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ORIGINAL ARTICLE

Degradation of crude oil-associated polycyclic aromatic hydrocarbons by marine-derived fungi

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Abstract

One of the major environmental concerns today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Crude oil is a complex mixture of hydrocarbons like alkanes, naphthene and polycyclic aromatic hydrocarbons (PAHs). PAHs are known to be highly toxic to humans and animals due to their carcinogenic and mutagenic effects. PAHs are environmentally recalcitrant due to their hydrophobicity which makes them difficult to degrade, thus making them persistent environmental contaminants. The mechanical and chemical methods in practice currently to remove hydrocarbon contaminants have limited effectiveness and are expensive. Bioremediation is a cost-effective technology for treating hydrocarbon-contaminated sites as it results in the complete mineralisation of the pollutant. This study demonstrates the degradation of crude oil and associated PAHs using ten fungal cultures isolated from the aquatic environment. The current study reported a 98.6% and 92.9% reduction in total PAHs in crude oil by *Fusarium* species, i.e. isolate NIOSN-T4 and NIOSN-T5, respectively. The fungal isolate, NIOSN-T4, identified as *Fusarium equiseti,* showed maximum PAH degradation efficiency of LMW PAHs 97.8%. NIOSN-M126, identified as *Penicillium citrinum*, exhibited a 100% removal of HMW PAHs. Microorganisms possess an untapped potential for various applications in biotechnology, and the current study demonstrated the potential of marine fungi for use in the bioremediation of xenobiotic hydrocarbons in the environment.

Keywords Bioremediation · Crude oil · Fungi · Polycyclic aromatic hydrocarbons · Xenobiotics

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a group of aromatic compounds composed of two or more aromatic rings fused in a linear, angular or clustered arrangement (Kadri et al. 2017; Agrawal et al. 2018). PAH can be produced either by natural or anthropogenic activities; nevertheless, the anthropogenic input of PAHs to the environment far exceeds the natural sources, which include emissions from incomplete combustion and industrial processes, ship traffic, leakage of petroleum products near refineries and accidental land spills and burning of agricultural waste (Park et al. 2019; Mahajan et al. 2021). PAH get sorbed onto soil particles and organic substances present in the soil, thus reducing their bioavailability and rendering them recalcitrant (Ghosal et al. 2016; Abdel-Shafy and Mansour 2016). The presence of PAHs in contaminated soil and sediment poses a significant risk to the soil because many PAH compounds are known or suspected to be toxic, mutagenic and, in some cases, carcinogenic (Park et al. 2019; Hadibarata et al. 2022). Based on abundance and toxicity, the United States Environmental Protection Agency (US EPA 2007) enlisted 16 PAHs as priority environmental pollutants. They include naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, benzo[g,h,i]perylene, indeno[1,2,3-c,d]pyrene and dibenz[a,h]anthracene.

PAHs are also important pollutants threatening the health of the marine ecosystems. The main source of PAHs input to the marine environments is natural oil seeps; however, accidental oil spills occurring sporadically could result in an enormous input of PAH pollutants into the marine systems

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(Duran and Cravo-Laureau 2016). Consequently, eliminating PAHs from contaminated sites has become essential to restore the environment. Microbial degradation can be considered as an economical and eco-friendly biotechnological alternative for achieving possible mineralisation of the pollutant (Raghukumar et al. 2006) and its biotransformation into less toxic derivatives with greater water solubility, which can then be degraded by the action of other microorganisms (Cerniglia and Sutherland 2001). Fungi enzymatically degrade PAHs to products like quinones, dihydrodiol epoxides, trans-dihydro diols and phenols, which may conjugate to form less toxic metabolites like glucosides, xylosides, sulphates and glucuronides (Cerniglia and Sutherland 2010).

Hydrocarbon degradation using marine-derived fungi was first reported by Ahearn and Meyers (1972); however, their application as potential bioremediation agents still needs to be better studied. In recent years, the interest in biotransformation and degradation of PAHs using fungi has increased (Mineki et al. 2015; Morales et al. 2017; Alvarez-Barragan et al. 2021). A few previously reported non-ligninolytic fungi capable of PAH degradation include *Penicillium* sp., *Aspergillus* sp., *Trichoderma harzianum* and *Fusarium solani* (Rafin et al. 2000; Saraswathy and Hallberg 2002; Wang et al. 2008; Gao et al. 2010). Fungi are ubiquitous in all marine environments (Orsi et al. 2013), belonging mainly to the phyla Ascomycota and Basidiomycota (Alvarez-Barragan et al. 2021). Although fungi isolated from marine habitats have similar characteristics to their terrestrial counterparts, they possess advantageous properties such as salinity tolerance and the ability to accumulate and degrade PAHs, which are less bioavailable due to adsorption onto marine sediments/organic matter (Trincone 2010; Bonugli-Santos et al. 2015).

