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Polycyclic Aromatic Hydrocarbons (PAHs) Biodegradation by Basidiomycetes Fungi, *Pseudomonas* Isolate, and Their Cocultures: Comparative In Vivo and In Silico Approach

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Abstract The polycyclic aromatic hydrocarbons (PAHs) biodegradation potential of the five basidiomycetes' fungal monocultures and their cocultures was compared with that of a *Pseudomonas* isolate recovered from oil-spilled soil. As utilization of hydrocarbons by the microorganisms is associated with biosurfactant production, the level of biosurfactant production and its composition by the selected microorganisms was also investigated. The *Pseudomonas* isolate showed higher ability to degrade three of the five PAHs but the isolate did not produce biosurfactant higher than *C. versicolor* and *P. ostreatus*. Among the PAHs, the most effective biodegradation of PAH—pyrene (42%)—was obtained with the fungus *C. versicolor*. Cocultures involving the fungi and *Pseudomonas* could not significantly degrade the selected PAHs compounds above that degraded by the most efficient monoculture. A slight increase in pyrene degradation was observed in cocultures of *C. versicolor* and *F. palustris* (93.7% pyrene). The crude biosurfactant was biochemically characterized as a multicomponent surfactant consisting of protein and polysaccharides. The PAH biodegradation potential of the basidiomycetes fungi positively correlated with their potential to express ligninolytic enzymes such as lignin peroxidase (Lip), manganese peroxidase (Mnp), and laccase. The present study utilized in silico method such as protein–ligand docking using the FRED in Open Eye software as a tool to assess the level of ligninolytic enzymes and PAHs interactions. The in silico analysis using FRED revealed that of the five PAHs, maximum interaction occurred between pyrene and all the three ligninolytic enzymes. The results of the in silico analysis corroborated with our experimental results showing that pyrene was degraded to the maximum extent by species such as *C. versicolor* and *P. ostreatus*.

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Introduction

Bioremediation is a process by which living organisms degrade or transform hazardous organic contaminants to less toxic compounds. Polycyclic aromatic hydrocarbons (PAHs) are formed from the incomplete combustion or pyrolysis of organic material such as oil, petroleum gas, coal, and wood. PAHs have attracted much attention in the studies on air pollution recently because some of them are highly carcinogenic or mutagenic [1]. They have been classified by the Environmental Protection Agency (EPA) of the United States as priority pollutants. The EPA lists 16 PAHs as hazardous because of their high mutagenic or carcinogenic potential [2–4]. Possible fates for PAHs released into the environment include volatilization, photooxidation, chemical oxidation, bioaccumulation, and adsorption on soil particles [5].

The study of the possible role of microorganisms in PAHs degradation revealed that two main groups of microorganisms are involved in the oxidation and subsequent mineralization of these compounds: soil bacteria and white rot fungi. The degradation of PAHs is limited by their solubility [6] as soil bacteria were found to effectively degrade low molecular weight PAHs. White rot fungi can oxidize more condensed PAHs molecules with up to six aromatic rings, limit water solubility [7, 8], and decrease their toxicity [9].

Ligninolytic enzymes produced by basidiomycetes fungi such as lignin peroxidase (Lip), manganese peroxidase (Mnp), and laccase have been suggested to play a key role in lignin degradation [10]. It was assumed that PAHs and other organopollutant degradation can be catalyzed by the extracellular ligninolytic enzyme system of basidiomycetes fungi [11–13].

A major factor controlling the biodegradation of PAHs is their bioavailability to microbial degradation which may be limited because of low aqueous solubility of the contaminants and their sorption on to soil [14]. Surfactants can help by solubilization or emulsification, to release hydrocarbons sorbed to soil organic matter and increase the aqueous concentration of hydrophobic compounds, resulting in higher mass transfer rate [15]. Contradictory results are found in the literature about the effects of the addition of synthetic and biologically produced surfactants on PAHs biodegradation [16]. However, recent studies indicate that they can enhance hydrocarbon biodegradation by increasing microbial accessibility to insoluble substrate [17]. Several researchers have investigated the addition of biosurfactants to enhance biodegradation of hydrocarbons [18].

