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Fungi with high ability to crunch multiple Polycyclic Aromatic Hydrocarbons (PAHs) from the pelagic sediments of Gulfs of Gujarat

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ABSTRACT

Marine ecosystem harbors diverse microbial diversity adapted to varied environmental conditions and stress. Gujarat possesses a wide coastline with unique and diverse niche in its two Gulfs. PAHs enter marine environments through various anthropogenic discharges and act as a threat to environment due to their xenobiotic nature. In the present study, sediment cores were collected across 4 coordinates, each from Gulf of Kutch and Khambhat; while one from Arabian sea. These samples were enriched for fungal growth in basal medium supplemented with naphthalene, pyrene, phenanthrene, anthracene and fluoranthene. Eight isolates were obtained from 3 samples and checked for tolerance against 5 PAHs followed by assessment of their biodegradation ability. *Penicillium ilerdanum* NPDF1239-K3-F21 and *Aspergillus versicolor* NPDF190-C1-26 showed >75% ability to degrade multiple PAHs. The results reveal the potential of fungal isolates from pelagic sediment for further in situ optimization and application in PAH removal from contaminated soil and sediment.

1. Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are one of the major xenobiotic compounds consisting of two or more benzene rings fused together in linear, angular or grouped forms. PAHs are widespread environmental pollutants generated due to natural sources and anthropogenic activities. There is a critical need to develop means for their remediation owing to their deleterious properties (Di Toro and McGrath, 2000; Velmurugan et al., 2017).

PAHs are released into the environment due to various activities such as oil spills, disposal of petroleum products, combustion of fossil fuels, burning of agricultural wastes and are thus a matter of environmental concern. PAHs in marine environments can be found in deep-sea sediments where they are stored for longer periods due to their high hydrophobicity and low volatility (Bouloubassi et al., 2006; Lang et al., 2015).

Due to lack of efficient technologies, bioremediation stands out as a promising technology for removing these hazardous pollutants, in which microorganisms are being intensively investigated for this purpose as they are safe, eco-friendly and economic. In similar context, several bacteria, fungi (both filamentous forms and yeasts) and algae from different niche have been explored for their PAH biodegradation ability (Chen et al., 2011; Huang et al., 2010; Márquez-Rocha et al., 2000). However, exploration of the microbial communities from marine ecosystems, with special focus on fungi as potential candidates for complete degradation of these pollutants is still one of the thrust research areas (Jones, 2011). Marine environment as a source for fungal isolates has been accepted as a potential alternative to plant derived bioremediation agents (Passarini et al., 2011).

The present study aimed at exploring the hitherto less studied pelagic sediments from different locations of the two Gulfs of Gujarat, which are well known for their unique sedimentation process and discharge rates (Deshkar et al., 2010). Nine deep sediment cores were collected from 4 different locations each in the Gulf of Kutch and Gulf of Cambay/Khambhat along with one location from the Arabian sea. The collected sediment samples were used for screening and isolation of marine fungi for PAH tolerance and degradation potential by enrichment approach. The isolates were assessed for their growth rate on five PAHs and further checked for *in-vitro* PAH degradation potential. The isolates were microscopically identified and taxonomy was confirmed by sequencing

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of the ITS region. The culture-based results were also correlated with the metagenomic analysis from the same sediment.

2. Materials and methods

2.1. Sample collection

Nine sediment samples GOCS1-4, GOKS1-4 and A (Supplementary material S1) were collected by Small Free Fall Corer (#13.570, KC Denmark) as described earlier (Mootapally et al., 2019; Nathani et al., 2019) and the sediments were divided into sterile containers for DNA isolation and culturing approach as described in the study design (Fig. 1).

2.2. Isolation of marine fungi

2.2.1. Enrichment in media with PAH

Enrichment of marine fungi tolerant to PAH was performed using Bushnell Haas Broth (BH), supplemented with 100 ppm (0.001 g in 10 ml acetone) of five PAHs (Naphthalene, Phenanthrene, Anthracene, Pyrene and Fluoranthene) respectively. Five gram of each of the nine sediment sample was individually suspended into 10 ml of distilled water. The sediment suspension was kept overnight on shaker and allowed to settle for 1 h before inoculation of 2 ml supernatant into the enrichment medium and incubated at 20 °C for 7 days.