This study explored the potential of cultivable marine fungi for their PAH degradation capacity. For this purpose, fungal strains were isolated from various marine environments, identified using ITS sequence analysis, and their PAH degradation potential was determined using GC–MS analysis.

Materials and methods

Fungal cultures used in the study

Ten previously isolated marine-derived fungal cultures (Barnes et al. 2018) were tested for their ability to utilise and biodegrade PAHs in the crude oil obtained from Bombay High. The details of ten fungal cultures were as follows: NIOSN-M126 (*Penicillium citrinum*), NIOSN-M109 (*Acremonium sclerotigenum*), NIOSN-M142 (*Aspergillus polyporicola*), NIOSN-M113 (*Aspergillus versicolor*), NIOSN-T4 (*Fusarium equiseti*), NIOSN-T5 (*Fusarium* sp.), NIOSN-SK56C42 (*Aspergillus sydowii*), NIOSN-SK56S32 (*Aspergillus* sp.), NIOSN-SK56S22 (*Aspergillus flavus*), NIOSN-SK56S57 (*Aspergillus sydowii*).

Preparation of fungal inoculum for biodegradation studies

The individual fungal cultures were inoculated into 100 mL Erlenmeyer flasks containing 20 mL Czapek Dox broth to prepare the starter inoculum. The flasks were incubated for five days under static conditions at room temperature (28 °C) to obtain a mycelial mat.

Biodegradation studies using marine-derived fungi

The mycelial mat formed was physically sheared using sterile glass beads for the biodegradation studies. The fragmented mycelia were then used to inoculate 20 mL of mineral salt medium containing 1% (w/v) crude oil as the sole carbon source. The experimental flasks were incubated with constant shaking at 80 rpm, 28 °C for 23 days. Following the 23-day incubation period, the mycelial mats were separated from the culture broth by skimming the mats and the residual crude oil from the culture broth was extracted with hexane and dichloromethane (1:1) in a separating funnel with continuous shaking. On settling, two layers were formed: an aqueous layer and an organic layer containing the residual crude oil. The organic layer was decanted into a round bottom flask, and the solvent was dried using a rotary evaporator.

Analysis of PAH degradation using gas chromatography-mass spectrometry

Following extraction, the residual crude oil was re-dissolved in 1 mL hexane and loaded onto a silica column. The PAH fraction was then eluted through the column using 12 mL hexane as the eluent. The hexane fraction obtained was partially dried in a rotary evaporator before GC–MS analysis. Dried samples were re-dissolved in 1000 µL hexane and quantified via capillary gas chromatography using Shimadzu GC 2010 plus Chromatograph equipped with a single quadrupole mass spectrometer (GC–MS-QP2010 SE). A fused silica capillary column of 30 m length, 0.32 mm internal diameter and 0.25 µm film thickness was employed. The column temperature programme was set as follows: 70 °C held for 2 min, column temperature ramp to 150 °C with an increase of 30 °C/min and further column temperature settle from 150 to 310 °C with a stepped temperature increase of 4 °C/min and held for 20 min. The GC injector was held isothermally at 280 °C for 3 min. Helium was used as the carrier gas at a flow rate of 1 mL/min. The GC/MS interface temperature was maintained at 280 °C. The MS was

operated in electron impact (EI) ionisation mode with an electron energy of 70 eV, and the MS ion source temperature was held at 260 °C. To increase sensitivity, the selected ion monitoring (SIM) mode was used to analyse the peaks quantitatively (Supplementary Table 1).

Toxicity assay using *Vigna radiata* (Mung bean)

The toxicity of the degradation by-products was tested on the germination efficiency of *Vigna radiate* using the culture broth separated from fungal mycelia from the test and control flasks. Mung beans used in this study were obtained from the local market. The seeds were soaked in a beaker filled with water, and only those that sunk to the bottom were used for the assay. The seeds (10 numbers) were kept moist and wrapped in a paper towel soaked in tap water for the control group and the media from the respective experimental flasks. The seeds were kept in a Petri dish, maintained at room temperature under moist conditions and observed daily for 3 days. The per cent germination of the seedlings was recorded over 3 days.

