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Isolation and screening of the various fungal isolates for the remediation of fuel oil derived polycyclic aromatic hydrocarbons (PAHS)

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Abstract

Polycyclic Aromatic Hydrocarbons (PAHs) are the environmental contaminants which are known to be mutagenic and carcinogenic. The removal of these contaminants has been a concern due to their harmful effects. High concentrations of PAHs are commonly found in the crude oil and its biproducts. Along with crude oil forest fire, oil seepage and volcanic eruption are the common natural source of PAHs. There are various methods to clean up these contaminants out of which bioremediation is considered to be the cost-effective and sustainable to clean up the contaminants. This study focuses on finding suitable fungal strains to remediate PAHs from the environment by using qualitative and quantitative analyses. To find the potential fungal strains 22 strains were isolated and screened on fuel oil contaminated media. Out of all the fungal strains 3 isolated belonging to the *Aspergillus* sp. and *Penicillium* sp. were found to be efficient in remediating the contaminant.

Keywords: Polycyclic aromatic hydrocarbons (PAHS), bioremediation, fungal isolates, *aspergillus* spp, *penicillium* spp

Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) are compounds which are made up of two or more fused benzene rings. They are both hazardous to the environment as well as to the organism by being carcinogenic (Bamforth & Singleton., 2005) ^[7]. As there is increase in the molecular weight of PAHs the water solubility and vapour pressure decreases while hydrophobicity increases. Long term accumulation of PAHs in the nature can lead to the negative impact on natural environmental receptors (Haritash & Kaushik., 2009) ^[17]. Fossil fuel, coal tar, lubricating oil, crude oil and municipal solid waste are the common anthropogenic source of PAHs, while natural sources include forest fire, oil seepage and volcanic eruptions (Kadri *et al.*, 2017) ^[19]. There are various techniques to clean up the contaminants which includes physical, chemical, thermal and biological remediation. Bioremediation is widely regarded as less damaging, less energy intensive and cost-effective method to clean up the contaminants from the environment as compared to the conventional methods (Alegbeleye *et al.*, 2017) ^[2]. The ability of various fungi and bacteria have been reported to degrade PAHs (Mao & Guan., 2016) ^[20].

Previous research has stated the advantage of fungal bioremediation over bacterial remediation in the following ways: (i) release of enzymes such as laccase, lignin peroxidase and manganese peroxidase having poor substrate specificity; (ii) many fungal species are osmo- and xerotolerant which allow them to live in harsh environment. (iii) the mycelia which is hydrophobic in nature, helps to improve the access of hydrocarbon contaminants (Medaura *et al.*, 2021) ^[22]. These properties are especially necessary when it comes to the less water-soluble heavy PAHs, which are strongly adsorbed with the organic compounds and are less accessible for microbial degradation (Ghosal *et al.*, 2016) ^[14].

According to earlier studies, the degradation of PAHs due to native fungal strains are studied which showed various fungal strains of genera like *Cladosporium*, *Alternaria*, *Fusarium*, *Trichoderma*, *Aspergillus*, *Penicillium*, *Paecilomyces*, *Coriolus*, *Pycnoporus*, *Pleurotus*, *Cephalosporium*, *Mucor*, *Fomitopsis*, and *Daedalea* (Marchand *et al.*, 2017) ^[21]. Due to the success rate, researchers are currently working to identify suitable fungal strains that can

degrade the PAHs. Hence, majority of studies now focuses on the use of native fungal isolates and its ability to break down PAHs in the environment (Benguenab & Chibani., 2021) [8]. The objective of this study is to find out capability of 22 fungal strains to degrade fuel oil and find the best suitable fungal isolate by a step-by-step method to find the best strain that can help to degrade these environmental contaminants.

Materials and Methods

Collection and Isolation of Fungal Strains

A variety of fungal isolates were collected from the various regions of The Maharaja Sayajirao University of Baroda and some strains were also procured from The Seed Anatomy Lab of The Maharaja Sayajirao University of Baroda. The collected fungal strains were isolated on Potato Dextrose Agar (PDA) by the method given by Sharma and Pandey (2010) [26] and the fruiting body was isolated using the method described by Cheng *et al.* (2004) [9].

Screening of Suitable Fungal Isolates

Screening of potential fungal strain for the remediation of PAHs were done using qualitative and quantitative methods.

