% of 2,4-DCP and 70% of PCP transformed by enzymes (laccase and Mn-peroxidase), 5% 2,4-DCP and 28% PCP adsorbed by the biomass and 10% 2,4-DCP and 2% PCP retained in the medium at the termination of the fermentation after 1020 hrs. In contrast free-cell cultures in the same medium with the same addition regime of PCP and 2,4-DCP, transformed 20% 2,4-DCP and 12% PCP by enzyme action, adsorbed 58% 2,4-DCP and 80% PCP by the biomass, and retained 22% 2,4-DCP and 8% PCP in the medium. The use of nylon mesh as an immobilization matrix for removal of PCP and 2,4-DCP facilitates more efficient removal of chlorophenols and can be adapted to scale-up for application of large volumes of chlorophenol-containing aqueous effluents.

Trametes versicolor is one of the most studied of the white-rot basidiomycetes with regard to transformation of recalcitrant pollutants such as chlorophenols. Chlorophenols, one of the most dangerous classes of

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environmental pollutants, have been produced in thousands of tons annually by the pulp and paper and agrochemical industries (Steiert et al. 1985).

In its natural habitat, *T. versicolor* grows mainly in dead wood, producing lignin-degrading enzymes to breakdown lignin in the wood cell walls in order to access the cellulose, which the fungus degrades to cellobiose and ultimately glucose (Evans et al. 1994). Fungal reactivity with chlorophenols is due to the production of lignin-degrading enzymes, particularly laccase and Mn-peroxidase that are secreted in increased quantities by the fungus during ligninolytic activity.

Chlorophenols are known substrates for the oxidative enzyme laccase, that can react with all the chlorophenols including the most recalcitrant pentachlorophenol (PCP) (Roy-Arcand and Archibald, 1991; Bollag et al. 1992; Milstein et al. 1992; Kadhim et al. 1999; Ullah et al. 2000a). They are also substrates for Mn-peroxidase that requires hydrogen peroxide and Mn(II) for reaction (Milstein et al. 1992).

Previous studies have shown that after adaptation of the white - rot fungus *Panus tigrinus* to high concentrations (up to 2000 mg l^{-1}) of 2,4,6-trichlorophenol (2,4,6-TCP), transformation of a mixture of 2,4-DCP, 2,4,6-TCP and PCP each at 500 mg l^{-1} was achieved in a 72- L bioreactor using free cell cultures of *P. tigrinus* (Leontievsky et al. 2002a). Extending the lifetime and reactivity of the biomass in the bioreactor would enable continuous transformation of chlorophenols in liquid effluents. This can be achieved by immobilization of free cells on to a support matrix, that produces higher cell densities, higher and time-extended enzyme activities, greater enzyme stability and frequently a reduction in treatment cost (Ruggiero et al. 1989).

The aim of the present work was to demonstrate that the catalytic activity of *T. versicolor* could be adapted for transformation of high concentrations of chlorophenols similar to that described for *P. tigrinus* and to develop specifically for chlorophenol removal, a simple immobilization method to extend and improve further the catalytic activity of *T. versicolor* in transforming chlorophenols in liquid effluents. Immobilization of mycelia has been used previously for production of ligninolytic enzymes, but has not been applied to the transformation of chlorophenols (Kirkpatrick and Palmer, 1987; Linko, 1988; Kirkpatrick et al. 1990).

MATERIALS AND METHODS

Cultivation of Microorganisms

Trametes (Coriolus) versicolor (FPRL 28A) was originally obtained from IMI (now CABI; Egham, Surrey, UK). Stock cultures were maintained on 3% malt - 2% agar plates grown at 26ºC for 7 days. A single 3 mm agar plug from the growing margin of a colonized plate was kept in sterile water in an Eppendorff tube at 20ºC in the dark, and sub cultured every 6 months. Medium for liquid cultures (seed cultures) was a glucose-amino acids- salts medium as previously described (Fahraeus and Reinhammar, 1967; Evans, 1985), inoculated with mycelia fragmented by shaking 5 mm plugs from malt-agar plates in universal bottles containing glass beads and sterile distilled water.

