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► **To cite this version:**

Rachel Rama, Christian Mougin, C. Malosse, J.C. Sigoillot, Véronique Chaplain, et al.. Biotransformation of PAHs by the white rot fungus *Trametes versicolor*. Bioremediation of contaminated soils, Marcel Dekker, 2000. hal-02841596

HAL Id: hal-02841596

<https://hal.inrae.fr/hal-02841596>

Submitted on 7 Mar 2023

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BIOTRANSFORMATION OF PAHS BY THE WHITE ROT FUNGUS
TRAMETES VERSICOLOR

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Key words

polycyclic aromatic hydrocarbons, filamentous fungi, screening, degradation,
volatilization, industrial polluted-soil, microcosm.

ABSTRACT

Five strains of *T. versicolor* were cultured on wet sand in the presence of phenanthrene and benzo[a]pyrene. Biodegradation studies and metabolite determination were performed. Microcosms containing industrial soil polluted by PAHs and supplemented with organic carriers overgrown with the fungi were then evaluated for fungal growth, laccase production and pollutant degradation.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants which result from fossil fuel combustion and industrial by-products. These carcinogen and mutagen chemicals are dispersed in the environment. Around industrial sites, such as coal gasification and liquefaction companies, high levels of PAHs are detected in the soil. Polluted sites are a health risk to human populations because of their long persistence. Widespread occurrence and the implications for public health are the motivating factors for soil remediation.

Due to the high cost of classical remediation processes which require for example soil excavation, incineration and secure landfilling, bioremediation is a promising and cost-effective technological development for cleaning up soil contaminated by PAHs. Increasing attention has been paid to filamentous fungi, which act as efficient tools in the bioaugmentation process.

AIMS AND OBJECTIVES

In a previous paper, we developed wet sand as a triphasic screening system mimicking natural soil, which also allowed for rapid and straightforward analysis [1]. In these culture conditions, the white rot fungus *Trametes versicolor* was shown to be one of the best degraders of phenanthrene and benzo[a]pyrene in mixture. We also described a method for improving the propagation of filamentous fungi in soil [2].

We now report for the first time experiments conducted with several strains of *T. versicolor* cultured on wet sand. Our aim is to reveal more efficient degraders from the species. Biodegradation studies and metabolite determination were carried out. Fungal growth and PAH transformation were then studied in microcosms, which contained industrial soil polluted by PAHs and supplemented with organic carriers overgrown with fungi.

EXPERIMENTAL

Microorganisms

Trametes versicolor Mic 209, ATCC 12679, ATCC 32745, ATCC 34758 and ATCC 42530 were taken from the culture collection of UBCF (INRA Marseille-Luminy).

Screening on wet sand

The culture support was river sand with 3 to 6 mm diameter grains. The medium used in our experiments with *T. versicolor* has been described previously [3,4]. Biomass was produced in Roux flasks containing 200-ml culture medium developed from 5 agar discs of mycelium and cultured for 7 days at 30°C in darkness. For degradation assays, two mats were harvested, mixed with 100 ml sterile water and blended. A 1 ml sample of this suspension was introduced into 150-ml erlenmeyer flasks supplemented with 10 ml of medium and 35 g of sand. The erlenmeyer flasks were sealed with cotton stoppers and incubated in 1-liter sealed flasks in the presence of vials containing 1N sodium hydroxide and water. Cultures were maintained without shaking at 25°C in the dark. The flasks were flushed with ambient air at the beginning of the incubations, and then aerated twice a week.

Each erlenmeyer was supplied with 10 mg kg⁻¹ (dry sand) phenanthrene and 10 mg kg⁻¹ (dry sand) benzo[a]pyrene consisting of a mixture of labeled [9-¹⁴C]Phenanthrene (2200 MBq mmol⁻¹), [7-¹⁴C]benzo[a]pyrene (980 MBq mmol⁻¹) and unlabeled chemicals.

