ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Mycoremediation of Hydrocarbon and its products using *Fusarium oxysporum*

S. Ivo Romauld[1]*, R. Venkataraghavan[1], D. Yuvaraj[2], Ishwarya Devi. V[2], Hashika. S[2]

[1]Department of Bio-Engineering, VISTAS, Pallavaram, Chennai-117

[2]Department of Biotechnology, Vel Tech High Tech Dr. Rangarajan, Dr. Sakunthala Engineering College, Avadi, Chennai

*Corresponding Author E-mail: **ivoromauld@gmail.com**

ABSTRACT:

Oil spillage is one of the major causes of environmental pollution which diversely affects the ecosystem. Mycoremediation plays a significant role in degrading or removing substances like hydrocarbons, polychlorinated compounds, heavy metals and others toxicants from the affected environment using fungi. The aim was to isolate the fungi from the contaminated soil and evaluate its degradative potential. The contaminated soil sample was collected from an automobile shop in Padi, Chennai. The fungi were grown in Bushnell Haas Agar medium and isolation of pure colonies was done. Two fungi species were grown. Hydrocarbon degradation assay was performed with the isolated fungi and the hydrocarbon chosen for degradation was naphthalene. The growth profiles were monitored and the absorbance was measured up to 9 days. One of the isolated fungi STRAIN-2 (C1-P) efficiently degraded 1% naphthalene and was confirmed by methylene blue assay. The molecular characterization of STRAIN-2 (C1-P) was done to generate DNA barcode for identifying the organism. The organism was identified as *Fusarium oxysporum* using morphological observations and genomic DNA sequencing. The identified fungi achieved 98% similarity with accession number KR0470601 from NCBI.

KEYWORDS: Oil spill, Mycoremediation, hydrocarbons, polychlorinated compounds, Naphthalene degradation, methylene blue.

INTRODUCTION:

There is an increase in the demand for the use of crude oil as a source of energy, unintended and related oil spill have been greater familiar during the manner of exploration, production, transportation and storage of oils. The oil spills on land are usually localized and their impact is restricted to the certain vicinity (Patin*et al*., 2004). As oil spills on land intercept the water absorption by the soil. Moreover, spills on agricultural farms or grasslands have destructive effects on the cultivation area results in damage to the crops[1].

Received on 18.12.2018 Modified on 11.01.2019
Accepted on 09.02.2019
Research J. Pharm. and Tech 2019; 12(9):4216-4224.
DOI: 10.5958/0974-360X.2019.00725.X

If there is an oil spill in these environments, the predominant best responsive method is to prevent the oil from leaching into groundwater or mixing up with the waterbodies surface as run-off as soon as possible since these hydrocarbons are toxic and have harmful effects on the lives which consume this contaminated water. In urban areas, spill response strategies provide primary shielding on the health of human beings thereby re-establishing the usability of the location of the spill as soon as possible.[1,2] Two standard tactics can be undertaken: to use surfactants or fertilizers to promote the microbial wreckage process of spilled oil and to seed microorganisms that are active chemical hydrocarbon degraders (Mulkinsphillips and Stewart, 1974). Several types of research are ongoing in order to degrade the oil spills efficiently, which includes the illumination on both the abiotic and biotic factors that influence the rate of degradation of the hydrocarbons.[2,3]

……

Mycoremediation:

Mycoremediation is the process of degradation of oil spills and hydrocarbon contaminants from the

contaminated area which uses the technique that involves fungal microorganisms for in-situ and ex-situclean up and of the contaminated land area. This technique is to degrade the specific contaminants, like petroleum hydrocarbons, pathogens, metals, and organophosphates with an augmented efficiency under specific and selective environmental regimes (Thomas *et al*., 1999).[3,4] They can survive in every habitat and develop under toxic conditions intolerable for most bacteria (Aust*et al.,* 2003). Fungi are exogastric in nature, which generate an opportunity for the metabolism of new substrates which are nonpolar, insoluble toxic chemical compounds which are not persuadable to intracellular process like cytochrome p450 (Reddy and Mathew, 2001; Levin *et al.,* 2003).[5] The growth rates of fungi are also fast enough which makes them the best ever possible bioremediators. Moreover, apart from their capability to penetrate through contaminating substrates, fungi are considered more superior to bacteria since they can survive under stressed environmental conditions including poor nutrient availability and low pH (Davis and Westlake, 1979). On the basis of involvement in remediation process of pollutants, the fungi can be classified as ligninolytic fungal degradation, Soil fungal biosorption, Mycorrhizal fungal degradation.[6-10] Strategies for the detection of molecular characteristics include Polymerase Chain Reaction (PCR), followingAgarose Gel Electrophoresis (AGE), Restriction Fragment Length Polymorphism for PCR (RFLP), In-situ hybridization (ISH). Polymerase Chain Reactions are widely used to amplify the recombinant DNA sequences selectively to deduce the total fungal diversity and their taxonomy using short oligonucleotide single-stranded specific primers complementary to the target DNA sequence (White *et al.*, 1990).Fungal species such as *Talaromyces, Neosartorya*, *Amorphoteca* and *Graphium*and yeast species such as *Candida, Pichia* and*Yarrowia* were isolated from oil and hydrocarbon contaminatedsoil and have shown potential to degrade the hydrocarbon efficiently (Bury *et al.,* 2004). A group of terrestrial fungal genera such as *Cephalosporium, Pencillium,* and *Aspergillus* was also shownto be the potential hydrocarbon degraders from the crude oil contaminated sites.[11-15] Yeast species such as *Trichosporonmucoides, Rhodotorulamucilaginosa, Candida lipolytica, and Geotrichum*sp, isolated from contaminated water have also shown the potential to degradethe petroleum compounds effectively (Dabrowski*et al*., 2004).[16,17]

