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Abstract

The transformation of pentachlorophenol (PCP) by *Phanerochaete chrysosporium* I-1512 in relation to pentachloroanisole (PCA) was studied in two different modes of culture. The degradation of PCP by immobilized cells in agitated medium was compared with that by static cultures. Results clearly established the advantage of an immobilized culture for mineralisation of PCP: 23% of CO_2 released for fungus immobilized on stainless steel mesh rings as compared with 11 % for static culture. PCA, a metabolite of PCP, was formed only in static cultures and underwent a limited mineralization. Moreover, experiments performed with mycelium and culture supernatant (in the presence of cycloheximide) clearly demonstrated that PCA formation was catalyzed by the biomass in static cultures of *P. chrvsosporium*. Assays in vitro did not establish any involvement of lignin or manganese peroxidascs in PCP or PCA transformation.

Keywords: filamentous fungi, *Phanerochaete chrysosporium*; pentachlorophenol; pentachloroanisole.

Résumé : La dégradation du pentachlorophenol (PCP) par *Phanerochaete chrysosporium* 1-1512 et la formation de pentachloroanisol (PCA) ont été étudiés selon deux modes de cultures différents. La degradation du PCP avec des cellules immobilisées dans un milieu agité est comparée à des conditions statiques. Les résultats mcttcnt en evidence l'avantage des cellules immobilisées pour la minéralisation du PCP : 23 % de CO_2 piégé avec le champignon immobilisé sur des anneaux de grille metallique et 11 % seulemcnt avec les cultures statiques. Le PCA, un métabolite du PCP, est produit uniquement dans les cultures statiques et ne subit qu'une faible minéralisation. De plus des essais réalisés avec du mycelium ou du surnageant de culture (en présence de cycloheximide) montrcnt claircmcnt que la biomasse est à l'origine de la formation de PCA dans les cultures statiques. Des essais réalisés in vitro n'ont pas permis d'impliquer directemcnt les lignine et manganèse pcroxydases dans la transformation du PCP ou du PCA.

Mots clés : champignons filamenteux; *Phanerochaete chrysosporium*; pentachlorophenol; pentachloroanisol.

Introduction

The wood rotting fungus *Phanerochaete chrvsosporium* is the subject of intensive investigations (Higson 1991). The main interest arises from the ability of *P. chrysosporium* to degrad lignin, a wood polymer, and an extremely diverse range of aromatic compounds. This has led to research on using this fungus to degrade organic pollutants in contaminated wastewaters and soils. Numerous xenobiotic compounds can be transformed or mineralized by fungal ligninolytic cultures including atrazine (Mougin et al. 1994), polycyclic aromatic hydrocarbons (Bumpus 1989; Sutherland el al. 1991), DDT (Bumpus and Aust 1987a), TNT (Stahl and Aust 1993), and several chlorophenols (Valli and Gold 1991). Enzymes of the ligninolytic system, lignin peroxidase and manganese-dependent peroxidase, are produced by the fungus in response to carbon, nitrogen, or sulfur limitation (Jeffries et al. 1981), and addition of inositol or phospholipids (Moukha et al. 1991). The lignin-degrading system is suspected to be a major factor responsible for the multistep biodegradative pathways of organic chemicals (Bumpus and Aust 1987b; Hammel and Tardone 1988). Nevertheless. the involvement of lignin peroxidase or manganese peroxidase in the degradation of small aromatic compounds is still unclear. In this study we focused on pcntachorophenol (PCP), a major industrial chemical used as a wood preservative. Milesky et al. (1988) reported PCP degradation by *P. chrysosporium* BKM-F-1767. They used purified ligninase to oxidize PCP to 2,3,5,6-tetrachloro-2.5-cyclohexadiene-l,4-dione (TCHD). Lin et al. (1990) modeled the degradation kinetics of PCP by *P. chrysosporium*. PCP was mineralized with culture supernatant and biomass. If we take into consideration the problem caused by the toxic and recalcitrant chemicals in the environment, it is necessary to find convenient methods to cultivate the fungus to enhance the degradation of xenobiotics. Toward this end, Alleman et al. (1995) have developed a rotating tube reactor to degrade PCP by fungal biofilms, and some coimmobilized systems have been reported by Lin et al. (1991). A few studies have been done in soil, and the transformation of pentachlorophenol to pentachloroanisole (PCA), a methylated metabolite, was observed (Lamar al. 1990a, 1990b)

