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Full Length Research Paper

Benzo[*a*]pyrene degradation by soil filamentous fungi

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The fungal ability to biotransform xenobiotics had received attention due to their dominance, ubiquity and different pathways that detoxificate aromatic hydrocarbons. The filamentous fungi *Aspergillus flavus* and *Paecilomyces farinosus* showed a significant degradation activity on benzo[*a*]pyrene with and without C_{16} as cosubstrate. $^{14}CO_2$, ^{14}C-volatile organic, ^{14}C-extractable, ^{14}C-nonextractable, ^{14}C-biomass and ^{14}C-aqueous fractions were determined with [7, 10]^{14}C-BaP assays, with *A. flavus, Cladosporium cladosporioides, Gliocladium viride, P. farinosus* and *Talaromyces rotundus*. However, the activity of *A. flavus* and *P. farinosus* were higher. These non-ligninolytic fungi degraded BaP by cometabolism in C_{16} presence, were adapted to toxicants and dominant in polluted habitats, so they could play an important role in self- bioremediation processes.

Key words: Benzo[*a*]pyrene biodegradation, cometabolism, mycoremediation, PAHs mixture, polluted sites, soil filamentous fungi.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) and their derivatives are widespread products of incomplete combustion of organic materials arising from natural combustion such as forest fires and volcanic eruptions (Da Silva et al., 2003; Pang et al., 2003). The major PAH pollutions are industrial activities, transportation, refuse burning, gasification and plastic waste incineration (Mrozik et al., 2003). Benzo[*a*]pyrene (BaP), 5-ring hydrocarbon, is formed during pyrolysis of organics being a petroleum, coal tar and fuels-oil component (Kanaly, 1997). Due to its chemical structure is highly recalcitrant and resistant to microbial degradation (Sutherland et al., 1995; Sack et al., 1997).

The fungal PAHs-degradation is an effective strategy to remove pollutants from the environment by bioremediation (Lowborn and Ekwonu, 2009). Diverse ligninolytic fungi had been confirmed as BaP degradersjavascript: popRef ('end-a1') (Bogan and Lamar, 1996; Kotterman et al., 1998; Pointing, 2001; Zheng and Obbard, 2002). These fungi did not compete for prolonged time and were not frequents species in heavy polluted habitats, so, their contribution to BaP detoxification was limited (Steffen, 2002; Tortella et al., 2005).

The knowledge about non-ligninolytic fungi is scarce, although the biomass and diversity of SFF were higher in contaminated sites (Sack and Gunther, 1993; Romero et al., 2001). Therefore, the aims of this study were to assess the ability of SFF isolated from industrial polluted sediments to metabolize BaP and to evaluate the incorporation into biomass, $^{14}CO_2$, extractable metabolites, nonextractable, volatile organic and aqueous phases.

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Abbreviations: BaP, Benzo[*a*]pyrene; **BM,** basal medium; **C_{16}**, *n*-hexadecane; **DMF,** N,N-dimethylformamide; **PAHs,** polycyclic aromatic hydrocarbons; **SFF,** soil filamentous fungi.

MATERIALS AND METHODS

Sampling area, isolation and identification of BaP degrading fungi

SFF were isolated from contaminated sediments of the industrial area, near an oil refinery, La Plata, Argentina; the sediments features were previously published (Romero et al., 1998). The isolate methodology, the basal medium (BM) and culture conditions were described by Massaccesi et al. (2002). The SFF were