Industrial soil characterization and culture conditions for microcosm studies

PAH-polluted industrial soil for microcosm studies was collected from a coal gasification plant in Rouen (France). It was mixed and sieved at 15 mm. The soil was

a silt loam (57% sand, 27% silt, 16% clay) comprising 55.6% CaCO₃ with 3.64% organic carbon, 0.16% total nitrogen and 0.18 g kg⁻¹ P₂O₅, at a pH of 7.2. Industrial soil contained 2560 mg kg⁻¹ total PAHs (based on 16 hydrocarbons typically analyzed) and 218 mg kg⁻¹ cyanides.

Microcosm consisted of 2-liter reactors refilled with 1.5 kg dry industrial soil and 150 g pellets overgrown with Mic 209 [2]. Reactor A had a 4-cm high layer of overgrown pellets in between two 15-cm layers of soil. In reactor B, the pellets were dispersed within the soil. In both cases, soil and pellets were stacked on a 4-cm layer of gravel (1 cm diameter), including an inlet of compressed air. Soil moisture was checked every 7 days and water was added to achieve 80% moisture holding capacity (MHC). The solution also contained nutrients (C/N/P) at a ratio of 100/5/1 at the beginning of the experiment. The reactors were continuously air-flushed, and incubated in the dark at room temperature. The air outlet from the reactor was plugged with stoppers containing XAD-2 resin to trap volatile compounds.

Analytical procedures for PAH compounds

We measured ¹⁴CO₂ by liquid scintillation counting of the NaOH solution.

Cultures were first extracted at the beginning of the assays, and then after 28 days of incubation.

Once the wet sand was screened, parent compounds and related metabolites were extracted with dichloromethane. Organic solvent (20 ml) was added to each erlenmeyer sealed with a rubber stopper, and the extraction was carried out twice for 1 hour at room temperature on a shaking table in the dark. The cultures were filtered on a stainless steel sieve (0.5 mm) to retain sand and mycelial fragments. Aqueous and organic phases were then transferred into a separatory funnel and separated. Organic phases were pooled, concentrated and dissolved in acetonitrile (500 µl). Aliquots of 100µl were filtered on a Whatman 0.2µm nylon membrane and injected in HPLC. Aqueous phases were pooled prior to HPLC analysis. Aliquots (10 ml) were filtered on a Whatman GF/D prefilter and concentrated on a C₁₈ guard column as described previously [5].

The PAHs in microcosm studies were Soxhlet-extracted in the presence of 100-ml acetonitrile over 8 hours. After cooling, the extracts were brought to volume and filtered on a Whatman 0.2 µm nylon membrane. Separation and quantification were achieved by injections in HPLC

Metabolite deconjugation experiments

Each polar fraction eluted by HPLC was concentrated under a stream of nitrogen and subjected to enzymatic deconjugation. The concentrates were mixed with 1 ml buffer and incubated overnight at 37°C in the presence of: 1) 1000 U β -glucuronidase in 0.1 M phosphate buffer pH 6.8; 2) 15 U β -glucosidase in 0.1 M citrate-phosphate buffer pH 5.0; 3) 500 U arylsulfatase in 0.1 M citrate-phosphate buffer pH 5.0. The resulting solutions were then centrifuged, and the supernatants were concentrated on the C₁₈ guard column and analyzed as described above. The efficiency of enzymes was assayed using similar incubation conditions in the presence of phenolphthalein-glucuronide, salicin and nitrochatechol-sulfate for β -glucuronidase, β -glucosidase and arylsulfatase, respectively.

Volatile compound trapping

The trapping of volatile radioactivity was performed using erlenmeyers sealed with stoppers allowing for airflow (50 ml min⁻¹) through the headspace. Air leaving the erlenmeyer was passed through a column (8 mm *id* x 25 cm) fitted with 5g XAD-2 resin. The airflow was regulated to 10 min every hour. The resin was rinsed every day with 40 ml dichloromethane, and radioactivity was measured by liquid scintillation counting of 1-ml aliquots. The organic fractions were pooled, reduced under light vacuum in the presence of 30 ml pure water. After total evaporation of the organic solvent, the aqueous fractions were concentrated on a guard column [5]. The elution of PAH compounds was then achieved in HPLC.

