

Bioremediation potential of hydrocarbon-utilizing fungi from select marine niches of India

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Abstract

Ten fungal isolates with an ability to degrade crude oil were isolated from select marine substrates, like mangrove sediments, Arabian Sea sediments, and tarballs. Out of the ten isolates, six belonged to *Aspergillus*, two to *Fusarium* and one each to *Penicillium* and *Acremonium* as identified using ITS rDNA sequencing. The selected ten fungal isolates were found to degrade the long chain n-alkanes as opposed to short chain n-alkanes from the crude oil. Mangrove fungus #NIO SN-M126 (*Penicillium citrinum*) was found to be highly efficient in biodegradation of crude oil, reducing the total crude oil content by 77% and the individual n-alkane fraction by an average of 95.37%, indicating it to be a potential candidate for the development into a bioremediation agent.

Keywords: Bioremediation; Degradation; Mangrove Fungi; Marine Pollution; Oil Spills; Tarballs

Introduction

Oil spillages into the environment have become one of the biggest problems in the last century. The hydrocarbons spread horizontally on the ground/water surface and partition into groundwater, soil pore airspace and on the surfaces of soil particles (Plohl et al. 2002), causing contamination of large volumes of soil and groundwater. Oil spills, tank leakages, and wastewater disposal are the key routes for the entry of tons of hydrocarbons into the environment, and which occur at every stage of production, transportation, and handling of petroleum products. Crude oil is a complex mixture of hydrophobic components such as n-alkanes, aromatics, resins, and asphaltenes. Some fractions of crude oil are toxic to living organisms (Costello 1979). The leakage of crude oil into soil damages the biota residing in the soil, including microorganisms and plants, and becomes increasingly toxic beyond a concentration of 3% (Onuoha et al. 2003).

In recent years, various technologies have emerged to manage hydrocarbon contamination. Bioremediation comprises a set of technologies that aid in the removal of contaminants or render them less harmful using biological activity (Silva et al. 2015). This can be achieved with the help of unique microorganisms, such as bacteria, microalgae, and fungi, either isolated from a different environment or introduced into the contaminated sites or by enriching the organisms already present at the contaminated sites (Adebusoye et al. 2007). Bioremediation of hydrocarbons is preferred to chemical and conventional treatment for several reasons. In bioremediation, the selected and potential microbes utilize crude oil as carbon substrate and biodegrade the hydrocarbons into products that are non-toxic and comparatively safer to the environment and living beings, such as carbon dioxide and water (Toledo et al. 2006). The process is economical and can be directly applied to the contaminated site. Thus microbial remediation is a promising, practical and economical method for complete mineralization of hydrocarbons into carbon dioxide and water (Wang et al. 2015). Hence the present study aimed at harnessing the potential of fungi isolated from three marine niches of India, towards efficient hydrocarbon degradation and detoxification.

Materials and methods

Fungal isolates used in the study

In our previous studies, a total of 342 marine fungi were isolated. Out of these 342 fungal isolates, 167 were isolated from sediments collected from Divar mangroves (out-skirts of Panaji, Goa, on the shores of Mandovi estuary) and 165 from off-shore sediments collected from the Arabian Sea (68°E 21°N) when cultured on Czapek Dox agar, malt extract agar, corn meal agar, Sabouraud dextrose agar and potato

dextrose agar (all media prepared in seawater). A total of 10 marine fungi were isolated from tarball samples (collected from Betul beach, South Goa) cultured on Czapek Dox agar and malt extract agar. Sediment samples from mangrove vegetated area were collected during low tide, and approximately 0.5 g of soil was suspended in sterile seawater, and 100 μ L aliquots were spread-plated on various growth media as mentioned above. Crude oil used in this study was obtained from off-shore drilling site operated by Oil and Natural Gas Corporation (ONGC) at Bombay High, India.

