

## Removal of polycyclic aromatic hydrocarbons from aqueous media by the marine fungus NIOCC # 312: involvement of lignin-degrading enzymes and exopolysaccharides

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The removal of polycyclic aromatic hydrocarbons from aqueous culture medium by the lignin-degrading marine fungus NIOCC # 312, obtained from decaying seagrass from a coral reef lagoon is reported here. We estimated the percentage of phenanthrene removed from the culture supernatant and the fungal biomass after 6 days. About 60-70% of phenanthrene, at a concentration of 12 mg l<sup>-1</sup> (12 ppm) was removed from the culture medium containing live or heat-killed fungus, as estimated by fluorescence spectroscopy method. Nuclear magnetic resonance spectra of the phenanthrene extracted from the fungal biomass revealed that in the heat-killed fungal biomass, the phenanthrene remained undegraded till day 6. On the other hand in the live fungal biomass, no phenanthrene was detected on day 6 suggesting that it was metabolized or transformed into non-aromatic fragments. We conclude that the disappearance of phenanthrene from the aqueous culture medium is due to its instant adsorption to the fungal biomass owing to the presence of the exopolymeric substance (EPS) around the fungal hyphae. The EPS produced by the fungus was partially characterized. We further hypothesize that phenanthrene thus adsorbed by the live fungal biomass was subsequently degraded by the lignin-degrading enzymes present in the cell wall and the EPS envelope. Thus, the heat-killed fungal biomass could be used only for adsorption of PAHs from contaminated sites whereas use of the live fungal biomass would result in degradation of PAHs.

**[Key words:** Polycyclic aromatic hydrocarbons, degradation, marine fungi, exopolymeric substance, lignin-degrading enzymes, fungal isolate, isolate NIOCC # 312]

### Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as the three-ringed phenanthrene and four-ringed chrysene are commonly found as pollutants in sediments, estuarine waters and other aquatic sites<sup>1</sup>. Phenanthrene has been shown to be toxic to marine diatoms<sup>2</sup>, gastropods<sup>3</sup>, mussels, crustaceans and fish<sup>4</sup>. These biomagnified contaminants ultimately pose a serious threat to human health. Due to the increasing importance of coastal zones, environmental agencies are imposing strict regulations for mitigation of pollution from industries.

There has been an increasing interest in using marine microorganisms to degrade recalcitrant organic compounds in the field of wastewater treatment<sup>5-7</sup>. Several bacteria and cyanobacteria are known to metabolize phenanthrene<sup>8,9</sup>. The rapidly

growing and bulk biomass-producing white-rot fungi of terrestrial origin are known to metabolize phenanthrene besides several other PAHs<sup>10</sup>. They also metabolize several xenobiotic compounds through extracellular lignin-degrading enzymes<sup>11</sup>. Most research concerning bioremediation by these fungi is focused on a single species, the wood-degrading white-rot fungus *Phanerochaete chrysosporium*<sup>12</sup>. Comparatively, very little is known about lignin-degrading enzymes of marine fungi and their role in degradation of xenobiotics. Raghukumar *et al.*<sup>13</sup> reported for the first time the presence of the lignin-degrading enzymes, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase in a marine isolate of the white-rot fungus, NIOCC #312 obtained from decaying seagrass in a coral lagoon. This fungus efficiently decolorized bleach plant effluent from paper and pulp mill, a range of synthetic dyes as well as molasses spent wash from alcohol distilleries under marine conditions<sup>13-15</sup>. Therefore, our objective in the present study was to test this fungus for

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degradation/removal of PAHs under similar conditions.

## Materials and Methods

### Growth conditions

A low-nitrogen medium (LNM) was prepared with seawater and supplemented with 2% agar and the synthetic polymeric dye Poly-R-478 (Sigma Chemicals, USA) for the selective isolation of lignin-degrading fungi from various marine sources such as mangrove, seagrass and algal detritus, sediments and driftwood<sup>16</sup>. Bacterial growth was suppressed using a cocktail of equal volume of streptomycin and penicillin at a final concentration of 1%. One of the fungal isolates, NIOCC # 312 obtained from decomposing seagrass leaves of *Thalassia hemprichii* (Ehrenberg) Ascherson, showed strong Poly-R decolorizing activity indicating presence of ligninolytic enzymes<sup>13</sup>. This isolate (NIOCC # 312) tentatively identified as *Flavodon flavus* (Klotzsch) Ryvar den belonging to the class Basidiomycetes has been deposited in the ARS Patent Collection, (USDA, Illinois, USA) under the accession number NRRL 30302. The culture was maintained on malt extract agar (MEA) slants.