2.2.2. Fungal isolation and Morphological studies

Fungal isolation was carried out by serial dilution (10^{-1} to 10^{-4}) from the 7-day old broth. 100 μl of each dilution was spread on PDA (Potato Dextrose Agar) plates containing antibiotic Erythromycin (0.0015 g/100 ml) and plates were incubated at 20 °C for five days. Isolates were further sub cultured and observed for microscopic and morphological features.

2.3. Molecular identification

Eight distinct fungal isolates were obtained from three out of the nine sediment samples viz., GOCS1, GOCS4 and GOKS3 and these were proceeded for molecular identification. Genomic DNA from the pure cultures was extracted by DNeasy Plant Mini Kit (Qiagen, Germany). After DNA extraction, the ITS region was amplified with the primer pair ITS1 and ITS4 (Eurofins Genomics). DNA sequencing was performed using the BigDye® Terminator Cycle Sequencing kit v.3.1 (Life Technologies) according to the manufacturer's protocol (Eurofins Genomics). Forward and reverse sequences were aligned into contig by BioEdit v7.1.3. The contigs were subjected to BLASTn against the nr/nt database for homology search.

2.4. Metagenomic analysis of sediment samples

2.4.1. Screening of PAH degrading genes and pathways

Shotgun metagenomics of the 9 samples was performed using Illumina HiSeq 4000 and 101 × 2 paired end chemistry. The raw data output had been already summarized in our earlier study on the ARG profiling from the data (Mootapally et al., 2019; Nathani et al., 2019). The data from all the studied samples was uploaded on EBI (Mitchell

