Biodegradation **15:** 79–85, 2004.

Pyrene and benzo(a)pyrene metabolism by an *Aspergillus terreus* strain isolated from a polycylic aromatic hydrocarbons polluted soil

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Accepted 15 October 2003

Key words: Aspergillus terreus, benzo(a)pyrene, bioremediation, metabolites, pyrene

Abstract

A strain of *Aspergillus terreus* was isolated from a polycyclic aromatic hydrocarbons (PAHs) polluted soil. The metabolism of pyrene and benzo(a)pyrene by this fungus was investigated in liquid submerged culture added of 50 and 25 ppm respectively of each compound. Depletion of pyrene and Bnzo(a)pyrene was evident during the first stages of growth and was 60% and 27.5% respectively of the added amount after nine days of culture. Solvent extracts of the fermentation broth and mycelium were analysed for presence of metabolites by HPLC-MS technique. Under the present cultural conditions pyrene was mainly metabolised to pyrenylsulfate similarly to benzo(a)pyrene that led to benzo(a)pyrenylsulfate. The structure of 1-pyrenilsulfate was determined after purification of extracts and H-NMR analysis. The result show that the isolated *A. terreus* strain metabolises PAHs by reaction similar to those previously reported for non lignolinolytic fungi with a mechanism that suggests the hydroxylation by a cytochrome P-450 monooxygenase followed by conjugation with sulfate ion.

Introduction

The PAHs may accumulate in high concentration in terrestrial environments near coal gasification sites and tar oil distillation plants. Many of these compounds are mutagenic and carcinogenic and thus pose a health risk to wild life and humankind (Jacob et al. 1986; Keith & Telliard 1979). Several physical-chemical techniques of reclamation of PAHs polluted soils are possible like incineration, thermal desorption, soil washing etc. but, because of high costs and legislation constraints, often these are not methods of choice. On the other hand there is an increasing interest in the use of microorganism for PAHs decontamination and detoxification as environmental well accepted and economically competitive technique (Atlas & Cerniglia 1995; Lamar et al. 1994; Nicholas 1987).

As a rule, PAHs in soil are biodegraded with much difficulty as their molecular weight increases, and their water solubility becomes extremely low. Unfortunately, this hydrophobicity usually correlates with increasing genotoxicity and decreasing biodegradability (Nicholas 1987; Gramss et al. 1999). Bacteria are reported to be able to mineralise only PAHs from 2 to 4 rings. For PAHs of higher molecular weight, fungal metabolism has been widely examined for the potential of detoxification (Gramss et al. 1999; Bumpus 1989; Field et al. 1992). Fungi are not able to utilise PAHs as sole source of carbon and energy but rather perform oxidation of these molecules under cometabolic conditions and in same case can mineralise them (Field et al. 1992; Bazelel & Cerniglia 1996).

Most attention has been focused on ligninolytic fungi that can cause the oxidation of PAHs by their non specific enzymatic extracellular complex, normally used to depolimerise lignin, including lignin peroxidase (LiP), manganese peroxidase (MnP), laccases and several H_2O_2 generating enzymes (Bumpus 1989; Field et al. 1992; Barr & Aust 1994). PAHs also bind tightly to soil particles and organic matter reducing

their bioavailabilty and the possibility to be uptaken and biodegraded by microorganism (Weissenfels et al. 1992). For this reason basidiomycetes of the group of the white rot fungi (WRF) such *Phanerochaete chrysosporium*, *Trametes versicolor*, *Bjerkandera* sp. and *Pleurotus ostreatus*, capable of extracellular attack of the PAHs, have been proposed as good candidate for bioremediation of soils (Field et al. 1992; George & Neufeld 1989; in der Wiesche et al. 1996; Morgan et al. 1991; Novotny et al. 1999). But, despite good results in laboratory experiments, the WRF technology have proved to be difficult to scale up (Boyle 1995) and in coal tar contaminated soil it has been found not to be beneficial, compared to the natural attenuation performed by indigenous microflora (Canet et al. 2001).

Another known metabolic pathway of PAHs, present in fungi, is the hydroxylation by cytochrome P-450 monooxygenase through a sequence of reactions similar to those reported in mammalian metabolism of these hydrocarbons (Cerniglia 1981; Cerniglia et al. 1982). This pathway is shared by many non ligninolytic mycromycetes, isolated from polluted soils, that could have a potential in PAHs degradation (Krivobok et al. 1998; Ravelet et al. 2000).