Results

PAH degradation using marine fungi

The residual PAHs were analysed using GC–MS. The 16 unsubstituted PAHs (EPA 16 PAHs) on a priority pollutant list are the most commonly analysed PAHs, and their concentrations differ from oil to oil. Of the EPA 16 PAHs, acenaphthene, naphthalene, acenaphthylene, fluorene, phenanthrene, pyrene, benzo[a]anthracene and indeno[1,2,3-c, d]pyrene were found to be present in the crude oil used in the current study. Among the PAHs analysed, the crude oil had a higher concentration of phenanthrene, followed by naphthalene.

Among the ten isolates studied, isolate NIOSN-T4 demonstrated a 100% removal of phenanthrene (Table 1). Four isolates, NIOSN-SK56S32, NIOSN-SK56S57, NIOSN-M142 and NIOSN-T5, degraded phenanthrene with an efficiency greater than 85%. Naphthalene was 100% degraded in broth with isolate NIOSN-SK56S32. NIOSN-T4 and NIOSN-T5 showed 92% and 85% degradation of naphthalene, respectively. Acenaphthene was completely degraded by isolate NIOSN-T4. NIOSN-SK56S32 was able to completely degrade fluorene from the crude oil mixture. Among the high molecular weight PAHs, isolates NIOSN-T4 and NIOSN-M126 were able to degrade pyrene, benzo[a] anthracene and indeno[1,2,3-c, d]pyrene by 100%. Of the ten isolates used in the current study, NIOSN-T4 (*Fusarium equiseti*) showed the highest efficiency in degrading the total PAHs in crude oil by an average of 98.6%, followed by NIOSN-T5 (*Fusarium* sp.) and NIOSN-SK56S32 (*Aspergillus* sp.), which demonstrated degradation of 92.9% and 91.3%, respectively.

Toxicity assay using mung bean germination

The tendency for PAHs to bind to particulate matter allows them to be transported by air and water and settle out in soil and sediments, which serve as contaminant sinks. Polycyclic aromatic hydrocarbons can accumulate to dangerous levels near their industrial sources. Several PAHs are probable or known carcinogens. Investigations for the production of toxic by-products were undertaken using the plant model *Vigna radiata* (Wulandari et al. 2021). The germination percentage of *Vigna radiata* for the treated and untreated experimental conditions is given in Table 2. The results

Table 1 The per cent degradation obtained from gas chromatographic mass spectrometry analysis for the individual components of the PAH fraction of crude oil as shown by the ten fungal isolates

Per cent degradation*										
Compound	Isolate									
	C42	M109	M113	M126	M142	S22	S32	S57	T4	T5
Acenaphthene	0	59.7	70.8	76.2	34.5	65.4	56.7	73.3	100	84.9
Naphthalene	0	68.8	80.7	71.5	49.6	63.1	100	80.9	91.9	85.1
Acenaphthylene	0	100	63.6	100	32.9	66.9	100	100	100	100
Fluorene	0	65.3	78.2	85.7	53.7	73.4	100	81.5	96.9	93.1
Phenanthrene	31.6	45.6	82.2	22.1	88.7	64.2	88.2	88.6	100	94.8
Pyrene	0	45.7	70.4	100	56.8	60.1	85.7	63.0	100	85.4
Benzo[a]anthracene	100	100	100	100	0.00029	100	100	100	100	100
Indeno[1,2,3-c,d]pyrene	0	100	99.9	100	100	100	100	100	99.9	100

*Per cent degradation is calculated as = (initial concentration- final concentration/initial concentration) × 100

Initial concentration is the value obtained from the control flask after the 23-day incubation period

Final concentration is the value obtained from the experimental flask after the 23-day incubation period

Table 2 The per cent germination obtained from the toxicity assay using *Vigna radiata* (Mung Bean) ($n=10$ seeds)

Per cent germination												
Isolate	C42	M109	M113	M126	M142	S22	S32	S57	T4	T5	Crude oil	Control
Day 1	70	80	80	80	70	70	80	70	90	80	50	90
Day 2	80	90	80	90	90	90	90	90	90	80	60	100
Day 3	90	100	100	100	100	90	90	100	100	90	60	100

indicate there are no toxic intermediates or bioremediation products synthesised by the fungal degradation of crude oil.