Characterized fungi *Fusarium oxysporum:*

Classification of the species- Superkingdom: *Eukaryota*, Kingdom: *Fungi*, Phylum: *Ascomycota*, Class: *Sordariomycetes*, Order: *Hypocreales*, Genus: *Fusarium.* The fungal species *Fusarium oxysporum* is a ubiquitous microorganism, which is a fungal parasite *Formaespeciales* found in diverse regions across the globe. This organism is mainly responsible for tomato wilt infections in plants and has been found to infect plants and crops in 32 countries across the globe. This fungus infects various types of crops in North and South American continent, Africa, Europe and Asia. This fungal species requires an optimum temperature of 28°C for its essential survival in nature. This fungus predominantly affects the roots of the plants with an optimum temperature of 30°C for its infection and its infection through the seed requires an optimum temperature of 14°C. Some strains of *Fusarium oxysporum* are pathogenic to humans. *Fusarium oxysporum* is pathogenic to both humans and as well as animals due to the release of mycotoxins.[18]

MATERIALS AND METHODS:

Sample collection:

Non-sterile soil samples were collected from diesel oil contaminated sites, Padi, Chennai. This site is exposed to several soil contaminants over the past years. The contaminants in the polluted soil are less bio accessible when compared to artificially contaminated, "spiked" soil. Lastly, there is a competition between the inoculated and aboriginal (native) microorganisms in the non-sterile contaminated soil.

Media preparation:

23.27 grams of BHA along with trace elements such as calcium chloride, magnesium sulphate, and ferric chloride were suspended in 1000 mL distilled water. The mixture is boiled in a water bath at 90°C to dissolve the agar medium completely, followed by sterilization in an autoclave at 15 psi pressure at 121°C for 15 minutes. A white colour precipitate before sterilization becomes yellow to orange after sterilization.[19,20] This media contains all the essential nutrients for the survival of the microorganism and do not contain any carbon source, which is mandatory for the growth of microbes. Carbon source such as a hydrocarbon is added to the growth medium and their utilization by the microorganisms can be examined. Ammonium nitrate is an excellent nitrogen source to the microbes while potassium phosphate and monopotassium phosphate are buffers that maintain the pH of the medium.

Screening, isolation and maintenance of diesel degraders:

The fungus was isolated from oil contaminated soil by using the Bushnell Hass Mineral Salts medium with diesel oil as a carbon source. The diesel oil was sterilized by moist heat sterilization by using an autoclave at 121˚C for 15 minutes in a sealed conical flask. The fungus was isolated by using an enrichment culture and a single colony isolation technique. The isolated culture was preserved in Potato Dextrose Agar

(PDA) slants and stored at 4°C for further use.[20,21]. The microorganisms grown and cultured on the agar plate will develop into colonies on to the plate after incubation at 28°C for 24 to 36 hours. The resultant number of colonies appearing on the plates denotes the number of living microorganisms in the soil sample. Soil samples were subjected to fungal enumeration by serial dilution and spread plate method. The serial dilution was carried out and spread plate done using Bushnell Haas Agar (BHA) plates.[19] The most commonly used series of dilutions factors with each transfer are (1:2, 1:4, 1:8 and so on A measured volume of sample is transferred to series of other tubes and mixed with dilution solvent or sterile distilled water, thereby creating a lower concentration from the previous titer. For example, if 10 mL of sample volume is mixed with 90 mL of dilution solvent. In this case, the resultant is 1:10 dilution. For screening, 1 gram of oil-contaminated soil sample was mixed with 10 mL of distilled water and vortexed. This solution sample was diluted up to 10^{-9} serial dilution. By using a sterile pipette, 1mL of solution sample was transferred into 10^{-1} tube. The test tube was mixed properly. The dilution is continued with aseptic technique until 10^{-9} dilutions. One mL from 10^{-1}, 10^{-3}, 10^{-5} and 10^{-7} dilutions were used as an inoculum for isolating the hydrocarbon degrading fungi. Each of serial dilution (10^{-1}, 10^{-3}, 10^{-5} and 10^{-7}) was transferred to BHMS agar plate by using spread plate method. Then 0.5mL of diesel oil (hydrocarbon) was added to the Petri plates and incubated. The plates are incubated for 2-5 days at 29°C until the colonies appear. It is important that the surface of the plate be fairly dry so that the spread liquid soaks in. The medium selectively permits the hydrocarbon-degrading microorganisms to grow.The colonies were isolated on the basis of physical morphology and the isolated colonies of fungi were preserved in Potato Dextrose Agar as slants in test tubes and incubated at 4°C.

Screening: Potential for mycoremediation:

The potential for mycoremediation was assessed in three ways. The principle developed in this study was to test whether or not an isolated colony can grow when a hydrocarbon is used as the sole carbon source. If fungi can grow under this condition, it means that the colonies can use the hydrocarbon for their metabolism and biodegradation is likely to happen. Naphthalene was selected as the hydrocarbon source. Naphthalene is a prototypic Poly Aromatic Hydrocarbons and acts as a marker compound to identify and detect PAH contamination since their chemical structure is found in carcinogenic PAHs.[22,23] They have also been used as model PAHs to determine factors that affect the bioavailability, biodegradation potential and rate of microbial degradation of PAHs in the environment.[33]

UV- spectrophotometer assay:

Bushnell- Hass broth (Desai *et al.,* 1993) was used for confirming the biodegradation capability of indigenous fungal colonies. The isolated colonies were inoculated into 25 mL of screening medium into glass conical flasks. The screening medium and enrichment BHMS broth were the same and possess diverse concentrations of naphthalene dissolved in chloroform such as 1%, 2%, and 3%. Moreover, 0.1% tween 80 was added to each conical flask after autoclaving, as the only carbon source. Then the flasks were incubated at temperature 25°C for ten days. However, the flasks were intermittently shaken at 95 rpm using an orbital shaker to facilitate oil phase contact. The ability of each isolate to naphthalene was indicated by an increase in turbidity of the medium measured at 600 nm using a UV spectrophotometer for 9 days. The degradation of naphthalene was also measured by the decrease in absorbance value measured at 270 nm.