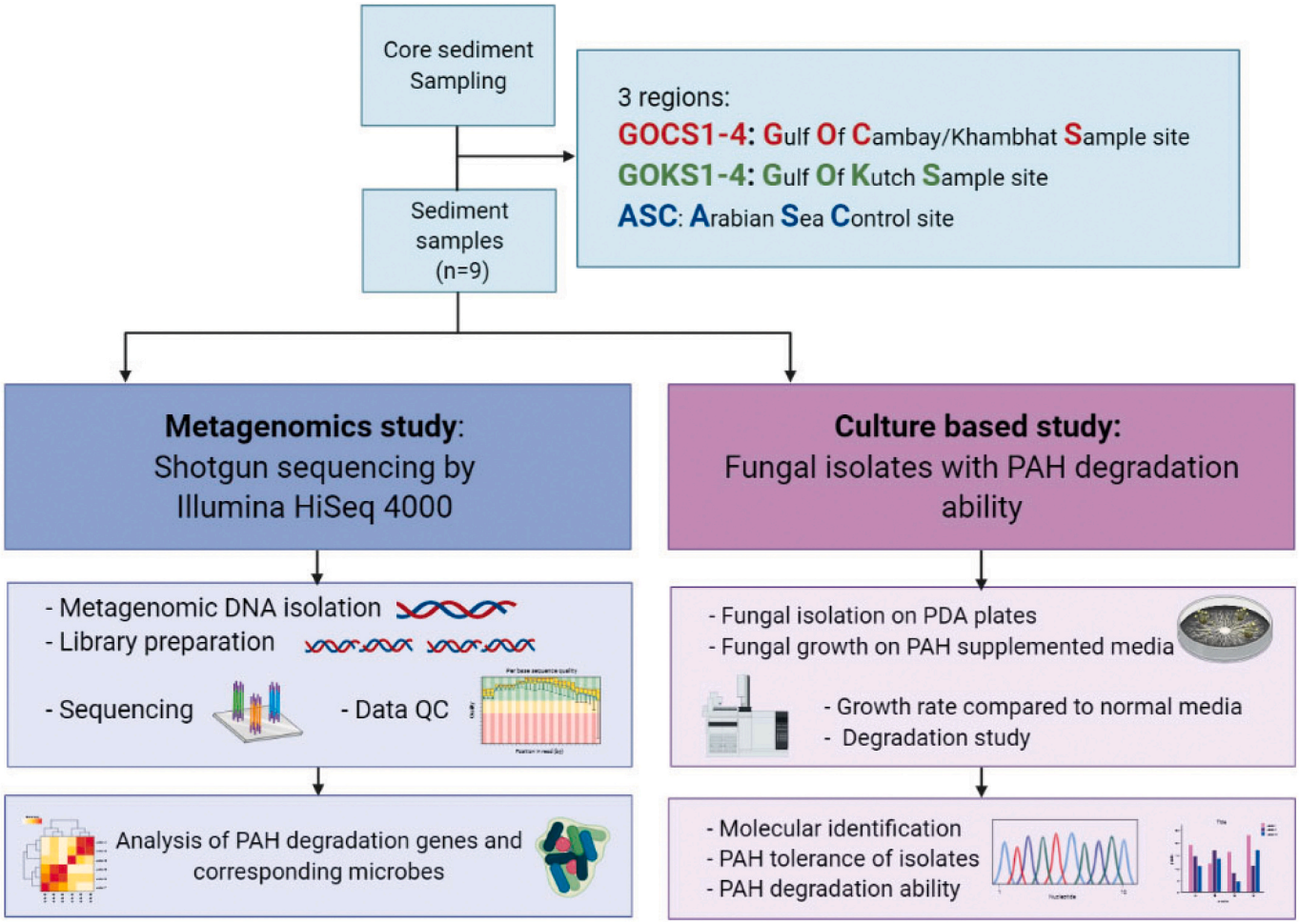

Fig. 1. Skeleton of experimental workflow of this study.

et al., 2020) and MG-RAST for functional and taxonomic overview (Meyer et al., 2008). The overall fungal diversity of the samples was observed by taxonomic analysis against the SSU database as predicted by MGnify, EBI. Further, functional annotation was performed using the subsystem database under default parameters in MG-RAST to screen the Coding Sequences (CDS) related to PAH degradation pathways and the observed genes were mapped to KEGG using KEGG Mapper v 3.1. The PAH degrading genes were filtered and taxa corresponding to them were assessed using the RDP database with default parameters in MG-RAST.

2.5. Growth rate study

To study the growth rate, the obtained isolates were grown in PDA plates as a control. For comparison against the utilization of the PAH, fungi were grown in plate with five different PAH on a BH agar plate. 5 μl inoculant of equal OD was plated onto PDA and BH plates and the plates were incubated for 5 days at 20 °C for sufficient growth. Fungal growth rate was calculated from the radial mycelial growth record, using following equation (Argumedo-Delira et al., 2012):

$$\textbf{\textit{Fungal Growth}}\ \textit{Rate} = \frac{\textbf{\textit{Diameter on BH Plate}}\ \textit{with}\ \textbf{\textit{PAH}}}{\textbf{\textit{Diameter on PDA plate}}} \times \mathbf{100}$$

2.6. PAH degradation study using GC–MS

Fungal isolates were inoculated in 100 ml Bushnell Haas broth supplemented with mixture of four PAHs (100 ppm each). Naphthalene was not considered for GC–MS study due to its highly volatile property. The flasks were incubated at 20 °C under 120 rpm rotation. Samples were harvested after 21 days for PAH extraction (Dudhagara et al., 2016). PAHs from medium were extracted using diethylether:acetone (2:1 *v*/v) followed by vigorous shaking on a reciprocating shaker at 150 rpm. After mixing, the samples were filtered through 0.2 μm nylon membrane filter for removal of fungal spores and PAHs were obtained from the organic phase by separating funnel. Samples were allowed to air dry up to 2 ml and processed for GC–MS analysis. For the standard, un-inoculated flasks with same concentrations was maintained for 21 days and PAH were extracted as above. Residual PAHs were estimated using GC–MS (COE, NFDD, Rajkot). The program included hold at 60 °C for 1 min, increased by 10 °C min-1 up to 160 °C then 10 min hold followed by further increase up to 280 °C at a rate of 5 °C min-1 hold for 10 min. For percent degradation, the peak area ratios of the samples to the internal standard were computed as a function of the concentration.

2.7. Data availability

The sequences are available in GenBank under the accession numbers: MH024085-88, MH025365-66, MH025368-69. The raw reads are submitted to EBI under the Bioproject Accession numbers: PRJEB26614 and PRJEB26615. The MG-RAST job IDs are as below: 328946, 331505, 33007, 331092, 329662, 330498, 330939, 331871 and 333033.

