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Research Article

BIODEGRADATION OF RESIDUAL PETROLEUM HYDROCARBONS BY USING FUNGAL ENDOPHYTES FROM THE MANGROVE ECOSYSTEM

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ABSTRACT

Residual oil comprising of toxic elements can contaminate soil and water bodies. The accumulation of such effluents has a severe impact on the plant ecosystem, mainly mangroves. To escape from the ill effects of such contaminants, plants attain resistance from mutual partners. Biodegradation of such contaminants using micro-organisms is widely used by researchers to reveal the impact of micro-organisms to degrade petroleum hydrocarbons. In the present study, seven dominant fungal endophytes *viz., Nigrospora* sp., *Aspergillus niger*, *Aspergillus* sp., *Curvularia* sp., *Pestalotiopsis adusta, Fusarium* sp. and *Cladosporium* sp. isolated from mangrove habitat were screened for their ability to degrade residual petroleum hydrocarbons. The study confirms the ability of mangrove endophytes to degrade hydrocarbons. Further FT-IR spectroscopy was used as a preliminary tool to analyze the degraded hydrocarbon groups by the tested fungal endophytes within residual oil. Among the fungal isolates, *Nigrospora* sp. had the highest ability to degrade petroleum hydrocarbons, followed by *Aspergillus* sp., *Curvularia sp.*, *Pestalotiopsis adusta, Fusarium* sp., *Aspergillus niger* and *Cladosporium* sp.

Keywords: Mangroves, Endophytic fungi, Petroleum hydrocarbon, Residual oil, FT-IR analysis.

1. INTRODUCTION

Petroleum is a viscous mixture of thousands of hydrocarbons, comprising of carbon and hydrogen [1]. It is a significant source of energy in many industries. It can be categorized as aromatic and aliphatic hydrocarbons. Aromatic hydrocarbons or arenes are organic molecules comprising of one or more aromatic benzene rings whereas, polycyclic aromatic hydrocarbons (PAH) are neutral non-polar organic molecules, consisting of three or more benzene ring [2].

Soil and water contaminated with these hydrocarbons cause extensive damage to the local system, including plants, animals, and other living organisms. Prolong accumulation of these contaminants add to pollutants and may cause mutation or even death in the plant system [3]. The subsequent release of these hydrocarbons into the environment, whether accidentally or through man-made activities, is a significant cause of air, water, and soil pollution [4]. The primary concern is when accidental spillage of such hydrocarbons, contaminating the environment while transportation, overflowing, or leakage in oil vehicles gets into nearby water bodies.

Petroleum degradation in the presence of hydrocarbon-degrading micro-organisms is a complex process of biodegradation that depends upon bacteria, yeast, and fungi. Fungi like *Aspergillus*, *Cladosporium*, *Corollasporium*, *Fusarium*, and *Penicillium* are reported to possess a beneficiary role in the degradation of hydrocarbons, which is mediated by the action of certain enzymes system like oxygenases and hydroxylases [5]. It is reported that these hydrocarbon-degrading micro-organisms detoxify the plant system by stimulating secondary metabolites. Without these microbes, plants would not have survived [6]. Hence micro-organisms have been reported to have a decisive role in biodegradation of oil and can be used as oil degrades. Filamentous fungi play a significant role in the degradation of diesel because of its fast growth, and extensive hyphal network [7]. PAHs (Poly Aromatic hydrocarbons) have been reported as ubiquitous xenobiotic environmental pollutants [8]. A diverse group of fungi such as Zygomycetes (*Cunninghamella elegans*), Ascomycetes (*Aspergillus niger* and *Penicillium* sp.), and white-rot Basidiomycetes (*Trametes versicolor*, *Pleurotus ostreatus*) is known to oxidize and degrade PAH's [9].

The identification of organisms plays a crucial role in *in-situ* biodegradation process. It is vital as the use of vehicles is increasing day by day. The residual oil is either

spilled or dumped at the vehicle service station, which gets later washed into nearby water bodies. Several physical and chemical techniques are employed to resolve the issue. However, it is a quite expensive and time-consuming method, and hence bioremediation using micro-organisms can be used as an alternative to degrade a wide range of hydrocarbon molecules.