Screening

A total of 51 fungal isolates were screened for their ability to utilize hydrocarbon as sole carbon source. The inocula were prepared by growing the fungus in Czapek Dox broth at 30°C under static conditions until a mycelial mat was formed. The mycelial mat was then broken down into fine fragments using sterile glass beads. This suspension was used as a 1% (v/v) inoculum for 20 mL Mineral Salt medium [0.83 g L⁻¹ potassium dihydrogen phosphate (KH₂PO₄); 10 g L⁻¹ sodium chloride (NaCl); 0.42 g L⁻¹ magnesium sulfate (MgSO₄ · 7H₂O); 0.29 g L⁻¹ potassium chloride (KCl); 1.25 g L⁻¹ disodium hydrogen phosphate (Na₂HPO₄); 0.42 g L⁻¹ sodium nitrate (NaNO₃)] containing 5% petrol as sole carbon source, without any other nutrient as additive. Petrol as C-source was used for initial screening and not crude oil as purified hydrocarbon products are more difficult to degrade as compared to crude oil. Fungal isolates capable of growing in the medium and showing a visible increase in biomass were selected for the further studies to estimate the quantitative and qualitative degradation of crude oil.

Identification of fungal isolates

The pure fungal isolates were sub-cultured and allowed to grow for seven days on Czapek Dox agar (CDA) medium (Fig.1). Total genomic DNA of the isolates was extracted from the mycelia using manufacturer specified protocol (ZR Fungal/ Bacterial DNA Microprep Kit, Zymo Research, USA). The isolates were identified by ITS and *beta*-tubulin sequence analysis. For ITS sequence analysis, the ITS gene region was amplified using PCR with primer set: ITS1 (5'-TCCGTAGGTGAACCTGCCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990). Amplification cycle involved an initial denaturation step at 95°C for 5 min followed by 35 cycles with a denaturing step at 95°C for 1 min, annealing temperature of 52°C for 45 s; elongation step at 72 °C for 1 min and final elongation step at 72°C for 10 min. The 600–750 bp amplicons obtained were stored at 4°C and then purified using manufacturer specified protocol (Wizard SV Gel and PCR clean-up system, Promega, USA). For *beta*-tubulin sequence analysis, the partial *beta*-tubulin gene was amplified using primer set: Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (5'- ACCCTCAGTGTAGTGACCCTTGGC) (Glass

and Donaldson 1995). Amplification cycle was the same as that used for ITS region, except the annealing temperature was set to 58°C. The 550–600 bp amplicons obtained were stored at 4°C and then purified using manufacturer specified protocol (Wizard SV Gel and PCR clean-up system, Promega, USA). The PCR purified products were then sequenced using Applied Biosystems (ABI) 3130 DNA stretch sequencer. DNA sequences obtained were analyzed using the BLAST tool to get the closest reference sequence (Altschul et al. 1990).

A multiple sequence alignment was carried out using the ITS sequences obtained in this study and the reference sequences obtained from NCBI-GenBank using with MEGA version 5.2 (Tamura et al. 2011). The phylogenetic trees were inferred using the Neighbour-Joining method (Saitau and Nei 1987), and bootstrap analyses were performed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al.2004)

Quantitative estimation of crude oil degradation using gravimetry

The comparative abilities of 10 fungal isolates (NIOSN-M113, NIOSN-M126, NIOSN-M142, NIOSN-M109, NIOSN-SK56S57, NIOSN-SK56S32, NIOSN-SK56S22, NIOSN-SK56C42, NIOSN-T5, and NIOSN-T4) to degrade crude oil were tested. Of the ten fungal isolates, four were obtained from mangrove environments (NIOSN-M113, NIOSN-M126, NIOSN-M142, and NIOSN-M127), four from marine environments (NIOSN-SK56S57, NIOSN-SK56S32, NIOSN-SK56S22 and NIOSN-SK56C42) and two from tarballs (NIOSN-T5 and NIOSN-T4). The fungal inoculum was prepared by sub-culturing into Czapek Dox broth as described above. For the biodegradation experiments, the fungal mycelia were cultivated under shaker conditions in 100 mL Erlenmeyer flasks containing 20 mL of mineral salts medium. Two mL of crude oil (corresponding to 2.67 g, which amounted to 13.35 % w/v) was used as the sole carbon source. The culture flasks were incubated at 28°C on a rotary shaker at 80 rpm for 23 days (till visible growth was observed). Uninoculated media served as a control. All the experiments were carried out in triplicates.