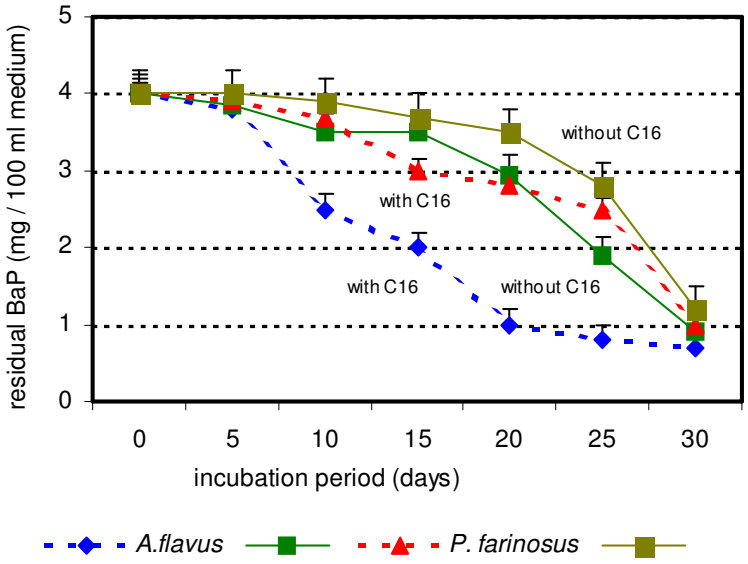

Figure 1. Residual BaP in *A. flavus* and *P. farinosus* cultures with and without C_{16} during the incubation period.

cultured in BM plus 100 µl of 40 mg BaP/2.5 ml N, N-dimethylformamide (DMF) solution, aseptically added on the agar-plate, pH 6.5.

The sporularing isolates were identified by different culture media according to Hanschke and Schauer (1996) and Romero et al. (2005). Non sporulating strains were rejected for further studies as in bio-remediation technologies are better to know the assayed species.

Culture conditions

Fungi were incubated in 100 ml flask with 20 ml BM plus 100 µl of 40 mg BaP/ l stock solution: (1) with 0.5% *n*-hexadecane (C_{16}) or (2) without C_{16} as cosubstrate, pH 6.5 and inoculated with 1 cm^2 plugs of well fungi developed on BaP-agar. The remaining BaP were deter-mined by extracting with 200 ml ethyl acetate at 5, 10, 15, 20 and 30 incubation days. The HPLC methodology was described by Romero et al. (2002). Benzo[*a*]pyrene was identified by its retention time (36.8 min), with a detection limit at 1 µg/ml solvent extract. The BaP-peak identity was confirmed by comparison with an authentic standard, by mass spectrometric identification of the molecular ion (m/z 252) and characteristic fragments (m/z 126) carried at Mass Spectrometry Center, UBA. BaP identification was also controlled with a calibration curve with different BaP levels in DMF (SD 1.5%).

Mass balance analysis

These assays were performed with 0.1 µCi of [7, 10^{14}C]-BaP (56 mCi./mmol specific radioactivity, radiochemical purity 98%) added to each fungal culture and the ^{14}C in the volatile organic substances, solvent extractable metabolites, nonextractable fractions, biomass and $^{14}CO_2$ fractions were obtained (Romero et al., 2002). The fungal growth was measured as mycelia dry weight, after vacuum filtration and dried at 90°C for 24 h.

Three controls were implemented; an inoculated and sterilized flask with BaP, a non-inoculated one and a third culture without BaP. The first and second controls showed the abiotic processes (Pal et al., 1994) and the third one controlled the contamination of the cultures. All the assays were done by triplicate and expressed at mean values.

CHEMICALS

[7, 10-^{14}C]-BaP (50 mCi./mmol specific radioactivity, radiochemical purity 97.1%) was purchased from Marshal Buchler (Braunschweig, Germany); BaP was obtained from Aldrich (Steinheim, Germany); DMF and chemicals were reagent grade and of the highest purity available from Merck (Darmstadt, Germany) and Fluka Chemie AG (Neu Ulm, Germany). The scintillation cocktail Optic-Fluor and Carbo Sorb were purchased from Packard (Meriden, Conn., USA) and Quickszint 212 from Zinsser Analytik (Frankfurt/Main, Germany).

RESULTS

Soil filamentous species were isolated and selected from chronical polluted sediments of an oil industrial area; *Aspergillus flavus, Cladosporium cladosporioides, Gliocladium viride, Paecilomyces farinosus* and *Talaromyces rotundus* were isolated on the basis of their prevailing growth on subsequent plantings on BaP-plates. But *A. flavus* and *P. farinosus* were only selected and assayed as BaP-degraders, due to its higher rates and increased activity after the 10th and 15th incubation day (Figure 1). Without C_{16}, BaP levels declined at 15th - 23rd day, the cosubstrate increased the SFF abilities. *A. flavus* BaP-uptake rate increased early respect to the *P. farinosus* ones; however, these differences diminished at the end of the experiment. The BaP consumed was 92.5 - 77.5% and 75.0 - 70.0% of the initial concentration, 4 mg BaP/100 ml medium, with or without cosubstrate, respectively.

