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Biodegradation of Fluoranthene by Basidiomycetes Fungal Isolate *Pleurotus Ostreatus* HP-1

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Abstract The biodegradation of fluoranthene, a high molecular weight polycyclic aromatic hydrocarbon (PAH), was investigated in submerged culture using the wood decaying fungus isolated from forest locality in Gujarat, India. The basidiomycete fungal isolate was found to have an ability to grow on sabaroud dextrose agar containing 50 mgl^{-1} of each naphthalene, anthracene, acenaphthene, benzo (a) anthracene, pyrene, flouranthene, carbazole, and biphenyl. The involvement of extracellular fungal peroxidases such as manganese peroxidase (MnP) and laccase (Phenol oxidase) in the degradation of fluoranthene was studied. On the eighth day of incubation 54.09% of 70 mg l^{-1} fluoranthene was removed. There after no PAHs removal was observed till the 20th day of the incubation period. The isolate was identified as *Pleurotus ostreatus* by 18S rRNA, 5.8S rRNA, and partial 28S rRNA gene sequencing. To the best of our knowledge this is the first time *Pleurotus ostreatus* have been reported to degrade such a high concentration of fluoranthene within much lower time period of incubation. Depletion in the residual fluoranthene in the culture medium was determined by HPLC. Attempts were made to identify the degradation product in the culture medium with the help of FT-IR, NMR, and HPTLC analysis. In the present study positive correlation between fluoranthene degradation and the ligninolytic enzyme (MnP and laccase) production is observed, thus this isolate can play an effective role for bioremediation of PAHs contaminated sites.

Keywords Basidiomycete · Degradation · Fluoranthene · Ligninolytic enzymes · Wood decaying fungus · *Pleurotus ostreatus*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of hazardous organic chemicals consisting of three or more fused benzene rings in linear, angular, and cluster arrangements.

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Many of the PAHs have been shown to be potentially genotoxic and carcinogenic [1, 2]. Human activities including fossil fuel combustion and industrial processing, is the primary source of PAH contamination. PAHs are also generated by natural phenomena, such as forest fires [3–5]. Some PAHs are classified as priority pollutants by the US Environmental Protection Agency (EPA) and fluoranthene is one of the 16 target compounds. Fluoranthene is also considered as a pollution indicator [6]. Biodegradation of PAHs by bacteria is well documented but such knowledge on fungi is limited [7–11]. A variety of bacteria can degrade certain PAHs completely to CO_2 and metabolic intermediates [12, 13]. Nonspecific oxidation reaction catalyzed by extracellualr enzymes of white rot fungi leads to the formation of variety of quinones and hydroxylated aromatic compounds [14, 15]. The degradation of PAHs by ligninolytic fungi includes the role of cytochrome P450 monooxygenase, lignin peroxidase, manganese peroxidase, and laccase [16].

To improve the biodegradation potential of white rot fungi, extensive research on the enzymes and their reactions involved in PAH degradation needs to be carried out. In the present study we report the ability of a wood decaying basidiomycetes fungus *Pleurotus ostreatus* HP-1 isolated from the forest locality of Gujarat, India, for degradation of fluoranthene and production of significant amount of ligninolytic enzymes.

Materials and Methods

Chemicals

Fluoranthene was gift from Atul Limited, Atul, Gujarat, India. 2, 2-Azino-bis (3-ethyl benzthiozoline-6-sulphonic acid) (ABTS) was purchased from Sigma, (St. Louis, MO, USA). 2, 6-Dimethoxyphenol was purchased from Lancaster, Loncs, UK. Bovine serum albumin (BSA) and agar-agar were purchased form Hi-media, Mumbai, India. Phenol reagent was purchased from Merck, Mumbai, India. All other chemicals used were of analytical grade procured from Qualigens, Mumbai, India.

Screening and Isolation of Fungal Strain

The rotted wood samples were collected from different forest localities in Gujarat, India. A small portion of wood sample was transferred in plates containing 2% malt extract agar added with chloramphenicol (0.05%) and incubated at 28±2 °C (Giraud et al., [4]). Further, isolation was carried out by subculturing these cultures on modified Sabaroud dextrose agar (SDA) containing (gl^{-1}) glucose, 20; peptone, 10; NaCl, 2.5; agar-agar, 30; penicillin G, 0.06; streptomycin sulfate, 0.0001; and fluoranthene, 0.05. After 7 days of incubation at 28±2 °C fungal cultures were transferred on the same medium without antibiotics until pure colonies were obtained. When all fungal cultures had similar microscopic characteristics a representative culture was selected for storage. SDA incorporated with 0.01% *w*/*v* orthodianisidine was used for screening of phenol oxidase.