### Degradation of polycyclic aromatic hydrocarbons

A 7-day old culture of # 312 grown in LNM was homogenized with sterile glass beads and was used for inoculating Erlenmeyer flasks of 100 ml capacity containing 10 ml LNM. The flasks were flushed with 100% oxygen for 1 min immediately after inoculation and every third day thereafter under sterile conditions. The flasks were stoppered with airtight rubber corks and incubated at room temperature in the dark for 7 days. To these, 20  $\mu$ l of PAHs, phenanthrene and chrysene (Sigma Chemicals, USA), dissolved in hexane and filter sterilized to a final concentration of 12 ppm (12 mg l<sup>-1</sup>), were added separately. Phenanthrene or chrysene added to heat-killed cultures (by autoclaving 7-day old culture at 120°C, 15 lbs pressure for 20 min) and medium without the fungal culture were used as control. Live and heat-killed cultures were filtered through GF/C filters (Whatmann, USA) at 3-day intervals up to 15 days to collect the fungal biomass and the culture supernatant. The culture supernatant was extracted with 5 volumes of distilled hexane twice, concentrated and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the residual phenanthrene was determined by measuring the fluorescence at excitation and emission wavelengths of 310 and

360 nm respectively<sup>17</sup> using a spectrofluorometer (Shimadzu, Model RF 1501, Japan).

The fungal biomass was extracted in a Soxhlet apparatus in 20 volumes of alkaline methanol (by addition of 1% KOH) twice, each for 3 h, pooled, concentrated, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and the residual phenanthrene was determined by fluorescence spectroscopy. The results were calculated as the difference between initial readings (day 0) and the day of measurement for the residual phenanthrene and represented as % removal/degradation.

The culture supernatant and the fungal biomass obtained from the culture on day 6 after addition of phenanthrene were treated as described above and the extracts were evaporated to dryness under nitrogen. Proton nuclear magnetic resonance (NMR) spectra of these were recorded with Bruker Avance 300 spectrometer (300 MHz) in deuterated chloroform (CDCl<sub>3</sub>) using TMS (tetramethylsilane) as an internal standard.

### Degradation of phenanthrene by the immobilized fungus

Polyurethane foam cubes of 1 cm<sup>3</sup> were boiled in water, washed repeatedly with distilled water and sterilized. These were soaked with LNM and inoculated with macerated mycelia of the fungus grown in LNM. Within 7 days, the foam cubes were covered with the fungal mycelium and were introduced into fresh LNM containing 12 ppm phenanthrene and incubated at room temperature for 6 days. Phenanthrene was extracted and estimated from the immobilized fungus as described above.

### Effect of phenanthrene on growth of the fungus

The effect of phenanthrene on the growth of # 312 was estimated as the fungal dry weight. For this purpose, cultures growing with and without phenanthrene were filtered over pre-weighed GF/C filter discs and were dried to a constant weight at 60°C. The difference in weight was calculated as fungal dry weight in mg.

### Characterization of exopolymeric substance (EPS)

The fungal mycelium was stained with acidic Alcian blue (1% Alcian blue in 3% acetic acid, w/v) pH 2.5 to test for acidic polysaccharides<sup>18</sup> and examined under a microscope (Olympus, BX 60, Japan). For examining EPS by scanning electron microscopy, the fungal mycelium was dehydrated serially through an increasing acetone concentration

by standard procedures and critical point dried<sup>19</sup>. Following gold-palladium sputter coating, the pieces were examined in a scanning electron microscope (JEOL JSM-5800LV, Japan).

The EPS of the fungus was prepared as follows: one liter of the culture supernatant from a 10-day old culture was frozen at  $-20^{\circ}\text{C}$  for 48 h. The frozen culture supernatant was allowed to thaw at room temperature during which, the EPS that appeared as strands was allowed to settle and collected by decanting the supernatant. Chilled methanol at a final concentration of 70% was added to the supernatant to further precipitate the remaining EPS. The precipitated EPS was collected by centrifugation, lyophilized and the dry weight was then determined. The carbon and nitrogen content were analyzed in a CNS analyzer (Model NCS 2500/S. no. 980813, Thermoquest Italia, S.P.A.) using MAG-1 and Sulphan standards. The sugar component of the EPS was determined by gas chromatography (at Central Food Technological Research Institute, Mysore, India).