Qualitative screening

Each fungal isolate was subcultured for 5 days on PDA media prior to the inoculation on the experimented media. The screening was done using the method described by Hamad *et al.* (2021) [16]. The subcultured fungal strain was inoculated on the Czapek Dox Agar (CZA) media [potassium chloride, 1; sodium nitrate, 2; dipotassium hydrogen phosphate, 1; ferrous sulfate heptahydrate, 0.01; magnesium sulfate hepta-hydrate, 0.5; and agar, 20 g/L.] devoid of sucrose and supplemented with 0.3ml fuel oil and incubated for 15 days. The plates were observed at 7th and 15th day, zone of clearance was taken into consideration for the screening.

Quantitative Screening

Quantitative screening was conducted by using Three Different Methods

a. Tolerance Test

Tolerance test was conducted to evaluate the ability of fungal strains to grow on the contaminated media. Tolerance test was carried out using the method described by Asemoloye *et al.* (2020) [6]. Fungal isolates were grown on Bushnell-Haas (BH) agar [magnesium sulphate, 0.20g; calcium chloride, 0.20g; monopotassium phosphate, 1.0g; dipotassium phosphate, 1.0 g; ammonium nitrate, 1.0g; ferric chloride, 0.05g in 1000ml] devoid of any carbon source and supplemented with 5% fuel oil while the control plates were supplemented with 10g/L dextrose. The experiment was performed in triplicate and the readings were taken on 7th and 15th day. The formula to calculate tolerance of each fungus is described below

$$\text{Fungal tolerance rate} = \frac{\text{Growth rate on hydrocarbon plate}}{\text{Growth rate on control plate}} \times 100$$

b. Paired Interaction Test

This method was used to find the compatibility of different fungal strains able to coexist with each other. An 8mm agar plug was taken from 7 days old sub cultured plate of different fungi and inoculated at the opposite corner of PDA media and allowed to grow. The plates were observed for 15 days. Monocultures of each strain were taken as control. Fungal interaction was classified based on the classification given by Stahl and Christensen (1992) [27] namely (i) Neutral

intermingling: one or both mycelia grows adjacent to one another without intermingling and causing adverse effect (ii) Replacement: one mycelium grows on other and replaces it. (iii) Deadlock: mycelium of both the strains does not enter each other's territory.

c. Colorimetric Assay

Fungal strains exhibiting more than 70% tolerance and a positive zone of clearance were selected for the assessment of degradation rate using 2,6-dichlorophenolindophenol (DCPIP) indicator. The selected strains were subcultured for 5 days before the inoculation. The strains were then inoculated on media [MgSO₄·7H₂O (0.2g), CaCl₂ (0.02g), KH₂PO₄ (1g), K₂HPO₄ (1g), FeCl₃ (0.05g), NH₄NO₃ (1g) and distilled water (1L).] containing 0.005%w/v DCPIP, 0.1% Tween 80 and 1% fuel oil and was continuously shaken at 120rpm according to the method by Dhar K., *et al.* (2013) till 15 days. The readings were taken at 7th and 15th day by using spectrometer at 600nm. The degradation rate was calculated using the method given below.

$$\text{Degradation rate} = \left(1 - \frac{\text{O.D. of experimental sample}}{\text{O.D. of control sample}}\right) \times 100$$

d. GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) was carried out to find the best fungal isolate to degrade the PAHs. Five days old culture was inoculated on the BH broth supplemented with 1% fuel oil according to the method described by Asemoloye *et al.* (2020) [6]. The cultures were kept for 15 days and continuously shaken at 120rpm. The tube without fungal isolate was kept as control. For GC-MS analysis the aliquots were mixed in dichloromethane in 1:1 ratio and mixed vigorously and vortexed for a minute and allowed to settle for 5 minutes for the phase separation. The organic layer was carefully extracted and centrifuged for 3000rpm for 3 minutes. The GC-MS was carried out at Gujarat Biotechnology Research Centre (GBRC), Gandhinagar. The instrument condition was set to split mode, with injection temperature of 260 °C, initial oven temperature was set at 50 °C for 5 minutes and then increased at the rate of 20°C/minute to 260 °C where it was held for 14.5 minutes.