Research on endophytic fungi has gained the attention of the scientific community in recent years because of its wide diversity particularly, for potential secondary metabolites. Evidence indicates that plant harbor endophytic contaminant degrading microorganisms as an adaptative response to improve its phytoremediation strategies [10]. Endophytes being a part of the host as an endosymbiont colonize plant without causing any adverse effect on the host. It improves host tolerance towards environmental stresses. Further FT-IR technique can be used as a preliminary tool to elucidate the impact of such endophytic organisms to screen the degradation performance of oil samples.

Being a part of the coastal belt, Goa is covered by dense mangrove vegetation. These mangroves are under threat due to constant additions of such hydrocarbons. In the present study, an attempt has been made to investigate the role of fungal endophytes in degrading these effluents, which gives an insight concerning the ecological role played by these endophytes in the ecosystem.

2. MATERIAL AND METHODS

2.1. Study site

The study was undertaken at Chorao (also known as Chodna), which is an island along the Mandovi River that extends from 15.30013 Latitude and 73.50013 Longitude. A small part of the Island covering 178 hectares declared as Reserved Forest under the Indian Forest Act (1927) is well known as Dr. Salim Ali Bird Sanctuary. The area is continuously under tremendous pressure of a hazardous influx of petroleum hydrocarbons, contaminating the aquatic flora (Fig. 1).

Fig. 1: Chorao water body with an influx of petroleum hydrocarbons

2.2. Isolation of endophytic fungi

The plant samples were washed by using running tap water to remove dirt and other surface contaminants followed by washing with sterile distilled water. The samples were then surface sterilized by using the modified method of Bayman et al., [11], as mentioned in Table 1.

Table 1: Plant material sterilization using a three-step sterilization method.

Steps	Sample	Treatment	Duration
1	Stem/Leaf/Root	90% Ethanol	1 min
2		0.5% Sodium hypochlorite	3 min
3		90% Ethanol	1 min
4		Distilled water	Two times

Each of the samples was cut into thin sections (3-4mm) and inoculated into sterile pre-autoclaved Petri plates containing PDA medium, followed by incubation for 5-6 days at 23-25°C. The fresh emerging hyphal mycelia were sub-cultured onto fresh PDA slants, and dominant cultures were selected to study the effect of endophytic fungi in the degradation of petroleum hydrocarbon.

2.3. Preparation of media for the study

Endophytic fungal growing tips were inoculated in the modified Bushnell-Hass (BH) broth medium [12] (Table 2).

Three agar plugs containing endophytic fungal culture were inoculated into 50 mL of sterilized modified BH broth along with 1mL residual oil and 2,6-Dichlorophenol indophenol indicator. Control flasks were also maintained separately for each test. The details of residual oil collected from the service station are depicted in Table 3. The inoculated flasks were incubated at 37°C with constant shaking on Scigenics Biotech shaker for 15 days with a 12 hour dark and 12 hour light period. The aliquots in the flasks were monitored daily for color change. Once the color changed from deep blue to colorless, the sample was filtered by using filter paper to separate mycelia mat, followed by centrifugation for 15 minutes at 8000 rpm.

2.4. Separation of the immiscible oil layer

Separating funnel was used for the separation of immiscible liquids of different densities. The immiscible liquid was placed into the flask and allowed to stand for a few minutes before separation. The two liquids formed distinct layers with the lighter fluid appearing on top.

The degraded oil was separated, and the total amount of leftover oil was recorded. The collected oil was subjected to FT-IR analysis for the identification of specific groups of functional compounds along with control.

Table 2: Composition of modified Bushnell-Hass Broth medium

Sr. No.	Molecular Formula	Compound	Amount
1	$MgSO_4$	Magnesium sulfate	0.2 g/l
2	$CaCl_2$	Calcium chloride	0.2g/l
3	KH_2PO_4	Mono Potassium phosphate	1 g/l
4	K_2HPO_4	Dipotassium phosphate	1 g/l
5	$FeCl_2$	Ferric chloride	0.5 g/l
6	NH_4NO_3	Ammonium nitrate	1 g/l
7	Tween 80	Tween 80	0.1%
8	NaCl	Sodium chloride	0.5 g/l
9	2,6- Dichlorophenol indophenols	2,6-Dichlorophenol indophenols	2%

Table 3: Details of residual oil collected from the motor vehicle service station

No.	Source of sample	Residual oil color	pH	Boiling point ˚C
1	Two-wheelers	Blackish brown	7.1±0.1	246
2	Four-wheelers	Dark black	7.1±0.1	230
3	Boat	Blackish grey	6.8±0.2	240

All values are mean of 3 readings

2.5. FT-IR analysis

FT-IR analysis was performed by using Vertex 80 FT-IR system to record the IR spectra of the oil samples at SAIF IIT Bombay, India. The IR detection was recorded between 4000 to 450 cm^{-1} with the spectral resolution of 0.2 cm^{-1}. The spectroscopic method detects the vibrations and rotations of molecules that help to characterize specific functional groups in the sample [13].