### Results and Discussion

About 70-80% of phenanthrene was removed from the culture supernatant (CS) by day 6 in both live as well as heat-killed cultures, inoculated with varying concentrations of the inoculum (0.5, 1.25 and 1.5 mg of the inoculum). The fungal biomass obtained on day 6 in these sets of cultures was 12, 26 and 33 mg (in 10 ml medium) respectively. Removal of phenanthrene was directly proportional to the amount of live fungal biomass (Fig. 1). The fungal biomass from the heat-killed cultures from these three inoculum concentrations adsorbed almost the same amount of phenanthrene from the culture medium (Fig. 1). About 71 and 78% of chrysene, a four-ringed PAH, was removed from the culture medium through adsorption by the live and heat-killed fungal biomass respectively. When a mixture of phenanthrene and chrysene was added to the culture, 81 and 78% of them were lost from the culture medium by live and heat-killed cultures respectively. As the spectrofluorometric measurement does not distinguish between phenanthrene and chrysene, the loss indicated cumulative removal of PAHs. The growth of the fungus was not inhibited in the presence of phenanthrene (Table 1). In fact, the fungus used phenanthrene as a carbon source, as its biomass increased by over 5% and remained high till day 12

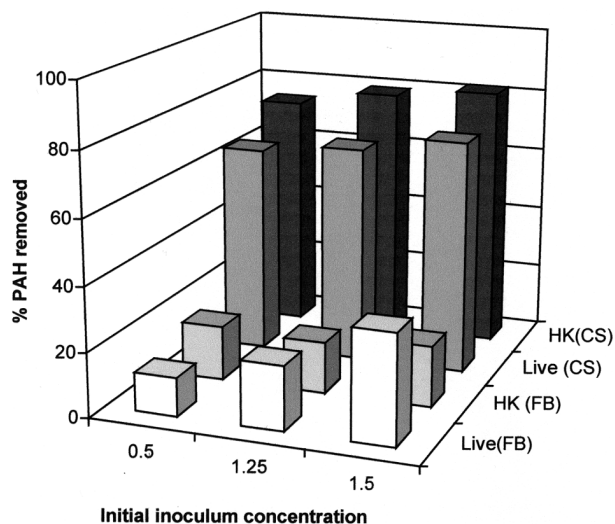


Fig. 1—Percentage of phenanthrene removed from the culture supernatant (CS) and the fungal biomass (FB) on day 6 containing live and heat-killed (HK) fungal biomass obtained by using different concentrations of the initial inoculum. Phenanthrene was added to 6 day-old culture when the fungal biomass (started with different initial inoculum concentrations) showed distinct difference in weight.

Table 1—Effect of phenanthrene on growth of the fungus *Flavodon flavus*.

Days	Growth (mg dry weight in 10 ml medium)	
	Without phenanthrene	With phenanthrene
0	10 ± 1.2	10 ± 1.2
3	23 ± 0.9	26 ± 1.8
6	28 ± 1.1	38 ± 1.6
9	20 ± 1.5	26 ± 2.1
12	19 ± 2.1	22 ± 1.8
15	23 ± 1.5	21 ± 1.9

(Table 1). The fungus immobilized in polyurethane foam was as effective as the free mycelium in degrading phenanthrene (66% removal after 6 days as estimated by fluorescence spectroscopy).

The H NMR peaks of phenanthrene were visible on day zero and six (Fig. 2A-C) in the extracts from the heat-killed fungal biomass with about 27% loss on day 6. On the other hand, phenanthrene peaks that were detected in the live fungal biomass on day 0 (Fig. 2D) were absent on day 6 (Fig. 2E) suggesting that it was metabolized or transformed into more polar derivatives. The culture supernatants from the live as well as the heat-killed cultures showed the presence of phenanthrene as confirmed by NMR studies (spectrum similar to that of Fig. 2B) indicating that it was not completely metabolized.