Result and Discussion

Collection and Isolation of Fungal Strain

A total of 22 fungal strains were collected and isolated from the above-mentioned places and labelled as SA 1 and SA 22. The isolates were cultured on PDA media in triplicate and tentative identification was carried out using the morphological and microscopic characteristics. Based on the characters observed five distinct species of *Aspergillus*, three species of *Penicillium*, and three species of *Trichoderma* were identified. Additionally, single species of *Phanerochaete*, *Flavodon*, *Agaricus*, *Fusarium*, *Mucor* and *Rhizopus* were also identified.

Screening of Suitable Fungal Isolates

Qualitative screening

The collected fungal strains were cultured for the qualitative screening, zone of clearance around the fungal biomass was taken into consideration for finding the potential strains for PAHs degradation. Out of 22 fungal isolates only 8 strains showed positive result for zone of clearance, while 12 strains showed no results. Additionally, two strains i.e., SA 19 and SA 20 failed to grow on the contaminated media. The detail finding and the images is given in table 1 and

figure 1 respectively. Among the fungal isolate that exhibited the zone of clearance, SA 4 demonstrated the earliest result, followed by SA 8, SA 5 and SA 15. Although SA 2, SA 14 and SA 17 exhibited positive result, the intensity was comparatively lower.

Positive result for the zone of clearance was observed in *Penicillium sp.* which was aligning with the results of Hamad *et al.* (2021) [16] and Adigwe *et al.* (2022) [1] who found zone of clearance for *Penicillium chrysogenum*. However, according to Daâssi and Almaghribi (2021) [11] smaller zone of clearance was observed for *Penicillium sp.*, contradicting to the present results. *Aspergillus sp.* i.e., SA 5 and SA 15 also showed significant clearance zone while SA 2 and SA 14 showed minimal results. This contrasts the

previous results of Ogbonna *et al.* (2013) [23] and Adigwe *et al.* (2022) [1] where *Aspergillus flavus* and *Aspergillus niger* exhibited higher degradation.

Trichoderma sp. showed no zone of clearance, contradicting to the earlier reports of *Trichoderma harzianum* exhibiting degradation potential (Osazee *et al.*, 2019; Ameko *et al.*, 2013) [24, 4]. Similarly, *Fusarium solani* showed positive result which was consistent with the study by Al-Otibi *et al.* (2023) [3]. No zone of clearance was observed in *Mucor sp.* and *Rhizopus sp.*, which was differing from the previous studies (Osazee *et al.*, 2019; Ferdinand *et al.*, 2020) [24, 13]. There was absence of zone of clearance for *Flavodon sp.* and *Agaricus sp.* with the lack of the prior reports for both.

Table 1: Zone of clearance shown by various fungal strains. (✓) indicates the fungi have shown the zone of clearance, (-) indicates there is no zone of clearance observed in the fungi

Sr No.	Name of fungal strain	Day 7	Day 15
1.	SA 1	-	-
2.	SA 2	✓	✓
3.	SA 3	-	-
4.	SA 4	✓	✓
5.	SA 5	✓	✓
6.	SA 6	-	-
7.	SA 7	-	-
8.	SA 8	✓	✓
9.	SA 9	-	-
10.	SA 10	-	-
11.	SA 11	-	-
12.	SA 12	-	-
13.	SA 13	-	-
14.	SA 14	✓	✓
15.	SA 15	✓	✓
16.	SA 16	-	-
17.	SA 17	✓	✓
18.	SA 18	-	-
19.	SA 19	No Growth	No Growth
20.	SA 20	No Growth	No Growth
21.	SA 21	✓	✓
22.	SA 22	-	-