In this study we report the isolation from a PAHs polluted soil of the deutoromycetes *Aspergillus terreus*, which is able to metabolise pyrene and benzo(a)pyrene to the corrispondent hydroxy and sulfate metabolites. The formation of 1-pyrenylsulfate metabolite suggests a cytochrome P-450 monooxygenase oxidation mechanism followed by sulfate conjugation.

Materials and methods

Microorganism isolation

The soil, by which mold and fungi were isolated, was a collected in a former gas plant manufactured area located in North Italy and it had the following characteristics: soil structure: coarse sand (500–2000 μm) 35%, fine sand (200–50 μm) 32%, silt (50–2 μm) 23%, clay ($<$ 2 μm) 2%; organic matter 0.75%; pH 7.1; total C & N content, determined by CHN analyzer: 10.91and 0.26% respectively (the high total C content was justified by presence of carbon black); PAHs content (the 16 listed by US EPA (Keith &Telliard 1979)), by GC-MS analysis: 2–4 rings 462 mg Kg^{-1}, 5-6 rings 1353 mg kg^{-1}; microbial population: total heterotrophes 1.5×10^7 cfu, counted by serial diluition on Nutrient Agar (Difco) Petri dishes, fungi and molds 10^5 cfu, counted by serial diluition on Malt Agar (MA) with 20 ppm streptomycine sulfate.

10 g of soil was suspended in a solution of 20 ppm streptomycine sulfate and 0.18% Na-pyrophosphate (ratio 1/10 w:v) under magnetic stirring for 1 h. Three serial diluitions 1/10 of this slurry were smeared on MA with 20 ppm streptomycine sulfate and incubated at 24 °C for a week. After this period of time about 10 different mold and fungi species developed on the basis of visible aspect of the colony, with a predominance of a highly sporogenic mold with yellow ochreous spores. This mold was axenically propagated on the same medium and periodically transferred on fresh plates.

PAHs degradation in liquid culture

A suspension of 1 ml of *A. terreus* spores, collected from 30 days old MA plates, with optical density 0.7 at 650 nm, was dispensed in 50 ml Czapek Dox Broth (CD) having the following composition: 30 g/l saccarose, 3 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 0.5 g/l KCl, 0.01g/l Fe $SO_4 \cdot 7H_2O$.

Pyrene was chosen as model compound for the study of the metabolism. After the inoculum 100 μl of a stock solution of pyrene (Aldrich) (25 g/l in acetone) was added. The cultures were incubated at 30 °C in orbital shaker at 100 rpm. At scheduled times (2, 5, 7 and 9 d) three flasks for each treatment were extracted by the following method derived by Lange et al. (1994): samples were filtered to separate mycelia from culture filtrate; the filtrate was extracted with 2 equal volume of ethyl acetate and the extract was dried with anhydrous sodium sulfate. The mycelia was extracted twice with acetone by stirring for 20 min each. Then mycelia were oven dried (105 °C for 1 day) and weighted. The extracts were evaporated under reduced pressure at 45° in rotavapor apparatus and the residues were dissolved in 1 ml of methanol for the analyses.

To ensure that the disappearance of pyrene was not caused by reduced extraction efficiency resulting from adsorption on fungal mycelium, 100 μl of the stock solutions was spiked on three replicates of 7 days grown culture and extracted after 4 h of incubation. The recovery in the control was the 93% of the added pyrene. The 1-hydroxypyrene and benzo(a)pyrene (Aldrich) used in further metabolism experiments had a recovery efficiency of the 90% and 85% respectively. Therefore the extraction technique employed was con-

sidered adequate to detect biotic elimination of the added chemicals.

Batch culture of the same type were prepared using as target molecule, benzo(a)pyrene, spiking 100 μl a stock solution (12.5 g/l in acetone), processed and extracted as above.

Extracts of mycelia and culture filtrate were analysed in GC-FID calibrated with external standards at the following conditions: instrument Chrompack CP9002; T injector 280 °C; capillary column Supelco PTE-5^{TM} 30 m 0.32 mm ID 0.25 μm; T detector FID 350 °C; injection of 1 μl sample in splitless mode, T gradient: from 70 °C (2 min) – to 220 °C (3 °C/min) – to 300 °C (2 °C/min) – 300 °C (4 min).