Discussion

Crude oil is a complex mixture of hydrocarbon compounds, viz. paraffin, naphthenes and aromatics (Kerr et al. 1999). PAHs may find their way into the environment through oil seeps and produced water (PW). PW comprises of water naturally occurring in the oil reservoir or the water added into the reservoir to facilitate the oil recovery and downstream production processes (Pampanin and Sydnes 2013). Remediation of PAH is a critical need due to the toxicogenic, mutagenic and carcinogenic properties of some PAHs (Boonchan et al. 2000). Bioremediation is a promising technology due to its relatively low cost and environmentally friendly nature (Khandelwal et al. 2021). Many bacterial species are reported as PAH degraders (Márquez-Rocha et al. 2005), but recent research is directed towards PAH degradation by fungi isolated from terrestrial and aquatic environments. Fungi regulate the flow of nutrients and energy, thereby playing a key role in ecosystem functioning (Tisma et al. 2010). In addition to the production of extracellular enzymes, the fungal mycelial network provides deeper penetration and hence a larger surface area for absorption in soil (Mao and Guan 2016).

Studies have reported several fungal species with the capacity to degrade a series of PAHs, such as naphthalene, phenanthrene, fluoranthene, chrysene, pyrene and benzo[a] pyrene (Kiehlmann et al. 1996; Saraswathy and Hallberg 2002; Mollea et al. 2005; Mineki et al. 2015). The common white rot fungi used for PAH degradation through ligninolytic enzymes are *Phanerochaete chrysosporium, Pleurotus ostreatus, Trametes versicolor, Armillaria* sp., *Bjerkandera adusta*, *Antrodia vaillantii* (Ghosal et al. 2016). In this study, ten fast-growing fungi isolated from marine environments showed the potential to co-metabolise PAHs contained in crude oil while utilising crude oil as the sole carbon source. Another key feature of the isolates tested is their potential to metabolise crude oil and its components into non-toxic derivatives. Fungal species involved in the degradation of different PAH compounds are listed in Table 3. Compared with previous literature, the fungal isolates obtained in the current study show a greater potential to grow and utilise crude oil and associated PAHs as a carbon and energy source. Additionally, the fungi used in the present study have been derived from marine sources and hence have a better advantage to adapt to and survive when exposed to the contaminated environmental condition due to their tolerance to extreme conditions such as high salinity, broad pH and temperature and changes (Mahajan et al. 2021). The PW used for the oil extraction and downstream processing has high salinity, and thus, halotolerant microorganisms would be better suited to remove xenobiotics under such conditions (Gonzalex-Abradelo et al. 2019). Reyes-Cesar et al. (2014) studied the potential of seven fungi isolated from crude oil-contaminated soil in the biodegradation of a mixture of PAHs. They reported a reduction of 21% in PAH mix after incubation for 2 weeks by the fungi *Aspergillus terreus*, *Talaromyces spectabilis* and *Fusarium sp.* The current study reported a 98.6% and 92.9% reduction in total PAHs present in crude oil by *Fusarium* species, i.e. isolate NIOSN-T4 and NIOSN-T5, respectively.

In the current study, five LMW PAHs were present in crude oil, acenaphthene, naphthalene, acenaphthylene phenanthrene and fluorene. On average, isolate NIOSN-T4 (*Fusarium equiseti*) and NIOSN-T5 (*Fusarium* sp.) showed the highest efficiency in degrading LMW PAHs, 97.7% and 91.6%, respectively. The HMW PAHs pyrene, benzo[a] anthracene and indeno[1,2,3-c, d]pyrene were found to be efficiently removed by the isolates NIOSN-T4 (99.98%) and NIOSN-M126 (100%). Previous reports have suggested that *Aspergillus* sp., *Trichocladium canadense*, and *Fusarium oxysporum* can degrade LMW PAHs more extensively (Ghosal et al. 2016). LMW PAHs are more volatile and water-soluble and consequently more prone to degradation. In contrast, HMW PAHs tend to be more recalcitrant owing to their low bioavailability and sorption onto soil organic matter (Li et al. 2008). Previous studies have reported the utilisation of LMW PAHs as the sole carbon source; however, this phenomenon is relatively rare with respect to HMW PAHs (Nam et al. 2001), and studies have shown the degradation of HMW PAHs to occur co-metabolically in the presence of an alternate carbon source such as glucose (Mao and Guan 2016). This study assessed the fate of PAHs as a complex mixture of different hydrocarbons using crude oil as the sole carbon source. The ability of fungi to use PAHs as the sole carbon and energy source has been previously described (Rafin et al. 2000). However, reports are also available that