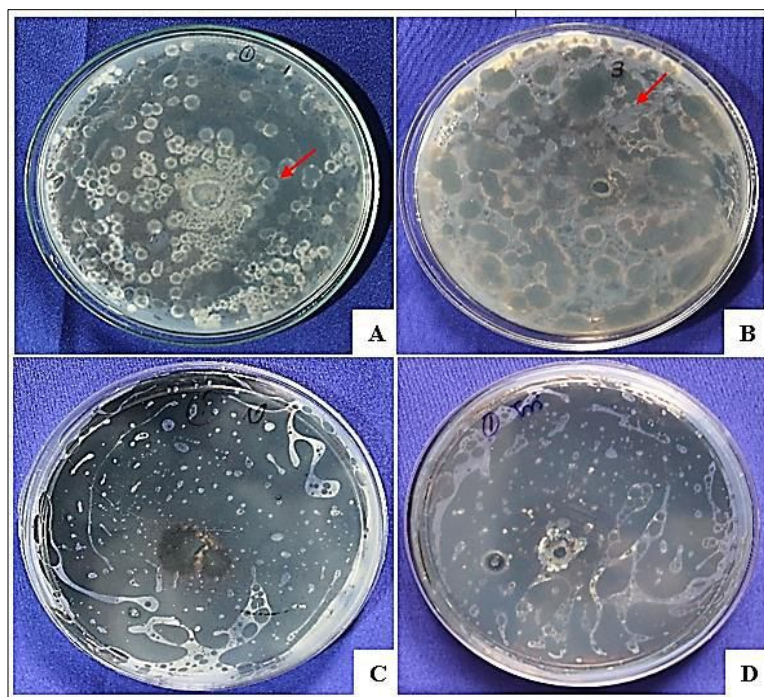


Fig 1: a and b- zone of clearance seen in SA 4 and SA 8. c and d- no clear zone in SA 6 and SA 18.

Quantitative screening

a. Tolerance test

The ability of fungal isolate to tolerate the contaminant was compared by monitoring their growth in 1% fuel oil contaminated media. Out of 22 fungal isolates tested eight strains i.e., SA 1 to SA 6, SA 11 and SA 15 showed tolerance value above 70%. Highest tolerance was recorded in SA 1 and SA 11, SA 2 and SA 3 also showed significant tolerance. These results suggest that certain strains can survive and grow in the contaminated area. Table 2 provides details of the tolerance test of various isolates while figure 2 shows rate of tolerance at different days.

Several strains in this study showed low tolerance. *Trichoderma* species reported less tolerance like the study done by Tiso *et al.* (2015) ^[28], who found no tolerance in

Trichoderma species despite positive result shown in previous other studies (Argumedo-Delira *et al.*, 2010; Shakam & Gaber, 2024; Zafra *et al.*, 2014) ^[5, 25, 30]. In contrast, high tolerance was recorded in *Aspergillus flavus*, *A. fumigatus* and *A. oryzae* which was consistent with previous studies. However, *A. oryzae* and *A. niger* showed moderate tolerance in contrast to Jawhari (2014) ^[18] where *A. niger* showed high tolerance. *Penicillium oxalicum* exhibited low tolerance rate while average tolerance was observed in *Penicillium chrysogenum* and *Penicillium* spp., which aligned with the research done by Zafra *et al.* (2014) ^[30] but contradicted with the results of Velez *et al.* (2019) ^[29]. Two studies conducted by Shakam and Gaber (2024) ^[25] and Cruz-Izquierdo *et al.* (2021) ^[10] reported low tolerance for *Fusarium solani* which was average for this study.

Table 2: Tolerance rate of all the fungal isolates. '++++': tolerance rate between 76-100%, '+++': tolerance rate between 51-75%, '++': tolerance rate between 26-50%, '+': tolerance rate between 1-25%, '0': No growth/ tolerance

Name of fungal strain	Tolerance rate
SA 1	++++
SA 2	++++
SA 3	++++
SA 4	++++
SA 5	+++
SA 6	++++
SA 7	++
SA 8	+++
SA 9	+
SA 10	+++
SA 11	++++
SA 12	+
SA 13	++
SA 14	+
SA 15	++++
SA 16	++
SA 17	++
SA 18	++
SA 19	0
SA 20	0
SA 21	++
SA 22	++

