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Molecular Characterization of *Pleurotus ostreatus* PO-3 involved in Mycoremediation of Benzo[*a*]Pyrene

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Abstract

Benzo[a]pyrene is a recalcitrant organic pollutant mainly because of the low water solubility which makes it unavailable for microbial degradation. Besides being ubiquitous in the environment, it is considered as a priority pollutant because of its carcinogenic, teratogenic and mutagenic effects. White-rot fungi such as Pleurotus ostreatus had been the organism of choice for the degradation of benzo[a]pyrene. The present study aims at the molecular identification of the selected fungal isolate by partial 18S rDNA sequencing and comparative evaluation of the benzo[a]pyrene biodegradation potential of both wild isolate and reference strain of Pleurotus ostreatus MTCC 142. Results indicated that the fungal isolate PO-3 was closely related to Pleurotus ostreatus strain Po-13 with 99% sequence similarity. The 703 bp 18S rDNA nucleotide sequence was provided a GenBank accession number KC986398. In terms of degradation potential, PO-3 isolate was a better degrader of 1µg/ml of benzo[a]pyrene. Level of degradation was 32% and 29% respectively for Pleurotus ostreatus isolate PO-3 and Pleurotus ostreatus MTCC 142. Thus based on the present finding, Pleurotus ostreatus PO-3 may prove to be a promising isolate for degradation of benzo[a]pyrene.

Keywords: Benzo[*a*]pyrene, *Pleurotus ostreatus*, degradation, 18S rDNA sequencing.

Introduction

Breakdown of natural polymers like lignin can be mediated by the ligninolytic enzymes of the white-rot fungi. Apart from finding their use in the removal of lignin from the lignocellulosic wastes, these ligninolytic enzymes of white-rot fungi are also reported to degrade different xenobiotic compounds including polycyclic aromatic hydrocarbons, polychlorinated biphenyls, synthetic dyes, etc[1].

Benzo[*a*]pyrene (B*a*P), a high molecular weight polycyclic aromatic hydrocarbon (HMW PAH) consists of five fused benzene rings. Since it behaves as a potent teratogen, mutagen and carcinogen, it is one of the sixteen PAHs specified as "priority pollutants" by the United States Environmental Protection Agency (US EPA)[2]. The relatively high octanol-water coefficient of B*a*P (K_{ow} of 6.04) makes it refractory to the soil and sediments, minimizes its partitioning into the water column and thus facilitates its unavailability for microbial degradation[3].

Several white-rot fungi can oxidize B*a*P and similar HMW PAHs with up to six aromatic rings[4]. Among the species tested so far, only a few are characterized by their strong competitive abilities towards indigenous microflora and seems to be promising. So far, most of the biodegradation experiments against B*a*P have involved basidiomycete fungi like *Phanerochaete chrysosporium*, *Trametes versicolor*, and especially *Pleurotus ostreatus*[5,6].

Morphological and biochemical distinctiveness of fungi are universally used for their identification, but differentiation of closely related cultures require extensive molecular techniques[7]. PCR amplification with universal primers targeted to conserved regions within the rRNA complex and subsequent DNA sequencing of the internal transcribed spacer (ITS) regions, shows a promising technique to identify a broad range of fungi to the species level[8]. The PCR primer sets routinely used for amplification of ITS regions and rDNA are known to be ITS1 and ITS4[9].

The objective of the study includes molecular identification of the selected isolate by partial 18S rDNA sequencing and comparative evaluation of the B*a*P biodegradation potential of both wild isolate and reference strain of *Pleurotus ostreatus* MTCC 142.

Material and Methods

Chemicals and reagents: Dehydrated media used during the course of the study were purchased from Himedia Laboratories Pvt. Limited (Mumbai, India). All the fine chemicals used were purchased from SRL Chemicals, India and were of the highest purity and analytical grade. HPLC grade B*a*P standard (98% pure) was procured from Spectrochem Pvt. Ltd., Mumbai, India. Pure distilled water was obtained with a Milli-Q system (Millipore, Tokyo, Japan).

Source of fungal cultures: Fruiting bodies of the Basidiomycete samples were collected from 3 different locations spread across Wayanad district, Kerala, India. Result from our previous study has shown that isolate PO-3 demonstrated the highest degradation, followed by PO-1 and PO-2. Hence isolate PO-3 was selected for further study[10]. Isolate PO-3 was maintained on malt extract agar. *Pleurotus ostreatus* MTCC 142 was obtained from Microbial Type Culture Collection (MTCC), Chandigarh, India. The fungus was propagated on glucose yeast extract agar.