In our study we investigated an immobilization method in which the fungus was fixed on stainless steel mesh rings, allowing a decrease of shear forces, a greater contact area with the culture medium, and therefore, a more efficient mass transfer (Laugero et al. 1996) We have comparcd the ability of the fungus to degrade PCP and PCA in this immobilized system with the static cultures usually used and then we focused on pentachloroanisole production and localization of the enzymatic system.

Materials and methods

Fungal strain

Phanerochaete chrysosporium I-1512 (CNCM, lnstitut Pasteur, Paris, France) was used in this study. It was grown at 37°C on MYA2 (20 g malt L^{-1}, 16 g agar L^{-1}, 1 g yeast extract L^{-1}).

Chemicals

High purity standards of unlabeled pentachlorophenol and pentachloroanisole were obtained from Sigma (Saint Quentin Fallavier, France). [ring-1-^{14}C(U)]PCP (98 MBq/mmol) was purchased from Sigma. The radiochemical purity was 99%. [ring-^{14}C(U)]PCA was obtained by methylation of I MBq [^{14}C]PCP (1 equiv.) with methyl iodide (10 equiv.) in 20 mL dry acetone, followed by the slow addition of potassium carhonate (5 equiv.). The solution was stirred overnight at room temperature and then filtered, and the resulting solution was evaporated to dryness. The reaction was complete, and the structure or the synthesized compound was confirmed by gas chromatography-mass spectroscopy (GC-MS). The radiochemical purity of PCA assayed by high pressure liquid chromatography (HPLC) was 99.4%.

Mass spectral analysis

Electron impact mass spectral analysis was performed on a Varian Saturn II ion trap instrument with an ionizing voltage or 70 eV. Samples were introduced by gas chromatograph y on a Varian 3400 chromatograph. The column was a J et W Scientific DB-5 (Varian. 30 m x 0.25 mm i.d.; 0.25-µm film thickness). The temperature of the septum-equipped programmable injector was programmed from 60 to 250°C al 190°/ min. The temperature of the column was programmed from 100 to 240°C (10°/min) and maintained at 240°C for 20 min. Helium carrier gas pressure was 12 psi (1 psi = 6.895 kPa).
Mass spectra of authentic and synthesized anisoles were compared. Both exhihited dissociation patterns with intense clusters near m/z 2l5.265 and 280 (M+) containing chlorine atoms, as well as minor ions or clusters at *m/z* 95, 130, 142, and 167.

Culture conditions

Phanerochaete chrysosporium was grown in a synthetic medium containing per litre: 2 g KH_2PO_4, 14 g $CaCl_2$ $2H_2O$.), 0.7 g $MgSO_4$ 7 H_2O, 0.07 g $FeSO_4$ $7H_2O$, 0.046 g $ZnSO_4$ $7H_2O$, 0.0355 g $MnSO_4$ H_2O, 0.007 g $CuSO_4$ 5 H_2O, 10 g glycerol, 1.842 g diammonium tartrate, 1 g yeast extract, 1 ml of a vitamin solution (Tatum et al. 1950), and 0.5 g soybean phospholipids (NAT 89) supplied by Natterman Phospholipid GmhH (Koln, Germany) (Capdcvila et al. 1990). The culture medium was buffered with a solution of disodium tartrate (pH 6.5, 2.3g). The different compounds were sterilized 20 min at 120°C, except the vitamin solution, which was filtered (0.2-µm pore size).