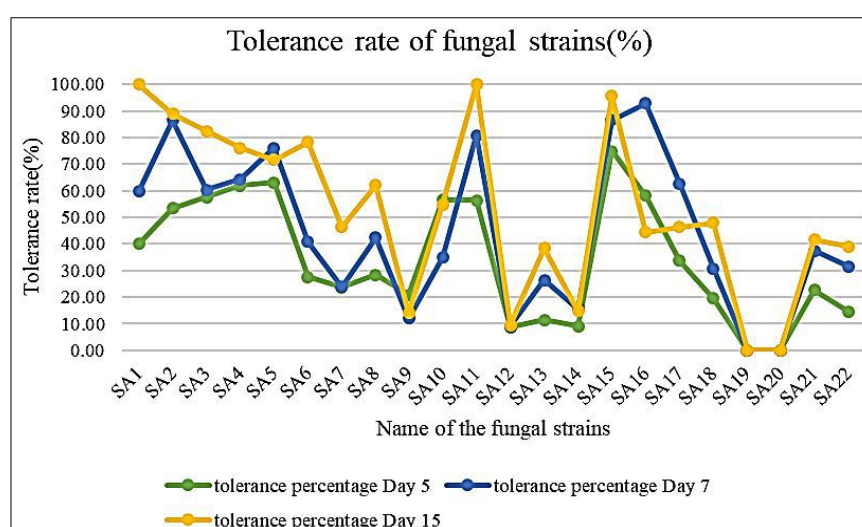


Fig 2: Tolerance percentage on various days for each fungal isolate

b. Paired interaction test

Compatibility of the selected fungal strains were tested as mentioned above. Each strain was co-cultured and observed on day 7 and day 15. Among all the isolates SA 2 showed

the highest growth rate while SA 5 shows the slowest growth rate. Almost all the isolates showed inhibition to each other. In the co-culture of SA 2 and SA 4, SA 2 overgrows SA 4 where SA 2 inhibited the growth of SA 4.

Similar result was seen in SA 4 and SA 17, where SA 17 overgrows on SA 4. SA 5 and SA 15 showed compatibility

through neutral intermingling. Table 3 describes the detail of co-culture test.

Table 3: Result of the compatibility test of the selected fungal isolates

Fungal strain	SA 2	SA 4	SA 5	SA 8	SA 15	SA 17
SA 2	-	Replacement, SA 2 grows over SA 4	Deadlock	Deadlock	Deadlock	Deadlock
SA 4	-	-	Deadlock	Deadlock	Deadlock	Replacement, SA 17 grows over SA 4
SA 5	-	-	-	Deadlock	Neutral intermingling	Deadlock
SA 8	-	-	-	-	Deadlock	Deadlock
SA 15	-	-	-	-	-	Deadlock
SA 17	-	-	-	-	-	-

c. Colorimetric assay

Six fungal strains were selected for the colorimetric assay using DCPIP test based on the results of tolerance test and qualitative assay. Optical density was taken at 600nm, visual result showed discoloration from blue colour to colourless. The highest degradation rate was seen in SA 15 (85.8%) while lowest was observed in SA 17 (65.6%). Positive colour change is seen in *Aspergillus flavus* (84.4%) which has been reported in various studies while certain studies show moderate colour change. Both strains of *A. oryzae*

showed significant colour change of approximately 85% which is consistent with the previous research. According to research by Gupta, S., & Pathak, B. (2019) [15] high colour change was noted for the *Penicillium* sp. which was similar to the results of the present study. According to the previous studies *Fusarium solani* showed the least colour change which was consistent with the present result. However, no reports on DCPIP test for *Phanerochaete chrysosporium* was found which was shown to be positive in this study.