PAHs metabolites characterisation

Extracts of pyrene degradation experiments were combined and purified on RP18 preparative silica gel column by elution with H_2O/MeOH (3:7) For the isolation of the metabolites.

Metabolites from PAHs were determined by HPLC-MS on liquid chromatograph HP 1090, column LiChrocart 250-4 Purospher RP18 (4 mm diam., 250 mm length); flow rate: 1 ml/min; eluent: elution gradient H_2O/MeOH from 30 to 99%; λ detector: 254 nm.

Mass spectral analyses were performed on a Finningan LCQ mass spectrometer. The conditions were APCI (Atmospheric Pressure Chemical Ionization) with an ion source temperature at 450 °C, sheath gas (N_2) 90, aux gas (N_2) 15, discharge current 5 μA, capillary temp. 160 °C, capillary voltage 15V, tube lens 10V. UV-visible absorption spectra were recorded with a Spectra System UV6000 diode array (ThermoFinnigan).

^{1}H nuclear magnetic resonance (NMR) spectra were recorded at ambient temperature on a Varian VXR-300 spectrometer in deuterated acetone.

Results

Strain classification

The isolated strain cultivated on MA plates formed a mycelium highly sporogenic with yellow ochreous colony. It was attributed to the specie *Aspergillus terreus* by morphological analysis of the conidiophore (Raper & Fennel 1965). This classification was then confirmed by analysis of 16s rDNA, through total DNA purification and amplification of a specific fragment corresponding to the central portion of the gene coding for the ribosomal RNA 25–28 S of the "large" subunity. The sequence was compared to those available on databases using the BLAST program at the NIH/Ncbi site. The isolated strain showed 100% similarity to *A. terreus* (ATU28842) and 99% to others *A. terreus* strains (ATU28844, ATU28846, ATU28847, ATU28848).

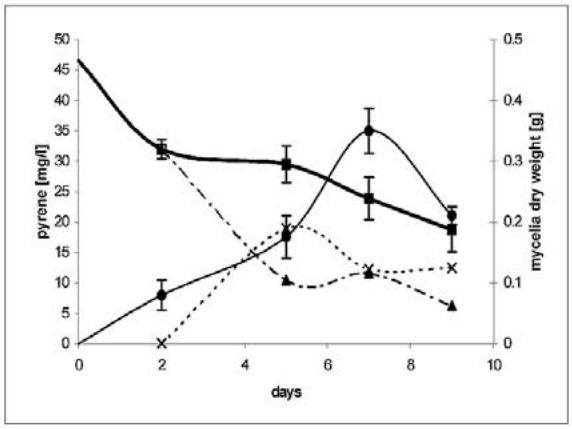

Figure 1. Growth of *A. terreus* and disappearance of pyrene on 50 ml batch scale. Symbols: ●, dry weight of mycelia; ■, total concentration of pyrene; ▲, conc. pyrene in cultural filtrate; ×, conc. of pyrene in mycelia. Error bars indicate standard deviation (n = 3).

Pyrene metabolism

A. terreus transformed pyrene cometabolically when growth in CD medium in submerged culture. Pyrene and others PAHs as sole source of carbon could not support the growth. Figure 1 shows the growth of *A. terreus* in CD medium and the reduction of pyrene concentration on a 50 ml scale batches. The added pyrene had no toxic effect on the fungus because the growth was similar in not spiked control (data not shown). The pyrene degradation occurred mainly in the first two days when fungal biomass was still negligible while in the following days the degradation rate significantly decreased, probably because of a partial absorption of pyrene on the mycelia, as indicated by the separate analysis of the extracts.

The HPLC-MS elution profiles of filtrate and mycelium extracts, of batches experiments with and without added pyrene, were compared in order to identify the peaks derived from pyrene metabolisms. The comparison led to the detection of two main pyrene metabolites (Figure 2) identified as hydroxypyrene and pyrenylsulfate by presence of mo-