Seed cultures were grown in 200 ml medium in 1-L shaken flasks, before transfer to a 2-L stirred tank reactor (STR) with a working volume of 1.5 L. Cultures were maintained at 26ºC, agitated at 150 rpm using two Rushton turbine impellers, with an airflow rate of 2 ml min^{-1}. In immobilized cultures, a nylon mesh was mounted on a metal baffle around the internal perimeter of the stirred tank reactor. Bioreactors were operated in batch mode.

Chlorophenols were added as four batches of 200 ppm, 200 ppm, 1000 ppm, and 2000 ppm PCP in 30% aqueous ethanol at 96, 192, 456 and 816 hrs of cultivation with a single batch addition of 2000 ppm 2,4-DCP at 816 hrs. Triplicate samples were removed by syringe periodically for analysis of chlorophenols and enzyme activities. The experiment was repeated twice. Data presented is of a representative run, with <7% variation in data points.

Biomass dry weight was determined at the end of the run as dry cell weight per litre of culture broth after filtration, washing of the biomass and drying at 105ºC to constant weight.

HPLC analysis of chlorophenols

A sensitive, fast and selective method for detection and quantification of chlorophenols was required. The use of preconcentration techniques such as solid phase extraction (SPE) aids in sample clarification and thus increases the sensitivity of detection by HPLC. Chlorophenols (PCP and 2,4-DCP) were extracted from the culture broth using SPE through C18 cartridges assembled as a manifold of 24 cartridges. The cartridges were then connected to the outlet of the extraction system. Vacuum through the cartridge was set to 5" Hg by applying air using a prostaltic pump. The SPE cartridges were then activated by addition of 1 ml of methanol. When all solution had passed through the cartridge, it was washed with 1 ml of water and dried for 2 min. The 2 ml of culture filtrate were acidified with 1 ml sulphuric acid to pH 2 and applied through each cartridge, washed with water and eluted with methanol. The methanol extract was then analysed by HPLC.

An HPLC system (Merck-Hitachi L series) equipped with a Dionex Acclaim ® using a 120 Å 5 µm C 18 reversed phase column (4.6 x 250 mm) was employed using isocratic elution with acetonitrile: water: acetic acid (75:25:0.125) at a flow rate of 1 ml min^{-1}. Detection was at 254 nm. Linear calibration of authentic PCP and 2,4-DCP were used in the range of 20 - 120 ppm. Standard samples of PCP and 2,4-DCP of known concentration were injected regularly to

determine chlorophenol concentrations in spiked culture liquid samples.

To evaluate the chlorophenol content adsorbed on the mycelia, the biomass was filtered from culture broth harvested after 1020 hrs (42.5 d), washed with distilled water, and immediately frozen at -72ºC before freeze-drying. The resulting dried extract was ground to small particle-size before addition to methanol in a 100 ml flask that was shaken at 120 rpm in an orbital shaker for 24 hrs. The filtrate was separated by centrifugation (15 min at 5000 g) and the extract used for solid phase extraction prior to analysis by HPLC. Repeat extractions were made until no further chlorophenols were extracted from the mycelia. Extractions were performed on triplicate samples.

Enzyme assays

Laccase activity in samples of culture broth was measured by the rate of oxidation of 0.5 mM 2,2' -azinobis- (3-ethylbenzothiazolline-6-sulphonic acid) (ABTS) in 20 mM sodium acetate buffer (pH 5.0), and the increase in absorbance at 420 nm was detected (Eggert et al. 1996). Mn-Peroxidase (MnP) activity was detected by the rate of oxidation of 1.5 mM 2,6-dimethoxyphenol (2,6-DMOP) in 25 mM Na-tartrate buffer at pH 5.0 with 10 mM $MnSO_4$. The reaction mixture contained in 1 ml: 50 µl $MnSO_4$, 800 µl DMOP and 50 -100 µl culture supernatant. The reaction was initiated with 50 µl H_2O_2 (1 mM) and the increase in absorbance at 470 nm recorded (Martinez et al. 1996). The molar extinction coefficient for coerolignone was 6,500 M^{-1} cm^{-1}. The amount of enzyme that converted 1 µmole of substrate or generated 1 µmole of product over 1 min was taken as one unit of activity (U).