The integrated approach of using the knowledge on enzymology and bioinformatics such as protein–ligand docking tools offers a rapid means of identifying new potential targets for bioremediation. These in silico approaches are being evaluated in other fields of science such as medicine and drug discovery [19]. But studies related to screening targets for a pollutant for bioremediation are limited [20].

In the present study, the PAHs biodegradation capabilities of the five basidiomycetes' fungal pure cultures were compared to that of *Pseudomonas* isolate recovered from oil-spilled soil and to their corresponding cocultures. As the production of biosurfactants seems to be a prerequisite for the ability of the microorganism to grow on poorly soluble hydrocarbons, the capability of the biosurfactant produced by the basidiomycetes fungi and bacterial strains were studied. The in vivo results were compared with extent of PAH biodegradation by the ligninolytic enzymes using in silico approach involving the docking tool, FRED in Open Eye software.

Materials and Methods

Organisms

Five basidiomycetes' fungal cultures such as *Pycnoporus sanguineus* (MTCC-137), *Coriolus versicolor* (MTCC-138), *Pleurotus ostreatus* (MTCC-142), *Fomitopsis palustris* (MTCC-169), and *Daedalea elegans* (MTCC-1812) were obtained from the Institute of Microbial Technology, Chandigarh, India. The PAH biodegrading bacteria was isolated from oil-spilled soil, Oil Collection Tank, Vilangudi, Madurai, Tamil Nadu, India.

Media

All fungi were precultured on 2% malt extract agar for 14 days. The basal media used for both agar plates and liquid cultures consisted of 10 g l^{-1} glucose, 2 g l^{-1} KH_2PO_4, 0.5 g l^{-1} $MgSo_4 \cdot 7H_2O$, 0.1 g l^{-1} $CaCl_2$, 0.5 g l^{-1} ammonium tartarate, and 2.2 g l^{-1} 2,2-dimethyl succinate [21]. PAHs degradation was carried out using the liquid medium mentioned above.

The liquid mineral medium used for biosurfactant production consisted of 4 g l^{-1} Na_2HPO_4, 1.5 g l^{-1} KH_2PO_4, 1 g l^{-1} NH_4Cl, 0.2 g l^{-1} $MgSo_4$, 0.005 g l^{-1} iron ammonium citrate, 0.01 g l^{-1} $CaCl_2$, and 2% glucose [22].

Chemicals

Five PAHs, naphthalene, acenaphthene, fluorene, anthracene, and pyrene with the minimum purity of 98% as determined by high-pressure liquid chromatography (HPLC), were obtained from Sigma. The chemical formula and molecular weight (in parenthesis) of the above PAHs were as follows: $C_{10}H_8$ (128.18); $C_{12}H_{10}$ (154.21); $C_{13}H_{10}$ (166.22); $C_{14}H_{10}$ (178.20); $C_{16}H_{10}$ (202.26). All solvents were of HPLC grade, and all other chemicals were of the highest purity available and purchased from HiMedia, India.

Enrichment, Isolation, and Identification of PAH Biodegrading Bacteria

Enrichment and isolation of PAH-degrading bacteria from oil-spilled soil was carried out as described by the method of Boochan et al. [23]. The bacterium was identified based on its microscopic, morphological, and biochemical characters [24].

Enzymatic Activity of Basidiomycetes Fungi

Secretion of ligninolytic enzymes by the selected basidiomycetes fungi in liquid cultures was determined using 50 ml Ehrlenmeyer's flasks containing 15 ml of basal medium. Flasks were aseptically inoculated with three mycelial agar plugs (10 mm diameter) of 14-day-old fungal preculture on malt extract (2%). The flasks were incubated at 24°C in the dark. After 20 days of incubation, the entire flasks contents were harvested and the activity of ligninolytic enzymes in the broth was measured [25–27].