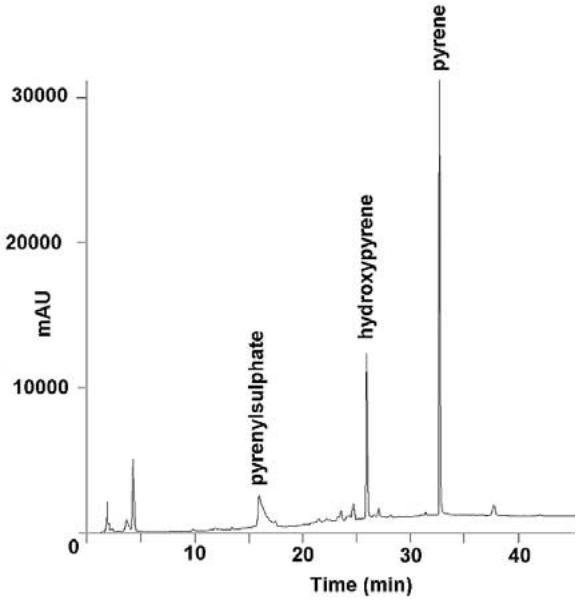

Figure 2. Typical HPLC elution profile of pyrene and pyrene metabolites formed by *A. terreus.*

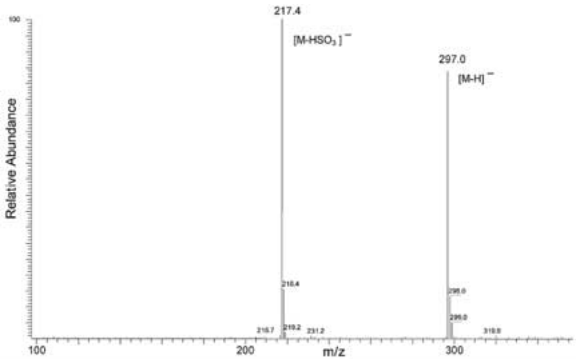

Figure 3. Mass spectra of pyrenylsulfate produced from pyrene by *A. terreus.*

lecular ions $[M\text{-}H]^-$ at m/z 217 and 297. MS/MS experiments show a molecular ion at m/z 297 and a peak at m/z 217 with a loss of a fragment of m/z 80 (Figure 3) that indicated a sulfate group as the substituent.

Quantification of the two metabolites, at day 5 and 7 of batch experiment, was performed both in the broth and in the mycelium (Table 1). The relative amount of the identified metabolites suggested that hydroxypyrene is a transient metabolite and that is quickly converted to sulfate-conjugate. In order to verify this hypothesis the depletion of hydroxypyrene was studied in batch experiment, at the conditions previously described, and a rapid desappearance of the

Table 1. Metabolites quantification in broth and mycelium extracts after 5 and 7 days of culture

Day	Extracts (mg/l)	Hydroxypyrene (mg/l)	Pyrenylsulfate Pyrene	Residual (mg/l)
5	broth	0.62	3.8	10.5
	mycelium	0.05	5.4	19
7	broth	0.074	9.6	11.6
	mycelium	0.075	6.8	12.3

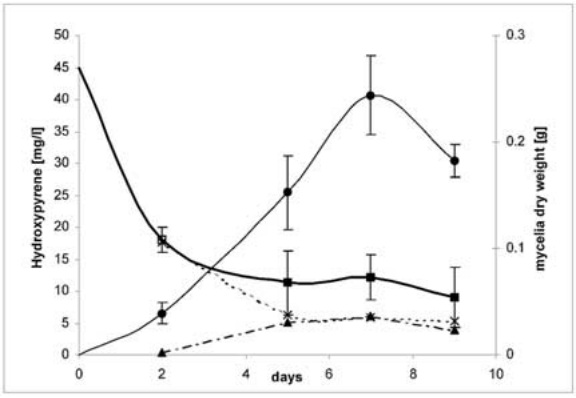

Figure 4. Growth of *A. terreus* and disappearance of hydroxypyrene on 50 ml batch scale. Symbols: ●, dry weight of mycelia; ■, total concentration of hydroxypyrene; ▲, conc. of hydroxypyrene in cultural filtrate; ×, conc. of hydroxypyrene in mycelia. Error bars indicate standard deviation (n = 3).

added chemical was found (Figure 4). The pyrenylsulfate was the only metabolite detected on HPLC-MS after 9 days (35 ppm in mycelium and 6 ppm in the broth).

UV spectra of pyrenylsulfate (Figure 5) shows a strong similarity to that of pyrene, indicating that the metabolite still contain the four rings structure.

The structure of the pyrenylsulfate was determined by ^{1}H-NMR analysis of purified extracts and confirmed that the sulfate group is linked in position C1. The 300-MHz spectral assignments of this metabolite are reported in Table 2.