3. RESULTS

3.1. Isolation of endophytic fungi

In all, 57 fungal isolates were recovered by using a three-step sterilization method. Of these, seven dominant pure cultures were employed for the biodegradation study (Fig. 2).

3.2. Residual oil degradation using selected fungal endophytes - Visual Qualitative Analysis

Experimental sets were monitored every day for color change. The preliminary visual interpretations indicated the color change from blackish-blue to colorless (Fig. 3). The continuous decrease in the amount of medium was observed in the experimental flask. From the observations, it is interpreted that the media constituents are absorbed by the growing hyphal tips while degrading the oil. In the experimental flask, the tiny drops of oil adhering to the fungal mass were noticed, suggesting the encapsulation of oil drops by endophyte for degradation. A similar observation has been recorded in an earlier study [3]. They suggested that the fungal endophytes degrade oil by microdroplet encapsulation at the hydrophobic microbial cell surface, which further converts the substrate into carbon dioxide and water.

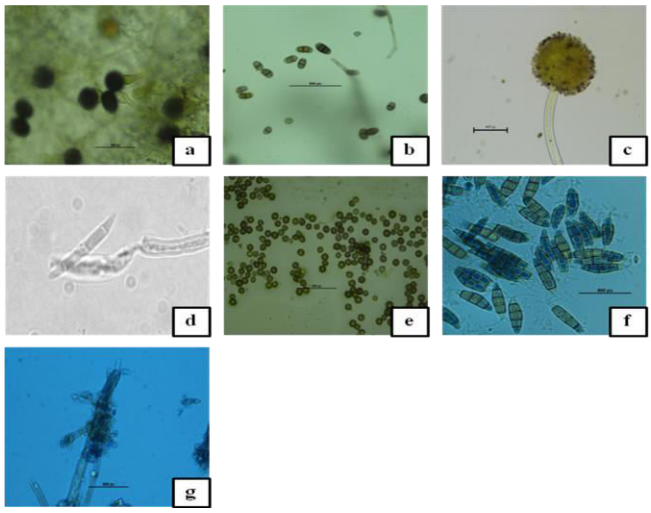

Fig. 2: Fungal isolates used for hydrocarbon degradation assay. a) *Nigrospora* sp., b) *Curvularia* sp., c) *Aspergillus* sp., d) *Fusarium* sp., e) *Aspergillus* niger, f) *Pestalotiopsis adusta*, and g) *Cladosporium* sp.