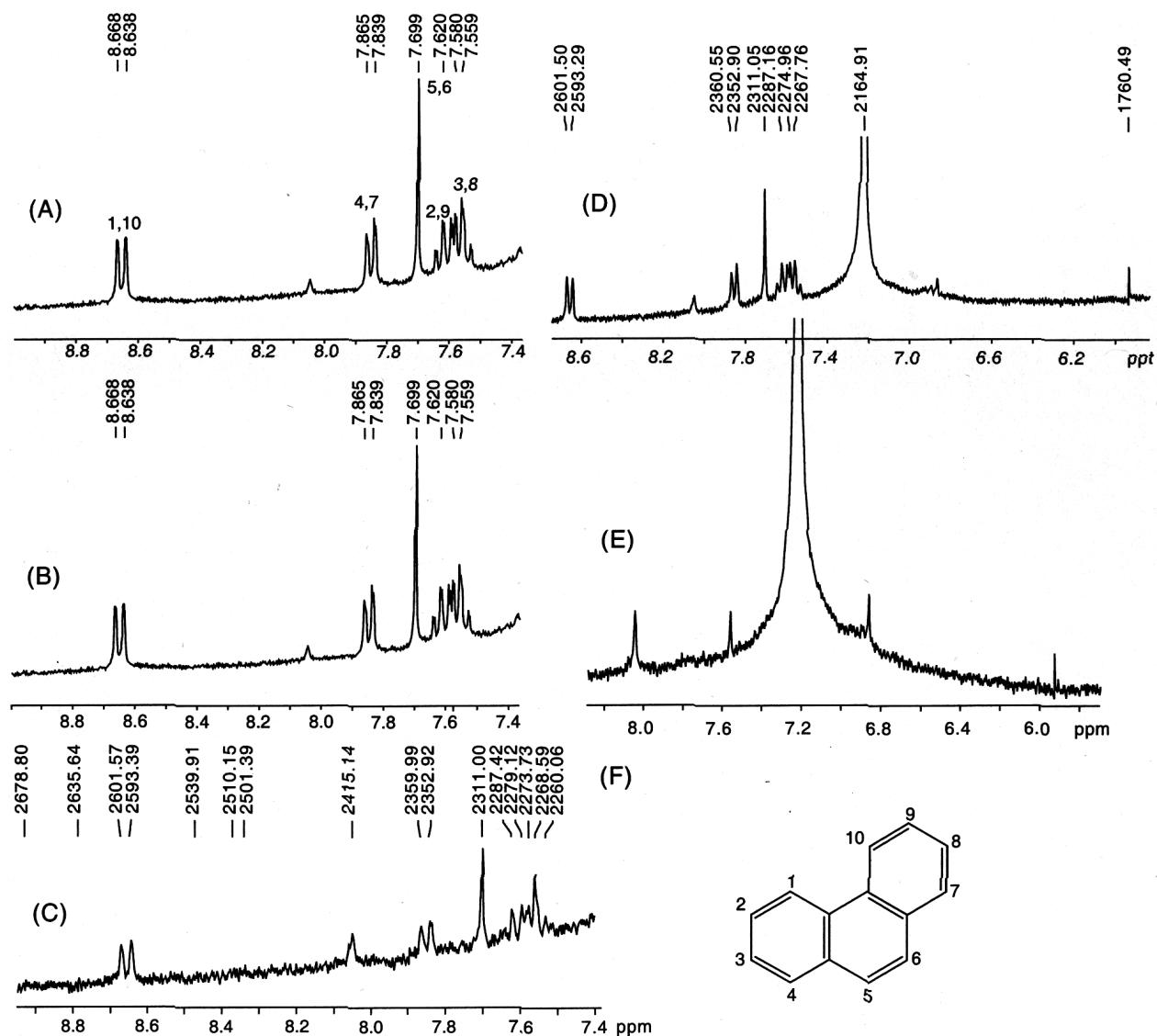


Fig. 2—(A) 300-MHz Proton NMR spectra of the standard phenanthrene, (B) phenanthrene extracted from the heat-killed fungal biomass on day 0 and (C) day 6, (D) phenanthrene extracted from the live fungal biomass on day 0 and (E) day 6, (F) the chemical structure of phenanthrene: the numbers in the structure correspond to the peaks numbered in the standard phenanthrene spectrum as seen in “A”.