Lignin peroxidase (LiP) activity was measured by the oxidation of veratryl alcohol to veratraldehyde in the presence of H_2O_2 at pH 2.7, according to the method of Tien and Kirk, 1984. Enzyme activity was expressed as ΔA min^{-1} ml^{-1}.

Cell lysis was observed by UV microscopy noting autofluorescence of the hyphae.

RESULTS

Transformation of chlorophenols by free-cell cultures

To evaluate the ability of *T. versicolor* to transform large quantities of PCP and 2,4-DCP, a free-cell culture was grown in a 2 L bioreactor (working volume 1.5 L) to which varying concentrations of PCP and 2,4-DCP were added during the 1020 hrs time period of the fermentation. Figure 1 shows the sequence of additions from 200 ppm PCP (twice over 192 hrs [8 d]) to 1000 ppm PCP (at 456 hrs [19 d]), and 2000 ppm PCP and 2000 ppm 2,4-DCP (both added at 816 hrs [34 d]). Chlorophenols were removed from the culture medium at different rates as shown in Table 1, with increasing rates of removal recorded when higher concentrations of chlorophenols were added to the culture. For the first two additions, there was a similar removal rate of PCP, approximately 1.0 ppm h^{-1}. This indicated that the culture had not become fully acclimatised to these moderate concentrations of PCP. When 1000 ppm of PCP was added (after 456 hrs) the removal rate of PCP from the culture increased to 2.8 ppm h^{-1}, and increased further to 7.9 ppm h^{-1} when the final addition of 2000 ppm of PCP was added after 816 hrs. At this time the removal rate of 2,4-DCP was 6.0 ppm h^{-1} when 2000 ppm of 2,4-DCP was added after 816 hrs, showing that the culture had acclimatized to these high concentrations of chlorophenols.

Transformation of chlorophenols by immobilized cell-cultures

When cells of *T. versicolor* were immobilized on nylon mesh around the internal perimeter of the bioreactor vessel, and the same additions of PCP and 2,4-DCP were made to the cultures as were made to the free cell cultures, a similar pattern of removal of chlorophenols was observed but at a faster rate than in the free-cell cultures (Table 1). The rate increased from 2.0 ppm h^{-1} following the first addition of 200 ppm PCP, to 2.6 ppm h^{-1} after the second addition of 200 ppm PCP, to 8.2 ppm h^{-1} after addition of 1000 ppm PCP and finally to 10.3 ppm h^{-1} after the final addition of 2000 ppm PCP. The rate of removal of 2,4-DCP added at 816 hrs was 7.8 ppm h^{-1}.

The addition of chlorophenols to the cultures induced the production of laccase and MnP, but no LiP (Figure 2). In the free-cell cultures, the first addition of 200 ppm PCP was accompanied by a 10-fold increase in laccase activity and a 6-fold increase in MnP, activities declining gradually until the next addition of PCP when they increased slightly, before tailing to basal levels. When 1000 ppm of PCP was added to the culture, both laccase and MnP activities increased from 0.3 to 0.5 units and from 0.001 to 0.18 units respectively. The final addition of both chlorophenols stimulated a rise in both enzyme activities from 0.1 to 0.5 units for laccase and 0 to 0.3 units for MnP before both activities disappeared completely. Lysis of the fungal hyphae occurred at this stage, as observed by UV microscopy of autofluorescence (Figure 3) indicating cell death. Autofluorescence indicates the release of intracellular components including nucleic acids, by the lysed cells. Release of intracellular enzymes due to cell lysis would account for some of the observed increase in enzyme activity in the culture liquid at the end of the fermentation.