Following incubation, the residual crude oil was sequentially extracted three times from the culture medium with dichloromethane (1:1) for exhaustive separation. Sample with dichloromethane was placed in a separating funnel with continuous shaking, after which the contents were allowed to settle; two layers were formed: aqueous layer and organic dichloromethane layer containing the residual crude oil. The organic layer was decanted in pre-weighed vials and air dried. After dichloromethane evaporation, the residual oil was quantified gravimetrically (modified from Elshafie et al. 2007). The percentage degradation of the crude oil was then calculated as:

$$\frac{[\textit{weight of crude oil (initial)} - \textit{weight of crude oil (after treatment)}]}{\textit{weight of crude oil (initial)}} \times 100$$

Qualitative analysis of crude oil degradation using gas chromatography

After gravimetric quantification, the residual crude oil was fractionated into alkane fractions on a silica gel column. For this purpose, samples were dissolved in n-hexane and the soluble fraction loaded onto a silica column and alkane fraction eluted with n-hexane (modified from Mishra et al. 2001). The solvent fractions were then evaporated to partial dryness with a rotary evaporator followed by complete drying using nitrogen gas. Dried samples were re-dissolved in 500 μ l n-hexane and spiked with internal standard n-methyl nona-decanoate and quantified via capillary gas chromatography using Shimadzu GC 2010 plus Chromatograph equipped with a Flame-Ionization Detector (FID), fused silica capillary column of 30 m length, 0.32 mm internal diameter, and 0.25 μ m film thickness. The detector and injector temperatures were maintained at 325°C and 300°C, respectively. The column temperature was programmed to increase from 60°C to 280°C with a rate of 5°C min⁻¹ and a final time of 15 min. High purity nitrogen (99.9995 %, oxygen-free) was used as a carrier gas at a flow rate of 3 mL min⁻¹. The method was validated using C₇–C₃₀ n-alkane standards obtained from Sigma Chemicals.

Toxicity assay using *Artemia salina* larvae

The bioremediation products were tested for their toxicity using *Artemia* larvae. The *Artemia* cysts were allowed to hatch in seawater 24 hours before conducting the assay. Following the incubation period of 23 days, the culture broth spiked with crude oil was tested for toxic biodegradation by-products. For this, *Artemia salina* (10 numbers) were inoculated into mineral salts medium from the test as well as the control flasks and microscopic observations were made daily to record the percent survival of the animals.

Results

Screening of fungi for hydrocarbon degradation

Of the total 342 marine fungi isolated in our previous studies, 51 fast-growing fungal isolates were screened for their ability to utilize and degrade hydrocarbons. Among the fifty-one fungal isolates, ten isolates exhibited a potential to use crude oil as the sole carbon source and selected for quantification of the degradation.

Identification of fungal isolates

The evolutionary relationships of the fungal isolates are presented in the NJ tree (Fig. 2, 3).

The trees as obtained from MEGA software are reproduced here. Fig. 2 involves 34 ITS sequences in all, out of which ten were obtained in this study, while Fig. 3 involves 30 β -tubulin sequences, out of which ten

are from this study. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the

branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Fig. 4 shows the generic distribution of the selected 51 isolates where more than half are contributed by *Aspergillus* (12), *Cladosporium* (11) and *Penicillium* (11).

Quantitative estimation of crude oil degradation using gravimetric methods

The selected ten fungal isolates were able to grow and degrade crude oil. Percent degradation estimation following gravimetric analysis demonstrated that isolate *Penicillium citrinum* NIOSN-M126, obtained from Divar mangrove sediments showed maximum ability to utilize crude oil, giving the highest percent degradation of 77 %, followed by *Aspergillus flavus* NIOSN-SK56S22 isolated from Arabian Sea sediments indicating a 62 % degradation (Fig. 5).

Qualitative analysis of crude oil degradation using gas chromatography

The residual oil at the end of the incubation time was analyzed qualitatively and quantitatively using gas chromatography. The chromatograms showed a number of peaks, each representing the residual hydrocarbon in crude oil (Supplementary Figure). The n-alkanes detected in crude oil were in the range C₈–C₃₀. C₇ n-alkane was not detected in any of the samples. In general, the higher alkanes (C₃₀–C₁₄) showed over 80% degradation for all the isolates (Supplementary Table). *Penicillium citrinum* NIOSN-M126 showed the highest efficiency of degradation of the long chain n-alkanes (C₂₉–C₂₀), while *Aspergillus sydowii* NIOSN-SK56C42 was least efficient. Carbon chain C₃₀ was completely degraded by isolate *A. flavus* NIOSN-SK56S22 giving 100 % degradation (Supplementary Table). Among the short chain n-alkanes, *Aspergillus* sp. NIOSN-SK56S32 and *Acremonium sclerotigenum* NIOSN-M109 showed an increase in the concentration of carbon number 12. This could be due to the degradation of higher chain alkanes to C₁₂ increasing the final concentration of Carbon 12 (Supplementary Table). Among the ten isolates screened, *P. citrinum* NIOSN-M126 proved to be most efficient in degradation of the higher chain mutagenic n-alkanes and a promising bioremediating agent.