Degradation Medium

Submerged culture experiments were performed in 250 ml Erlenmeyer flasks containing 25 ml of degradation medium prepared according to Tien and Kirk [15] with modifications containing (gl^{-1}) glucose, 10; ammonium tartrate, 0.2; sodium acetate, 3.28; thiamine, 2; KH_2PO_4, 2; $MgSO_4.7\ H_2O$, 0.53; $CaCl_2$, 0.1; $CuSO_4$, 0.001, $MnSO_4$, 0.005; H_3BO_3, 0.001;

$NaMoO_4.2H_2O$, 0.0001; $ZnSO_4.7H_2O$, 0.0001; $AlK(SO_4)_2.12\ H_2O$, 0.0001; and yeast extract 0.5.The pH of the medium was adjusted to 5.0 with 2 N HCl. Flasks were sterilized at 15 lbs and 121 °C for 15 min. Separately filter sterilized thiamine solution was added to the medium. All flasks were inoculated with four mycelial discs of 8-mm diameter from the edge of actively growing culture and incubated at 28±2 °C at 100 rpm for 6 days. The fluoranthene prepared in acetone stock solution was sterilized by filtration through 0.2 μm millipore membrane, and added to 6-day-old culture to a final concentration of 0.1 gl^{-1}. All flasks were reincubated at 28±2 °C at 100 rpm in orbital shaking condition for 15 days.

Extracation of Residual Fluoranthene and Fungal Biomass Estimation

Extraction of residual fluoranthene from the medium was performed on second, fourth, sixth, eighth, tenth, 12th, and 15th day of incubation. The liquid media with mycelia containing fluoranthene were extracted with one volume of bidistilled ethyl acetate by incubating for 30 min on rotary shaker. Mycelia were filtered off with Whatman No.1 filter paper and rinsed with ethyl acetate. The media and the rinse were transferred in a separating funnel. The mixture was shaken for 2 min. The organic phase was collected and saved. The extraction was then repeated twice. The crude extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness at 40 °C under reduced pressure [17]. Estimation of fungal biomass and determination of fluoranthene degradation was carried out according to method described by Verdin et al. [18]. To determine the fungal biomass the filtered mycelia was dried to constant weight at 60 °C. Dry weights were corrected for organic and inorganic components in the medium by subtracting measurements made for filtered uninoculated (abiotic) control flasks.

PAH degradation rate was calculated by subtracting the amount of PAH extracted from the abiotic control from the other samples. The rate of degradation was calculated as:

$$\text{PAH degradation rate } (\%) = \text{abiotic control extraction } (100\%) - (\text{sample extraction } + \text{ adsorption losses})$$

Where sample extraction=filtrate extraction+mycelium extraction.

Extracellular Enzyme Activity

Total extra cellular protein was determined by the method of Lowry et al. [19]. Bovine serum albumin was used as a standard.

Laccase activity (E.C. 1.10.3.2) was determined by measuring the oxidation of 2,2-Azino-bis-3-ethyl-benzthiozoline-6-sulphonic acid. Increase in absorbance was measured spectrophotometrically (Elico BL-198, Hyderabad, India) at 420 nm (ε=36,000 $cm^{-1}\ M^{-1}$) [20]. The reaction mixture contained 100 μl of 50 mM ABTS and 800 μl of 20 mM Na-Acetate buffer (pH 4.5) and 100 μl of appropriately diluted enzyme extract. Manganese peroxidase activity (E.C 1.11.1.13) was measured by oxidation of 2,6-dimethoxy phenol (DMP) at 469 nm (ε=27,500 $cm^{-1}\ M^{-1}$). The reaction mixture contained 1 mM DMP, 0.1 mM $H_2\ O_2$, 1 mM $MnSO_4$, and 100 mM Sodium Tartrate buffer (pH 4.5). MnP activity was corrected for manganese independent peroxidase activity by subtracting the activity obtained at pH 3.25 in absence of $MnSO_4$ at 469 nm [21]. All enzyme activities were carried out at room temperature. One unit of enzyme activity was defined as amount of enzyme that oxidized 1 μM of substrate per minute.