Cultures with agitated medium and immobilized cells

Four-day-old cultures, grown from three agar discs (5 mm) of mycelium on the synthetic medium (without NAT 89 and additives) and incubated at 37°C without agitation, were used to inoculate agitated immobilized cultures. Five mats were harvested and grounded with an Ultra Turax (10000 rpm, green position, 1 min) in 250 ml distilled water. The mycelial homogenate (2.5 mL or 0.01 g dry weight) was used to inoculate 500-mL Erlenmeyer flasks. Fungus was immobilized on four stainless steel mesh rings (20 mm i.d., 30 mm height arranged vertically in the Erlenmeyer flasks with 100 mL culture medium. Agitated flasks were incubated on a rotary shaker operating at 120°C, 37°C. Cultures were grown under an oxygen atmosphere and flushed with pure oxygen at each sampling. After 3 days of growth, the culture medium was supplemented with 0.1 g NAT 89 L^{-1} and 0.42 g veratryl alcohol L^{-1}. At day 6, 10, 15, 20 and 25, the medium was supplemented with glycerol and yeast extract.[^{14}C]PCP or [^{14}C]PCA in acetone (100 µL or less) was added to cultures at the beginning of the incubation (1 mg L^{-1}; 40 kBq) and then after 3 days growth (0.5 mg L^{-1}; 20 kBq). Unlabeled PCP was also added on days 3, 4 and 5. The final concentration of PCP in the culture was 2.4 md L^{-1}.

For each sample, 2-mL aliquots of culture media were analyzed. After 3, 10, 15 and 20 days of growth, mycelia were harvested by scraping off the rings.

Static cultures

Cultures were incubated at 37°C with 10 mL culture medium. The 150-mL Erlenmeyer flasks were inoculated with three agar discs of mycelium (5 mm). [14C]PCP or [I4C]PCA (1 mg L^{-1}; 3.9 kBq) were added at the beginning of the culture. At each sampling point, culture supernatant and mycelium were separated by filtration through Whatman GF/A glass-fiber filters (Prolabo, Paris, France) layered on a 1.2-µm cellulose membrane (Millipore, Saint Quentin Yvelines, France). The filtration system was subsequently rinsed with 10 mL Milli-Q water and the eluate was pooled with the supernatant.

Mineralization of PCP and PCA

Erlenmeyer flasks (six for each experiments) were incubated in 1-L sealed jars in the presence of vials containing 10 mL of 1 N NaOH and 10 mL of water, respectively. The headspaces of the flasks were flushed with pure oxygen. The amount of $^{14}CO_2$ trapped in NaOH was determined daily by liquid scintillation counting.

PCP and PCA determination

Analysis of the radioactivity in the culture media was assayed by HPLC, following the determination of

the total radioactivity by liquid scintillation counting. For static cultures, 15 mL of filtered medium and 2 mL of supernatant of agitated culture were concentrated on a C18 guard column MCH- I0 (3 cm x 4 rnm i.d.)(Varian, Les Ulis, France) at a flow rate of 1 mL min^{-1} with an isocratic pump Varian 9001. The elution of labeled compounds onto the analytical column ODS-80TM (25 cm x 4.6 rnm i.d.) (Varian) was achieved with a Varian 9010 pump delivering a solvent system composed of acetonitrile and water, acidified with 0.05% H_3PO_4. The solvent gradient was 1% acetonitrile for 3 min, followed by a linear increase to 100% acetonitrile over 15 min and a stationary phase of 10 min. The radioactivity of the column eluate was monitored with a HPLC LB 507 A radioactivity monitor (EG et G, Evry, France). UV absorbance was also monitored with a Varian 9050 variable wavelength detector. To analyze the radioactivity associated with the biomass, mycelia were ground in 5 mL methanol. The methanolic extracts were analyzed by HPLC as described above.