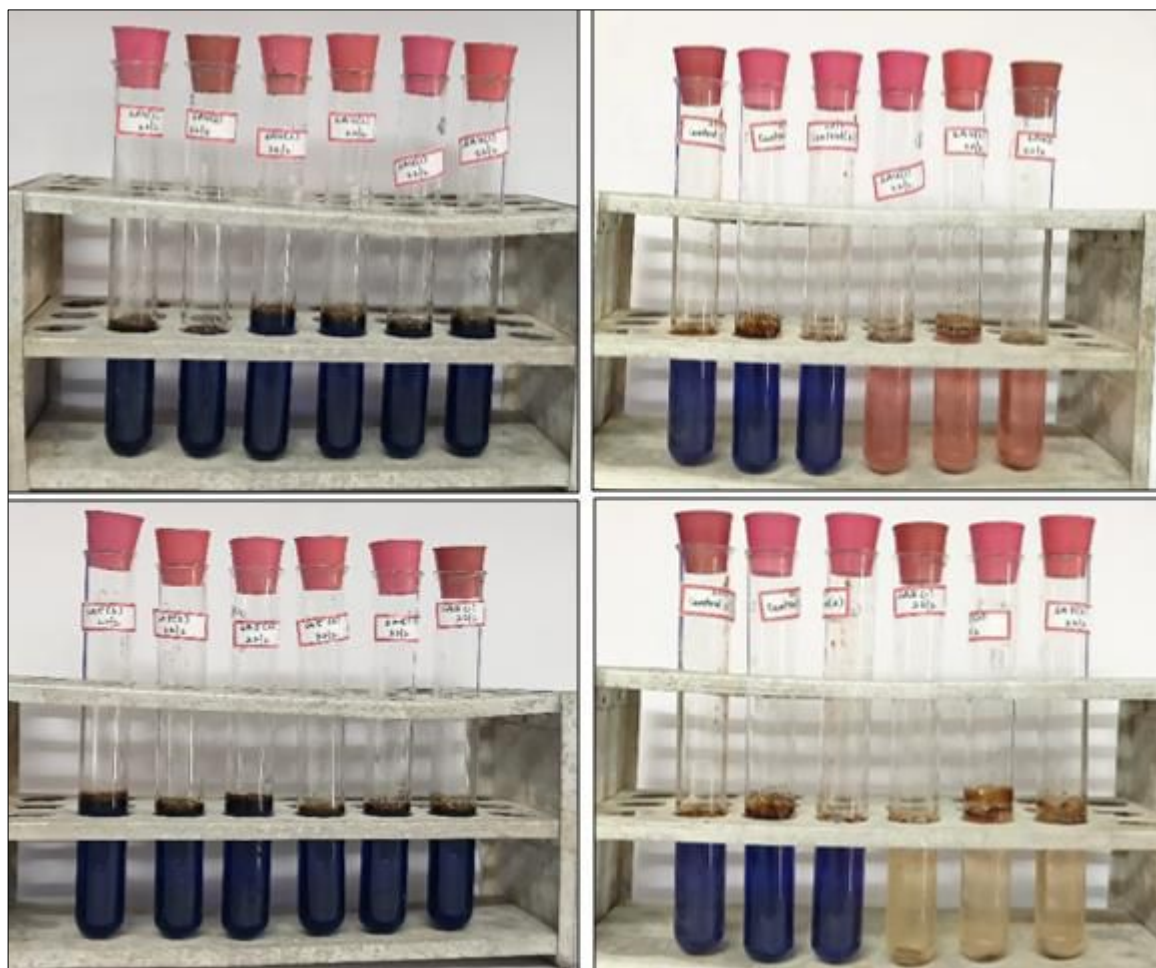


Fig 3: DCPIP assay; (A): control sample of SA 4, (B): colorimetric assay of SA 4, (C): control sample of SA 5, (D): colorimetric assay of SA 5