Screening in solid agar plate method:

In the first experiment, the growth potential of each colony that was sub-cultured was determined in a plate containing 20 mL of Bushnell Haas Mineral salt Agar (BHMS).[19] The BHMS agar was prepared and following autoclaving at 121°C for 15 minutes, different concentration of naphthalene (1%, 2%, and 3%) was added to the agar medium. The naphthalene solution was prepared by solubilizing in chloroform. To the agar medium containing naphthalene 0.1%, tween 80 was added and the medium was mixed well for complete solubility. The agar was poured onto Petri plates at 40°C and allowed to solidify.The pure fungal colonies isolated were aseptically inoculated onto the plates containing naphthalene and tween 80. The plates were incubated for 5 to 10 days at 25°C in the incubator. Control experiments were set up by incubating BHMS medium with the test aromatic hydrocarbon without the microorganisms.

Screening in broth: Methylene blue assay:

Preparation of methylene blue solution (Redox indicator): 2% (w/v) methylene blue solution as a redox indicator was prepared. 1 gm of methylene blue was dissolved in 50 mL of distilled water. The prepared solution was sterilized separately at temperature 121˚C at 15 psi pressure for 20minutes. On the basis of results obtained from Screening on solid agar plates and Absorbance value, screening in broth (Bushnell - Haas) was carried out. Based on this, a specific concentration of carbon source for screening of isolates in broth for naphthalene was fixed to be 1% (10µg/mL). Bushnell – Haas broth was used for the screening test. Medium was sterilized at 121˚C, 15 psi pressure for 20 minutes. Agar discs of a pure culture of each fungal isolate were inoculated into 50 mLof sterilized Bushnell – Haas broth in a flask containing 1% of hydrocarbon (Naphthalene)

and Tween 80 (0.1%) and 0.05 mL of methylene blue solution. Control flask was also prepared to have no organism. The control and the sample flask were incubated at temperature 28˚C at 120 rpm for 10 days in an orbital shaker. The flasks were constantly monitored daily for the colour change from deep blue to colorless due to the action and degradation potential of fungi.

Percentage of methylene blue reduction:

After incubation, broth in the flask was subjected to filtration by filter paper to separate biomass and others, followed by centrifugation at 8000 rpm for 15 minutes. Supernatant thus obtained were analyzed spectrophotometrically at 609 nm (for methylene blue). Percentage of biodegradation was calculated by a formula

$$\text{Percentage of Degradation} = \frac{[1\text{-Absorbance of treated sample} \times 100}{\text{Absorbance of control}]}$$

RESULTS AND DISCUSSION:

Isolation of fungus:

According to the quantification of indigenous fungal isolates, the total number of hydrocarbon using fungi in the media were expressed as (X 10 3 CFU /G soil). The count for hydrocarbon utilizing fungal count was from 30 to 100 with an average of 50±1.7 for the sample. Two fungal isolates namely (STRAIN-1(A1-D) and STRAIN-2 (C1-P) were obtained from Bushnell–Haas medium. Of the two isolates obtained, the STRAIN-2 (C1-P) isolate had shown potential for hydrocarbon biodegradation.Serial dilution plating was performed using diesel and petrol, after incubating the plates were observed. The fungal growth was witnessed in 10^{-1} concentration using diesel and in 10^{-1}, 10^{-7} concentration using petrol. Only two strains showed satisfactory results, STRAIN-1(from plate-A containing diesel [A1D]) and STRAIN-2 (from plate-C containing petrol [C1P]). These two strains were further selected for degradation and identification.

Fig.1 Pure culture of the two fungal isolates

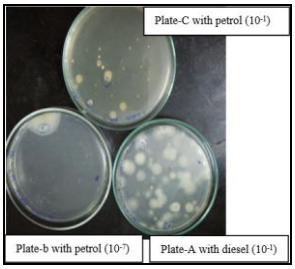


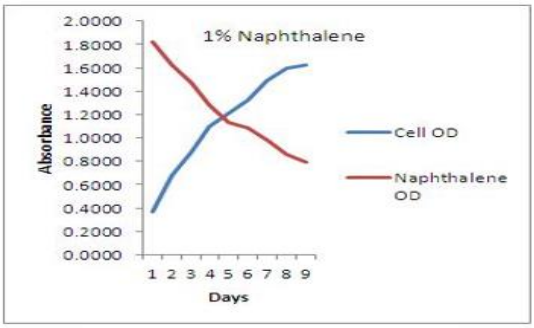
Fig. 2 Isolation of PAH-degrading fungal strains

Fungal growth and naphthalene degradation[24]:

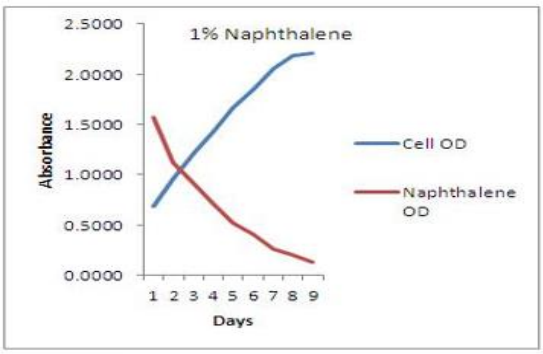
During the first step of confirming the biodegrading capability of fungal isolates, the absorbance (Optical Density) of the enrichment BHMS broth decreased with respect to the fungal degradation capability in each flask. The growth profiles of all the isolates were monitored and the absorbance measured on each day for 9 days. Naphthalene absorbance was measured at the range of 290 nm. In the optimal conditions, the observed highest degradation rate could be related to the increase in cell population under aerobic condition. Growth on naphthalene containing media indicated the ability of a selected isolate to utilize naphthalene as the sole source of carbon and energy. The continuous increase in O.D. at 600 nm on naphthalene supplemented media at concentrations (1%, 2%, and 3%) confirmed that these isolates have the ability to utilize Naphthalene. Maximum degradation and maximum growth potential of fungus were exhibited at a concentration of 1% Naphthalene[25].