Transformation by purified lignin peroxidase (LiP) and manganese peroxidase (MnP)

LiP activity was determined by the rate of oxidation of veratryl alcohol to veratraldehyde at 30°C by the method of Tien and Kirk (1984). MnP activity was also determined spectrophotometrically by the method of Paszczynski et al. (1986) with vanillylacetone as a substrate at 30°C. Similar conditions were used for assaying PCP and PCA trans- formation by purified enzymes. The pesticide was dissolved in 20 µL ethanol, the reaction was initiated by adding H_2O_2, and the mixture was analysed after 10, 30, 60, and 90 min at 30°C. The final solvent concentration did not affect the activities. Controls were supplemented with the same volume of solvent.

Role of mycelium and extracellular fluid in PCA formation

Experiments were carried out with 2-, 3-, or 5-day-old static cultures treated with unlabeled PCP (1 mg L^{-1} added at the beginning of culture).

Assays with culture supernatant

Mycelium was removed and labeled PCP (3.3 kBq) was added to the culture supernatant. Flasks were incubated 0.5 or 1 day at 37°C and the culture medium was analyzed.

Assays with washed mycelium

Mycelium and extracellular fluid were separated. The mycelium was washed with sterile water and resuspended in supernatant which was previously boiled for 10 min. Cycloheximide (1.46 mM) and phenylmethylsulfonyl fluoride (PMSF; 0.5 mM) were added to suppress de novo synthesis of proteins and to inhibit proteases, respectively (Armenante et al. 1994). Labeled PCP was added (3.3 kBq). Mycelium and extracellular fluid were analyzed separately after l or 2 days of incubation at 37°C.

Experimental error

Each experiment was done at least three times. Results are expressed as means. The standard deviation was less than 10% of the mean.

Results

PCP degradation in relation to PCA formation

In agitated cultures, PCP was rapidly removed from the medium (Fig. 1A). Radioactivity in the mycelium was mainly attributed to PCP. We never observed PCA either in cell biomass (harvested at day 3, 10, 15, or 30) or in the supernatant. No PCA or other metabolites could be detected in the cultures treated with PCP. In static cultures, all the PCP was removed from the medium within the first 4 days of incubation. This corresponded to an increase of the radioactivity in the mycelium. Nine percent of the initial radioactivity incorporated in the mycelium was attributed to pentachloroanisole (Fig. 1B). The radioactivity associated with mycelium from static cultures corresponded to PCA, which appeared in cell biomass at day 2 and then decreased at day 6 before a complete disappearance at day 15. Minor amounts of PCA were also detected in the supernatant (3.6% at day 3).

In static cultures, almost 27 % of the total radioactivity in medium remained unidentified at the end of the experiments. This might be attributed to very polar compounds unretained on C18 cartridges, and unextractable by organic solvent.

Mineralization of PCP and PCA by *P. chrysosporium*

Amounts of $^{14}CO_2$ evolved from PCP- or PCA-amended cultures under static and agitated conditions are reported in Fig. 2. Mineralization levels were quite different according to the chemicals and the culture conditions tested. PCP mineralization started after 1 day of incubation under both conditions, 13 and 23% of the total radioactivity were converted to CO_2 in static and agitated cultures after a 30-day incubation

Mineralization of PCA also occurred but at lower rates. Mineralization reached 1.2 and 3.2% of the total radioactivity by day 15 in static and agitated cultures, respectively, and went no higher by day 30. No $^{14}CO_2$ was trapped from uninoculated sterile controls.

Total radioactivity recovered in cultures of *P. chrysosporium* incubated with PCP or PCA

Total radioactivity recovered in cultures incubated in the presence of PCP or PCA are shown in Fig. 3. This comprised the radioactivity found in extracellular fluid, in mycelium, and released as $^{14}CO_2$.The recovery was calculated on days 3, 10, 15, and 30 for the agitated cultures and every day for static cultures. With regard to PCP degradation, the radioactivity recovered represents between 91.7 and

101.54% of the initial radioactivity for agitated cultures. In static cultures, it decreased to 48.7% at the end of the incubation. In the uninoculated sterile controls, PCP content remained constant in agitated cultures and decreased to approximately 70% in static cultures.