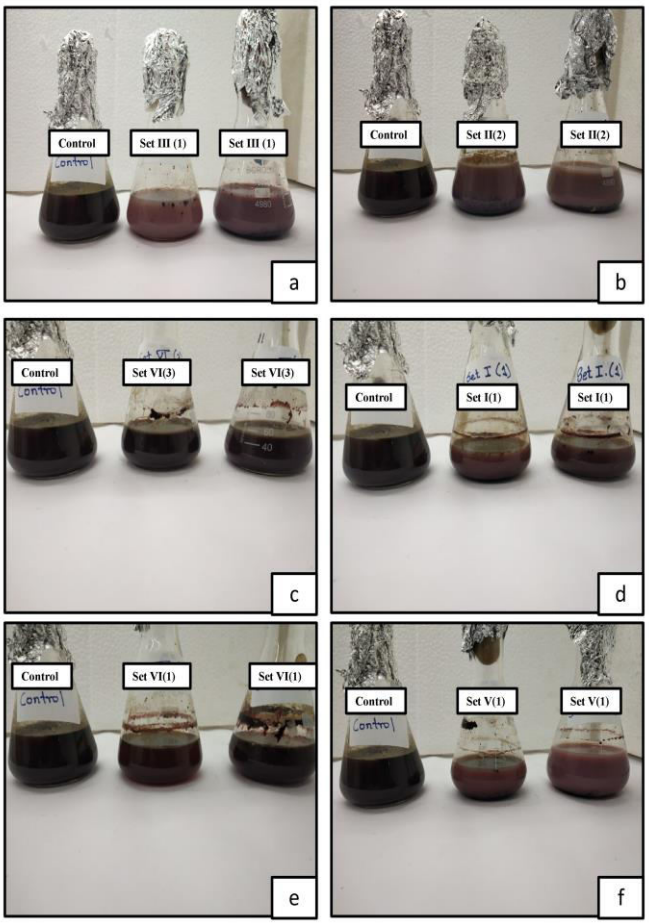

Fig.3: Qualitative analysis of *in vitro* biodegradation

Four wheeler residual oil by a: Fusarium sp., b: Pestalotiopsis sp., c: Curvularia sp.Two wheeler residual oil by d: Aspergillus niger; e: Aspergillus sp., f: Curvularia sp.

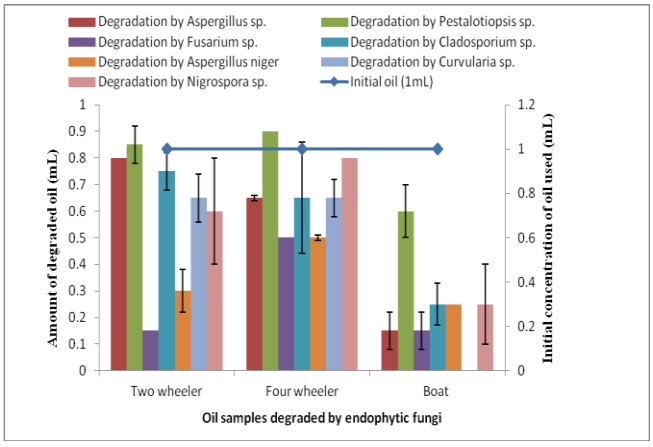

Fig. 4: Quantitative interpretation of oil degradation by mangrove endophytes

The results of the quantitative degradation of oils are depicted in Fig. 4. The study revealed a constant change in the oil quantity inoculated with fungal isolates. The data show the amount of degraded and non-degraded oil. The remaining oil was subjected to FT-IR analysis to check the degradative pattern of the sample along with the control. The interpretation of the degraded hydrocarbon groups was identified using standard literature by Nakamoto, [14].

3.3. FT-IR analysis of leftover oil

The leftover oil was subjected to FT-IR analysis along with control to understand the degradation of hydrocarbons. The results indicated the difference in the peak/band formation between each tested organism against the control. It can be interpreted that endophytic isolates utilize petroleum hydrocarbons as the only source of carbon to retain energy. It was also noted that the different species showed variation in their ability to degrade oil hydrocarbons (Fig. 5 and Fig. 6).

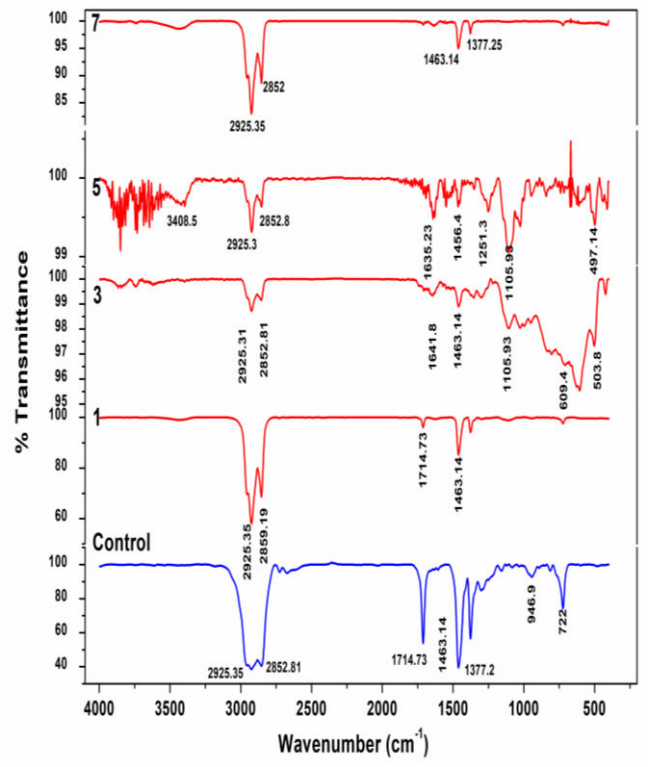

Fig.5: FT-IR spectrum of two wheeler residual oil degradation *by Aspergillus sp. (chromatogram 1); Aspergillus niger (chromatogram 3); Pestalotiopsis sp. (chromatogram 5) and Fusarium sp. (chromatogram 7).*