Table 1 Fungal growth and Naphthalene degradation at 1% of naphthalene

	STRAIN-1 (A1-D)		STRAIN-2 (C1-P)	
Day	**Cell OD at 600 nm**	**Naphthalene OD at 290 nm**	**Cell OD at 600 nm**	**Naphthalene OD at 290 nm**
1	0.3702	1.824	0.6820	1.573
2	0.6801	1.628	0.9515	1.1245
3	0.8756	1.4693	1.2202	0.9024
4	1.0971	1.2836	1.4308	0.7041
5	1.2099	1.1296	1.6614	0.5127
6	1.3207	1.0901	1.8544	0.3945
7	1.4893	0.9841	2.0533	0.2631
8	1.5931	0.8562	2.1818	0.1945
9	1.6214	0.7965	2.2060	0.1263



STRAIN-1 (A1-D)



STRAIN-2(C1-P)

Fig. 3 Cell and naphthalene absorbance at 1% concentration

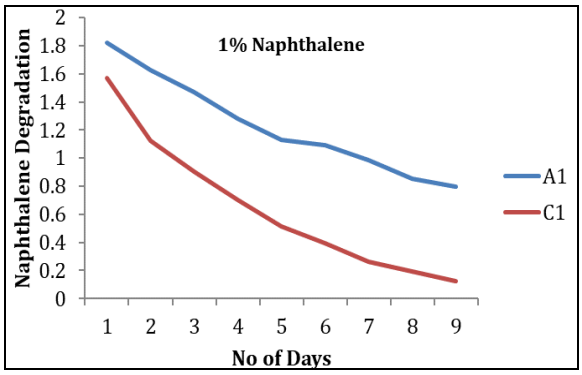
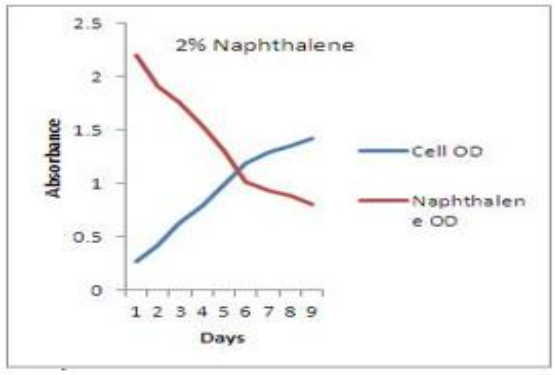


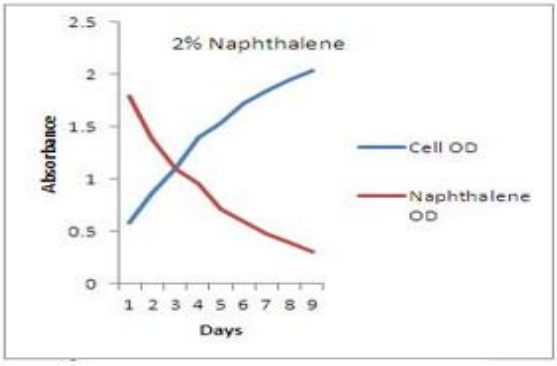
Fig. 4 Naphthalene degradation at 1% concentration
A1-STRAIN1with diesel; C1- STRAIN 2 with petrol

Table 2 Fungal growth and Naphthalene degradation: 2% Naphthalene

	STRAIN-1 (A1-D)		STRAIN-2 (C1-P)	
Day	Cell OD at 600 nm	Naphthalene OD at 290 nm	Cell OD at 600 nm	Naphthalene OD at 290 nm
1	0.2754	2.196	0.5863	1.797
2	0.4192	1.9108	0.8635	1.392
3	0.6281	1.7569	1.1068	1.102
4	0.7949	1.5369	1.3979	0.9584
5	0.9928	1.3058	1.5368	0.7145
6	1.1929	1.0147	1.7265	0.5947
7	1.2949	0.9378	1.8365	0.4832
8	1.3575	0.8897	1.9456	0.3963
9	1.4225	0.8014	2.0333	0.3021



STRAIN-1(A1-D)



STRAIN-2 (C1-P)

Fig. 5 Cell and naphthalene absorbance at 2% concentration

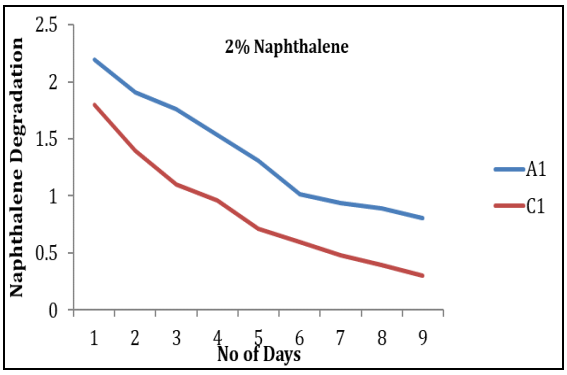
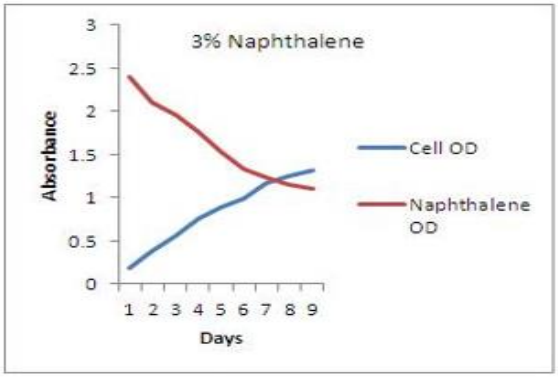


Fig. 6 Naphthalene degradation at 2% concentration
A1-STRAIN1with diesel; C1- STRAIN 2 with petrol

Table 3 Fungal growth and Naphthalene degradation:

	STRAIN-1 (A1-D)		STRAIN-2 (C1-P)	
Day	Cell OD	Naphthalene OD	Cell OD	Naphthalene OD
1	0.1907	2.3987	0.4559	1.9913
2	0.3803	2.1032	0.7427	1.6385
3	0.5552	1.9617	0.9536	1.4047
4	0.7589	1.7659	1.1402	1.2649
5	0.8955	1.5237	1.3133	1.0264
6	0.9825	1.3289	1.5139	0.8038
7	1.1647	1.2281	1.6224	0.7054
8	1.2573	1.1479	1.7253	0.6081
9	1.3179	1.0967	1.8002	0.5135

From the Fig. 3 and Fig. 5; in 1% and 2% naphthalene concentration STRAIN-2 showed high fungal growth rate than STRAIN-2. Degradation rate was also high in STRAIN-2 (Fig.4 and Fig.6). In 3% naphthalene concentration, STRAIN-1 didn't show high fungal rate as other concentrations. Strain-2 showed average fungal growth and degradation rate. Thus, among the 2 isolates, STRAIN-2 C1-P with 1% naphthalene content displayed the highest extent of biodegradation. It showed maximum degradation against all the concentrations of Naphthalene. From these, STRAIN-2 C-1P were selected as potential strain and used for Identification.