Removal of phenanthrene and chrysene from the culture supernatant containing live or heat-killed fungal culture appears to be due to its adsorption to the fungal biomass. This adsorption is facilitated by the exopolysaccharide sheath seen around the fungal mycelium. The blue-stained polymeric substance around the mycelium after staining the fungal biomass with acidic Alcian blue, indicated the presence of exopolymer (Fig. 3A). Scanning electron microscopy also revealed fungal mycelium covered with EPS (Fig. 3B) and presence of sheath-like EPS around the hyphae (Fig. 3C). The carbon and nitrogen analysis of

the exopolymer indicated 32% of C and 7.8% of N. The polymer had glucose as the major monomeric sugar as analysed by gas chromatography (Fig. 4). About 370 mg of EPS (dry weight) was produced in 1 liter of the culture medium. It has been documented that the lignin-degrading enzymes secreted extracellularly are trapped in this EPS-matrix in some of the lignin-degrading fungi<sup>20</sup>. Therefore, we hypothesize that the lignin-degrading enzymes present in the EPS-matrix around the fungal mycelium further aided in the degradation of phenanthrene adsorbed by the live fungal biomass.

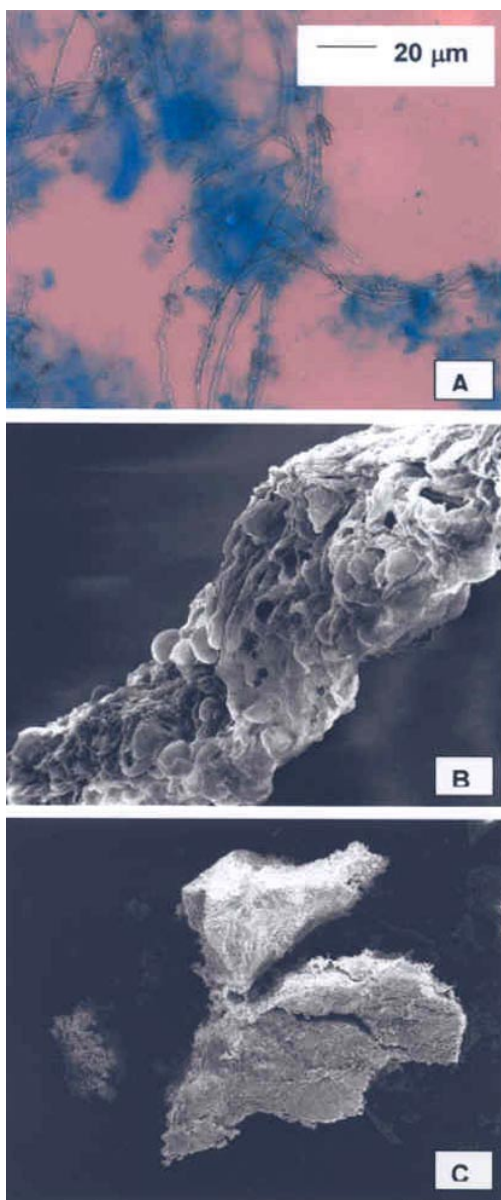


Fig. 3—(A) Alcian blue-stained EPS (arrow) around fungal hyphae, (B) SEM of the EPS covering the fungal hyphae ( $\times 950$ ), (C) SEM of the EPS-matrix around the fungal hyphae ( $\times 110$ ).

The fungal mycelium had completely covered the entire surface area of 10 ml culture medium in a 100 ml flask in 6-7 days. Therefore, when phenanthrene was added to such cultures, most of it was immediately adsorbed to the fungal mycelial mat. The live and heat-killed fungal mycelial biomass behaved similarly and therefore the amount of phenanthrene lost from the culture medium on the day of addition was similar. On the other hand, on day 6 the amount of phenanthrene degraded by the fungal

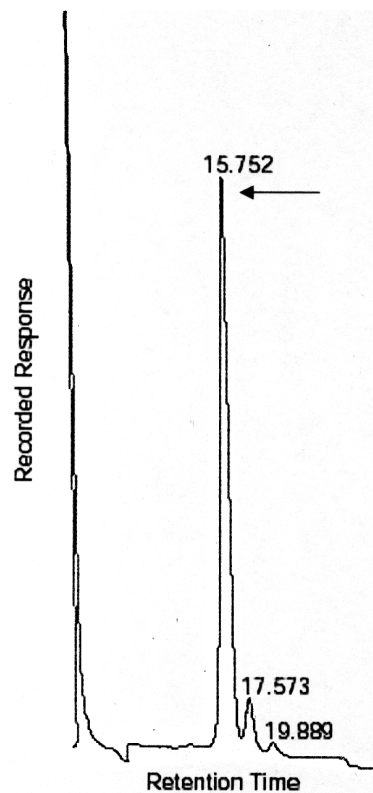


Fig. 4—Gas chromatography of the hydrolyzed EPS showing the glucose peak (arrow).

biomass due to the presence of lignin-degrading enzymes was more in the live than in the heat-killed biomass.