SPME was also performed by using a manual holder with a 100 μ m polydimethylsiloxane fiber assembly. Volatile compounds were collected in flask headspaces for 30 min at the beginning of the incubations, and then after 7, 14, 21 and 28 days of incubation in the presence of the mixtures of the two PAHs. Chromatographic analysis was performed using a gas chromatograph fitted with a flame ionization detector.

Fungal biomass determination

Fungal biomass in soils was estimated from extractable ergosterol as described [6]. Ergosterol was quantified by HPLC. The ergosterol-to-biomass ratio was in the range of previously reported values for filamentous fungi.

PAH BIODEGRADATION IN WET SAND CULTURES

Evidence for biodegradation

Table 1 reports the mass-balance analysis of the wet sand cultures for the control and the six strains in 28-day incubations treated with the mixture of PAHs.

Recovery of ^{14}C in the sterile controls after 28-day incubations was 80.4%. The loss of radioactivity could be mainly attributed to the volatilization of phenanthrene and the adsorption of benzo[a]pyrene on the erlenmeyer-glass. When fungi were present, ^{14}C recovery generally gave values of between 64.2 and 94.8% of initial radioactivity. A low value was noted in Mic 209 cultures (34.9%), whereas the highest recovery was achieved with ATCC 12679 (94.8%).

PAHs were poorly mineralized in the presence of two strains. No metabolites were detected after extraction by dichloromethane in both organic and aqueous phases from control cultures, which also suggested that the loss of radioactivity may be attributed to volatilization and adsorption. For all strains, solvent fractionation and HPLC analyses showed that most of the radioactivity was associated with organic extractable compounds, mainly as residual benzo[a]pyrene. Very small amounts of organic soluble metabolites (0 to 6.4%) were also noted during the incubations. Water-soluble compounds ranged from 1.5 to 11.3% of the initial radioactivity, the highest value being obtained with ATCC 32745. Radioactivity associated with sand and mycelial fragments after solvent extraction was 9.0% in the controls, and 6.4 to 11.6% in the presence of fungi. This may have included large amounts of phenanthrene and benzo[a]pyrene.

These studies on wet sand cultures helped us to reveal two strains, Mic 209 and ATCC 32745, as being efficient fungi in both phenanthrene and benzo[a]pyrene degradation. These two strains behaved differently: Mic 209 produced the sharpest decline in the contents of both PAHs due to low levels of organic and water-soluble metabolites and poor ^{14}C recovery. ATCC 32745 induced a lower decrease than Mic 209 in the contents of both PAHs, but produced the highest levels of water and organic soluble. It would be interesting to characterize these metabolites. Analyzing the gaseous phase of the cultures is essential to reveal volatile degradation products.

Volatilization

Figure 1 represents volatile amounts in the gaseous phase of the erlenmeyer flasks incubated with phenanthrene, benzo[a]pyrene and Mic 209, over 28 days.

Data indicated a rapid increase in the radioactivity trapped by the XAD-2 resin during the experiment, which reached 21.2% of the initial radioactivity on day 14. It then climbed more slowly to 26.6% of the initial radioactivity by the end. This result takes the ^{14}C recovery from 35.0% (Table 1) to 65.0-70.0% in Mic 209 cultures. Concentration of the pooled radioactive extracts from the resin on the guard column led to the retention of about 75.0% of the radioactivity, suggesting the presence of very polar compounds or compounds exhibiting no affinity with the C_{18} phase. HPLC analysis of the radioactivity retained by the guard column revealed the presence of phenanthrene (85.0% of the injected radioactivity) and of several minor compounds (15.0%).