Benzo(a)pyrene metabolism

Once established the capability to metabolise pyrene, we investigated the *A. terreus* biotransformation in CD medium of the benzo[a]pyrene, that was among the molecules of major environmental concern in the soil from which the mold was isolated. A batch experiment similar to that previously described was performed

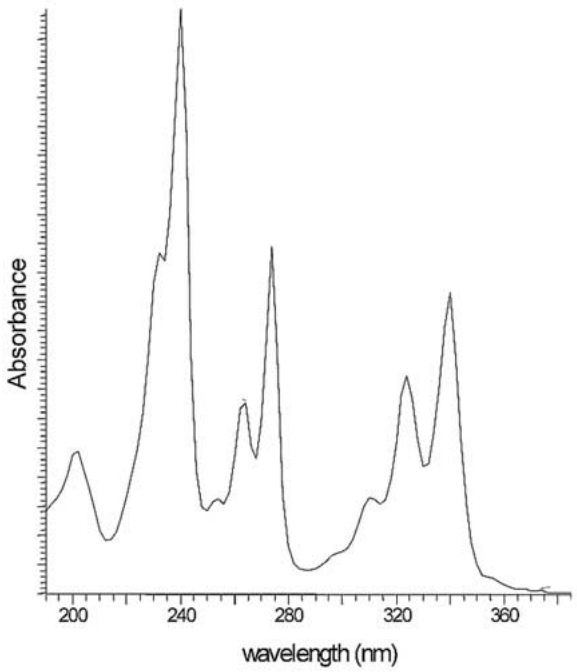

Figure 5. UV absorption spectra of the pyrenylsulfate produced from pyrene by *A. terreus.*

Table 2. NMR data for 1-pyrenyl-sulfate, in CD_3COCD_3

H (n)	σ (ppm)	J (n,m) Hz
2	8.37 (d)	8.4 (2.3)
3	8.186 (d)	8.4 (3.2)
4	8.096 (d)	9 (4.5)
5	8.0165 (d)	9 (5.4)
6	8.195 (d)	6.9 (6.7)
7	8.00 (t)	7.5 (7.6/8)
8	8.195 (d)	6.9 (8.7)
9	8.085 (d)	9 (9.10)
10	8.59 (d)	9 (10.9)

adding 25 mg/l of BAP. After 9 days of incubation period the residual extractable BAP was reduced to 15.4 mg/l (13.7 in mycelium and 1.7 in culture filtrate). The benzo(a)pyrene culture extracts were analyzed by HPLC-MS that showed a molecular ion at m/z 347 and a peak at m/z 267 (hydroxybenzo[a]pyrene) with lost of a fragment of m/z = 80, attributable to sulfate group (Figure 6). Registration of UV spectra of the metabolite identified as benzopyrenylsulfate showed similarity to that of pyrenylsulfate denoting that the BAP metabolisms takes place without ring fission (Figure 7). It

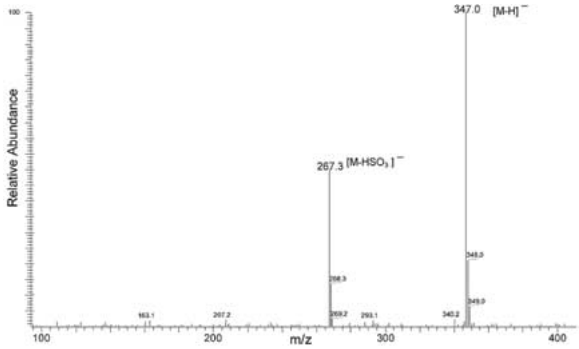

Figure 6. Mass spectra of benzopyrenylsulfate produced by benzo(a)pyrene by *A. terreus.*

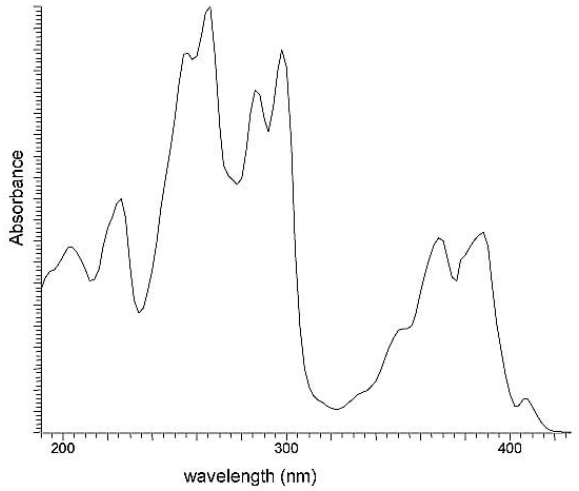

Figure 7. UV absorption spectra of the benzopyrenylsulfate produced from benzo(a)pyrene by *A. terreus.*

was therefore possible to suppose a metabolic pathway similar to that of pyrene.