Analytical Procedures

Residual Fluoranthene Estimation

Residual fluoranthene was quantified by reverse-phase high-performance liquid chromatography (HPLC) (Perkin Elmer, USA, Series 200 model) equipped with Spheri 5 column (RP18, 5 μm, 250×4.6 mm) and Series 200 pump. Detection was done using photo diod array detector. Extracted dried samples were redissolved in 1 ml of mobile phase (Acetonitrile, Water, 85: 15, *v*/*v*), filtered through 0.2 μm filter and 20 μl was used for HPLC analysis.

Identification of Metabolites

Detection of extracellular degradation product was done by using TLC Silica gel 60 F_{254} plates (Merck, Germany). A 15 μl of sample was spotted on TLC plates using a microsyringe (HPTLC, Camag, Lanomat 5). The solvent system used was Propanol/Acetic acid/Water, 90:9:1 (*v*/*v*/*v*). The chromatogram was observed under ultraviolet light, (Camag, TLC Scanner 3). The ^{1}H nuclear magnetic resonance spectroscopy was done on Varian Mercury spectrophotometer (YH 300) and fourier transform infrared (FT-IR) spectroscopy was done on Spectrum GX FT-IR model, Perkin Elmer, USA.

Data Analysis

The data in subsequent sections represent arithmetic mean values of three experimental repetitions (each one was made in duplicate).

Results and Discussion

The new isolate was found to be nonsporulating, with abundant clamp connection in mycelia, under microscopic observations which are characteristics of basidiomycete. The isolate was identified as *P. ostreatus* by 18S rRNA, 5.8S rRNA, and partial 28S rRNA gene sequencing from Bangalore GENEI, India, and was identified as *P. ostreatus* HP-1 (GenBank Accession No. EU420068).The isolate was able to grow on SDA having 50 mgl^{-1} of each flouranthene, anthracene, naphthalene, pyrene, benzo(a)anthracene, and carbazole but showed poor growth on biphenyl and acenaphthene (Table 1). A good growth on solid medium did not always imply a good biodegradation in liquid medium. This phenomenon has been previously reported with growth on solid medium containing

Table 1 The zone of proliferation of *Pleurotus ostreatus* HP-1 on eighth day of growth on SDA having different PAHs (50 mg l^{-1} each).

Name of compound	Zone of Proliferation[a]
Naphthalene	8.8
Anthracene	8.8
Acenaphthene	4.0
Biphenyl	3.8
Benzo(a)anthracene	8.5
Carbazole	8.3
Pyrene	7.5
Fluoranthene	8.0

[a] All above zones were measured in cm

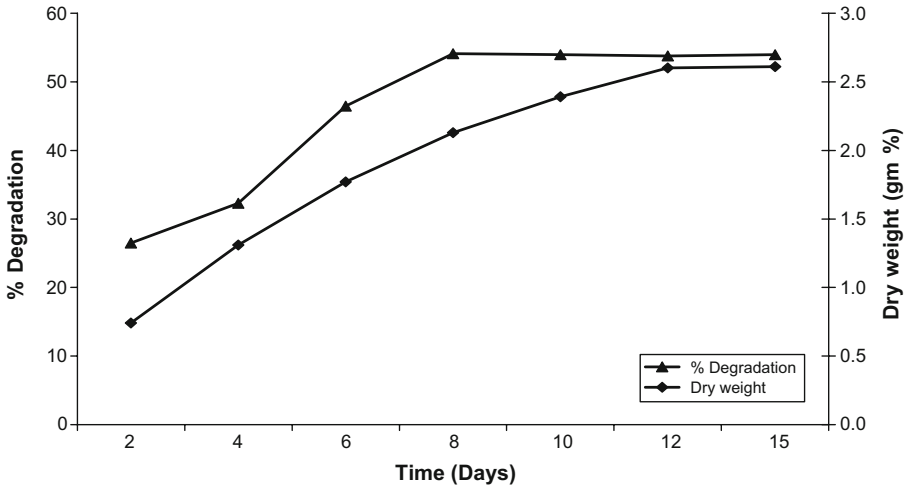

Fig. 1 Comparison of % degradation and dry weight during degradation of fluoranthene

biphenyl oxide and 2-acetylthiophene biotransformation [22, 23]. The isolate showed dark brown colored zone surrounding mycelial growth on orthodianisine containing SDA because of Bavendamm's reaction. Previously several researchers demonstrated that the different white rot fungi are able to degrade variety of PAHs [24–28]. Present study demonstrates potential of basidiomycetes fungal isolate for fluoranthene degradation.