SPME-GC analysis confirmed the immediate presence of phenanthrene in the headspace of the flasks (Figure 1). Phenanthrene amounts trapped were high at the beginning of the experiments. Afterwards, their increase was moderate until day 21, before a decrease which lasted until day 28. As expected, benzo[a]pyrene was never detected in the headspace of the flasks. Light volatile compounds were also separated during the GC analysis of control samples. A first group was comprised mainly of acetone (used to introduce the PAHs in the culture medium) and solvent impurities. A second group of compounds, referred to as PAH contaminants, was comprised of dibenzothiophene and fluorene, identified in the flask headspace by SPME-GC-MS. All these compounds were omitted for area calculations. Levels of other unknown volatile compounds, referred to as PAH metabolites, increased ten-fold during the first week of incubation, before a return to control values after 4 weeks.

Characterizing the metabolites

The metabolites were analyzed in both the organic and aqueous phases from ATCC 32745 cultures. The retention times were compared to the times of transformation compounds of phenanthrene available commercially, and the metabolites obtained after incubation of benzo[a]pyrene with purified laccases of *T. versicolor*.

In the organic phase, a metabolite was co-chromatographed with the standard of phenanthrene dihydrodiol.

An unresolved radioactive cluster (retention time of 12 min) was noted in the aqueous phases isolated from ATCC 32745 incubations. In order to identify the polar compounds, aqueous phases were subjected to hydrolysis by β -glucosidase, β -glucuronidase and arylsulfatase. Hydrolysis and subsequent release of additional apolar compounds was not noticed after one-night assays at 37°C in the presence of each hydrolase, whereas the known substrate of these enzymes was totally hydrolyzed under the same incubation conditions. All the fractions also remained stable under acidic conditions. These results clearly established that the polar metabolites were not conjugates of benzo[a]pyrene.

MICROCOSM STUDIES WITH INDUSTRIAL SOIL

Physicochemical parameters

Microcosm temperature was between 17°C and 23°C during the experiments. Flushing the reactors with air lowered the soil temperature by 4-5°C with regard to room temperature. Soil pH was not modified during our incubations, remaining in the 7.1-7.4 range.

In general, soil moisture was reduced to 55-65% of the MHC every week in the topsoil, and consequently brought to 80% of the MHC by adding water; moreover, soil moisture also depended on reactor design (Table 2). In reactor A, soil moisture was reduced twice from the top to the bottom of the soil column after 28-day incubations. Conversely, soil moisture was maintained regular in reactor B, and remained higher than in reactor A. Dispersed lignocellulosic pellets probably ensured an homogenous soil moisture. They also reduced soil packing.

Fungal growth and enzymes

Fungal inoculum was comprised of 1.5 g dry biomass and represented 0.1% of soil weight. The proliferation of white mycelium throughout the soil from the pellets demonstrated Mic 209 growth, which was also confirmed by ergosterol measurements (Table 2). Fungal biomass was highest in the pellet layer in reactor A,

while fungal proliferation was 5-times lower in the two adjoining layers of soil after 28-days. Fungal biomass was similar in the three layers of reactor B.

Laccase activity was also measured and appeared closely linked to fungal biomass in reactor A. Conversely, the principal laccase activity was detected at the bottom of the soil in reactor B, where fungal biomass was smallest.

PAH degradation

Quantities of PAHs were measured after 28-day incubation periods in the same soil layers presented above. The results suggest low transformation during that period (Table 2), as extracted PAHs amounted to between 82.7 and 97.0% of their initial levels. In reactors A and B, data seemed to indicate that the smallest amounts of chemicals were recovered in the soil layers which contained the highest biomass.

No volatilized compounds were evidenced from the resin-containing stoppers.

CONCLUSIONS

Three conclusions can be drawn from this study:

First, our screening system shows major differences in the ability of fungal strains from the *T. versicolor* species to transform selected chemicals. They also demonstrate volatilization of parent and degradation products.

In addition, the method of soil inoculation by fungus governs some soil physicochemical properties, as well as fungal growth and development. Mixing the soil with the pellets overgrown by the fungus is the most efficient process for soil colonization.

Pollutant degradation should also be increased for a pilot application.