Discussion

The capability to degrade pyrene, and other PAHs, could be widely expressed among fungi different from ligninolytic basidiomycetes, and till now not fully investigated for its bioremediation potential. A recent paper showed that among 41 indigenous strains isolated from a contaminated sediment 40 were pyrene degraders (Ravelet et al. 2000); the authors reported zygomycetes, especially *Mucor racemosus*, as the best pyrene degraders followed by several Moniliacae (*Penicillium* and *Aspergillus* groups in the order), including an *Aspergillus terreus* isolate. The reported pyrene depletion of this latter strain (1.97 mg g^{-1}

dry weight) was markedly lower than that we have found with our *A. terreus* strain in the first two days of fermentation (9 mg g^{-1} dry weight) and this difference could be ascribed both at strain specificity and experimental conditions.

Our isolate of *A. terreus* cultivated in vitro can metabolises pyrene and benzo[a]pyrene with different efficiency, probably due to water solubility, and/or enzymatic selectivity that led to 60% and 27.5% of removal respectively after a week of incubation. Final products of the reaction were identified as pyrenylsulfate and benzo(a)pyrenylsulfate while the corresponding hydroxyderivatives were detected as intermediates. Therefore we stated that *A. terreus*, grown on CD medium, metabolises polyaromatic hydrocarbons like other non ligninolytic soil fungi, in which a cytochrome P-450 oxidation mechanism and a transconjugation was demonstrated. The formation of 1-pyrenylsulfate was previously found in *Crinipellis stipitaria* (Lange et al. 1994), a plant-inhabiting basidiomycete; glucoronide and sulfate conjugation of 1-naphtol formed by naphthalene metabolism was also reported in *Cunnighamella elegans* (Cerniglia et al. 1989). A strain of *A. niger* isolated from hydrocarbon-contaminated soil formed 1-methoxyphenantrene and 1-methoxypyrene as conjugates derived from the corresponding hydroxyderivatives. In this paper the authors suggested that the formation of conjugates could be medium dependent and may be a way of detoxification (Sack et al. 1997). Even in the WRF fungus *Pleurotus ostreatus* the oxidation of pyrene anthracene fluorene and dibenzothiophene by cytochrome P-450 monooxigenase was found (Bazelel et al. 1996a). The same authors suggested that the PAHs degradation by ligninolytic enzymes probably occurs after a first oxidation step by intracellular monooxigenases e.g. cytochrome P-450 (Bazelel et al. 1996b). In addition the cytochrome P-450 is involved in the fungal metabolism of phenantrene even if it is not a direct substrate for ligninolytic peroxidases due to its high oxidation potential (IP) of 8.19 eV (Bazelel et al. 1996c). In fact it is reported that LiP and MnP can oxidise PAHs up to a threshold IP value of 7.55 and 7.8 respectively, but oxidation of compounds having higher IP can be accomplished by MnP only with the aid of cooxidants e.g. lipids (Bogan & Lamar 1995).

The conjugation reaction of the hydroxylated derivatives of PAHs is generally considered a detoxification step (Cerniglia et al. 1982; Wunder et al. 1994), and the demonstrated pathway could be an important reaction in polluted wastes decontamination. However, the free hydroxylates metabolites of PAHs are often more toxic than parent compounds towards many organisms (Lambert et al. 1995) and are proved to be poorly biodegradable (Hwang et al. 2001). Therefore an in-depth study of the environmental fate and toxicity of the metabolites formed by fungi is required to evaluate their potential in bioremediation of PAHs contaminated soils.

Metabolism of PAHs different from pyrene and benzo(a)pyrene by this fungus and ecotoxicology of its metabolites is currently under investigation.

Aknowledgements

We thank Prof. Bianca Maria Ranzi of the University of Milan for the helpful suggestions and discussion and Ileana Gagliardi who contributed to most of the fermentation study. This work is part of the Chemistry degree thesis of Dr Paolo Digianvincenzo.

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