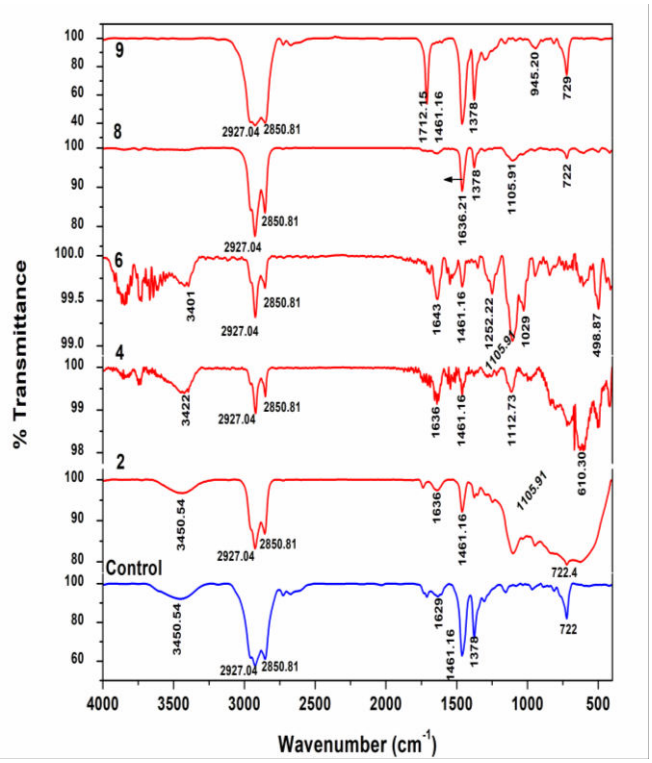

Fig. 6: FT-IR spectrum of four wheeler residual oil degradation *by Aspergillus niger (chromatogram 4); Curvularia sp. (chromatogram 6); Aspergillus sp. (chromatogram 8) and Nigrospora sp. (chromatogram 9).*

3.4. Analysis of two-wheeler residual oil.

The FT-IR analysis of the control (untreated) data set revealed a broad peak at 2925.35 cm^{-1}, and 2852.81 cm^{-1} that corresponds to the -C-H stretching of the aliphatic compound of phenols and alcohols attributed to the presence of saturation due to the -C-H vibrations of phenols and alcohols. A distinct sharp band at centered at 1714.73 cm^{-1} confirms the presence of C=O stretching vibration due to the acid. The absorption bands at 722 and 946.9 cm^{-1} are attributed to the C-H out-of-plane bending mode of the aromatic components. The C=C vibrations are observed between 1600-1400 cm^{-1}, while the symmetric and anti-symmetric vibrations of the carboxylate group for the organic acid (ν_{COO^-}) appear at 1463 and 1377 cm^{-1} respectively.

3.4.1. Chromatogram 1 (oil degradation by Aspergillus sp.)

Compared to the above control data set, *Aspergillus* sp. revealed the presence of sharp bands at 2925.35 cm^{-1} and 2859.19 cm^{-1} indicating =C-H stretching vibrations of the aliphatic components (phenol and alcohol). It was observed that in control, the peaks had a broad spectral signature compared to the experimental set indicating the degradation of some hydrocarbons in the presence of endophytic inoculum. At 1714.73 cm^{-1}, a distinct sharp peak was observed in the control spectrum, which was negligible in the experimental set. The presence of a distinct sharp peak at 1463.14 cm^{-1} for the control set, was seen as a fading peak in the experimental set as a result of degradation by endophytes. In control, absorption bands detected at 1377.2 cm^{-1}, 946.9 cm^{-1} and 722 cm^{-1}. The fading of the above absorptions in the experimental set was due to degradation by fungal endophyte.