ACKNOWLEDGMENTS

R. Rama is grateful to KREBS for a Ph.D. scholarship. We thank Michael Dever for proof-reading the manuscript.

REFERENCES

- [1] Rama-Mercier, R., Mougín, C., Sigoillot, J.-C., Sohier, L., Chaplain, V. and Asther, M. Wet sand cultures to screen filamentous fungi for the biotransformation of PAHs. *Biotechnol. Techniques* (In press).
- [2] Rama, R., Mougín, C., Sigoillot, J.-C., Chaplain, V. and Asther, M. A method for improving the propagation of filamentous fungi in industrial PAH-polluted soils. Submitted to *Soil Biol. Biochem.* (SBBKEG11.98).
- [3] Gross, B., Yonnet, G., Picque, D., Brunerie, P., Corrieu, G. and Asther, M. 1990. Production of methylanthranilate by the basidiomycete *Pycnoporus cinnabarinus*. *Appl. Microbiol. Biotechnol.*, 34:387-391.
- [4] Lesage-Meessen, L., Delattre, M., Haon, M., Thibault, J.-F., Colonna Ceccaldi, B., Brunerie, P. and Asther, M. 1996. A two-step bioconversion process for vanillin production from ferulic acid combining *Aspergillus niger* and *Pycnoporus cinnabarinus*. *J. Biotechnol.*, 50:107-113.
- [5] Mougín, C., Laugero, C., Asther, M. and Chaplain, V. 1997. Biotransformation of β -triazine herbicides and related degradation products in liquid cultures by the white rot fungus *Phanerochaete chrysosporium*. *Pestic. Sci.*, 49:169-177.
- [6] Mougín, C., Pericaud, C., Dubroca, J. and Asther, M. 1997. Enhanced mineralization of lindane in soils supplemented with the white rot basidiomycete *Phanerochaete chrysosporium*. *Soil Biol. Biochem.*, 29:1321-1324.

Table 1 Percentage distribution of initial radioactivity in fractions of 28-day cultures treated with a mixture of [9-¹⁴C]phenanthrene and [7-¹⁴C]benzo[a]pyrene in a 1-to-1 ratio.

Fungal strains	Organic soluble compounds (%)			Water soluble compounds (%)	Compounds associated to solid phase (%)	CO ₂ (%)	¹⁴ C recovery (%)
	Phe	B[a]P	Metabolites				
Initial amounts	50.0	50.0	0.0	0.0	0.0	0.0	100.0
Sterile controls	29.4.0	42.0	0.0	0.0	9.0	0.0	80.4
White-rot fungi							
<i>T. versicolor</i> Mic 209	1.0	18.9	0.5	2.9	11.6	0.0	34.9
<i>ATCC 12679</i>	39.4	49.9	0.0	1.5	3.5	0.0	94.8
<i>ATCC 32745</i>	6.8	27.6	6.4	11.3	11.6	0.5	64.2
<i>ATCC 34758</i>	28.0	39.9	0.0	2.8	6.4	0.0	77.1
<i>ATCC 42530</i>	21.5	34.2	2.4	6.8	6.5	0.3	71.7

Table 2. Parameters measured in the different layers isolated from reactors A and B, containing industrial soil and Mic 209.

Parameter	Unit	Reactor A			Reactor B		
		1	2	3	1	2	3
Soil moisture	% DW	18.7	15.8	9.2	24.8	23.5	20.1
Fungal biomass	mg g ⁻¹ soil DW	1.0	5.5	1.1	4.1	3.1	2.5
Laccase activity	U	0.3	4.7	1.1	0.7	0.3	3.1
Remaining PAHs	% of initial amount	90.0	82.8	97.0	87.7	91.3	94.2

Columns 1, 2, and 3 corresponded to 5-cm layers from the top, the middle and the bottom of the reactors, respectively. They were separately considered for analyses.

Figure 1. Radioactivity (●) trapped by the XAD-2 resin, phenanthrene (■) and metabolites (σ) trapped by SPME in flask headspaces during incubations in the presence of phenanthrene, benzo[a]pyrene, and Mic 209.