The rate of degradation was determined from second to 15th day of incubation after addition of fluoranthene to the previously grown 6-days-old culture. The mean amount of maximum degraded fluoranthene was found to be 54.09% on the eighth day of incubation (Fig. 1).Thereafter, no further significant level of fluoranthene elimination was observed.

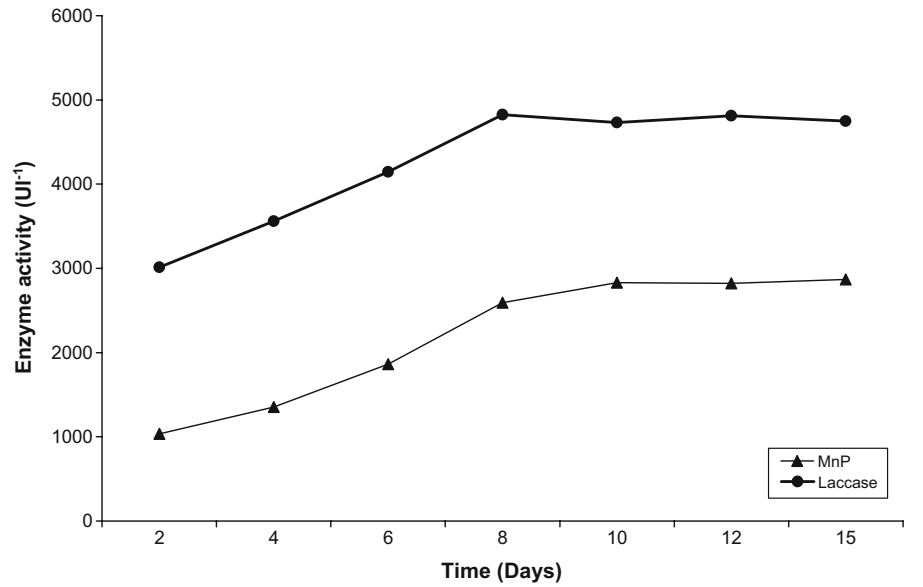

Fig. 2 Activity of ligninolytic enzymes after addition of fluoranthene to 6 days grown culture

Our findings report a considerably higher fluoranthene degradation by our isolate compared to earlier reported lignin-degrading fungi [29], which showed 15% and 30% of fluoranthene degradation with *P. ostreatus* and *Bjerkandera adusta,* respectively, after seven to 10 days of incubation. The decline in fluoranthene removal suggests the possibility of nutrient limitations. The decrease in fluoranthene removal rate and the ligninolytic enzyme production were results of low nutrient availability in the system (i.e., cosubstrate carbon). Joner et al. [30] reported the bacterial degradation of four ring PAHs were preliminary limited by availability of mineral nutrients in system. Collin et al. (1996) also reported the