In the immobilized cell-cultures, enzyme activities (Figure 2) showed similar responses to chlorophenol additions as in the free cell cultures though higher levels of laccase were maintained throughout the culture time after the initial rise to 8 units when the first addition of 200 ppm PCP was made. MnP activity was similar in the free and immobilized

cell-cultures. Laccase activity has been reported previously as the main enzyme activity in *T. versicolor* responsible for transformation of chlorophenols (Roy-Arcand and Archibald, 1991; Kadhim et al. 1999; Ullah et al. 2000a).

When 1000 ppm PCP was added directly to cultures of *T. versicolor* without the fungus becoming first acclimatized to increasing concentrations, complete lysis of the hyphae was observed and the fungal culture died.

The increase in the PCP transformation rate from 2.6 to 8.2 ppm h^{-1} in the immobilized culture coincided with almost constant enzyme activities, indicating that the culture had acclimatized to the PCP concentrations added. However after the final addition of 2000 ppm PCP and 2000 ppm 2,4-DCP, with the transformation rate at its highest, the enzymatic activity then declined rapidly. The cell wall breakage observed at this stage (Figure 3) may have been due to the production of phenoxy free radicals from 2,4-DCP degradation by laccase, as suggested by Alberti and Klibanov, 1981 and Shuttleworth and Bollag, 1986.

Adsorption of chlorophenols to fungal biomass

Fungal biomass was extracted and analyzed at the end of the fermentations for adsorbed PCP and 2,4-DCP. Figure 4 shows the fate of the chlorophenols in both the free cell and immobilized cell-cultures, including that adsorbed to the biomass, that retained in the culture medium and that enzymatically degraded. More PCP and 2,4-DCP were adsorbed to the fungal biomass in the free-cell culture than in the immobilized cell-culture, while more PCP and 2,4-DCP were transformed by laccase and MnP in the immobilized cell-culture compared with the free cell culture. Laccase was the predominant enzyme for reaction with the chlorophenols.

Immobilization of the fungal biomass on nylon mesh increased the level of production of laccase activity, up to 29% greater than in the free-cell cultures under similar conditions. A 23% increase in biomass also occurred in the immobilized system (3.2 g dry wt L^{-1}), compared with the free-cell system (2.6 g dry wt L^{-1}) by the end of the fermentation. Comparisons at intermediate times during fermentation were not possible without destroying the immobilized fungus.

DISCUSSION

Previous reports of transformation of chlorophenols by white rot fungi have addressed a concentration of up to 50 ppm (Reddy et al. 1998; Kadhim et al. 1999; Leontievsky et al. 2000), though there are isolated reports of transformations of 200 - 500 ppm of some chlorophenols (Mileski et al. 1988; Ullah et al. 2000b). The most recent study of Leontievsky et al. 2002a has demonstrated that the white rot fungus *P. tigrinus* could be acclimatized to higher concentrations of 2,4,6-trichlorophenol by addition of increasing concentrations into the culture medium over a period of time. The data presented here shows that this process of acclimatization can be applied to other white rot fungi, namely *T. versicolor,* in order to remove higher concentrations of chlorophenols (2000 ppm PCP and 2000 ppm 2,4-DCP added simultaneously) from the culture. The choice of microorganism for bioremediation of chlorophenol-containing effluents is clearly now in favour of white rot fungi rather than with bacteria that are much more sensitive to the toxicity of these pollutants (Bollag, 1992; Leontievsky et al. 2002a).

Immobilization and acclimatization of biomass of *T. versicolor* enabled more effective transformation of PCP and 2,4-DCP compared with free-cell cultures, due to increased biomass with higher enzyme activity (laccase). For concentrations of PCP up to 1000ppm the immobilized system removed the PCP more rapidly than in the free cell culture. Less of the chlorophenols (5% 2,4-DCP, 28% PCP) were adsorbed on to the mycelia of the biomass in the immobilized system, causing less environmental stress to the mycelia in contrast with the free-cell culture (80% 2,4-DCP, 56% PCP). In previous studies on chlorophenol transformation by wood-rotting fungi, substantial quantities of chlorophenols have been found adsorbed to the fungal biomass (Roy-Arcand and Archibald, 1991; Leontievsky et al. 2002a). Immobilization of biomass has reduced this effect, increasing the rate of transformation of the chlorophenols.