Biodegradation of PAHs

Ehrlenmeyer's flasks (50 ml) containing 15 ml of basal media were inoculated aseptically with monocultures and cocultures of three agar plugs (10 mm diameter) of active fungal

preculture and 100 μl of 24 h grown bacterial isolate. The cultures were pregrown as stationary culture for 3 days at 24°C in the dark after which the PAHs stock solutions were aseptically added. The PAHs stock solutions consisted of naphthalene, acenaphthene, fluorene, anthracene, and pyrene at a concentration of 1 mg/ml. Uninoculated flasks were treated as control. The whole flask contents were harvested on the 28th day after PAHs addition. The residual PAHs were extracted [28], and aliquots of 1 ml were transferred to HPLC vials.

Analytical Methods

HPLC (SHIMADZU, SPD-10 A VP) with silicon C_{18} column was used to separate and analyze PAHs under isocratic condition (solvent—acetonitrile:water=80:20 *v*/*v*; detection wavelength=254 nm).

One milliliter of the extracted samples was added to 5 ml of methanol, and from this, 20 μl was injected to the HPLC analyzer for the analysis of PAHs. (The concentration of each PAHs in 20 μl of the sample is 50 μg.)

The PAHs present in the culture liquid were identified by the comparison of retention time with authentic chemicals. Based on the remaining PAHs present in the sample, the percentage degradation of PAHs by the organisms was calculated.

Biosurfactant Production and Quantification

The biosurfactant-producing capabilities of the organisms in the plate were assessed according to the method followed by Mulligan et al. [29]. Organisms with high biosurfactant-producing potential were used for the production, quantification, and characterization of biosurfactants. Biosurfactant production by the organism was followed by the method described by Bodour and Miller [22] in duplicates and was extracted [30]. One set of extracted samples was dried using a dessicator and weighed. Another set of extracted samples was used for the estimation of biosurfactants using the oil spreading technique [31], emulsion test [32] and for the determination of biochemical composition of the biosurfactant such as protein [33] and carbohydrates. One percent SDS was used as the control.

In Silico Experiments

X-ray crystal structures of ligninolytic enzymes laccase (1 Gyc), manganese peroxidase (MNP), lignin peroxidase (ILLP) were collected from the Brookhaven Protein Data Bank in Pdb format. The structures of various PAHs were drawn in chemdraw (Version—Ultra vision—6.0, Cambridge Soft Corporation) and saved as MDL MOL file format. The enzyme–ligand docking was carried out using FRED (Version—VIDA—2.1.2, Open Eye structure software).The scoring function employed was Chem gauss 3.

Results and Discussion

The bacterium isolated from oil-spilled soil by enrichment culture was identified as *Pseudomonas* sp. based on its macroscopic, microscopic, and biochemical characteristics. All the five basidiomycetes fungi and *Pseudomonas* sp. were able to degrade the mixture of naphthalene, acenaphthene, fluorene, anthracene, and pyrene—although considerable differences in their abilities were observed.

The ligninolytic activities of the enzymes secreted by the fungi were determined [21, 34]. *P. ostreatus* and *C. versicolor* produced relatively high amounts of the enzyme laccase and Lip in the order of 2.0 and 2.2 U ml^{-1}, respectively. Both showed the highest Mnp activity (3 U ml^{-1}). Of the five basidiomycetes fungi used, *C. versicolor* showed higher amounts of ligninolytic activity in Lip and Mnp whereas *P. ostreatus* showed higher Mnp and laccase enzymatic activity (Table 1).

A substantial improvement in the rate of pyrene degradation by all the monocultures was seen, *Pseudomonas* sp. was found to be the most efficient strain in that it showed the highest 92.3% pyrene degradation but it could degrade only 28% and 24.4% of acenaphthene and fluorene, respectively. Similar results were observed by Yuan et al. [35] (Table 2). PAHs degradation by *Pseudomonas* has been reported in other species such as *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas vesicularis*, *Pseudomonas cepacia*, *Pseudomonas paucimobilis*, and other microorganisms such as *Acenitobacter calcoaceticus*, *Alcaligenes denitrificans*, *Mycobacterium* sp., *Rhodococcus* sp., *Corynebacterium renale*, *Moraxella* sp., *Bacillus cereus*, *Beijerinckia* sp., and *Sphingomonas.* sp. [36–38].