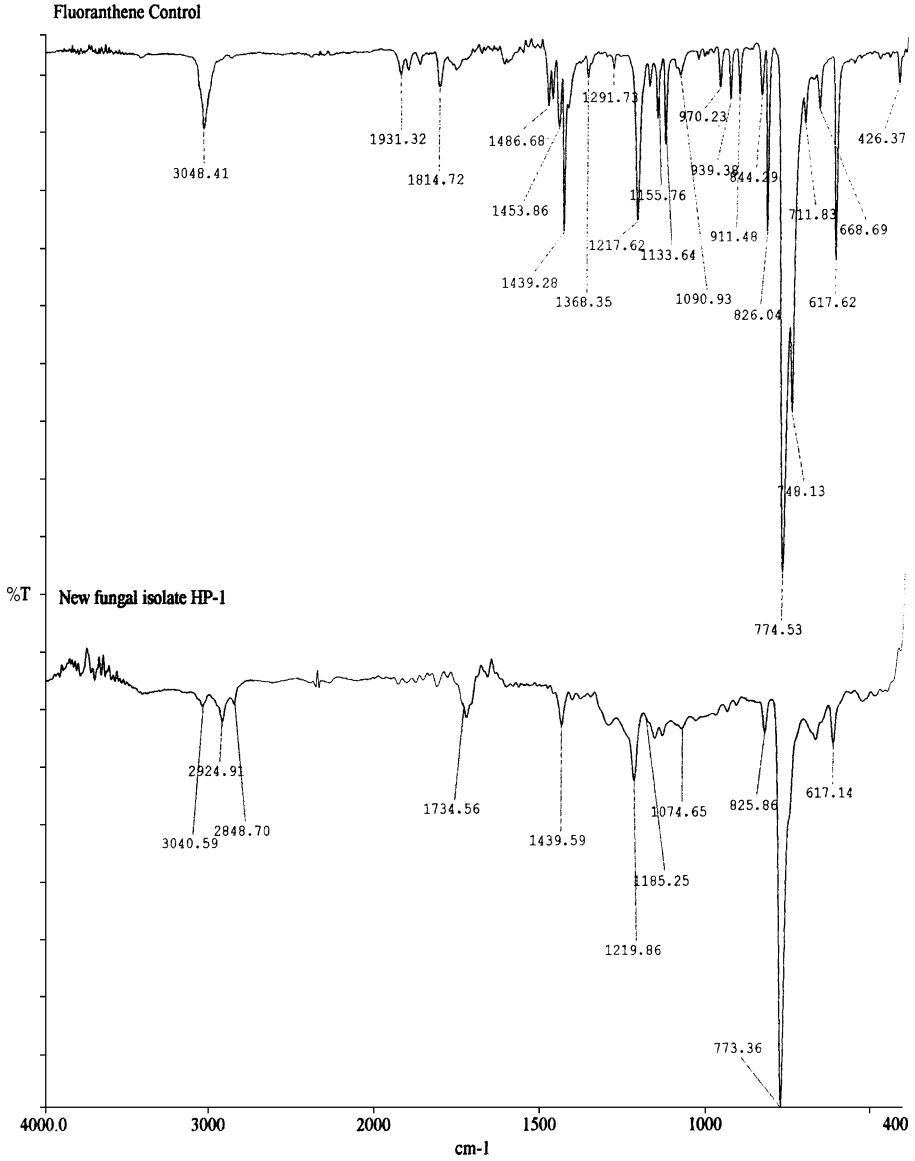

Fig. 3 Comparison of FT-IR spectrum of eighth day (after addition of FA to 6-day grown culture) extracted sample with uninoculated control

nutrient limitation enhance the production of ligninolytic enzymes and increase the PAHs degradation. At maximum degradation, ligninolytic enzyme activities were found to be 2,593 and 4,823 UI^{-1} for MnP and laccase, respectively (Fig. 2). Proportional increase in degradation rate with biomass development and enzyme production was in accordance with