3. Results

3.1. Marine fungi isolation, morphological studies and molecular identification

We observed morphologically distinct eight fungi, which were denoted by Lab IDs as given in Table 1. These were observed to have distinct hyphae structures when observed for microscopic features. These were further identified by molecular ITS based sequencing (Table 1).

3.2. Metagenomic analysis of sediment samples

The shotgun metagenomic data of samples submitted under the MG-RAST (Data under private as on date), revealed the presence of genes involved in various aromatic degradation pathways, and all the genes required for complete Naphthalene degradation pathway were found to be encoded by the microbes in the studied samples as shown in Fig. 2a. Other predicted genes in all aromatic utilizing pathways were also assessed (Fig. 2b) for the genes encoding enzymes involved in their degradation.

Further, assessment of the corresponding microbial taxa involved in encoding the potential genes for degradation revealed the presence of major bacterial species and *Ascomycota* as the most abundant among the fungal taxa (Fig. 3). Similar observations were also reflected in the culturing based approach in the study wherein major fungal isolates obtained belonged to the same taxa. The overall fungal diversity in all the 9 samples as observed using SSU database is described in the Supplementary material S2.

3.3. Growth rate of the isolated marine fungi

Growth rate was calculated by comparing fungal growth on PDA (control) with PAH supplemented BH plate. All fungi grew in the presence of naphthalene. However, fungal growth rates were lower in presence of phenanthrene compared to other PAH indicating greater growth inhibition capacity compared to the control group. In absence of PAHs, no fungal growth was observed within 48 h. For naphthalene, the highest growth rate was shown by isolate F20 viz., 94.7% while the lowest growth rate was observed for the fungi with Lab ID F8B viz.,

Table 1
Morphological characteristics of the isolated marine fungi.

Fungi	Lab ID	Sample site	Shape	Appearance	Color	Hyphae	Molecular identification
1	F8A	GOKS3	Circular; Irregular	Granular	Green; Border greenish white	Sporangia; Not dense; Long	*Aspergillus stellatus* strain NPDF1239-K3-F8A
2	F21		Circular; Lobbed	Granular	Green colony; orange green border	Not dense; Branched; small	*Penicillium ilerdanum* strain NPDF1239-K3-F21
3	F8B		Irregular	Granular	Black with white margins; Pigmentation	Sporangium; Long; Not dense	*Aspergillus stellifer* strain NPDF1239-K3-F8B
4	F20		Circular	Cottony	White color; Back side orange white	Branched; Very thin; Small size; Not dense	*Sarocladium implicatum* strain NPDF1239-K3-F20
5	F24	GOCS4	Irregular	Granular	Grey color; Back side brown; White border	Not dense; Thick	*Aspergillus sp*. NPDF190-C4-24B
6	F25	GOCS1	Round; Lobbed	Granular	White colony	Dense; Branched; Thin; Small	*Engyodontium sp*. NPDF190-C4-25
7	F26		Round; Lobbed	Granular	Light brown; Backside brown; Brown liquid producing from middle part of the body	Not dense; Long; Thick; Engiospore	*Aspergillus versicolor* strain NPDF190-C1-26
8	F28		Irregular; Lobbed	Slightly hairy	Light brown; White border	Dense; Conidiospore; Medium	*Aspergillus nidulans* strain NPDF190-C1-28