3.4.2. Chromatogram 3 (oil degradation by Aspergillus niger)

Compared to the control, *A. niger* medium absorption bands centered at 2925.31 cm^{-1} and 2852.81 cm^{-1} were indicative of the -C-H stretching vibrations of the organic moieties (phenols and alcohols). The prominent peak was seen at 1714 cm^{-1}, which was fully degraded by the endophytic isolate. Traces of functional groups were noticed at 1463.14 cm^{-1} in the experimental set. A small absorption peak at 1105.93 cm^{-1} was noticed that may be involved in the formation of a new functional group while degrading hydrocarbons. It was also noticed that spectral peaks at 722 cm^{-1} (seen in control) were absent in the experimental isolate. However, new bands at 609.4 cm^{-1} and 503.8 cm^{-1} were noticed corresponding to alkyl halides.

3.4.3. Chromatogram 5 (oil degradation by Pestalotiopsis adusta)

Chromatogram 5 showed new spectral bands at 3408.5 cm^{-1}, indicating the presence of hydroxyl stretching of alcohols and phenols. A Broadband at 2925 cm^{-1} and 2852 cm^{-1} corresponding to -C-H stretching of the aliphatic phenols and alcohols was replaced by a small sharp peak indicating the degradation of hydrocarbons by the fungus. At 1635.23 cm^{-1} another small broadband was noticed corresponding to C=O of the carbonyl group of organic acid, which was absent in control, indicating the formation of new functional groups by the endophytes. The sharp spectral band at 1463 cm^{-1} was replaced by a small peak revealing the decisive role of *P. adusta* in degradation. However, at 1377.2 cm^{-1}, 946.9 cm^{-1} and 722 cm^{-1} bands were degraded fully by the isolates, while the presence of a new functional group at 1251.3 cm^{-1}, 1105 cm^{-1}, and 497.14 cm^{-1} was detected.

3.4.4. Chromatogram 7 (oil degradation by Fusarium sp.)

Fusarium sp. showed apparent degradation of several functional groups that were present in control. It was clear that broad spectral peaks at 2925.35 cm^{-1} and 2852 cm^{-1} were replaced by small sharp groups indicating the degradation of (-C-H) aliphatic compound of phenols and alcohols hydrocarbons. Prominent sharp bands at 1714 cm^{-1}, 946.9 cm^{-1}, and 722 cm^{-1} was fully degraded in the experimental set.

A sharp long band at 1463.14 cm^{-1} representing the methylene group of CH_2, CH_3 was degraded at the maximum level, whereas traces of hydrocarbons were degraded at 1377.2 cm^{-1}.

3.5. Analysis of Four wheelers residual oil

The FT-IR analysis of control (untreated) dataset showed the presence of spectral bands at 3450.54 cm^{-1} (alcohols and phenols), 29927.04 cm^{-1} and 2850 cm^{-1} (aliphatic -C-H vibrations), 1629 cm^{-1} (C=O), 1461.16 cm^{-1} (methylene group), 1378 cm^{-1} and at 722 cm^{-1} (alkyl halides).

3.5.1. Chromatogram 2 (oil degradation by Fusarium sp.)

Compared to the control, *Fusarium* sp. showed traces of alcohol and phenol degradation at 3450 cm^{-1} characteristics for the O-H functionality. Peaks at 2927 cm^{-1} and 2850 cm^{-1} (representing aliphatic compounds) were not fully degraded however small change in the size of the band was noticed. The sharp band at 1461 cm^{-1} was replaced by a short sharp band, however band at 1370 cm^{-1} was absent in the experimental set. The new band at 1105.91 cm^{-1} was noticed, indicating the C-O stretch of the alcohol and the production of new functional groups by the fungus. The sharp peak at 722 cm^{-1} in control was replaced by a small peak at 722 cm^{-1} in the experimental set, indicating the degradation of alkyl halides.

3.5.2. Chromatogram 4 (oil degradation by Aspergillus niger)

Results indicated that the bands at 2927 cm^{-1} and 2850 cm^{-1} corresponding to the -C-H stretching were degraded. The peak at 1461 cm^{-1} was seen degrading, while the formation of new functional groups at 1112.73 cm^{-1} comprising of the C-O band was noticed. The absorption peak at 1378 cm^{-1} and 722 cm^{-1} was absent in the experimental set, while a new peak at 488.57 cm^{-1} was observed.