In cultures treated with PCA, the total radioactivity recovered was relatively low. In agitated cultures, only 47% of total radioactivity was recovered after 2 days of incubation (Fig. 3). Total radioactivity recovered also fell below 20% in static cultures. In uninoculated sterile controls, PCA content decreased during incubation. The loss of 30 and 70% of the initial PCA in agitated and static cultures could be attributed to volatilization, because radioactivity could be detected in traps for organic volatiles (not shown).

In vitro transformation of PCP and PCA

Transformation of PCP and PCA was assayed with purified LiPs and MnPs: 5580 and 11 400 U L^{-1}, respectively. In vitro, the experiments did not establish any direct involvement of lignin or manganese peroxidases in PCP or PCA transformation. No metabolites were observed and no disappearance of PCP or PCA could be measured by using these enzymes.

Role of mycelium and extracellular fluid in PCA formation

PCP conversion to PCA was followed in culture medium and mycelium from 2-, 3-, and 5-day-old cultures treated with labeled PCP. When separated from the mycelium and supplied with PMSF to inhibit proteases, culture supernatant did not transform PCP to PCA. In contrast, mycelium treated with cycloheximide was able to convert PCP to PCA (Fig. 4). In all cases, PCP concentration decreased in the medium (mainly during a 1-day period) and increased in the mycelium. The rate of PCP decrease in the medium was apparently dependent on the amount of biomass in the cultures.

PCA increased first in the medium (Fig. 4) and then in the mycelium. After 1 day of incubation, the amount of PCA in both medium and mycelium was comparable in all cases. PCA seemed to accumulate linearly in the medium reaching 50% of the initial radioactivity for 5-day-old cultures. By contrast, it was constant or lower in the mycelium, whatever the age of culture. PCA incorporated in mycelium did not exceed 37 % of the initial radioactivity. This maximum was seen after 1 day of incubation and remained unchanged 1 day later. PCA formation appeared also to depend on the amount of biomass.

Discussion

Extensive degradation of PCP by *P. chrysosporium* has been reported previously (Milesky et al. 1988; Lamar et al. 1990a, 1990b). Usually, biotransformation of PCP was performed by inoculating soil or static liquid cultures. In our studies, PCP underwent transformation in two different liquid culture

conditions. This allowed us to compare and to improve the efficiency of an immobilized biomass in an agitated medium versus a static culture (i.e., mycelium developed as a shallow mat form). Milesky et al. (1988) previously reported the toxicity of PCP to spore germination as an inhibitor of oxidative phosphorylation. Inoculation with mycelium overcame this problem and the growth was not affected by the presence of PCP at the beginning of incubation.

The results obtained in our experiments showed some differences in the biodegradation pathway between the two culture conditions. In PCP degradation, agitated cultures enabled a higher percentage of radioactivity to be recovered than with static cultures. The total radioactivity recovered during PCP degradation in agitated cultures was twice as high as in static cultures where an important loss of initial radioactivity was noted. In addition, with static cultures supplied with PCP, an accumulation of PCA (10%) was observed during the 2 first days. This indicates that in each experiment involving PCA, an important amount of radioactivity was lost. In this case, the amount of volatile compounds was significant. Lamar et al. (1990a) reported that volatilization of PCA in liquid cultures is significant. Our studies confirmed that most radioactivity disappearance from the cultures was probably due to volatilization of nontransformed PCA. Nevertheless, volatilization of PCA was relatively minor in agitated cultures.