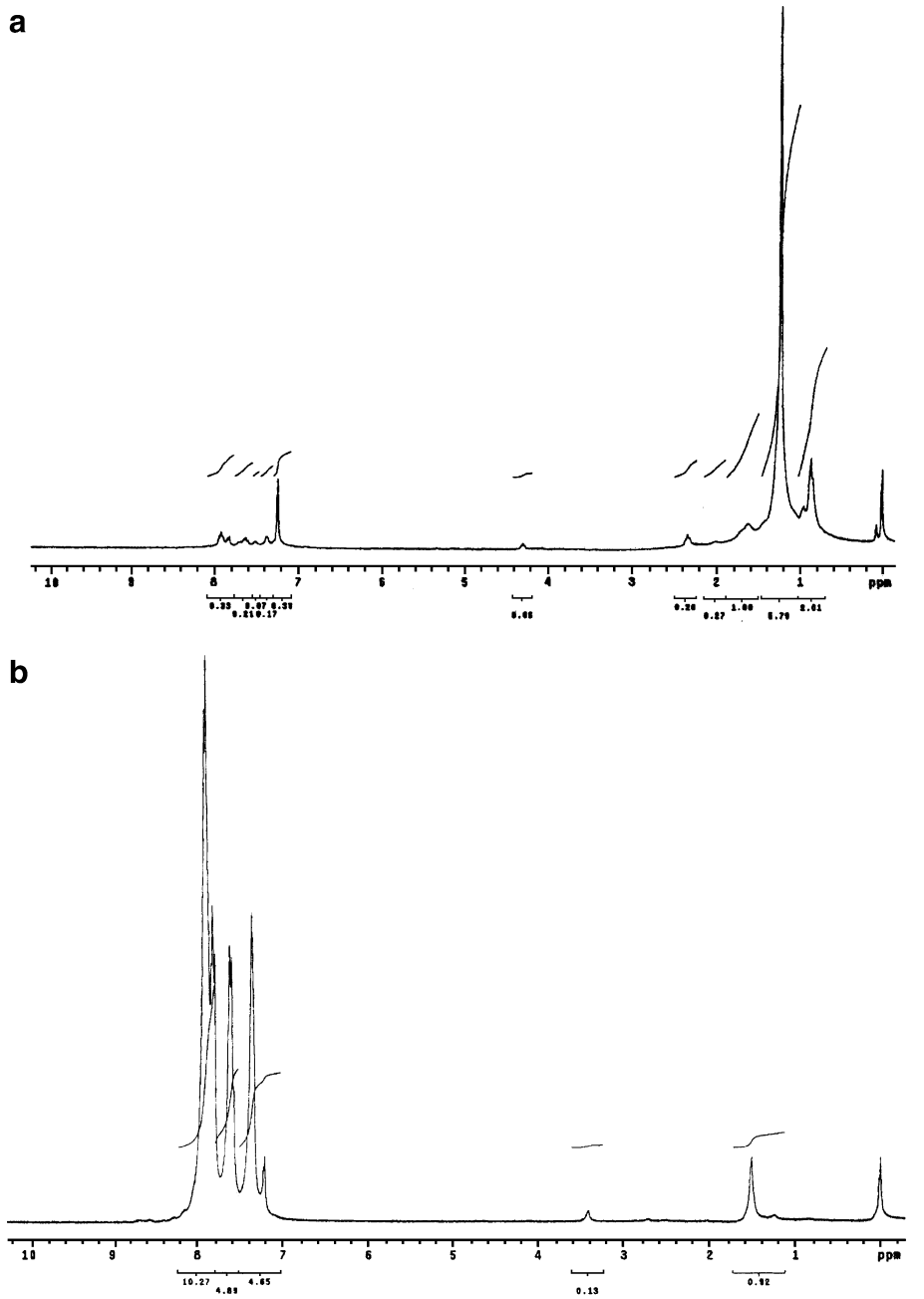

Fig. 4 Comparison of ^{1}H NMR Spectrum of 8-day ethyl acetate extracted sample (**a**) (after addition of FA to 6-day grown culture) with uninoculated control (**b**)

previous study by Novotny et al. [31] (Fig. 2). At this stage of experiment no attempt were made to differentiate between intra- and extracellular degradation products.

The Fig. 3 shows the FT-IR analysis of ethyl acetate extracted metabolites from degradation medium by comparing spectra of eighth day fungal treated flask with untreated flask. The peak at 3,048 cm^{-1} is assigned to C–H stretching frequency of aromatic ring. As expected for aromatic compounds there was very weak absorption in the region of 1,077–1,900 cm^{-1}. Three strong absorptions at 826–748 are assigned to C–H out of plane banding in the aromatic ring. Remarkable vibrational changes were observed in the finger print region (1,500–500 cm^{-1}) of fungal treated flasks (Fig. 3). The peak at 1,734 cm^{-1} may be due to the formation of carbonyl group because of biotransformation of fluoranthene on 8th day. This indicates the transformation of the compound.

^{1}H Nuclear magnetic resonance spectroscopic analysis showed the appearance of new peaks in the δ region of 1–3 ppm, which is characteristic region for aliphatic chain structure compounds (Fig. 4a). The intensity at δ region of 7–8 ppm in the control which is for aromatic ring structure compounds goes down indicating that the degradation of fluoranthene (Fig. 4b). This also indicates the aromatic compound is degraded to linear aliphatic compound. This degradation was further confirmed by HPTLC analysis, in which the spots of degraded products were seen other than the uninoculated flask (Fig. 5). At this stage no identification of the metabolites produced as a result of degradation of fluoranthene was carried out.