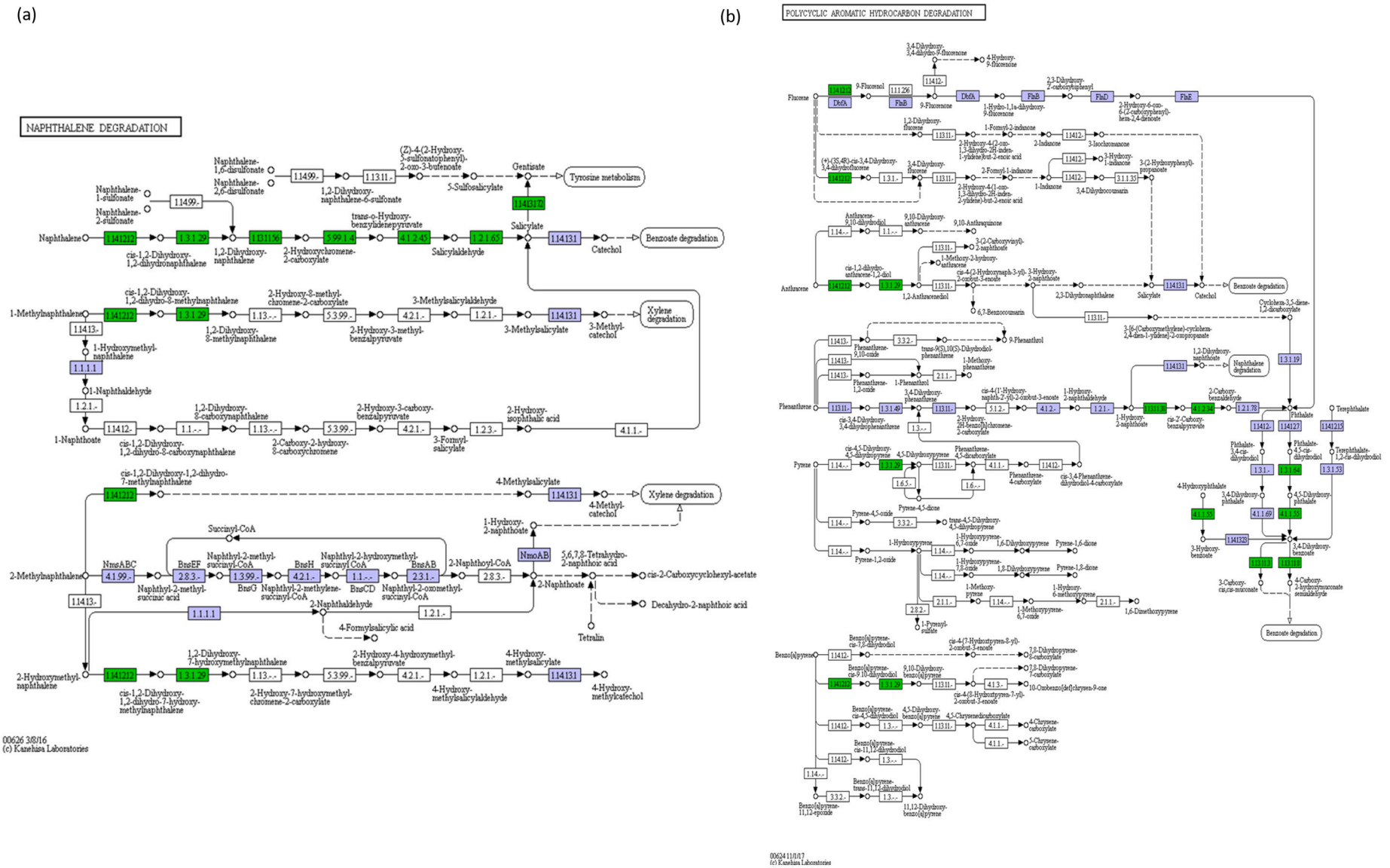

Fig. 2. Genes predicted in the samples for A) Naphthalene degradation and B) Polycyclic aromatic degradation pathways. Genes predicted in the samples are represented in green and purple indicate presence of putative partial gene sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