D. elegans and *F. palustris* were found to be most efficient strains as they degraded 35.8% and 31.7% of naphthalene and anthracene, respectively, in 28 days of incubation (Table 2). PAHs degradation by white rot fungi has been reported for the other genera such as *Phanerochaete* [39], *Pleurotus* [40], *Tyromyces* and *Gleophyllum* [41], *Coriopsis* [42], and *Stropharia* and *Trametes* [43]. Yuan et al. [35] observed that the PAHs-degrading microorganisms were ineffective in degrading anthracene. In our present study, similar results were observed using basidiomycetes fungi. Launen et al. [44] reported that microorganisms degrade the PAHs with the lower number of aromatic rings in the molecule. However, in the present work, pyrene with higher complexity was the most efficiently degraded PAH by most of the organisms used.

It has been suggested that the degradation of PAHs may be enhanced by the synergistic interaction between the inoculated wood rotting fungi and native microflora [45]. Lang et al. [18] have suggested that every fungal strain designated for bioremediation should be assessed for its degradation activity and survival in the presence of the competitive pressure from the native soil microflora. In our work, cocultures of a selected basidiomycetes fungus and *Pseudomonas* sp. isolated from oil-spilled soil were used. PAHs degradation by cocultures of white rot fungi–bacterial isolate has been reported by Lang et al., Boochan et al., and Yuan et al. [18, 23, 35]. Among the cocultures used in our work, *C. versicolor–Pseudomonas* sp. and *F. palustris–Pseudomonas* sp. cocultures efficiently degraded pyrene

Table 1 Ligninolytic activity of the selected basidiomycetes fungi.

Organism	Ligninolytic activity (U/ml)[a]		
	Lip	Mnp	Laccase
Pycnoporus sanguineus	1.3	2.0	1.0
Coriolus versicolor	2.2	3.0	1.8
Pleurotus ostreatus	2.0	3.0	2.0
Fomitopsis palustris	1.9	2.0	1.7
Daedalea elegans	2.1	2.0	1.8

[a] All values are the mean (n=3) with a standard deviation of <3%.

Table 2 PAHs biodegradation by bacterial and fungal monocultures.

Organism	PAH degradation (%)[a]					Reference
	Naphthalene	Acenaphthene	Fluorene	Anthracene	Pyrene	
Pseudomonas sp.	15.5	28.0	24.4	25.4	92.3	Present study
Pycnoporus sanguineus	12.0	7.0	17.6	15.6	4.4	Present study
Coriolus versicolor	27.4	2.0	23.0	22.4	42.0	Present study
Pleurotus ostreatus	29.4	20.6	20.6	19.0	32.0	Present study
Fomitopsis palustris	19.5	7.5	7.0	31.7	7.3	Present study
Daedalea elegans	35.8	5.9	5.9	2.4	26.1	Present study
Mixed culture	–	–	40.0	20.0	70.0	Yuan et al. [35]

[a] All values are the mean (n=3) with a standard deviation of <3%.

Table 3 PAHs biodegradation by bacterial–fungal cocultures.

Coculture	PAH degradation (%)[a]				
	Naphthalene	Acenaphthene	Fluorene	Anthracene	Pyrene
P. sanguineus–Pseudomonas sp.	13.5	29	24.2	11.4	17.4
C. versicolor–Pseudomonas sp.	15.5	27	24	25	93.7
P. ostreatus–Pseudomonas sp.	13	25	19	20	17
F. palustris–Pseudomonas sp.	13.1	16.3	16.3	12	93.7
D. elegans–Pseudomonas sp.	23	14.9	14.9	3.4	46.4

[a] All values are the mean (n=3) with a standard deviation of <3%.

Table 4 Quantification of biosurfactant produced by selected bacterial and basidiomycetes fungi.

Organism	Zone of hemolysis (cm)	Dry weight (g l^{-1})	Oil spreading technique[a] (mg/100 ml)	Emulsion index (%)
Pseudomonas sp.	0.6	1	24	117
C. versicolor	1.5	3	55	120
P. ostreatus	1.4	2.5	27	107
1% SDS	–	–	–	239

[a] Concentration of biosurfactant.