Different amount of laccase and MnP produced by the fungi did not affect its capability to degrade fluoranthene. The accumulation of metabolite in the culture medium indicates involvement of extracellular ligninolytic enzyme system in fluoranthene biodegradation. However, another fluoranthene biodegradation system may probably be involved in the organism simultaneously, i.e., intracellular Cytochrome P-450 monooxygenese that may contribute to degradation of fluoranthene [32]. But the relative participation of individual enzyme system in fluoranthene biodegradation is still not known.

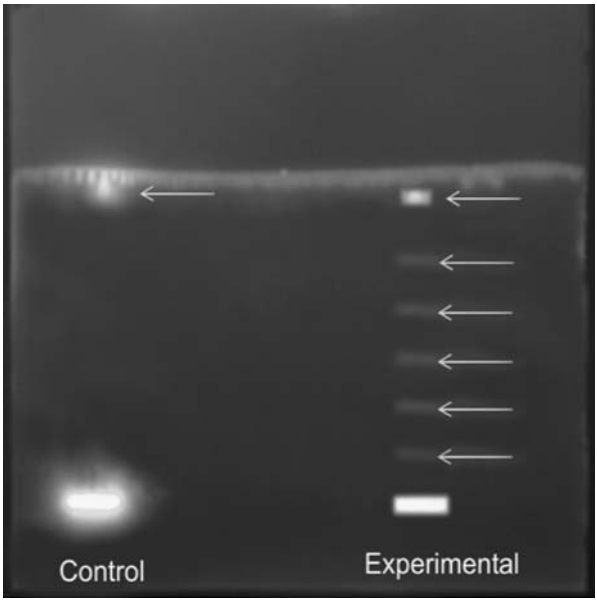

Fig. 5 Different spots of ethyl acetate extractable metabolites of fluoranthene on TLC plate under UV light (254 nm)

Conclusion

These promising results suggest the application of a basidiomycete fungal isolate *P. ostreatus* HP-1 for clean-up of contaminated sites with recalcitrant pollutants like PAHs. It is capable to grow and degrade them with its efficient extracellular enzyme system. High concentrations of fluoranthene are effectively degraded in short time period. A dense mycelial growth and enzyme expression are prerequisites for an ability of a fungus to degrade the pollutant molecules from the environment. Rate of fluoranthene degradation observed with new basidiomycete fungal isolate in liquid medium was significantly exceeded those found with other reported data for degradation of PAHs.