Toxicity Assay using *Artemia salina* larvae

Investigations for the production of toxic by-products from degradation of crude oil by the fungal isolates were carried out with *Artemia salina*, the brine shrimp, as a test organism. The chief advantage of using these organisms lies in the ease of handling, relatively small amounts of effluents required for tests and the

ease by which data may be obtained. The survival percentages of test organism for the treated and untreated experimental flasks are given in Table 2. The results indicate there were no toxic intermediates or bioremediation products produced by the fungi tested.

Discussion

Though there have been quite a few studies on hydrocarbon-degrading fungi, our understanding of the potential of fungi as bioremediation agents need to be improved. Fungi are the decomposers in the global cycle of nutrients. Fungi possess decomposing abilities to deal with an array of naturally-occurring compounds that serve as potential carbon sources. Hydrocarbon pollutants have similar or analogous molecular structures which enable fungi to act on them as well.

Fungal genera, namely, *Amorphoteca*, *Neosartorya*, *Talaromyces*, and *Graphium* and yeast genera, namely, *Candida*, *Yarrowia*, and *Pichia* were isolated from petroleum-contaminated soil and proven to be potential organisms for hydrocarbon degradation (Chaillan et al. 2004). Singh (2006) also reported a group of terrestrial fungi, namely, *Aspergillus*, *Cephalosporium*, and *Penicillium* which were also found to be the potential degraders of crude oil hydrocarbons.

Bik et al. (2012) demonstrated a shift in microbial communities at the shorelines of Dauphin and Mobile Bay, Alabama following the Deepwater Horizon Oil spill. Two fungal taxa belonging to the phylum *Ascomycota* dominated, in particular, members of the genera *Alternaria* and *Cladosporium* as well as *Aspergillus*, *Acremonium*, *Acarospora*, *Rhodocollybia*, and *Rhizopus*. Analysis of beach sands in Gulf of Mexico area isolated *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Cochliobolus* sp., and also provided evidence of their ability to degrade oil (Hussein 2012). In a study to isolate fungi that colonize tarballs on beaches in the Gulf of Oman, 10 fungal isolates were identified belonging to the genera *Aspergillus*, *Penicillium*, *Cladosporium*, *Rhizopus*, *Alternaria* and *Paecilomyces*, of which *Penicillium chrysogenum*, *Aspergillus niger*, and *Aspergillus terreus* were the most active crude oil degraders (Elshafie et al. 2007). Lotfinasabasl et al. (2012) isolated fungi from soil and tarball samples collected from mangrove forest of Alibaug and Akshi coastal area, Maharashtra, India and tested their ability to degrade kerosene. They reported *Aspergillus terreus* and *Rhizopus* sp. from the tarball samples and observed that *Rhizopus* sp. exhibited the highest growth diameter in 5% kerosene. Recently, Sanyal et al. (2016) isolated tarball-associated fungi belonging to the genera *Alternaria*, *Cladosporium*, *Curvularia*, *Issatchenkia*, and *Candida* from tarballs collected from Candolim beach in Goa, India.

In this study, we focused on the n-alkanes from C₇ to C₃₀. The higher molecular weight alkanes have greater toxicity as compared to the lower molecular weight alkanes. This is because lower molecular

weight alkanes do not persist for long periods in the contaminated site due to their ability to rapidly volatilize. Also, the vapour phase of short-chain alkanes is less toxic than the liquid phase (Bartha and Atlas 1977). Among the ten isolates screened *Penicillium citrinum* NIOSN-M126 proved to be most efficient in degradation of the higher chain mutagenic n-alkanes and a promising bioremediating agent. As opposed to the current study, previous studies available have reported the preferential degradation of short-chain n-alkanes. *Aspergillus* sp. isolated from flare-pit burning petroleum waste (April et al. 1998), *Aspergillus fumigatus* present in diesel storage tanks (Bento et al. 2005) and *A. terreus* and *Fusarium solani* isolated from oil contaminated soils (Colombo et al. 1996) have shown preferential degradation of short-chain alkanes. The marine fungal isolates *Gliomastix* sp., *Paecilomyces* sp., and *Verticillum* sp. have also shown preferential degradation of shorter chain n-alkanes (Fedorak et al. 1984). *Fusarium* sp., *Aspergillus* sp., and *Scopulariopsis* sp. isolated from shorelines of the Gulf of Mexico have also shown an increased degradation of short-chain alkanes as compared to longer chain alkanes (Simister et al. 2015).