Table 5 Characterization of biosurfactants produced by selected bacterial and basidiomycetes fungi.

Organism	Sugar (g l^{-1})	Protein (g l^{-1})
Pseudomonas sp.	0.85	0.81
Coriolus versicolor	0.95	1.08
Pleurotus ostreatus	0.70	0.85

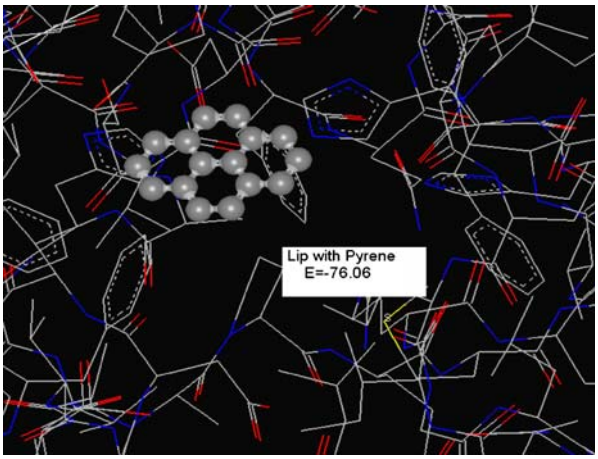

Fig. 1 In silico experiments: Lip with pyrene (E=−76.06)

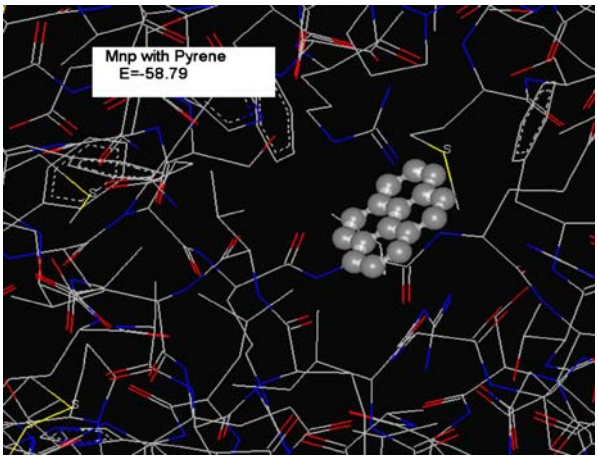

Fig. 2 In silico experiments: Mnp with pyrene (E=−58.79)

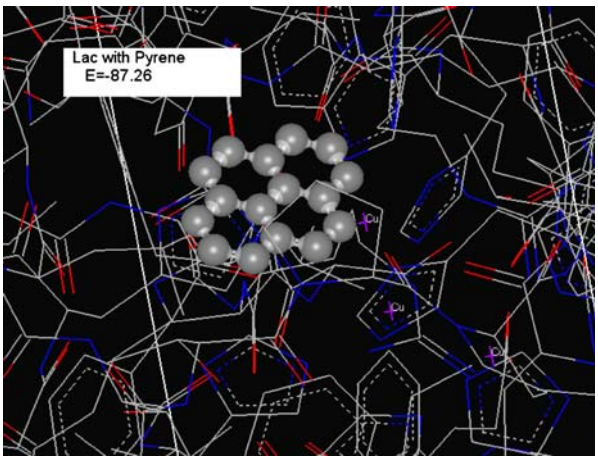

Fig. 3 In silico experiments: Lac with pyrene (E=−87.26)

up to a level of 93.7% (Table 3). Among the cocultures used, *C. versicolor* was found to be the most efficient strain in degrading all the PAHs except acenaphthene. In all the monocultures and cocultures treatments, pyrene was found to be maximum biodegraded in all culture conditions. The cocultures of *C. versicolor* and *F. palustris* were shown to improve the rates of degradation of pyrene (1.4%). The results are in agreement with the reports of Boochan et al. [23]. Our data has shown that defined bacterial–fungal cocultures can be used in the bioremediation of four-benzene ring PAHs such as pyrene.