A number of white-rot fungi are reported to produce exopolymeric substance that forms a sheath around the fungal mycelium and plays an important role in their natural environment. The lignolytic fungus *Phanerochaete chrysosporium* also produces polysaccharide sheath and dissolution of this sheath by addition of glucanase inhibited lignin degradation<sup>21</sup>. This suggested an active involvement of exopolysaccharides in lignin degradation processes. Decolorization of colored industrial effluents by EPS produced by another marine white-rot fungus (NIOCC # 2a) was reported recently<sup>22</sup>.

We hypothesize that EPS acts as an adsorbing matrix where the lignin-degrading enzymes can get concentrated. The enzymes in this matrix further break down PAHs and other pollutants. A model as shown in Fig. 5 depicts possible process involved in degradation/removal of phenanthrene by the isolate # 312.

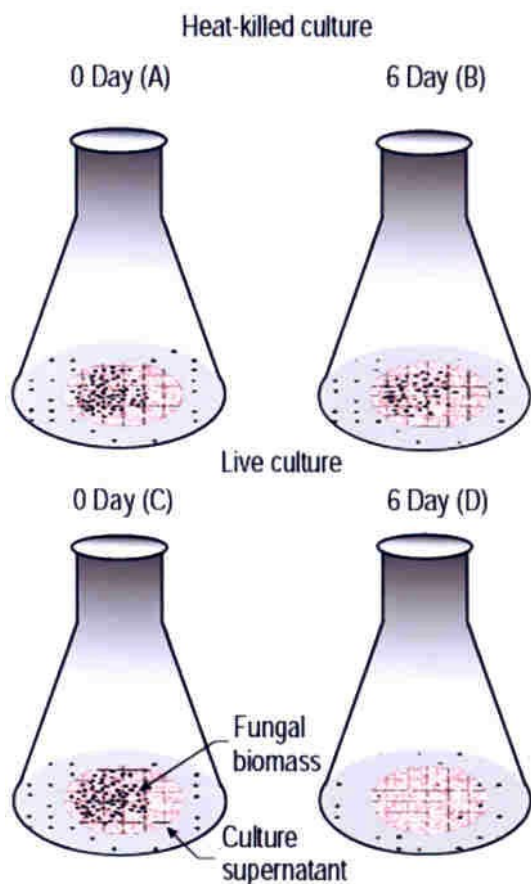


Fig. 5—Model showing the fate of phenanthrene in the live and heat-killed fungal biomass and the culture supernatant. The black dots and the large round disc represent phenanthrene and the fungal mycelial biomass respectively. The phenanthrene dots correspond to its distribution in the culture medium and fungal biomass, amounting to a total of 100%.

A similarly high rate of adsorption of highly chlorinated polychlorobiphenyls to hyphae of *Phanerochaete chrysosporium* was reported<sup>23</sup>. Degradation of phenanthrene by heat-killed and live culture (38 and 37% respectively) was reported in another white-rot fungus *Irpex lacteus*<sup>24</sup>. Bacterial adsorption for removal of PAHs is considered as one of the significant options for treatment of refinery wastewater<sup>25</sup>. However, PAH biosorption was not indicative of PAH degradation capabilities of bacterial strains. Usually, bacterial species and strains vary in their biosorption capacity<sup>26</sup>. The advantage in our process is that a large amount of PAHs were removed from the system instantly and the adsorbed PAHs were further degraded by the enzymatic process.

Intracellular cytochrome P-450 monooxygenase is implicated in degradation of PAHs by fungi in

general<sup>27</sup>. Lignin-degrading enzymes, manganese peroxidase (MnP) and laccase by white-rot fungi in particular are also known to degrade PAHs<sup>28</sup>. We have reported the production of these enzymes in low nitrogen medium<sup>13</sup> by this fungus. Maximum MnP was produced on day 6 when degradation of phenanthrene was observed to be maximum. Thus, a concerted action of the lignin-degrading enzymes of this fungus and EPS might help in degradation/removal of PAHs. In addition, live cultures aid continuous biotransformation of phenanthrene or such toxic organic molecules. Application of live cultures of marine fungi and their appropriate enzymes for bioremediation of such pollutants appear to hold great potential in pollution clean-up<sup>29</sup>.

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