STRAIN-1 (A1-D)

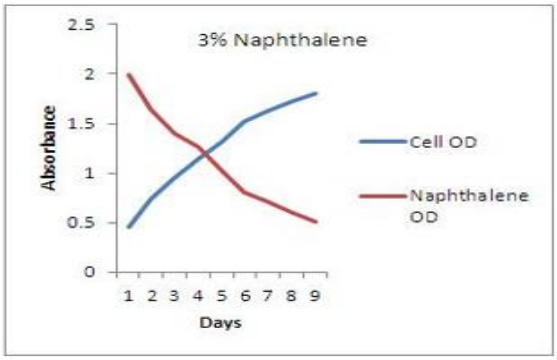


Fig.7 Cell and naphthalene absorbance at 3% concentration

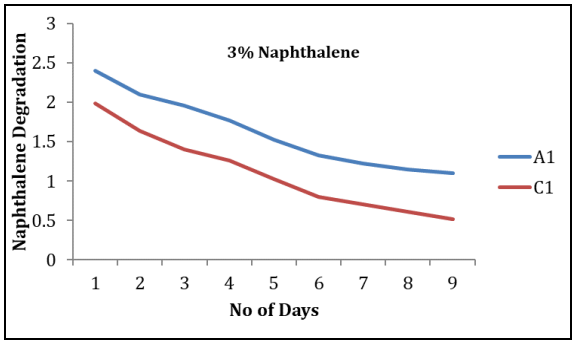


Fig. 8 Naphthalene degradation at 3% concentration
A1-STRAIN 1with diesel; C1- STRAIN2 with petrol

Comparison between Naphthalene utilization by fungal isolates:

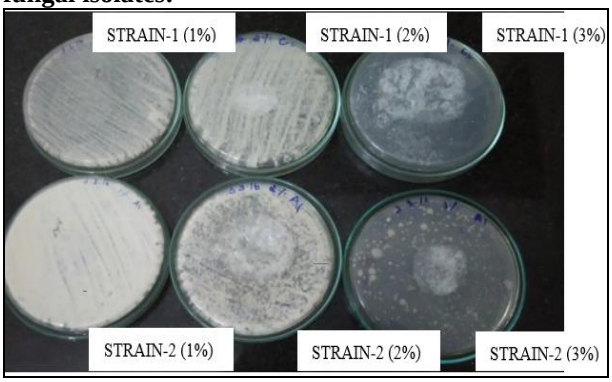


Fig. 9 Screening in soild agar plate

Methylene blue assay:

During the last step of confirming the biodegrading capability of fungal isolates, a color change occurred in the Bushnell- Haas broth medium (deep blue to colorless) which indicates the extent of fungal degradation. The greater in the variation in the colour indicates the augmented degradation ability of the microbes. This change in the colour of the medium is denoted by the potential of the fungal isolates is due to the reduction reaction of the indicator due to the oxidized products formed due to the degradation of hydrocarbons. The resultant color change (blue to colorless) report that the fact that the fungal isolate is a potential hydrocarbon oxidizer. Among the two fungal strains isolated, only one of them showed the potential to degrade the hydrocarbons. *Fusarium oxysporumoxysporum* (STRAIN-2 C1) had shown the faster rate of bioremediation.

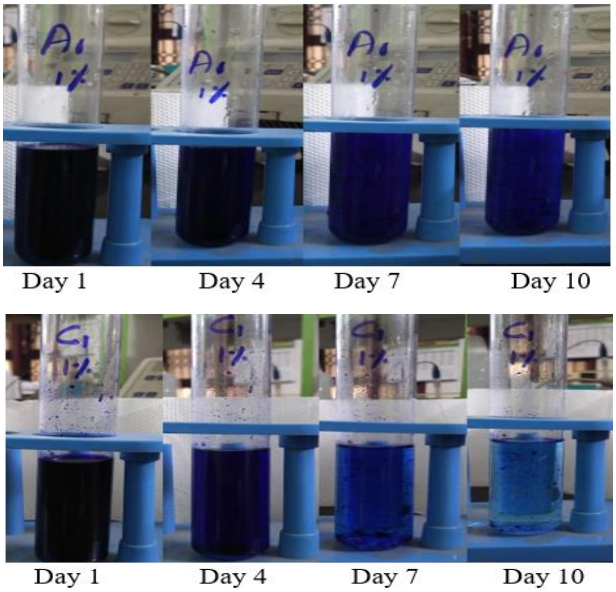


Fig.10 Methylene blue redox reaction in STRAIN-1 and STRAIN-2

Soil contaminated with PAHs poses serious environmental concerns because of their toxic nature and harmful effects. Naphthalene is known to be a model substrate for environmental studies due to their wide distribution, toxicity to biological functions and their presence as a structural part of carcinogenic PAHs, benzopyrene, benzoanthracene and 3–methylcholanthrene. Hence these results reveal the fact that the fungal isolate was able to efficiently degrade PAHs more effectively states that the biodegradation of the aliphatic moieties could be performed in an easier and rapid way than the degradation of their polycyclic aromatic moieties (Englert *et al*. 1993).[26,27] High biodegradation efficiency (>80%) exhibited by *Fusarium oxysporum* within 10 days of incubation showed that the cultural conditions were very appropriate for their growth and biodegradation. The utilization of 0.1% of Tween 80 in the assay was based on the earlier findings that enhanced biodegradation activities (George-Okafor *et al*., 2005). The hydrocarbon degrading abilities of *Fusarium oxysporum* are similar to the findings of (Silva *et al*., 2009) which showed that the organism along with a combination of *Aspergillus sp*. and *Trichocladium sp.* has different hydrocarbon degrading abilities towards PAHs.[28,29] Soil fungi were studied regarding their ability to degrade polycyclic aromatic hydrocarbons (PAHs) and produce ligninolytic enzymes under microaerobic and very-low-oxygen conditions.[30] Low-molecular-weight PAHs (LMW-