Molecular identification of the selected isolate: The genomic DNA isolation, PCR amplification and partial 18S rDNA sequencing of the PO-3 isolate were performed at Chromous Biotech Pvt. Ltd., Bangalore, India.

Extraction of fungal genomic DNA: Two fungal plugs from 5 days old culture of the selected isolate (grown on malt extract agar) were inoculated into 20 ml of potato dextrose broth and incubated at 25°C and 150 rpm for 5 days. Following incubation, fungal biomass was filtered through Whatman No.1 filter paper and used for genomic DNA isolation using Fungal Genomic DNA Spin-50 isolation kit (Chromous Biotech Pvt. Ltd., Bangalore, India) according to manufacturer's instructions. The eluted DNA was used for PCR amplification.

PCR amplification: DNA amplification by polymerase chain reaction (PCR) was performed in a total volume of 100 µl. Each reaction mixture contained the following solutions: 1 µl template DNA, 400 ng forward 18S rDNA primer (5'-GTAGTCATATGCTTGTCTC-3'); 400 ng reverse 18S rDNA primer (5'-GAAACCTTGTTACGACTT-3'); 4 µl of dNTPs (2.5 mM each); 10 µl of Taq DNA polymerase assay buffer and 1 µl Taq DNA polymerase (3 U/µl) (Chromous Biotech Pvt. Ltd., Bangalore, India) and water was added up to 100 µl. The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed as follows: 5 min initial denaturation at 94°C, followed by 35 cycles that consisted of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and extension at 72°C for 1 min and a final extension of 5 min at 72°C. PCR products obtained were eluted from the gel using Gel Extraction Spin-50 kit (Chromous Biotech, Bangalore, India) according to the manufacturer's instructions. The PCR amplified product was detected by 1.2% agarose gel (with ethidium bromide) electrophoresis.

Partial 18S rDNA sequencing and analysis of sequenced data: Sequencing of the PCR amplified product was performed using Big Dye Terminator Version 3.1 cycles sequencing kit and ABI 3500 XL Genetic Analyzer (Applied Biosystems, USA). 10 µl of the sequencing reaction mixture contained 4 µl of Big Dye Terminator Ready Reaction Mix, 1 µl of rDNA amplification product (100 ng/µl), 2 µl primer (10 pmol/λ) and 3 µl Milli Q Water. The ABI 2720 Thermal Cycler (Applied Biosystems, USA) was programmed to perform initial denaturation at 96°C for 1 min, followed by 25 cycles that consisted of denaturation at 96°C for 10 sec, hybridization at 50°C for 5 sec and elongation at 60°C for 4 min. The resultant nucleotide sequence was analyzed using the software Seq Scape version 5.2. The fungal species was identified by comparing the sequence with known 18S ribosomal sequences in the NCBI database using BLASTN[11]. The phylogenetic tree was constructed based on fast minimum evolution method[12] using BLAST pairwise alignment between the query and the database sequences. The nucleotide sequence was submitted to GenBank database (NCBI, USA) under an accession number.

Comparison of B*a*P degradation potential: Acetone solution containing B*a*P (1µg/ml) was placed in two 250 ml conical flasks overnight for complete volatilization of the organic solvent. After the acetone had become completely volatile, 50 ml sterile liquid mineral salt medium was added to both conical flasks and two fungal plugs were inoculated in individual solutions under aseptic condition. Uninoculated flasks served as "controls". Incubation of all the flasks, including the controls was done at 28°C, 120 rpm in the dark under ambient air for 30 days, following which the degradation efficiency was determined.

Analytical methods: Extraction of residual B*a*P: Following incubation, the extraction of residual B*a*P was performed using a modified method of Capotorti *et al.*[13]. The concentrated extracts were subjected to High Performance Liquid Chromatography analysis.

High Performance Liquid Chromatography Analysis: The condensed samples were subjected to filtration using 0.25 µ nitrocellulose membrane filter. The working standard solution of B*a*P (concentration of 5 µg/ml) was prepared using 80:20 (v/v) of acetonitrile: water. 20 µl of the eluate containing 0.1 µg of the standard B*a*P was injected into the HPLC system (Waters, USA, model number- 2487, with Dual λ absorbance UV detector and binary pump system, model number-1525). A reverse phase, C-18 column (150 x 4.6 mm) was used. The mobile phase used was acetonitrile: water (80:20 v/v). The flow rate was maintained at 1 ml/min. The concentration of the B*a*P standard solution was determined at 254 nm. Area under the absorbance peak was used to estimate the percentage of degradation using a formula: $[(C_i-C_f)/C_i]*100$, where C_i is the initial concentration of B*a*P and C_f is the final concentration of B*a*P.