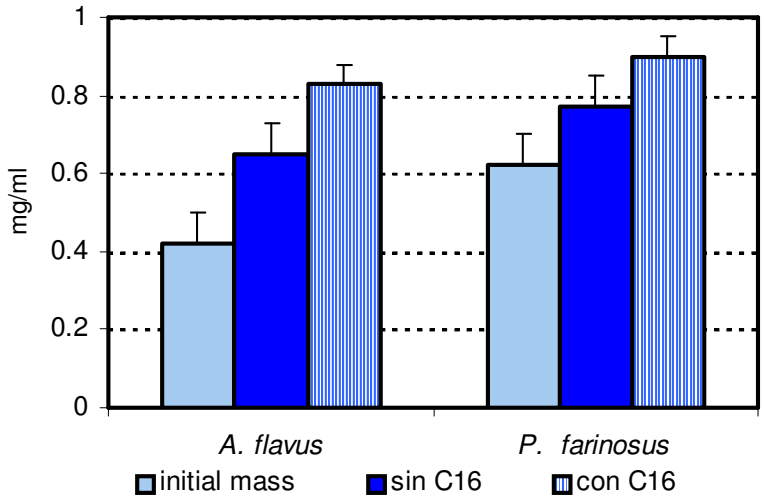

Figure 2. Biomass increase of *A. flavus* and *P. farinosus* with and without cosubstrate (C_{16}) in the BaP assays.

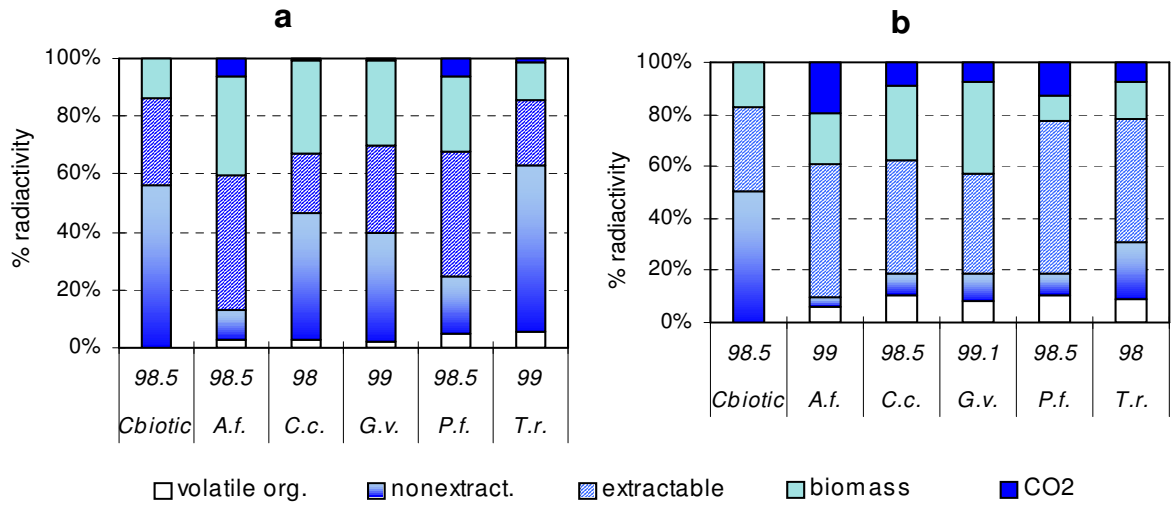

Figure 3. Balance activities among different fractions with ^{14}C (a) without C_{16}, (b) with C_{16}(C: biotic control; *A.f. A. flavus, C.c. C. cladosporioides, G.v. G. viride, P.f. P. farinosus* and *T.r. T. rotundus*).