PAHs, 2-3 rings) were degraded most extensively by *Aspergillus sp., Trichocladium canadense*, and *Fusarium oxysporum. Fusarium spp.* degraded 55% of 100 ppm anthracene after their incubation at 28°C at 100rpm under controlled laboratory conditions. Fungal strains to PAHs such as *Trichoderma viridi, Fusarium verticelloides* and *Aspergillus niger* isolated from the soil of petroleum refinery have shown to possess potential to degrade naphthalene hydrocarbons (Morasch *et al*., 2001).[26]

Morphological identification:

From the Fig.11,mycelial structures and spores can be observed. From the observation, it is identified as a filamentous fungi. A filamentous fungi possesses a long, threadlike, and branched filaments of cells producding out that looks like tiny hairs when seen through naked eye called hyphae that form a cluster called mycelium, a tangled mass aggregation of hyphae. Hyphae grows on the surface if the cell when grown in culture media. They form aerial hyphae, called reproductive hyphae, that bear asexual reproductive spores or conidia. Hyphae also grows beneath the surface of the culture media and are called rhizoidal hyphae. Examples for filamentous fungi includes *Alternaria sp., Aspergillus sp., Fusarium sp., and Penicillium sp.* From molecular characterization this unknown fungi was identified as *Fusarium oxysporum.*

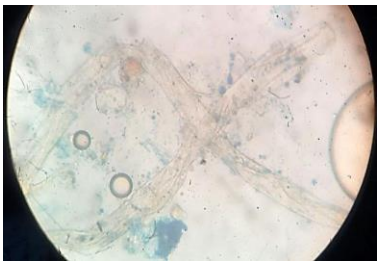


Fig.11 Microscopic view of the fungi

Identification and characterization of the fungi:

Genomic DNA isolation:

Method used for the isolation of genomic DNA was in accordance with a modified method of Strauss, W. M *et al (1995).* Fungal cultures were ground with lysis buffer in an eppendorf with a sterilized plastic pestle. Lysis buffer was used to free the DNA from polysaccharides and sarcosyl solution for the extra digestion of tissues. DNA precipitation was performed with potassium acetate and absolute ethanol. The precipitated DNA was washed with cold ethanol, dried and stored at -20 ºC after dissolving in TE Buffer water. Satisfactory results were obtained for isolation of genomic DNA from the fungal culture.

Table 4 DNA concentration

	OD at 260nm	OD at 280nm	Concentration (ng/µl)	Purity
Blank	0.000	0.000	--	--
Sample	0.209	0.118	10450	1.77

DNA quantification by spectrophotometric method:

DNA quantification:

The isolated DNA was quantified by spectrophotometer at two different wavelengths 260 nm and 280 nm. A260/A280 ratio gives purity of the DNA as proteins absorb at 280 nm due to tyrosine and tryptophan residues. The ratios were in the range of 1.67 which indicated a light protein contamination. DNA concentrations are given in Table 4.Concentration of DNA: A_{260} X 50µg/ml X dilution factor, Dilution Factor = 3ml/3µl = 1000

Polymerase chain reaction:

PCR was subsequently performed on DNA isolated from the fungal species. The required positive amplification was attained for the preferred parts of the gene ITSwith suitable pairs of primers. PCR was implemented by applying the cycling conditions as given in Materials and Methods. All the amplified PCR products were run on 1.5 % agarose at 100 V for 30 min. The ITS region of the fungal species (Lane 1) showed amplification of 600 bp. From the above experiments it was concluded that concentration of DNA template and primer set at optimized annealing temperature (50°C) proved to be best in use for required gene fragments.

DNA sequencing & identification of species:

The purified samples were sent to Eurofins, Bangalore, India for DNA sequencing. After fetching sequences, nBLAST (a database National Centre for Biotechnology Information, www.ncbi.nlm.nih.gov) analysis was performed against each sequence to obtain exact identification of species on the bases of maximum similarity among others. Using BLAST, the species can be identified by their maximum query coverage, a very low E value and maximum resemblance with pre-existing ones. Taxonomic determination of species *Fusarium Oxysporum* could be achieved, showing 98 % similarity with accession number KR047060.1already present on NCBI database.

Sequence:

AAAACACACCTGAATTTGATAACAAGGTCTCCG
TTGGTGAACCAGCGGAGGGATCATTACCGAGTT
TACAACTCCCAAACCCCTGTGAACATACCACTT
GTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAA
ACGGGACGGCCCGCCAGAGGACCCCTAAACTCT
GTTTCTATATGTAACTTCTGAGTAAAACCATAA
ATAAATCAAAACTTTCAACAACGGATCTCTTGA
TTCTGGCATCGATGAAGAACGCAGCAAAATGCG
ATAAGTAATGTGAATTGCAGAATTCAGTGAATC
ATCGAATCTTTGAACGCACATTGCGCCCGCCAG
TATTCTGGCGGGCATGCCTTTTCGAGCGTCATTT
CAGCCCTCAAGCACAGCTTGGTGTTGGGGCTCG
CGTTAATTCGCGTTCCCCAAATTGATTGGCGGT
CACGTCGGGCTTCCATAGCGTAGTAGTAAAACC
CCCGTTACTGGTAATCGTCGCGGCCACGCCGTT