Among the three different environments sampled for potential hydrocarbon degrading fungi, the isolate *Penicillium citrinum* NIOSN-M126, obtained from mangrove sediments has shown to be more efficient in total oil degradation when compared to those isolated from tarballs and deep-sea sediments. Mangroves are often situated in areas of high anthropogenic influence, being exposed to pollutants. Divar mangroves are located on the banks of Mandovi estuary, which is used for the barge movement, ferry-boats, and other tourist boats. No previous reports are available on hydrocarbon-degrading fungi from these regions. But in a previous study, Fernandes et al. (2014) have reported the presence of a bacterium, *Marinobacter* sp. from these mangrove sediments, which is known to utilize a variety of hydrocarbons as the sole source of carbon and energy.

Oil spills are the most destructive source of pollution impacting sandy beaches, affecting all trophic levels (Defeo et al. 2009). Persistence and breakdown of stranded oil depend on sand particle size, wave energy, temperature and other factors (Owens et al. 2008), including fungal degradation (Elshafie et al. 2007). Many isolated bacterial and fungal species have been reported to be capable of biodegrading petroleum hydrocarbons (Márquez-Rocha et al. 2005). Fungi, however, have been found to be better degraders of petroleum than traditional bioremediation techniques including bacteria (Batelle 2000). Also, fungi have an advantage over bacteria as fungal hyphae can penetrate the contaminated soil to reach the hydrocarbons that have spread beyond the top layer of the soil (Novotny et al. 1999).

Furthermore, fungi have a higher tolerance to the toxicity of hydrocarbons due to their physiology and adaptation to such variations in the environment and also the presence of mechanisms for the elimination of

spilled oil from the environment. A few research studies carried out in the screening, isolation and characterization of fungi capable of degrading hydrocarbons and their derivatives recognized the enormous potential of the fungal isolates for use and application in the bioremediation of hydrocarbons (Prenafeta-Boldu' et al. 2001; Clemente et al. 2001; Mesyami and Baheri 2003; Yamada et al. 2002; Santos et al. 2008). Also, the isolate *Penicillium citrinum* NIOSN-M126 is a sporulating culture, and the application of spores to contaminated sites is more feasible when compared to bacterial cells. The isolate *P. citrinum* NIOSN-M126 can be further studied for the possible use in clean-ups of oil spills and tarballs that appear on beaches, and also its amalgamation as a component in the Oil-Zapper (TERI, India) which currently comprises a bacterial consortium only. A better understanding of the metabolism, enzyme mechanisms, and genetics of hydrocarbon degrading fungi is critical for the optimization of bioremediation processes. It is not acceptable that in any clean-up process, the by-products generated as a result of the degradation turn out to be more toxic. Fathalla (2007) had shown that photo-oxidation of crude oil in seawater could lead to the destruction of existing toxic components leading to the generation of new toxic compounds. In this study, the break-down products generated by the fungal action were not toxic as shown by the toxicity tests using *Artemia* larvae, thereby rendering their use for degrading crude oil as safe.

Conclusions

Environmental pollution caused due to oil spillage is one of the major problems of this century which needs to be tackled with all possible means. Bioremedial measures using fungi can emerge as a key to control this problem owing to their natural abilities to degrade crude oil. Fungi isolated from different marine niches reported here have shown ability to utilize hydrocarbon as sole C-source and in the process degrade the toxic long-chain n-alkanes to short-chain alkanes which are easier to handle and volatilize. Fungal degradation can be considered as a key component in the cleanup strategy for petroleum hydrocarbon remediation. They need to be evaluated as possible bioremediation agents.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig.1 Growth of all the ten isolates used in the study on Czapek Dox agar. Inset

Fig. 2 The phylogenetic relationships of marine hydrocarbon-degrading fungi inferred from NJ analysis of the ITS sequences using MEGA.