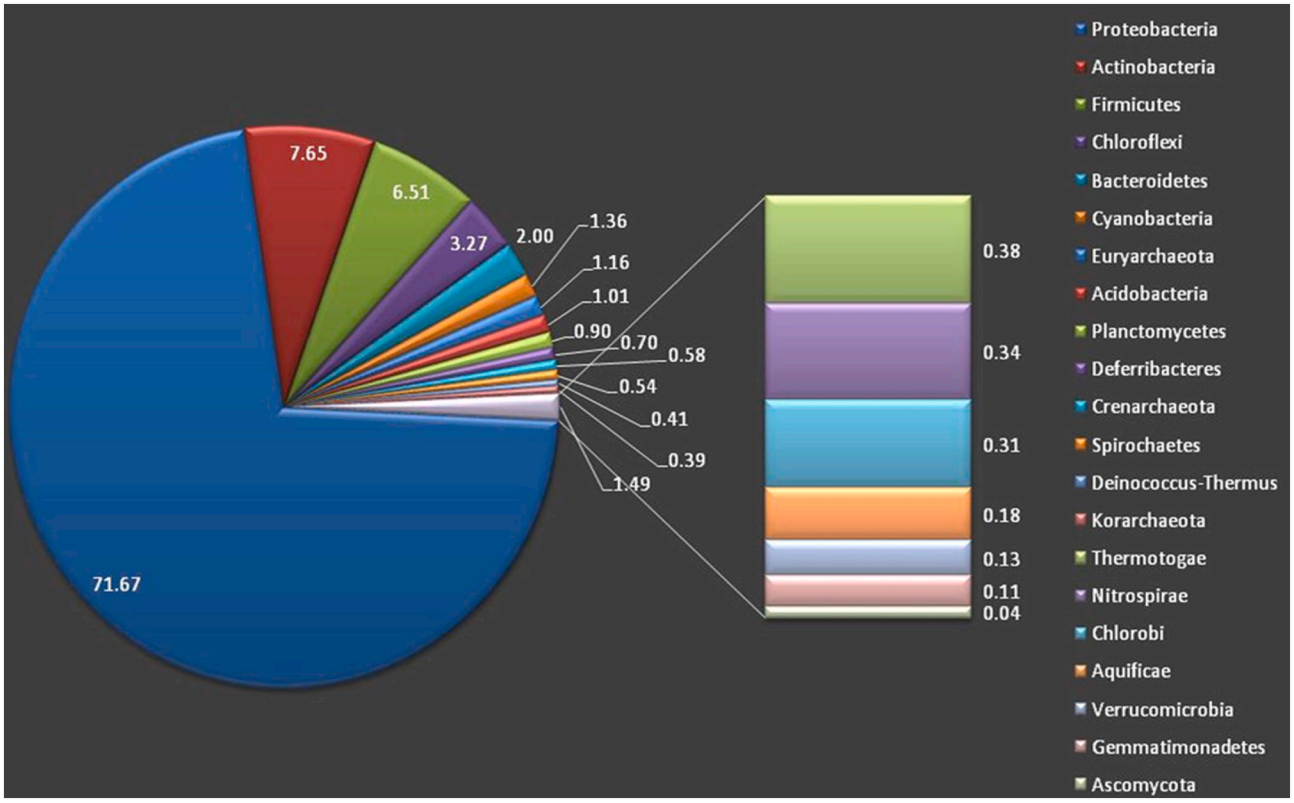

Fig. 3. Abundance of taxa encoding the genes for polycyclic aromatic degradation pathways.

26.92% (Fig. 4). The results indicated that the tested fungi had different responses to the presence of different PAHs. F20 showed highest growth rate in all the PAHs with highest growth towards Anthracene (336.84%). Isolate F24 had the lowest growth rate in presence of phenanthrene, fluoranthene and pyrene, whereas isolate F8A had lowest growth rate in anthracene supplemented media. Isolate F26 showed meager growth when it was exposed to fluoranthene Fig. 4.

3.4. PAH degradation by the fungal isolates as studied using GC–MS profiling

Based on the GC–MS result profile, it was observed that though *Sarocladium implicatum* strain NPDF1239-K3-F20 was most tolerant to 4 PAHs as revealed by the growth rate results, its degradation potential was limited (Fig. 5). The three isolates viz., F24, F25 and F28 from GOCS1 and GOCS4 locations across the Gulf of Khambhat did not show any significant change in the peak area as compared to the standard control and hence are not discussed further. While *Penicillium ilerdanum* strain NPDF1239-K3-F21 and *Aspergillus versicolor* strain NPDF190-C1-26 showed the maximum degradation >75% for all the PAHs under study (Fig. 5). The results revealed that the potential microbes were from the GOKS3 and GOCS1 sediment samples, which were having industrial activities at the shore. These activity discharges may have led to accumulation of complex compounds in the deeper sediments along the long run of time. However, there were fewer isolates from the GOCS4 sample, which leads to prediction that the activities in the Alang region are not leading to PAH deposits in the deeper sediments or there might be unculturable microbial load throughout the region that converts the complex compounds to simpler near the shore area itself.