d. GC-MS Analysis

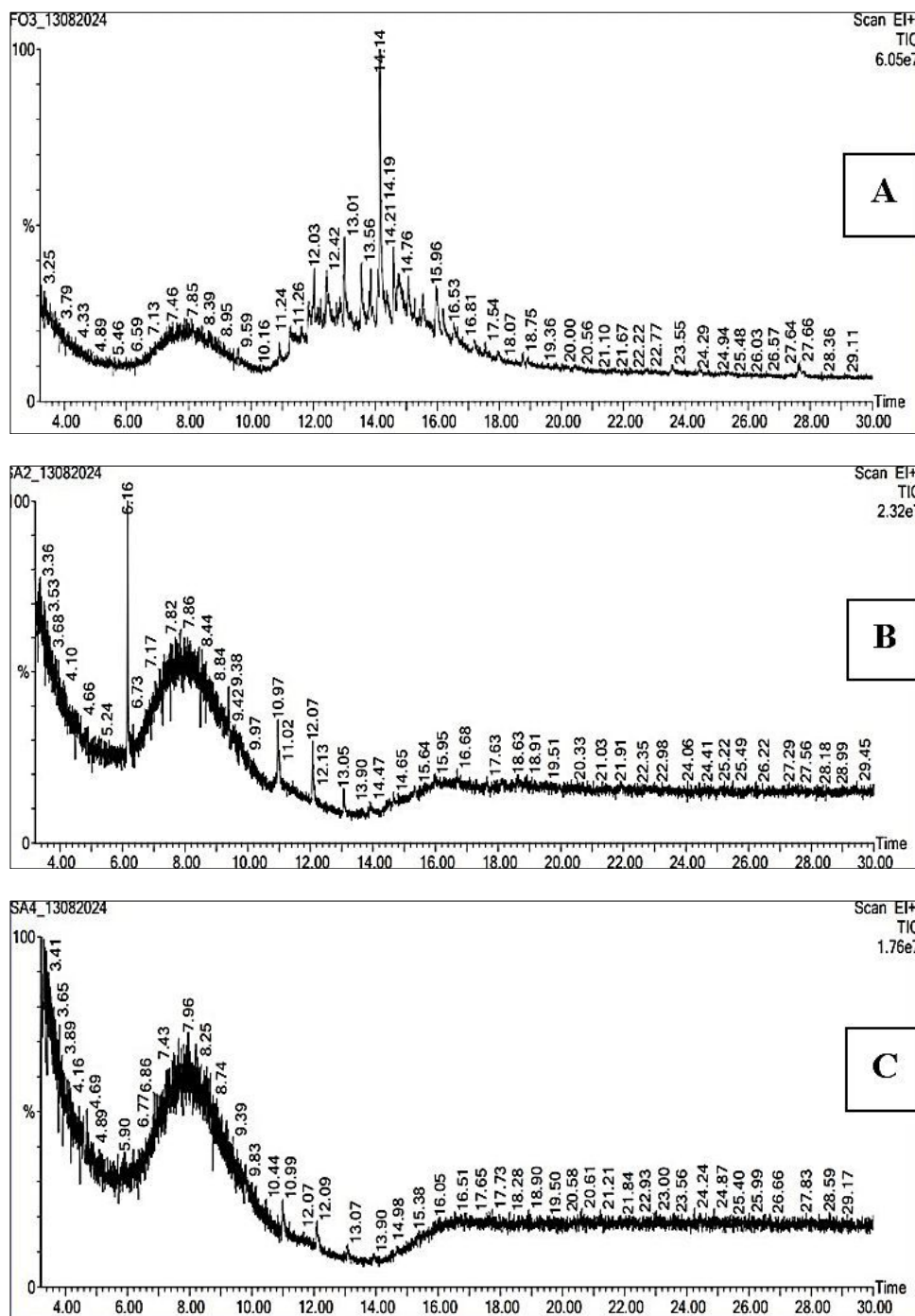
The efficiency of PAHs degradation by six fungal isolates namely SA 2, SA 4, SA 5, SA 8 and SA 15 was compared using GC-MS. The TIC of control sample was 6.05×10^7 which showed the highest number of compounds present in

it. Highest degradation was seen in SA 4, SA 5 and SA 15 with TIC of 1.76×10^7 , 1.67×10^7 and 1.64×10^7 respectively. While moderate degradation was seen in SA 8 with TIC of 1.80×10^7 and lowest was seen in SA 2 with TIC of 2.32×10^7 . The GC-MS data for the fuel oil

indicated peaks for the heavy PAHs at the RT between 11-18 while not much peak at the earlier RTs were seen for the lighter compounds as compared to the degraded samples. In contrast all the other degraded samples showed the peaks at the RT between 3-10 indicating the possibility of the formation of the lower weight compounds after degradation. SA 15 showed higher number of peaks at early RTs showing the possibility of higher number of lighter compounds formed after degradation of heavy weight PAHs. There was decrease in the peak of the heavy compounds which was seen in the control samples proving the ability of SA 15 to degrade PAHs. Similar results were observed in SA 4 and

SA 5 where there was disappearance of peaks from 10-18 RTs and increase in the size of peaks from 4-9 RTs depicting the degradation of heavy compounds to the lightweight compounds.

SA 2 and SA 8 showed almost similar degradation profile where there was disappearance of the higher weight compounds while most of the peaks were seen at low and mid RT range which shows the probability of partial degradation of the heavy compounds. From the above interpretation SA 4, SA 5 and SA 15 showed the highest potential for the degradation of PAHs as compared to the other samples.