Maintaining a higher laccase activity for a longer period of time in the immobilized culture allowed for a faster rate of transformation of the chlorophenols, and hence more effective removal from the medium. In cultures of *T. versicolor*, laccase is reported to be the dominant ligninolytic enzyme involved in chlorophenol degradation (Roy-Arcand and Archibald, 1991; Kadhim et al. 1999; Ullah et al. 2000b; Leontievsky et al. 2002b).

The simplicity of the immobilization matrix as a nylon mesh fixed to a metal frame around the perimeter of the bioreactor provides a potential method for scale-up of bioreactor design. It will permit ready removal of spent biomass and replacement with new, young mycelia already immobilized on replacement mesh, without serious interruption of effluent treatment. This design could be applied for treatment of chlorophenol-containing effluents from the pulp and paper, and pharmaceutical industries.

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APPENDIX

FIGURES

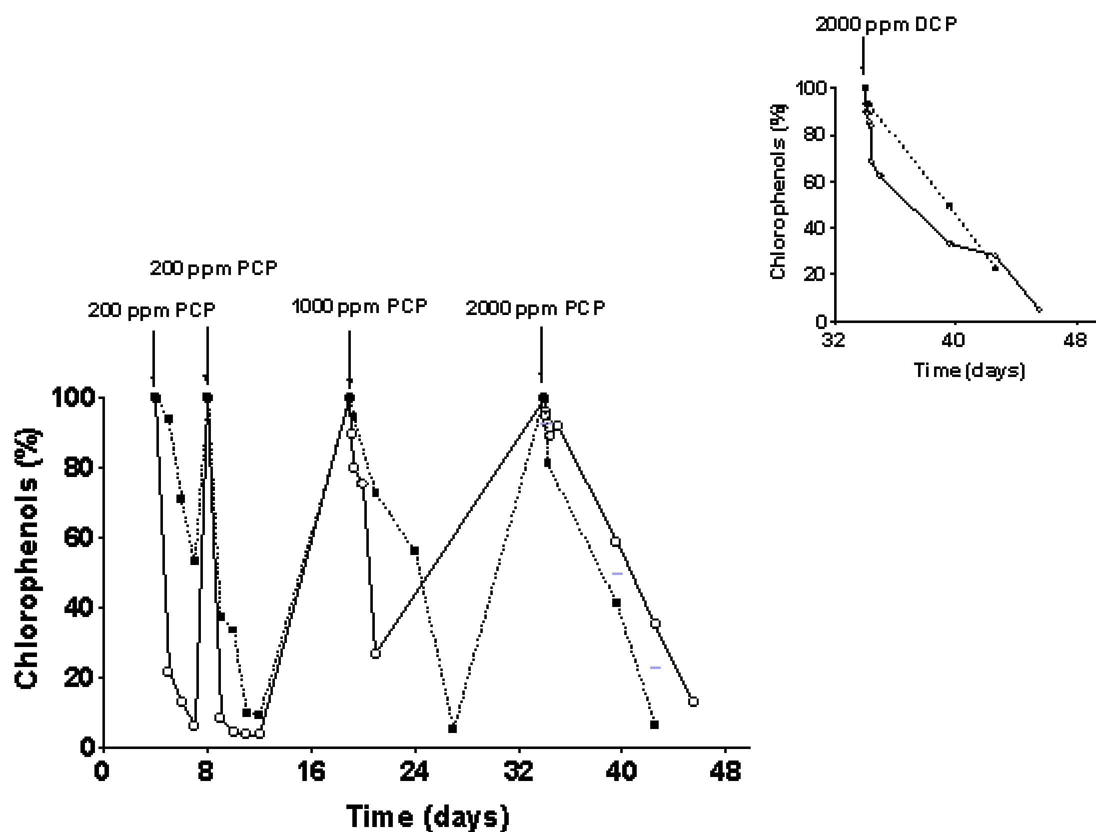
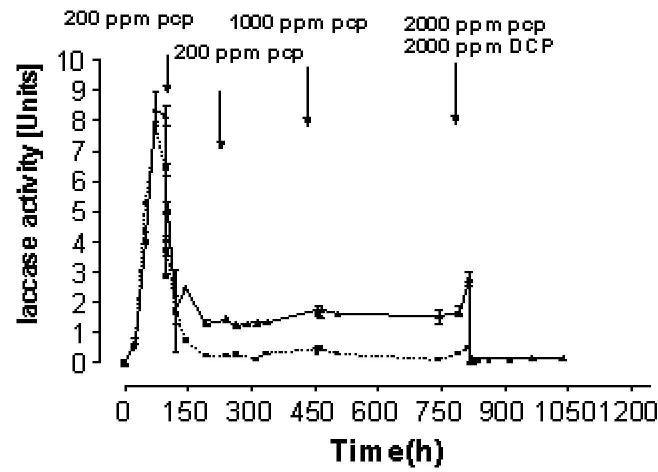