Statistical analysis: Experiment was done in triplicate and the data are graphically presented as the mean ± S.D. of triplicates (n = 3).

Results and Discussion

Chemical oxidation, photolysis and bioremediation are methods commonly employed for the removal of highly recalcitrant compounds from contaminated sites. However, among all these clean up strategies, bioremediation proves to be advantageous as it is less expensive and promotes the enzymatic products or

microbial growth necessary to transform the noxious pollutants to nontoxic end products[14].

Bioremediation is defined as a controlled or spontaneous process in which microbiological processes are used to degrade or transform contaminants to less toxic or nontoxic forms, thereby mitigating or eliminating environmental contamination[15]. Mycoremediation refers to fungal degradation or transformation of hazardous organic contaminants to less toxic compounds. Fungi display several advantages such as penetration of the hyphae and secretion of oxidative enzymes in the polluted sites[16].

Molecular identification of fungal isolate PO-3: Partial 18S rDNA sequencing was used for the molecular identification of the fungal isolate PO-3. The PCR amplicon had an apparent size of 1.5-1.8 kb, as shown in figure-1. Following PCR amplification, the obtained 703 bp 18S rDNA nucleotide sequence was compared with available 18S ribosomal sequences in the NCBI database using BLASTN. The PO-3 isolate has been enrolled into a cluster containing *Pleurotus* sp. and exhibited high sequence similarity (~99%) to that of *Pleurotus ostreatus* strain Po-13. Hence it was designated as *Pleurotus ostreatus* PO-3, figure-2 shows the submitted nucleotide sequence that was provided a GenBank accession number KC986398. Based on 18S rDNA sequences, a fast minimum evolution tree revealed that the isolate PO-3 shares a same clade with *Pleurotus ostreatus* and occupies a distinct phylogenetic position within the representative members of the genus *Pleurotus,* as illustrated in figure-3.

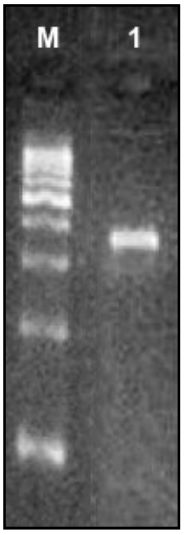

Figure-1
Agarose gel analysis of PCR amplification product using universal 18S rDNA primers, Lane M, 500 bp DNA molecular size marker; Lane 1, fungal isolate PO-3

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  1 tagtttattt gatggtacct tgctacatgg gataactgtg gtaattctag agctaataca
 61 tgcaatcaag ccccgacttc tggaaggggt gtatttatta gataaaaaac caacgcggct
121 cgccgcttcc cttggtgatt cataataact tctcgaatcg catggccttg tgccggcgat
181 gcttcattca aatatctgcc ctatcaactt tcgatggtag gatagaaggc ctaccatggt
241 ttcaacgggt aacggggaat aagggttcga ttccggagag ggagcctgag aaacggctac
301 cacatccaag gaaggcagca ggcgccgcaa attacccaat cccgacacgg ggaggtagtg
361 acaataaata acaatatagg gctcttttgg gtcttataat tggaatgagt acaatttaaa
421 tccctcaacg aggaacaatt ggagggcaag tctggtgcca gcagccgcgg taattccagc
481 tccaatagcg tatattaaag ttgttgcagt taaaaagctc gtagttgaac ttcagacctg
541 gctgggcggt ccgcttaacg gcgtgtactg tctggctggg ccttacctct tggtgagccg
601 gcgtgccctt tattggtgtg cgttggggaa ccaggacttt taccttgaga aaattagagt
661 gttcaaagca ggcctgtgcc tgaatacatt agcatggaat aat
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Figure-2
The 703 bp 18S rDNA nucleotide sequence of fungal isolate PO-3