A. flavus and *P. farinosus* initial biomass were 0.42 and 0.62 mg/ml, this parameter increased to 0.65 - 0.83 and to 0.77 - 0.90 mg/ml with and without C_{16}; therefore, the increment were 97 - 54% and 45 - 24% (Figure 2). The cosubstrate enhanced the mass production and BaP-uptake as C-source.

Although, *G. viride, T. rotundus* and *C. cladosporioides* grew on BaP-plates; they showed significantly less activity in relation to *A. flavus* and *P. farinosus* (Figure 3). Similar patterns were obtained between extractable and mineralization rates, being greater in C_{16} presence. If any BaP metabolites were produced during the incubation period they should be contained in this fraction, and volatile organics increased in all flasks.

^{14}C-biomass counts were a significant recovery fraction, although combusted mycelia could be due to unreacted BaP associated with fungal mass; so, this parameter was rather ambiguous to explain. Nonextract-able parts represented the ^{14}C-BaP solubilized in BM without biological reactions; so the BM had BaP enough to go on the hydrocarbon uptake.

The higher values of the C_{16}-fractions confirmed that these two species mineralized BaP by cometabolism (Figure 3b). Cultures of single species oxidized BaP but not mineralized data had been reported, so this ring cleavage intermediates were produced cometabolically in the presence of other PAHs. To date, BaP had not been reported to support microbial growth (Cerniglia, 1993),

Table 1. Distribution of ^{14}C-BaP in the abiotic controls[a].

Day	Extractable fraction	Irreversibly bound	Reversibly bound	% recovery
0	94.3	1.2	2.5	98.0
10	92.7	2.6	1.7	97.0
20	92.7	3.6	1.2	97.5
30	91.8	3.7	1.0	96.2

[a] Averages of the triplicate measurements expressed in percentages (%) of the total ^{14}C-BaP added (4 mgBaP).

therefore a time-dependent enrichment of BaP degraders is necessary to biotransform this PAH.

The c.a. 100% recovery of the originally ^{14}C-BaP added in both biotic and abiotic controls and triplicates indicated the general validity of the approach. Day-0 ^{14}C-binding in the biotic assays consisted of 7% tie but 6 - 5 - 5% were reversible binding, so only a 1 - 1.5% remained fixed in the biomass. In contrast, the abiotic controls showed 7.5% strong tie and 2.7% irreversible one. These values increased during the assays, probably due to the increment of dead mass particles (Table 1). The BaP amount did not change in non-inoculated controls, and a negligible BaP-portion (< 1%) adsorbed to the autoclaved media.

DISCUSSION

Based on our findings and previous reports, increasing biodegradation of xenobiotics by cometabolisms had been confirmed, even though these SFF lack ligninolytic activities and few of them were able to utilize the compounds as sole source of carbon and energy (Da Silva, 2003; Da Silva et al., 2004; Johnsen, 2005). BaP metabolism had been shown for several molds, deuteromycetes and zygomycetes. *Aspergillus ochraceae* (Datta and Samanta, 1988) and *Penicillium* spp. hydroxylated BaP via cytochrome P-450-dependent mono-oxygenases (Kapoor and Lin, 1984; Launen et al., 1995).

Other authors evaluated the PAHs-transformation by natural communities with chronical pollution and this systems degraded mixed PAHs (Oleszczuk and Baran, 2003; Owabor, 2007). In contaminated areas, the presence of different pollutants was the real situation, so, a natural simple organic could be the cosubstrate that contributed to detoxificate high condensed aromatic hydrocarbons by the autochthonous micro flora (Tortella, 2005; Najafi and Hajinezhad, 2008).

Therefore, our results showed that *A. flavus, C. cladosporioides, G. viride, P. farinosus* and *T. rotundus*, non-ligninolytic fungi, degraded benzo[*a*]pyrene by co-metabolisms. *A. flavus* and *P. farinosus* were adapted to mixed hydrocarbons and could be used in bioremedia-tion techniques. Even more, these SFF are active sure enough in natural decontamination and could play an important role for self- bioremediation processes.

In conclusion, fungal degradation of xenobiotics is looked upon as an effective method to remove chronic pollutants by remediation strategies. This study confirmed that a wide variety of SFF without ligninolytic enzymes, are capable of degrading an equally wide range of PAHs via cometabolism reactions.

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