3.5.3. Chromatogram 8 (oil degradation by Aspergillus sp.)

Endophyte isolates indicated full degradation of hydrocarbons evidenced by absorption at 3450 cm^{-1}, whereas a sharp peak was noticed at 2927 cm^{-1} and 2850 cm^{-1} due to the -C-H functionality. The spectral band at 1461 cm^{-1} was fully degraded, while the new functional group at 1105.91 cm^{-1} was seen. Prominent bands at 1636 cm^{-1}, 1370 cm^{-1}, and 722 cm^{-1} were degraded in the experimental set.

3.5.4. Chromatogram 9 (oil degradation by Nigrospora sp.)

The results indicated clear degradation of 3450.54 cm^{-1} & 1629 cm^{-1} absorption peaks followed by slight degradation of the spectral bands at 2927 cm^{-1} and 2850 cm^{-1}. Additional bands observed at 1712 and 945 cm^{-1} suggested the presence of -C=O and -C-H out-of-plane functional group modes.

4. DISCUSSION

Bioremediation using microorganisms is a result of microbial activity involved in degrading environmental pollutants into less toxic forms [15], one of such environmental pollutants are PAHs. Mixing of such PAHs into the water sources are known to pose various health and environmental risks. Conventional methods are often expensive and have limited purification rate. In such situations, researchers across the world tried a natural fungal source of bioinoculum to degrade effluents. Fungi like *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Coriolus versicolor* showed the ability to degrade organic pollutants through oxidation of PAHs by secreting lignolytic enzymes [16]. Endophytic fungi survive in diverse living habitats and are being reported to grow in effluent treatment plants. The potential of fungi in degrading effluents from polluted water bodies is being used in the bioremediation process [5]. Damare et al., [17], stated the potential of marine fungi in the bioremediation of water bodies contaminated with petroleum hydrocarbons. In the present study, it was observed that the endophytic isolates degrade petroleum hydrocarbon by either degrading the functional groups or by the formation of new structural compounds. It was also observed that *Aspergillus* sp. was able to degrade petroleum hydrocarbon from residual two and four-wheeler oil samples. Evidence reveals that *Aspergillus*, *Cephalosporium,* and *Penicillium* to have the potential to degrade hydrocarbons from crude oil [18]. In our study, maximum degradation was achieved by *Nigrospora* sp.,

which could degrade a large number of hydrocarbon molecules. However, few isolates showed least activity to degrade hydrocarbons. This could be attributed to some limiting factors affecting the rate of degradation. Literature indicates that biodegradation depends upon factors such as environmental temperature, oil viscosity, inherent biodegradative capability of fungi, and available nutrient content [19]. At low temperatures, the thickness of the oil increases by lowering the toxicity of molecular weight hydrocarbons, thereby directly reducing the rate of degradation. Atlas, [20] indicated the optimum temperature required for biodegradation is between 20-30°C in freshwater bodies and 15-20°C in the marine environment [21].

Nutrient availability is another limiting factor for hydrocarbon degradation in the mangrove ecosystem [22]. Marine water bodies are typically comprised of lower levels of nitrogen (N) and phosphorus (P) availability, and additionally, during oil spills, carbon level in the marine system increases, making it difficult for degraders to degrade hydrocarbons [20]. A high level of NPK decreases the amount of degradation, especially of aromatic hydrocarbons [23].

It is indicated that endophytes contribute to plant adaptation by activating mechanisms to withstand against contaminants [24]. The qualitative analysis showed the hydrocarbon degradation activity of seven endophytic isolates (in terms of volume) was higher in two and four wheeler oil. In contrast, partial degradation in the case of boat oil was observed. Further FT-IR analysis also showed similar observations, indicating the degradation of hydrocarbons. Studies reveal that within petroleum fractions, the most preferred substrate for biodegradations is n-alkanes and branched alkanes of length between C-10 to C-20 [25]. Similar results were obtained from the tested endophytic isolates that consumed alkanes and aromatic hydrocarbons of residual oil. *Nigrospora* sp. was the most promising isolate, involved in the degradation of maximum functional groups from two and four wheeler oil. *Fusarium sp*. also indicated a higher amount of two-wheeler hydrocarbon degradation as compared to other isolates.

5. CONCLUSION

From the present study, it can be concluded that mangrove endophytes have a significant role in the bioremediation process. Further, FT-IR can be used as a tool to study the performance of microbial degraders and enable selection of potential candidate for the biodegradation process.

Conflict of interest

There is no conflict of interest in the present study.

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