Figure 1. Removal of PCP and 2,4-DCP by free-cell (dashed line) and immobilized (solid line) cultures of *T. versicolor.*

a.



b.

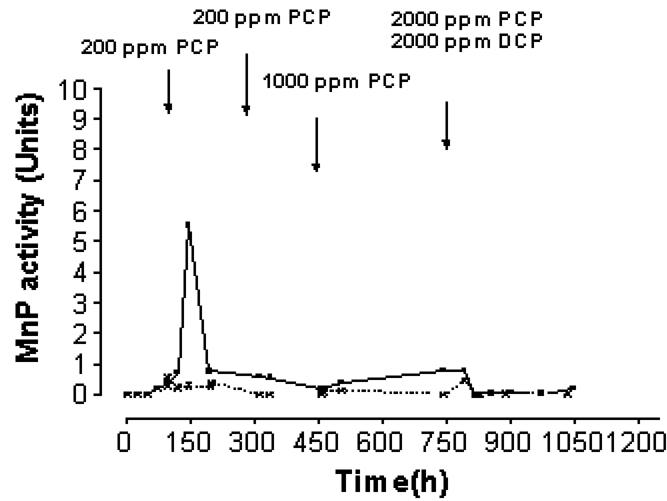


Figure 2. Enzyme activities in free-cell (dashed line) and immobilized (solid line) cultures of *T.versicolor.*

a. Laccase

b. Mn-peroxidase

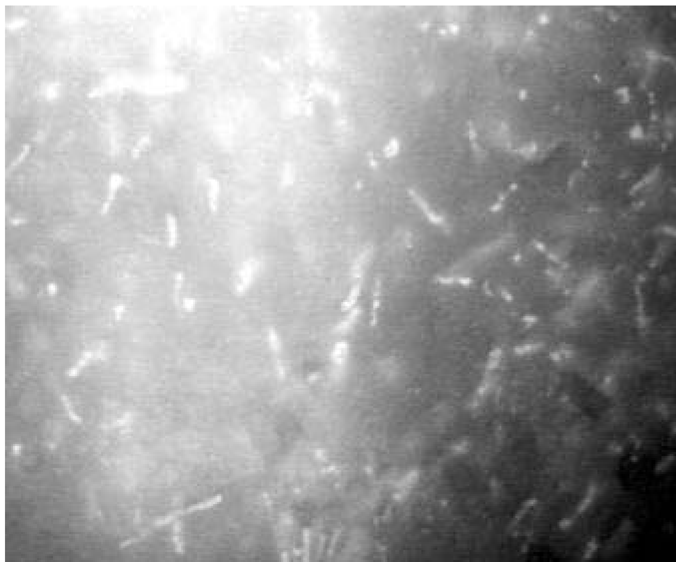


Figure 3. Lysed hyphae of *T. versicolor* after the final addition of chlorophenols, visible by autoflurescence under UV light. Magnification ×400.