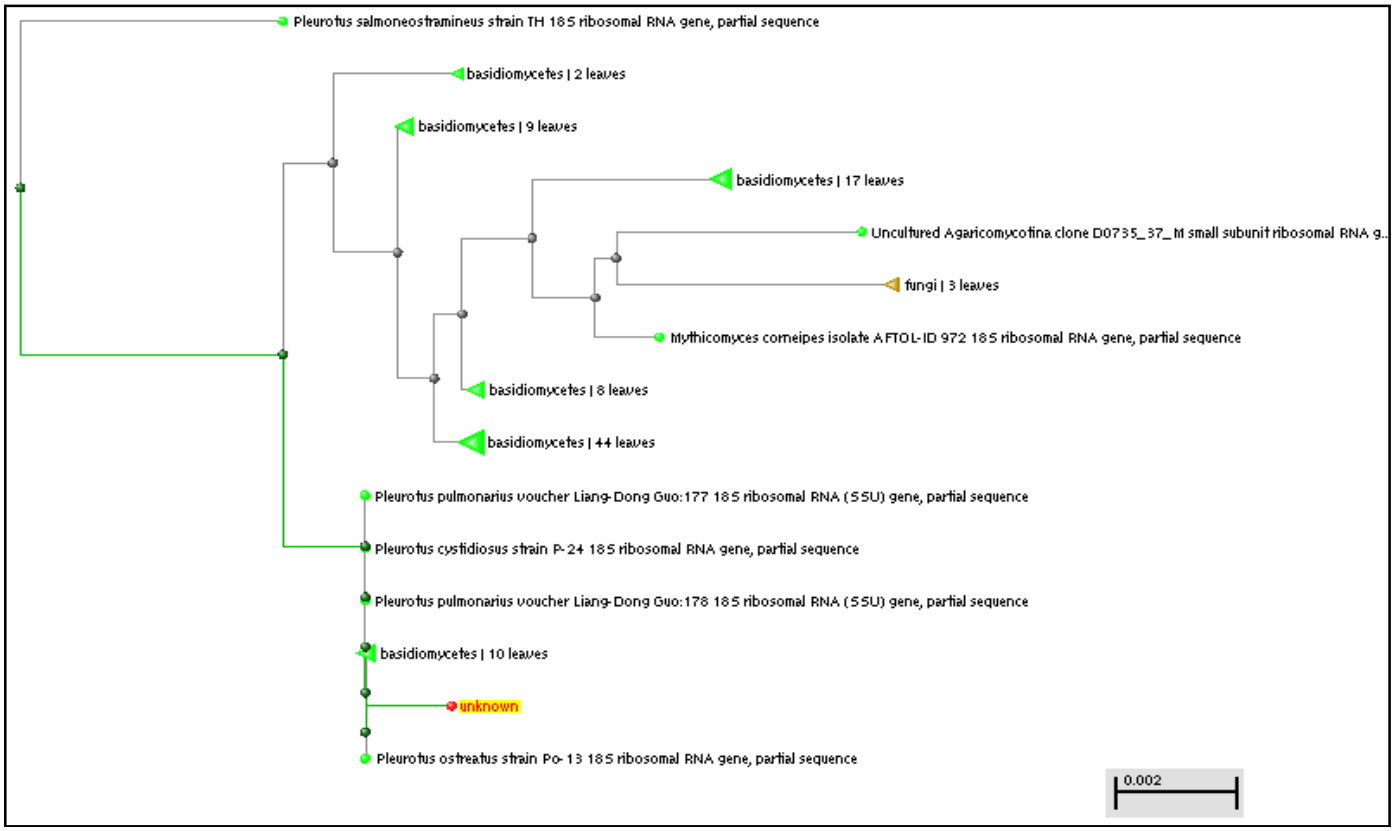

Figure-3
Phylogenetic tree showing the relationship between the fungal isolate PO-3 (unknown) and related basidiomycetes from NCBI database based upon fast minimum evolution analysis of partial 18S rDNA sequences

Analysis of morphological characters had been the basis of most taxomonic and phylogenetic studies on Basidiomycota. However, in recent time molecular techniques like RFLP studies and amplification of nuclear sequences by PCR are often used to establish the phylogenetic relationships among species in several genera of Basidiomycota[17].

Earlier, in order to determine phylogenetic relationship of strains of *Pleurotus* and allied genera, partial 18S ribosomal RNA gene and internal transcribed spacer region (ITS) were amplified by polymerase chain reaction and then sequenced. 383 bases of 1 SSrDNA and 179 bases of ITS-1 were compared and phylogenetic trees of *Pleurotus* were drawn. The 1 SSrDNA was very conservative, and similarities of 1 SSrDNA among all species examined were more than 90%. The phylogenetic tree showed *Pleurotus* was separated from other genera examined. In addition, it was shown that *Pleurotus tuberregium* (Fr.) Singer was distantly related to *Lentinus* and *Panus*. In the ITS-1 there were many insertions and deletions. The ITS-1 of *P. pulmonarius* was very close to that of *P. ostreatus*[18].