We have established that PCA transformation in agitated or static cultures did not produce a large quantity of CO_2 (ranging from 2 to 4% of total radioactivity added). Mineralization of PCA was limited, although volatilization dearly confounded our results. In contrast, PCP transformation released a significant amount of $^{14}CO_2$: 11 and 23% for static and agitated cultures, respectively. For static biotransformation of PCP where PCA is involved as a metabolite in the degradation pathway, mineralization was lower than in agitated cultures. We never obtained PCA as metabolite of PCP transformation in agitated cultures. This can explain the important level of mineralisation of PCP in this condition.

PCP transformation to PCA has been reported in many experiments in soil (Lamar et al. 1990b; Lamar and Dietrich 1990). PCP was first transformed to PCA and then the PCA was degraded or mineralized. Authors reporting the appearance of TCHD, another metabolite in PCP degradation with concentrated extracellular fluid, never observed PCA in the same conditions and vice versa (Milesky et al. 1988; Lin et al. 1990).

In the present study, one hypothesis that can be proposed is that two different PCP degradation pathways are involved, depending on culture conditions. Another hypothesis is that the degradation pathway involving PCA occurred in both culture conditions, but in agitated cultures metabolism did not allow any storage of intermediate metabolite. It is also possible that metabolism of PCA requires aerobiosis, and thus, PCA accumulates under static (partial anoxic) conditions. The latter possibilities appear less likely.

In agitated cultures, PCA did not undergo mineralization to an important extent. There was no storage of intermediate metabolites detectable with HPLC analysis. We could only observe an important amount of very polar compounds, which probably represented conjugated products not observed with the other mode of culture (data not shown). No loss of radioactivity has been observed in the recovery. In these conditions, therefore, the degradation pathway does not seem to involve PCA as an intermediate metabolite.

Concerning the role of mycelium and extracellular fluid in PCA formation, we have clearly established that biomass is necessary and sufficient for this step. We used cycloheximide to stop de novo synthesis of protein. PCA was found free or associated to mycelial biomass. Methylation of chlorophenols is known to increase their lipophilicity, and thus, their tendency to bioaccumulate. In our experiments we could suppose that an intracellular enzyme caused PCP methylation and PCA was excreted or lost into the extracellular fluid. However, PCA appeared in the medium before in the mycelium (Figs. 1 and 4). So, an alternative is that PCA formation occurred on mycelium surface and was realized by a parietal enzyme. Then, a PCA fraction might be bioaccumulated in mycelium before being degraded. Lin et al. (1990) have described a metabolic model for PCP degradation by *P. chrysosporium*. PCP can be mineralized into CO_2 through two mechanisms involving extracellular enzymes and biomass or only cell mass, respectively. Our results agree with this model. Cell mass is responsible for PCA formation, whereas mineralization is probably caused by mycelium and (or) extracellular fluid. Nevertheless, purified lignin and manganese peroxidases did not allow a direct transformation of PCP or PCA. Depending on the culture conditions, the two metabolic pathways did not appear concurrently. In static culture PCA formation occurred in preference to the mineralization pathway and the reverse was true in agitated conditions. This suggests possible advantages of agitated cultures versus stationary phase cultures. Agitated conditions creating a biofilm of mycelium developed a greater contact area between medium and biomass compared with static conditions. Moreover, Leisola et al. (1983) have reported that mycelium mat formation in static cultures limited oxygen transfer into the extracellular fluid. Therefore, stationary phase cultures did not allow an efficient mass transfer. This could be a possible reason why mineralization of PCP was improved in agitated cultures.

The different metabolic pathways depending on culture conditions and the superiority of the immobilized system with agitated medium for PCP mineralization seem to be important factors for the use of *P. chrysosporium* cultures in bioremediation.

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Fig. 1. Profiles of PCP (A) and PCA (B) content in static (growth medium, ● ; mycelium, ■) and agitated (growth medium, □; mycelium, O) cultures treated with PCP.