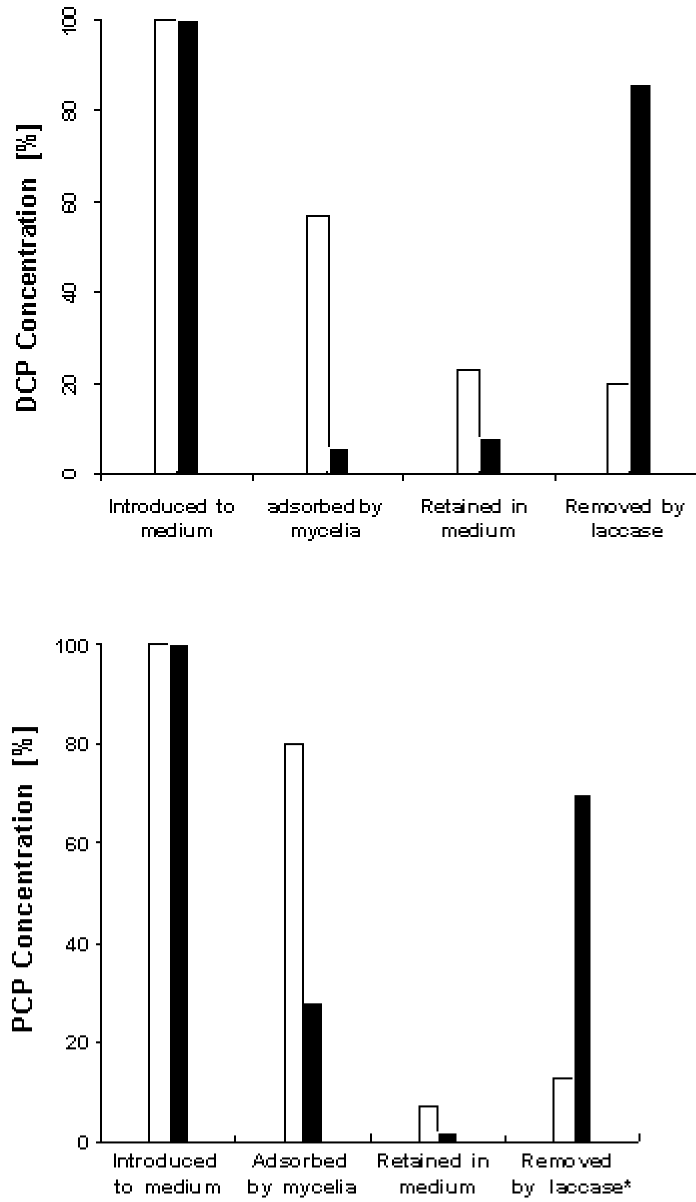


Figure 4. Fate of chlorophenols (2,4-DCP, PCP) in free-cell (open bars) and immobilized (solid bars) cultures of *T. versicolor.*

TABLE

Table 1. Rate of removal of chlorophenols in free-cell and immobilized cell-cultures of *T. versicolor.* Data show means and standard deviations of triplicate assays from duplicate experiments.

Cultures	**Rate of removal of PCP and DCP (ppm h^{-1})**				
	200 ppm PCP added at 96 h	200 ppm PCP added at 192 h	1000 ppm PCP added at 456 h	2000 ppm PCP added at 816 h	2000 ppm DCP added at 816 h
Free cells	1.3 ± 0.005	0.8 ± 0.03	2.8 ± 0.03	7.9 ± 0.3	6.0 ± 0.2
Immobilized	2.0 ± 0.03	2.6 ± 0.04	8.2 ± 0.14	10.3 ± 0.1	7.8 ± 0.25

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