TAACCCCAAATTCTGAATGTTGACCTCGGATCA
GGTAGGAATGCCCACTGAACTTAAGCATACTAT
GCCCGCCAA

Sequence alignment:

Phylogenetic analysis:

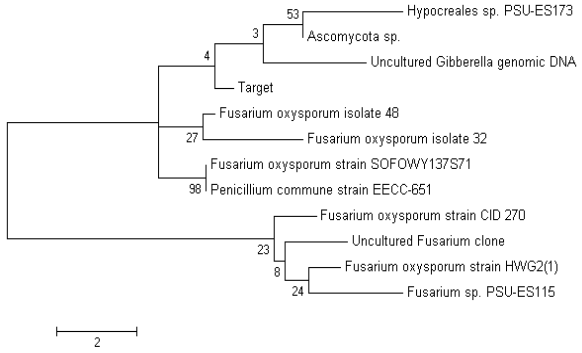


Fig.12 Phylogenetic analysis of *Fusarium oxysporum*

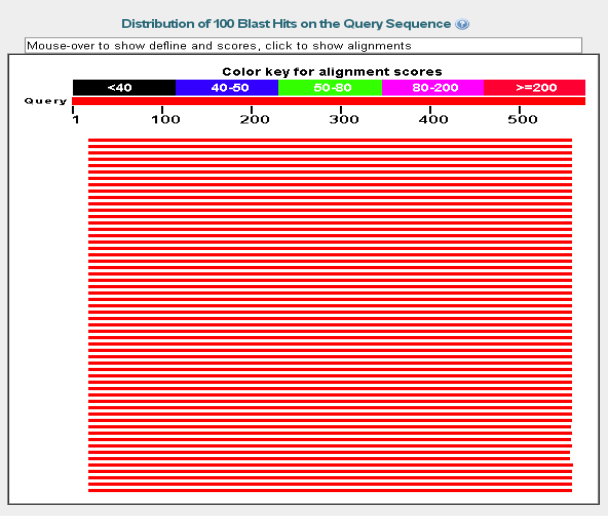


Fig.13BLAST analysis

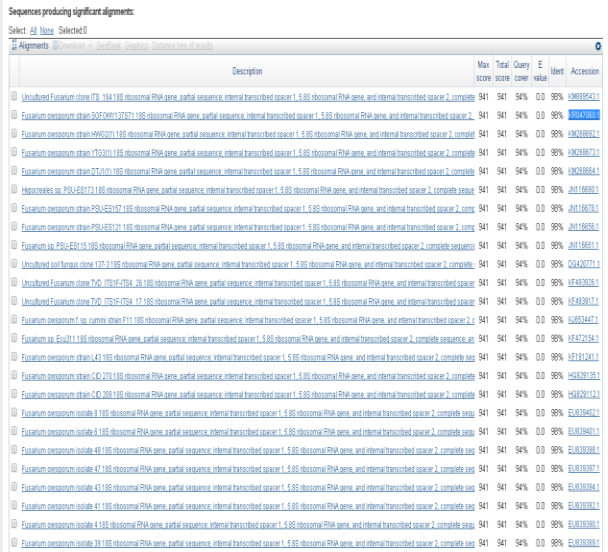


Fig.14 Sequences producing significant alignments

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 539 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

CONCLUSION:

The result shows that fungi isolated from the contaminated soil sample can be used as a potential bioremediator in thedegradation of naphthalene. From the pure culture, one of the fungal isolates (STRAIN-2 [C1-P]) showed potential for hydrocarbon degradation. Naphthalene was utilized as a sole source of carbon and energy by the fungi. The maximum growth was witnessed at a concentration of 1% naphthalene in STRAIN-2 (C1-P). This isolate displayed significant biodegradation capacity and thus selected as potential strain and used for identification. Using Basic Local Alignment Search Tool(BLAST) search engine, the species was identified as *Fusarium oxysporum.* It achieved 98% similarity with the accession number KR047060.1.However, the degradation of high molecular weight PAHs is limited. Thus, it is very important to screen isolate and apply fungi that can degrade high molecular weight PAHs efficiently. Genes encoding enzymes involving degradation of high molecular weight PAHs can be cloned, sequenced and characterized in order to enhance the bioremediation of naphthalene and petroleum products by increasing the expression of enzymes like cellulases and hemicellulases for breaking complex polymer of cellulose, hemicellulosic polymers.[31] The phylogenetic analysis of *Fusarium oxysporum* is done. Evolutionary analyses were conducted in MEGA6. There was a total of 539 positions in the final dataset. Thus, from these studies it is evident that *Fusarium oxysporum* is very potent in degrading naphthalene without any effect on the soil and the future aspect will be continued by bioengineering the microorganism through the novel genetic engineering strategies and recombinant DNA technologies and thereby widely using it in

mycoremediation.

ACKNOWLEDGEMENT:

We, the authors sincerely thank VISTAS and Vels Tech High Tech management for their support and facilities provided to carry out this research work.

CONFLICTS OF INTEREST:

The authors declare that they do not have any conflict of interest.

REFERENCES:

1. Rhodes CJ. Mycoremediation (bioremediation with fungi)–growing mushrooms to clean the earth. Chemical Speciation & Bioavailability. 2014 Jan 1; 26(3):196-8.
2. Adedokun OM, Ataga AE. Oil Spills Remediation Using Native Mushroom-A Viable Option. Research Journal of Environmental Sciences. 2014 Jan 1; 8(1):57.
3. Edema NE, Okungbowa FI. Bioremediation prospects of fungi isolated from water soluble fraction of crude oil samples. Ife Journal of Science. 2012; 14(2): 385-9.
4. Cerniglia CE, Gibson DT. Aromatic hydrocarbons: Degradation by bacteria and fungi. Am. Chem. Soc., Div. Fuel Chem., Prepr.; (United States). 1977 Jan 1; 22(3).
5. Črešnar B, Petrič Š. Cytochrome P450 enzymes in the fungal kingdom. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2011 Jan 1; 1814(1): 29-35.
6. Okwujiako IA, Okwulehie IC, Igbojionu VO. Mycoremediation of engine oil-polluted soil using the white rot fungus, Pleurotusflorida (Mont.) Singer, an edible fungus. Research Journal of Agriculture & Environmental Management. 2013; 2(11): 354-7.
7. Adongbede EM, Sanni RO. Biodegradation of engine oil by Agaricus campestris (a white rot fungus). Journal of Bioremediation & Biodegredation. 2014 Oct 1; 5(7):1.
8. Ali, M. I., Khalil, N. M., & El-Ghany, M. N. A. (2012). Biodegradation of some polycyclic aromatic hydrocarbons by Aspergillus terreus. African Journal of Microbiology Research, 6(16), 3783-3790.
9. Hadibarata T, Teh ZC, Zubir MM, Khudhair AB, Yusoff AR, Salim MR, Hidayat T. Identification of naphthalene metabolism by white rot fungus Pleurotuseryngii. Bioprocess and biosystems engineering. 2013 Oct 1; 36(10):1455-61.
10. Ogbo EM, Okhuoya JA. Biodegradation of aliphatic, aromatic, resinic and asphaltic fractions of crude oil contaminated soils by Pleurotus tuber-regium Fr. Singer-a white rot fungus. African Journal of Biotechnology. 2008 Dec 3; 7(23): 4291.
11. Ekundayo FO. Comparative studies on biodegradative abilities of Pleurotusostreatus and P. pulmonarius in soils contaminated with crude and used engine oils. Advances in Microbiology. 2014 Sep 15; 4(12): 849.
12. Thenmozhi R, Arumugam K, Nagasathya A, Thajuddin N, Paneerselvam A. Studies on mycoremediation of used engine oil contaminated soil samples. Advances in Applied Science Research. 2013; 4(2):1.
13. Olusola SA, Anslem EE (2010) Bioremediation of a Crude Oil Polluted Soil with *Pleurotus Pulmonarius* and *Glomus Mosseae* using *Amaranthus Hybridus*as a Test Plant. J Bioremed Biodegrad 1:113. doi:10.4172/2155-6199.1000113.
14. Isitua CC, Ibeh IN. Comparative study of Aspergillus niger and Penicillium sp. in the biodegradation of automotive gas oil (AGO) and premium motor spirit (PMS). African Journal of Biotechnology. 2010; 9(24):3607-10.
15. Abraham J, Mukherjee P, Bose D, Dutta A. Utilization of monocrotophos by Aspergillus sojae strain JPDA1 isolated from sugarcane fields of Vellore district in India. Research Journal of Pharmacy and Technology. 2016 Dec 6; 9(12): 2155-60.
16. Koul S, Fulekar MH. Petrochemical industrial waste: Bioremediation Techniques: An Overview. International Journal of Advanced Research Technology. 2003; 2(7): 211-57.
17. Kumar A, Bisht BS, Joshi VD, Dhewa T. Review on bioremediation of polluted environment:: A management tool. International journal of environmental sciences. 2011 Jan 1; 1(6):1079.
18. Zafra G, Cortés-Espinosa DV. Biodegradation of polycyclic aromatic hydrocarbons by Trichoderma species: a mini review. Environmental Science and Pollution Research. 2015 Dec 1; 22(24):19426-33.
19. Chanda A, Gummadidala PM, Gomaa OM. Mycoremediation with mycotoxin producers: a critical perspective. Applied microbiology and biotechnology. 2016 Jan 1; 100(1):17-29.
20. Bushnell LD, Haas HF. The utilization of certain hydrocarbons by microorganisms. Journal of Bacteriology. 1941 May;41(5):653.
21. Bhattacharya S, Angayarkanni J, Das A, Palaniswamy M. Evaluation of physical parameters involved in mycoremediation of Benzo [a] pyrene by Pleurotusostreatus. J Pure ApplMicrobio. 2012 Dec 1; 6: 1721-6.
22. Balch TH, inventor; Balch Thomas H, assignee. Method and apparatus for hydrocarbon-contaminated soil remediation. United States patent US 5, 228, 804. 1993 Jul 20.
23. Novotný Č, Cajthaml T, Svobodova K, Šušla M, Šašek V. Irpexlacteus, a white-rot fungus with biotechnological potential. Folia Microbiologica. 2009 Sep 1; 54(5):375-90.
24. Heitkamp MA, Freeman JP, Cerniglia CE. Naphthalene biodegradation in environmental microcosms: estimates of degradation rates and characterization of metabolites. Applied and environmental microbiology. 1987 Jan 1;53(1):129-36.
25. Pawar AN, Ugale SS, More MG, Kokani NF, Khandelwal SR. Biological degradation of naphthalene: a new era. J. Bioremed. Biodeg. 2013; 4:203.
26. Raïssa KR, Augustin M, Martin-Benoît N. Naphthalene biodegradation by microbial consortia isolated from soils in Ngaoundere (Cameroon). International journal of environmental sciences. 2012 Jan 1; 3(1):1.
27. Seo JS, Keum YS, Li QX. Bacterial degradation of aromatic compounds. International journal of environmental research and public health. 2009 Jan 13; 6(1):278-309.
28. Tao XQ, Lu GN, Liu JP, Li T, Yang LN. Rapid degradation of phenanthrene by using Sphingomonas sp. GY2B immobilized in calcium alginate gel beads. International journal of environmental research and public health. 2009 Sep 16; 6(9): 2470-80.
29. Vanishree M, Thatheyus AJ, Ramya D. Biodegradation of petro l using the fungus Penicillium sp. Science International. 2014; 2(1): 26-31.
30. Stanley HO, Immanuel OM. Bioremediation potential of Lentinussubnudus in decontaminating crude oil polluted soil. Nigerian Journal of Biotechnology. 2015; 29(1): 21-6.
31. Singh A, Sharma R. Mycoremediation an eco-friendly approach for the degradation of cellulosic wastes from paper industry with the help of cellulases and hemicellulase activity to minimize the industrial pollution. Int J Environ EngManag. 2013; 4(3):199-206.