Comparison of B*a*P degradation potential: The extent of biodegradation of PAHs is highly variable and does not depend only on PAH structure, but also on the physicochemical parameters of the site as well as the number and types of microorganism present[19].

P. ostreatus differs from *P. chrysosporium* in the fact that the ligninolytic enzyme machinery of *P. ostreatus* consists of laccase and manganese peroxidase and has good ability to colonize soil and remain unaffected by the presence of indigenous microflora[20].

While testing the ability of the isolate *P. ostreatus* PO-3 and *P. ostreatus* MTCC 142 to degrade 1µg/ml of B*a*P under liquid condition, it was found that isolate *P. ostreatus* PO-3 has better degradation potential than *P. ostreatus* MTCC 142. In the presence of the PAH, the biomass formation by the PO-3 isolate was higher than that by *P. ostreatus* MTCC 142. The ability of the fungus to degrade B*a*P is related to its ability to produce laccase and similar enzymes. *P. ostreatus* PO-3 isolate could degrade B*a*P better than *P. ostreatus* MTCC 142 possibly due to its ability to synthesize higher titers of laccase and manganese peroxidase. Extent of degradation were 32% and 29% and biomass formation were 51 and 43 mg/50 ml of media respectively for *Pleurotus ostreatus* isolate PO-3 and *Pleurotus ostreatus* MTCC 142, as shown in figure 4. Similarly, a native isolate of *P. ostreatus* HP-1 (Genbank Accession No. EU420068) was found to have an excellent laccase producing ability[21].

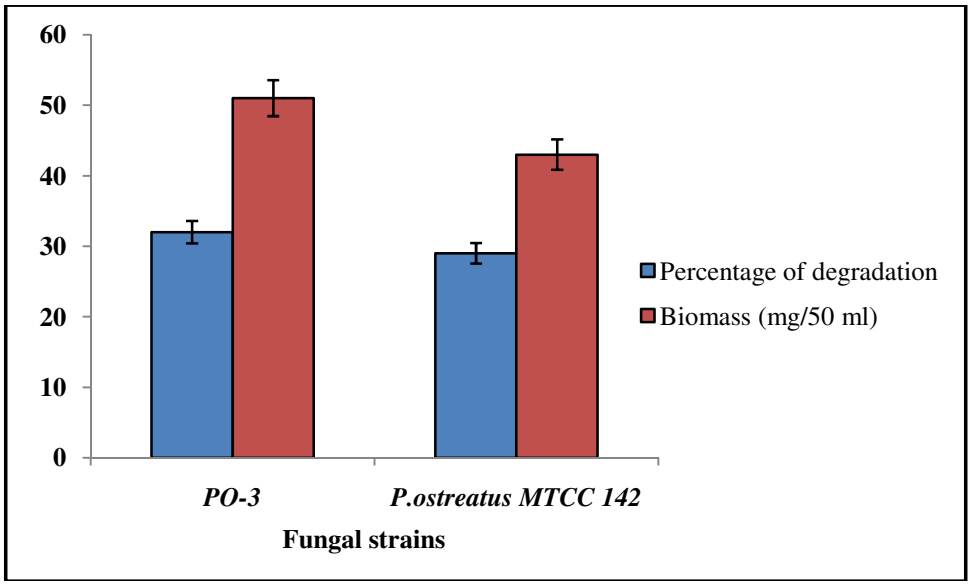

Figure-4
B*a*P degradation pattern and fungal biomass production by isolate PO-3 and *P. ostreatus* MTCC 142. Data represent mean ± S.D. (n=3)

In an attempt to study the enzyme activity against residual B*a*P, several peroxidases and oxygenases such as manganese peroxidase, lignin peroxidase, laccase, 1, 2-dioxygenase and 2, 3-dioxygenase were detected in the culture of *Polyporus* sp. S133. Laccase and 1,2-dioxygenase activities and B*a*P degradation by *Polyporus* sp. S133 were very similar. Both these enzyme activities increased rapidly and then decreased within 30 days of incubation, which was parallel to the rapid degradation period of 30 days incubation. It was evident that enzyme production during the B*a*P degradation by *Polyporus* sp. S133 can be correlated to the fungal growth[22].

Conclusion

Molecular identification technique like 18S rDNA sequencing was carried on for the potent isolate and a fast minimum evolution tree revealed that the isolate PO-3 shares a 99% sequence similarity to that of *Pleurotus ostreatus* strain Po-13.The wild isolate *P. ostreatus* PO-3 had better potential for degrading B*a*P in comparison to *P. ostreatus* MTCC 142.

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