Table 3 Fungal degradation of PAH compounds

PAH Compound	Fungus	Per cent degradation (%)	Incubation days	Reference
Phenanthrene	*Trichoderma sp.*	72	30	Hadibarata et al. (2007)
	Scopulariopsis brevicaulis	60	30	Mao and Guan (2016)
	Phomopsis liquidambaris	77	10	Fu et al. (2018)
	Pleurotus ostreatus	94	11	Bezalel et al. (1996)
	Fusarium sp.	**95**	**23**	**Present study**
Fluorene	*Mucor irregularis*	79.8	5	Bankole et al. (2021)
	Aspergillus sp.	**100**	**23**	**Present study**
Pyrene	*Penicillium simplicissimum*	60	28	Saraswathy and Hallberg (2002)
	Penicillium janthinellum	31	28	Saraswathy and Hallberg (2002)
	Penicillium funiculosum	40	28	Saraswathy and Hallberg (2002)
	Penicillium terrestre	67	28	Saraswathy and Hallberg (2002)
	Trichoderma harzianum	33	28	Saraswathy and Hallberg (2002)
	Scopulariopsis brevicaulis	64	30	Mao and Guan (2016)
	Coriolopsis byrsina	96.1	18	Agrawal and Shahi (2017)
	Fusarium equiseti	**100**	**23**	**Present study**
Benzo[a]anthracene	*Fusarium solani*	60	40	Wu et al. (2010)
	Fusarium equiseti	**100**	**23**	**Present study**
	Fusarium sp.	**100**	**23**	**Present study**
	Penicillium citrinum	**100**	**23**	**Present study**
	Aspergillus flavus	**100**	**23**	**Present study**

Bold values indicate that these observations are from the present study

state fungal degradation of PAH occurs as co-metabolism with another carbon source (Cerniglia et al. 1986).

The phytotoxicity of the PAH degradation products was assessed by observing its effects on the germination of *V. radiata* (Table 2). The seed germination of *V. radiata* in the PAH degradation products ranged from 70 to 100%, which is apparently not toxic. Previous studies have reported that seedling germination was not significantly affected by PAHs (Juhasz et al. 2010; Khan et al. 2012). Furthermore, it has been suggested that seedling germination alone may not predict PAH toxicity (Sverdrup et al. 2003). In accordance with this, a brine shrimp lethality assay was used to study the toxicity of PAH degradation derivatives on marine organisms. The *Artemia*-based toxicity assay results have been published for the same cultures in the previous publication (Barnes et al. 2018).

Among the ten isolates screened, NIOSN-T4, NIOSN-T5 and NIOSN-M126 showed the highest efficiency in degrading HMW as well as LMW PAHs. Isolates NIOSN-T4 and NIOSN-T5 were isolated from tar balls, due to which these isolates may have proven to be highly efficient for the degradation of crude oil-associated PAHs. Isolate NIOSN-M126 (*P. citrinum*) efficiently degraded the HMW PAH fraction by 100%. Previously, isolate NIOSN-M126 was reported to show an average of 95% reduction in the n-alkane fraction of crude oil (Barnes et al. 2018). Isolate NIOSN-M126 was obtained from Divar mangrove sediments situated on the banks of Mandovi estuary, which is used for the movement of barges, ferry boats and other tourist boats. Divar mangroves have been exposed to petroleum contamination due to the heavy traffic activity along the Mandovi estuary. Isolates NIOSN-T4, NIOSN-T5 and NIOSN-M126 are promising cultures for further study as potential bio-remediating agents for possible use in clean-up of hydrocarbon-contaminated sites. The synergistic effect of biostimulation combined with bioaugmentation using these fungal strains could be explored to improve the biodegradation efficiency in crude oil-contaminated soils.

Conclusion

Hydrocarbon pollution is one of the emerging threats to the environment. At present, most of the remedial measures taken are containment of the pollution. This study reveals that marine-derived fungal isolates were able to tolerate and degrade different PAH compounds like acenaphthene, naphthalene, acenaphthylene phenanthrene, fluorine, benzo[a] anthracene and indeno[1,2,3-c, d]pyrene. Overall, the results confirm that the isolated fungi have great potential for use in contaminated environments and further in in situ microcosm and mesocosm studies using the fungi with core consortia

from the site for potential applications in biodegradation and bioremediation of PAHs. With their ability to penetrate the substrate, fungi can emerge as a potent remedial option for remediating hydrocarbon pollution caused due to crude oil. A detailed study of the intermediates formed and the actual end product can be performed to further validate the studied PAHs' complete/partial degradation by time-based experiment as a future perspective of the study outcomes.

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Author contributions The corresponding author planned the work. The fungal cultures were isolated and identified by the last author as a part of her doctoral work. The first author carried out all the experimental work. The first, third and fourth authors carried out the GC–MS analysis. The first and corresponding authors wrote the manuscript, and all authors contributed towards improvising the manuscript.

Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Research involving human participants and/or animals Not applicable to this manuscript.

Informed consent Not applicable to this manuscript.

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