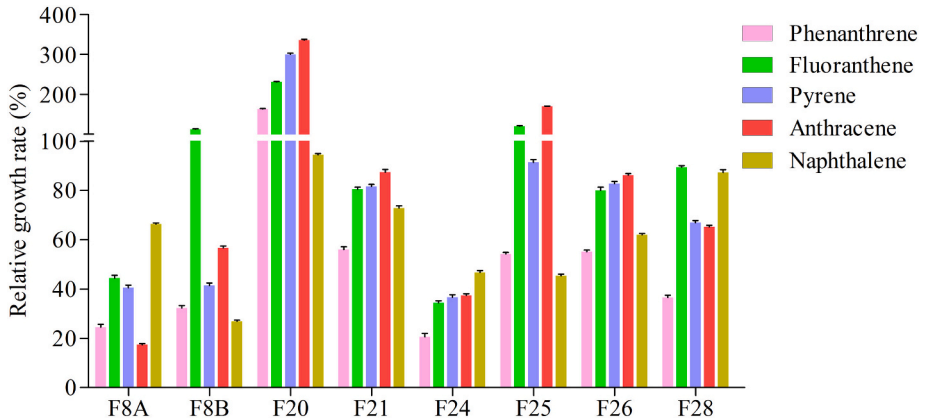

Fig. 4. Percent growth rate of the eight fungal isolates on the studied five PAHs against that on the control plate. Values are mean of triplicates and error bars represent SEM.

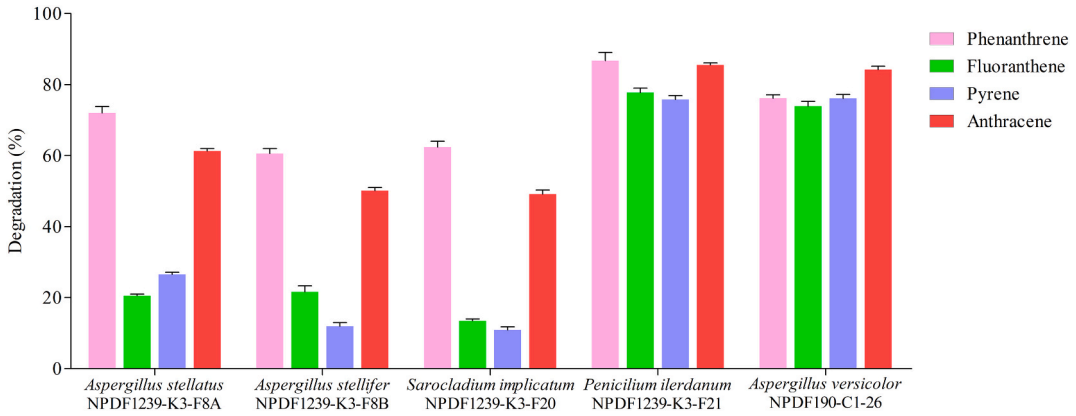

Fig. 5. Percent degradation of four PAHs by five fungal isolates as revealed by GC–MS profile. (Naphthalene was not considered due to its high volatility). Values are mean of duplicates and error bars represent SEM.

4. Discussion

Despite several cultural and molecular based studies on microbial degradation of PAHs, little is reported on biodegradation of environmental pollutants by fungi from the marine origin, especially from the Indian EEZ (Vieira et al., 2018). Fungi from the marine sources have been well characterized for their ability to degrade pollutants including polycyclic aromatic hydrocarbons (PAHs) (Passarini et al., 2011). The PAHs are commonly found in all the environmental areas including air, water, soil and sediments as they are released into them by various anthropogenic and non - anthropogenic sources (Cerniglia, 1992; Sutherland, 1992; Zang et al., 2007). The 4 and 5 ringed PAHs are known to cause potential health risk as they lead to toxicity and mutagenicity, even resulting into cancer (Baborová et al., 2006). Thus, it is necessary to remediate the PAH compound from environment. In context to this, our study showed potential fungal isolates that are active at different levels against 5 out of 16 United States Environmental Protection Agency (USEPA) listed PAHs as priority pollutants (Agrawal et al., 2018; Jin et al., 2007; Puglisi et al., 2007; Wu et al., 2016).