Carillo et al. [46] found an association between hemolytic activity and surfactant production and recommended the use of the method for primary screening for biosurfactant activity. Following the method of Mulligan et al. [40] based on red blood cell lysis, the organisms such as *C. versicolor*, *P. ostreatus*, and *Pseudomonas* sp. were identified as potential biosurfactant producers. Yonebayashi et al. [47] also recommended the red blood cell lysis method as a simple and easy method to test for biosurfactant activity. The strains which displayed the highest biosurfactant productivity were selected for a more detailed analysis. The biosurfactant production by the selected organisms shows a considerable difference.

There are several compounds produced by microorganisms which can cause red blood lysis without having surface-active molecules. For this reason, many authors suggested that the method should be supported by other techniques based on surface activity measurements [48]. The biosurfactant activities of the selected organisms were tested by the following methods: hemolysis, dry weight, oil spreading technique, and emulsion test assay (Table 4). *C. versicolor* was found to be the most efficient strain in producing biosurfactants. The biochemical characterization of biosurfactants produced by the selected organisms suggests that it is a multicomponent surfactant consisting of protein and polysaccharides (Table 5). The exact reason for the production of biosurfactant by some microorganisms is unclear. However, biosurfactant-producing microbes are found in higher concentrations in hydrocarbon-contaminated soil [49]. Few researches who have looked for surfactant production by PAHs-degrading microorganisms has been unsuccessful [50, 51]. The influence of PAH biodegradation by biosurfactants produced by several microorganisms has been reported by several researchers [52–56].

By comparing the results of PAHs biodegradation by monocultures and cocultures with biosurfactant production, it is evident that the best PAHs-biodegrading organism such as *C. versicolor* is having the high biosurfactant activity. Carmichael and Pfander, Kanga et al., and Willumsen and Karlson [57–59] have suggested that the surfactant-producing bacterial strains and basidiomycetes fungi were able to solubilize PAHs with the help of biosurfactants. Our results also indicate that biosurfactants could mediate in PAHs biodegradation.

Selected ligninolytic enzymes (Lip, Mnp, laccase) and PAH ligands—naphthalene, acenaphthene, fluorene, anthracene, and pyrene—were docked using FRED in Open Eye

Table 6 Scoring function for docked complexes between enzymes of basidiomycetes fungi and PAHs.

PAHs	Total energy (kJ/mol)		
	Lip (*E*)	Mnp (*E*)	Laccase (*E*)
Acenaphthene	−60.86	−41.84	−68.33
Anthracene	−64.03	−52.29	−72.17
Fluorene	−63.28	−48.88	−71.06
Naphthalene	−49.85	−39.18	−62.22
Pyrene	−76.06	−58.79	−87.26

software (Figs. 1, 2, and 3). The in silico results revealed that the pyrene ligand docked with all three enzymes (Table 6). The order of interaction of various PAHs with various enzymes decreased in the order of pyrene>anthracene>fluorene>acenaphthene>naphthalene.

Among the five PAH ligands, maximum interaction occurred between pyrene and all three ligninolytic enzymes. These results lend support to our experimental observation showing that pyrene was degraded to the maximum extent by species such as *C. versicolor* and *P. ostreatus*. Suresh et al. [20] also suggested that the in silico approach to bioremediation would be helpful to find putative pollutants for other biodegradative enzymes.

Conclusion

The day by day increase in the release of PAHs threatens our ecofriendly environment and human resources; and so, in view of the above problem, our present work propose that the oil-spilled soil isolate such as *Pseudomonas* sp. is found to be the most efficient strain in the degradation of pyrene. The basidiomycetes fungi such as *C. versicolor* and *P. ostreatus* also showed comparably similar efficiency of pyrene biodegradation. The present work underscores the need for isolation of potential PAHs-degrading microorganism from naturally polluted environment colonized by native microflora. Significant increase in pyrene biodegradation was shown by the cocultures of *C. versicolor* and *F. palustris* with the *Pseudomonas* isolate. Furthermore, our results lend support for the positive influence of biosurfactants in PAHs biodegradation. Comparison of the in silico and in vivo results of our study might be a milestone in bioremediation research. This approach will improve our ability to predict the fate of PAHs compounds and lead to the development of more effective bioremediation strategies for the reclamation of the polluted environment.

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