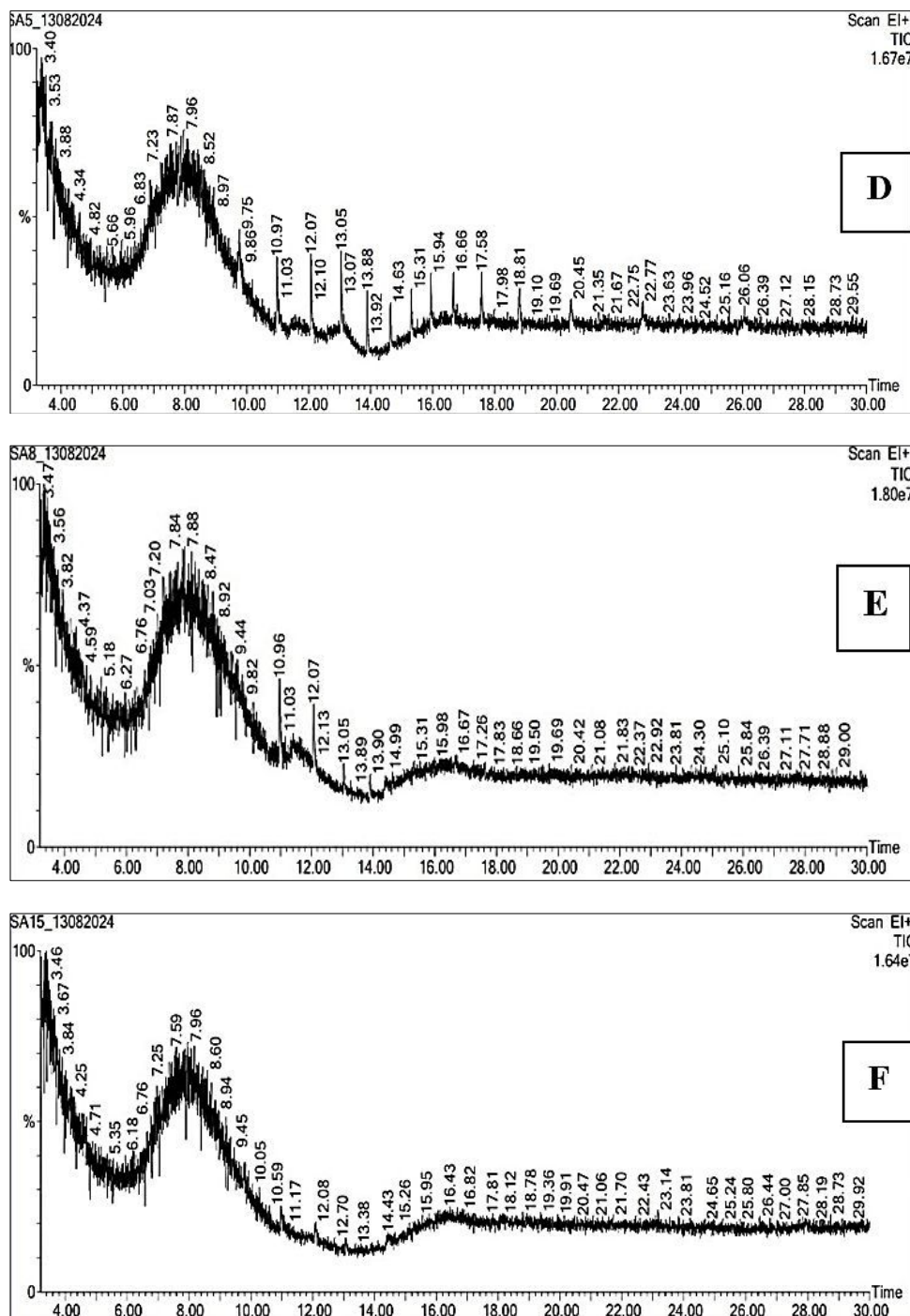


Fig 4: GC-MS chromatogram of the control samples with the chromatogram of treated samples; (A): chromatogram of control, (B): chromatogram of SA 2, (C): chromatogram of SA 4, (D): chromatogram of SA 5, (E): chromatogram of SA 8, (F): chromatogram of SA 15.

Conclusion

Overall, this study investigated the capacity of 22 fungal strains to bioremediate fuel oil contaminated media by breaking down hydrocarbons. The potential fungal isolates were selected based on the methodological process which included qualitative and quantitative analysis using tolerance test, colorimetric assay, compatibility tests, DCPIP assay, and GC-MS analysis. As indicated by the method three fungal isolates namely SA 4, SA 5 and SA 15 from *Aspergillus* and *Penicillium* sp. showed the potential to degrade the hydrocarbons. The degradation of the hydrocarbon was validated by the change in the coloration during the DCPIP assay as well as the shift in the retention time of the GC-MS analysis. As observed, the potential fungal isolates can be further evaluated for the field-based

application. Moreover, further research is needed to find out the enzymes responsible for the degradation of PAHs and its quantification. Furthermore, the potential of the strains can be increased by the optimization of the various parameters which can help in faster degradation of the contaminants.

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