The use of marine-derived fungi for the bioremediation of polluted marine or other environments is preferred due to their tolerance to extreme conditions such as high salinity, broad pH and temperature ranges. Earlier studies had reported marine Cnidarian with regard to the potential to degrade the PAH compounds pyrene and benzo[*a*]pyrene (Passarini et al., 2011). As reported earlier (Mootapally et al., 2019; Nathani et al., 2019), the pH of the sediment samples in the shore area have been reported in the similar range to be slightly alkaline. Observed value is similar to previous reports of sediments with PAH contamination (Dudhagara et al., 2016; Makkar and Rockne, 2003; Mrozik and Piotrowska-Seget, 2010)

Along with culturing approach, the study on the metabolic pathways involved in aromatic compound degradation allows us to get insight into the probable enzymes and intermediates that would be formed. Similar search was performed from the metagenomics data of the study samples and compared to the obtained culturing results for fungal taxonomy and the PAH degradation pathways. The comparison showed to be in accordance to each other. Among the isolated fungi, majority were falling under the *Ascomycota* lineage. *Ascomycetes* have been earlier reported to be promising candidates for bioremediation, and are known for performing the initial oxidative conversion of PAH aromatic rings by use of the intracellular monooxygenase enzyme. Further they were observed to act by epoxide hydrolases in presence of phenol to form conjugates like glucuronide, glycosides and sulfates (Hilton Marcelo de Lima et al., 2017)

The tolerance to PAHs were also assessed and the highest rate of fungal growth inhibition was observed by phenanthrene, which could be attributed to its higher toxicity. Earlier studies have also evaluated tolerance of estuarine sediment isolated filamentous fungi against phenanthrene and pyrene, which showed similar results (Hilton Marcelo de Lima et al., 2017). Microbial study from contaminated sites is gaining more interest for bioremediation research to improve the impacted areas. These sites act as a selective reservoir for the pollutant adapted microorganisms and thus provide ample variety of gene pool for the purpose of biodegradation (Hilton Marcelo de Lima et al., 2017). Similarly, in the current study, the marine derived fungal isolates, proved to be promising candidates in terms of its ability to tolerate PAHs. Here also, the samples were from sediments having on-shore anthropogenic activities and industrial effluent discharges (Deshkar et al., 2010). Overall, the results confirm that the isolated fungi have great potential for use in contaminated environments and further in situ microcosm and mesocosm studies using the fungi with core consortia from the site can be used for potential applications in biodegradation and bioremediation of PAHs.

5. Conclusion

Overall, the study reveals that the marine derived fungal isolates were able to tolerate and degrade different PAHs like naphthalene, phenanthrene, pyrene, fluoranthene and anthracene. Species like *Aspergillus*, *Engyodontium*, *Chaetomium* and *Sarocladium* showed high tolerance. Further work using these selected species should be conducted to assess the potential intermediates during the PAH degradation and the production of enzymes involved in the oxidation of aromatic rings. Based on the GC–MS result profile, we can conclude that though *Sarocladium implicatum* strain NPDF1239-K3-F20 was most tolerant to all the PAHs, its degradation potential was limited while *Penicillium ilerdanum* strain NPDF1239-K3-F21 and *Aspergillus versicolor* strain NPDF190-C1-26 showed the maximum degradation of >75% for all the PAHs under study. As observed, the potential fungal candidates can be further assessed for on field application and bioremediation in individual as well as combined cultures to develop strategy for PAH removal from contaminated sites. A detailed study of the intermediates formed and actual end product can be performed for further validation of complete/partial degradation of the studied PAHs by time-based experiment as a future perspective of the study outcomes. The less potent candidates can also be further optimized for increasing their potential by changing various conditions.

CRediT authorship contribution statement

Mayur Mahajan: Writing - original draft.
Devika Manek: Methodology, Formal analysis.
Nishant Vora: Methodology, Formal analysis.
Ramesh Kothari: Resources, Investigation.

Chandrashekar Mootapally: Conceptualization, Writing - review & editing, funding acquisition, Project administration.

Neelam M Nathani: Conceptualization, Writing - review & editing, funding acquisition, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2021.112293.

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