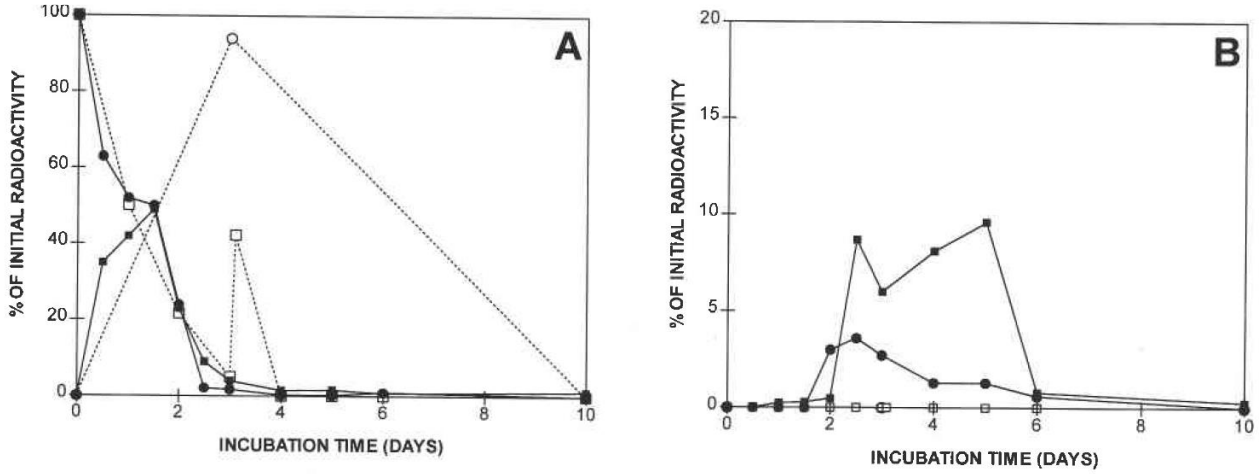

Fig. 2. Comparison of [ring-^{14}C(U)]PCP and [ring-^{14}C(U)]PCA mineralization in agitated (PCP, □; PCA, ▲) and static cultures (PCP, O ; PCA, ●)