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References

1. Menzie, C. A., Potocki, B. B., & Santodonato, J. (1992). *Environmental Science & Technology*, *26*, 1278–1284, doi:10.1021/es00031a002.
2. Nadon, L., Siemiatycki, J., Dewar, R., Krewaski, D., & Guerin, M. (1995). *American Journal of Industrial Medicine*, *28*, 303–324, doi:10.1002/ajim.4700280302.
3. Christian, K., & Juan, M. L. (1997). *Biotechnology Letters*, *16*, 759–764.
4. Giraud, F., Giraud, P., Kadri, M., Blake, G., & Steiman, R. (2001). *Water Research*, *35*, 4126–4136, doi:10.1016/S0043-1354(01)00137-3.
5. Guerin, T. F. (1999). *Journal of Hazardous Materials*, *B65*, 305–315, doi:10.1016/S0304-3894(99)00002-3.
6. Chaudhary, G. R. (1994). *Biological degradation and bioremediation of toxic chemicals*. Portland, OR, USA: Dioscorides Press.
7. Cerniglia, C. E. (1993). *Current Opinion in Biotechnology*, *4*, 331–338, doi:10.1016/0958-1669(93)90104-5.
8. Harayama, S. (1997). *Current Opinion in Biotechnology*, *8*, 268–273, doi:10.1016/S0958-1669(97)80002-X.
9. Dean-Ross, D., Moody, J. D., Freeman, J. P., Doerge, D. R., & Cerniglia, C. E. (2001). *FEMS Microbiology Letters*, *204*, 205–211, doi:10.1111/j.1574-6968.2001.tb10886.x.
10. Moody, J. D., Freeman, J. P., Doerge, D. R., & Cerniglia, C. E. (2001). *Applied and Environmental Microbiology*, *67*, 1476–1483, dooi:10.1128/AEM.67.4.1476-483.2001.
11. Sack, U., & Fritsche, W. (1997). *FEMS Microbiology Ecology*, *22*, 77–83, doi:10.1111/j.1574-941.1997.tb00358.x.
12. Kelly, I., Freeman, J. P., Evans, F. E., & Cerniglia, C. E. (1993). *Applied and Environmental Microbiology*, *59*, 800–806.
13. Muncnerova, D., & Augustin, J. (1994). *Bioresource Technology*, *48*, 97–106, doi:10.1016/0960-524(94)90195-3.
14. Bogan, B. W., & Lamar, R. T. (1996). *Applied and Environmental Microbiology*, *62*, 1597–1603.
15. Tien, M., & Kirk, T. K. (1988). *Methods in Enzymology*, *161*, 238–249, DOI 10.1016/0076-6879(88)61025-1.
16. Cerniglia, C. E. (1997). *Journal of Industrial Microbiology & Biotechnology*, *19*, 324–333, DOI 10.1038/sj.jim.2900459.
17. Schutzendubel, A. M., Majcherczyk, A., Johannes, C., & Hutterman, A. (1999). *International Biodeterioration and Biodegradation*, *43*, 93–100, doi:10.1016/S0964-8305(99)00035-9.
18. Verdin, A., Lounes-Hadj Sahraoui, A., & Durand, R. (2004). *International Biodeterioration and Biodegradation*, *53*, 65–70, doi:10.1016/j.ibiod.2003.12.001.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *Journal of Biochemistry*, *193*, 265–275.
20. Niku-Paavola, M. L., Raaska, L., & Itavaara, M. (1990). *Mycological Research*, *94*, 27–31.
21. Martinez, M. J., Ruiz-Duenas, F. J., Guillen, F. A., & Martinez, A. T. (1996). *European Journal of Biochemistry*, *237*, 424–432, doi:10.1111/j.1432-33.1996.0424k.x.
22. Seigle-Murandi, F., Krivobok, S., Steiman, R., Thiault, G. A., & Benoit-Guyod, J. L. (1991b). *Journal of Agricultural and Food Chemistry*, *39*, 428–430, doi:10.1021/jf00002a041.
23. Tekere, M., Read, J. S., & Mattiasson, B. (2005). *Journal of Biotechnology*, *115*, 367–377, doi:10.1016/j.jbiotec.2004.09.012.

24. Collins, P. J., & Dobson, A. D. W. (1996). *Biotechnology Letters*, *18*, 801–804, doi:10.1007/BF00127892.
25. Dhawale, S. W., Dhawale, S. S., & Ross, D. D. (1999). *Applied and Environmental Microbiology*, *58*, 3000–3006.
26. Moredo, N., Lorenzo, M., Dominguez, A., Moldes, D., Cameselle, C., & Sanroman, A. (2003). *World Journal of Microbiology & Biotechnology*, *19*, 665–669, doi:10.1023/A:1025198917474.
27. Morgan, R., Lewis, S. T., & Watkinson, R. J. (1991). *Applied Microbiology and Biotechnology*, *34*, 693–696, doi:10.1007/BF00167925.
28. Salicis, F., Krivobok, K., Jack, M., & Benoit Guyod, J. L. (1999). *Chemo*, *38*, 3031–3039, doi:10.1016/S0045-535(98)00504-9.
29. Seigle-Murandi, F., Krivobok, S., Steiman, R., Benoit-Guyod, J. L., & Thiault, G. A. (1991a). *Applied Microbial Biotechnology*, *34*, 436–440.
30. Joner, E. J., Corgie, S. C., Amellal, N., & Leyval, C. (2002). *Soil Biology & Biochemistry*, *34*, 859–864, doi:10.1016/S0038-0717(02)00018-4.
31. Novotny, C., Svobodova, K., Erbanova, P., Cajthaml, T., Kasinath, A., Lang, E., et al. (2004). *Soil Biology & Biochemistry*, *36*, 1545–1551, doi:10.1016/j.soilbio.2004.07.019.
32. Novotny, C., Erbanova, P., Sasek, V., Kubatova, A., Cajthamal, T., Lang, E., et al. (1999). *Biodegradation*, *10*, 159–168, doi:10.1023/A:1008324111558.