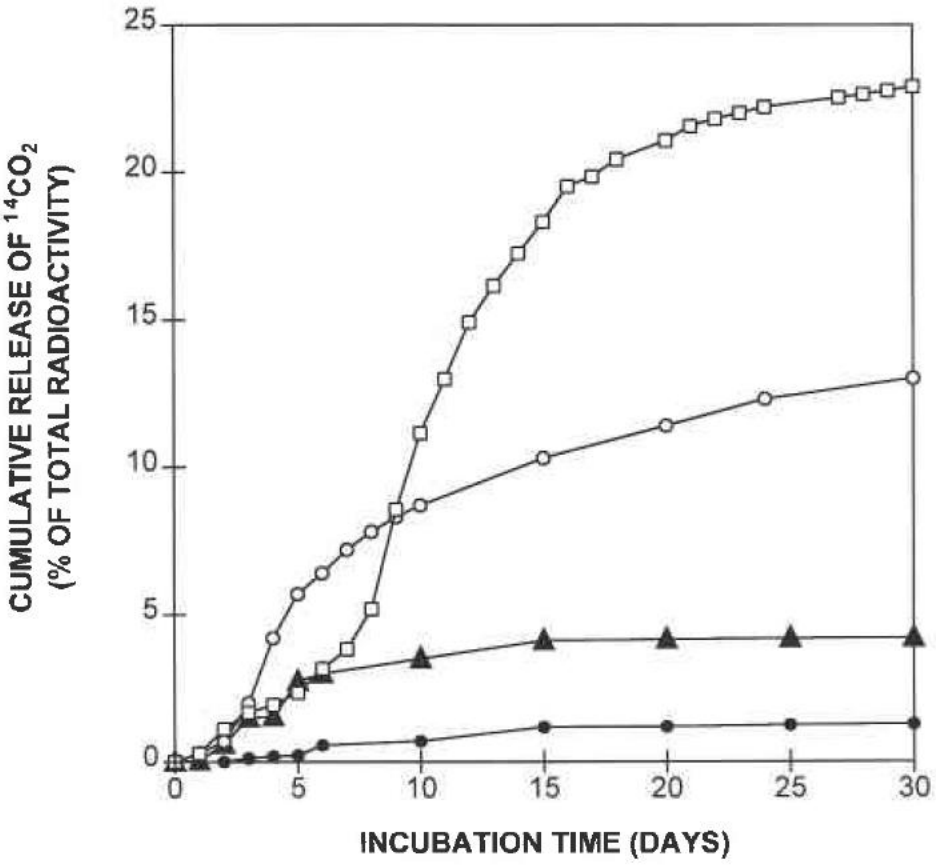

Fig. 3. Total radioactivity recovered in agitated (□)and static (O) cultures treated with [ring-^{14}C(U)]PCP and in agitated (▲) and static (●) cultures treated with [ring-^{14}C(U)]PCA.

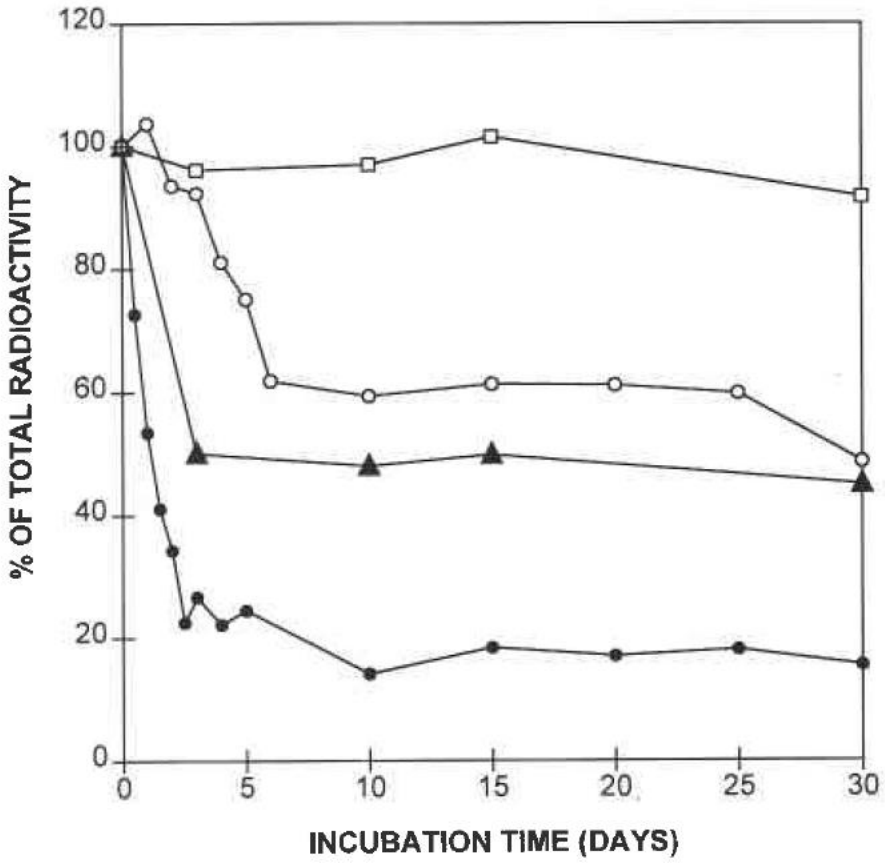

Fig. 4. Distribution of radioactivity from [ring-^{14}C(U)]PCP in medium (O) and mycelium (■). and [ring-^{14}C(U)]PCA in medium (∇) and mycelium ●) in experiments with 2-. 3-, and 5-day-old mycelium treated with cycloheximide and incubated 2 days more with boiled medium.