Fig. 3 The phylogenetic relationships of marine hydrocarbon-degrading fungi inferred from NJ analysis of the *beta*-tubulin sequences using MEGA.

Fig. 4 Pie-chart depicting the distribution of isolates at genus level for the selected 51 fungal isolates.

Fig. 5 The percent degradation of crude oil (2.67 g) by the ten selected fungal isolates after 23 days of incubation at 28°C on a rotary shaker at 80 rpm as estimated by gravimetric quantification. The error bars represent standard deviation for set of triplicate for each isolate.

KEY:



Deep Sea isolates



Tar ball isolates



Mangrove isolates

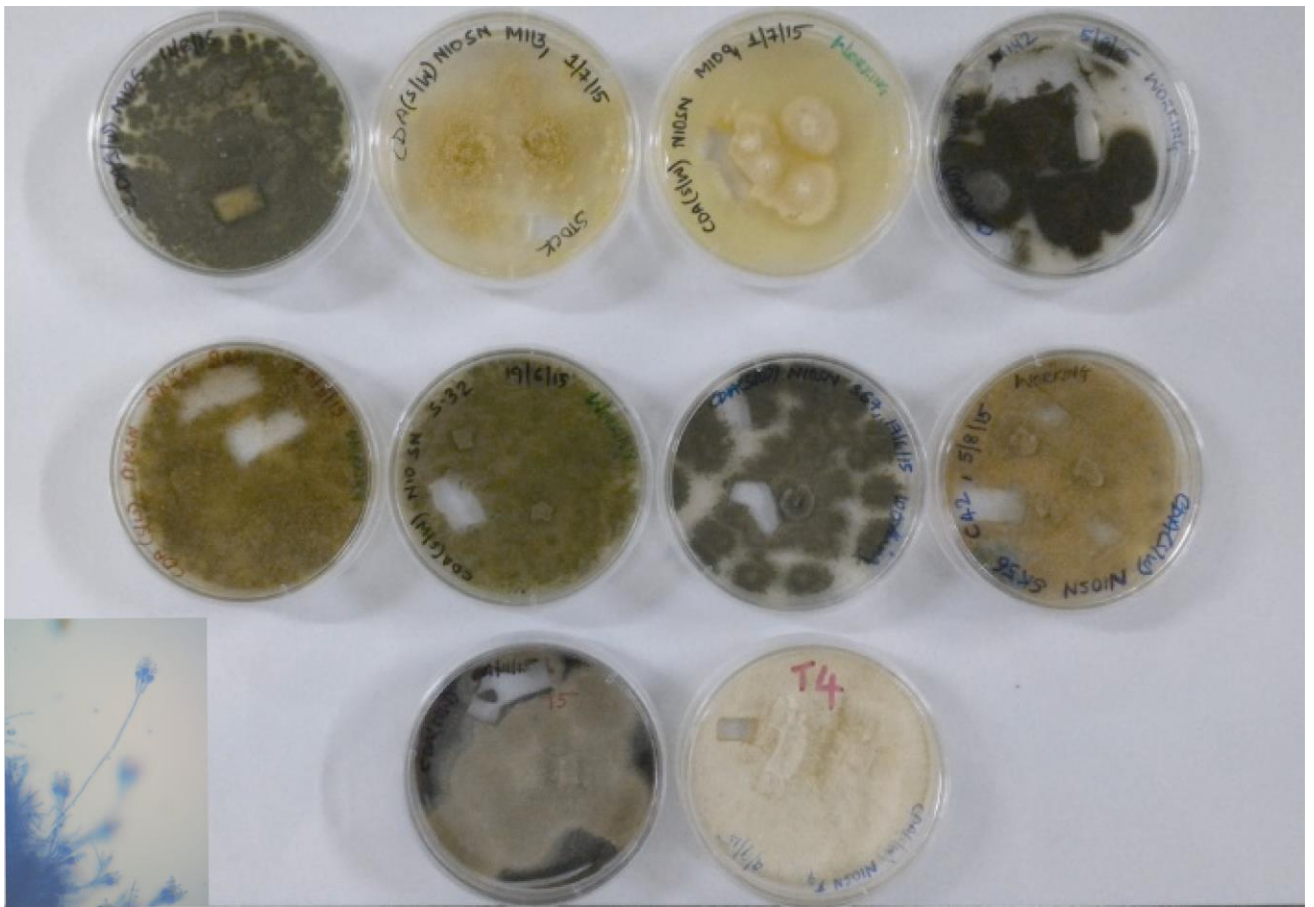
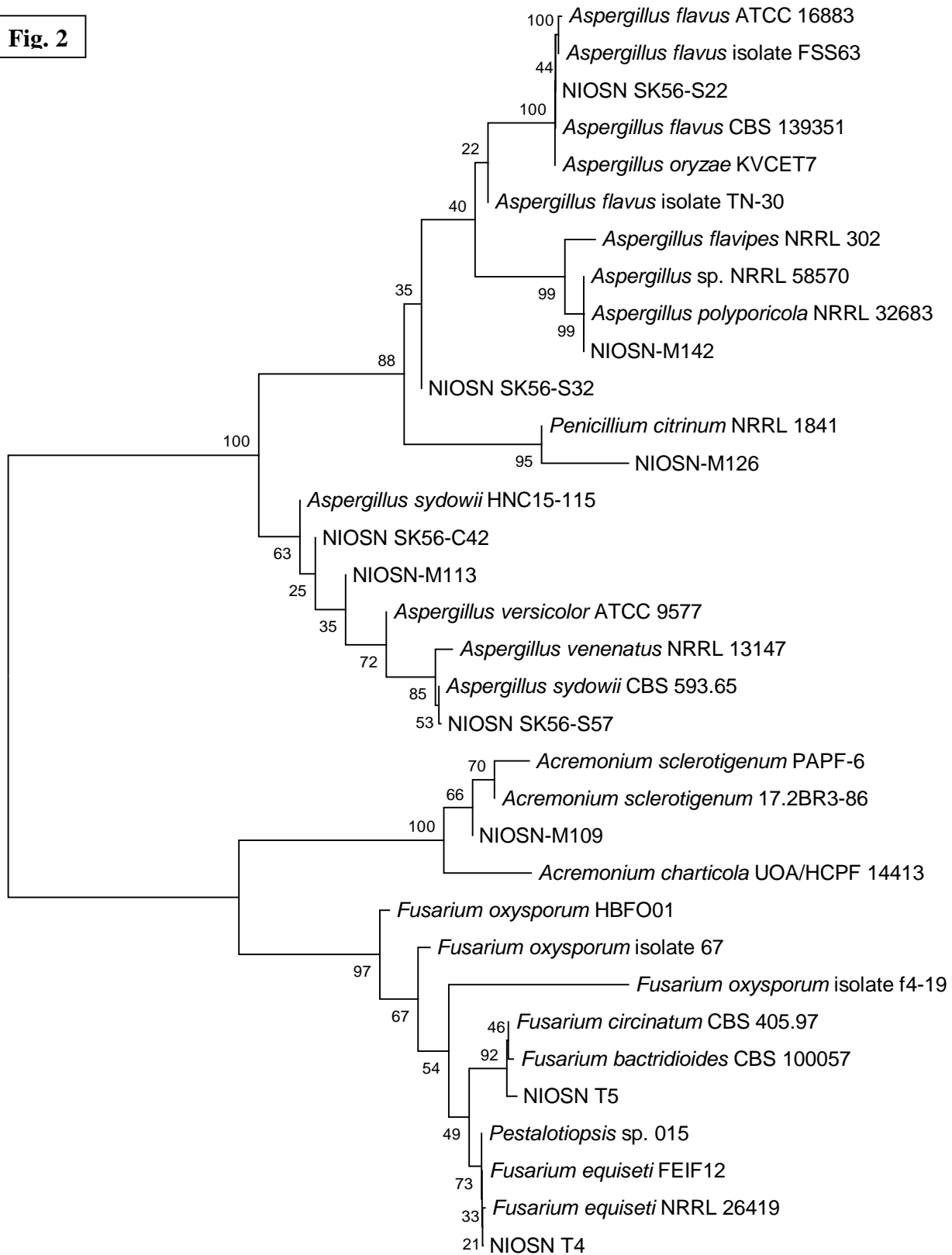


Fig. 1 Growth of all the ten isolates used in the study on Czapek Dox agar. Inset

Fig. 2



0.05

Fig. 3

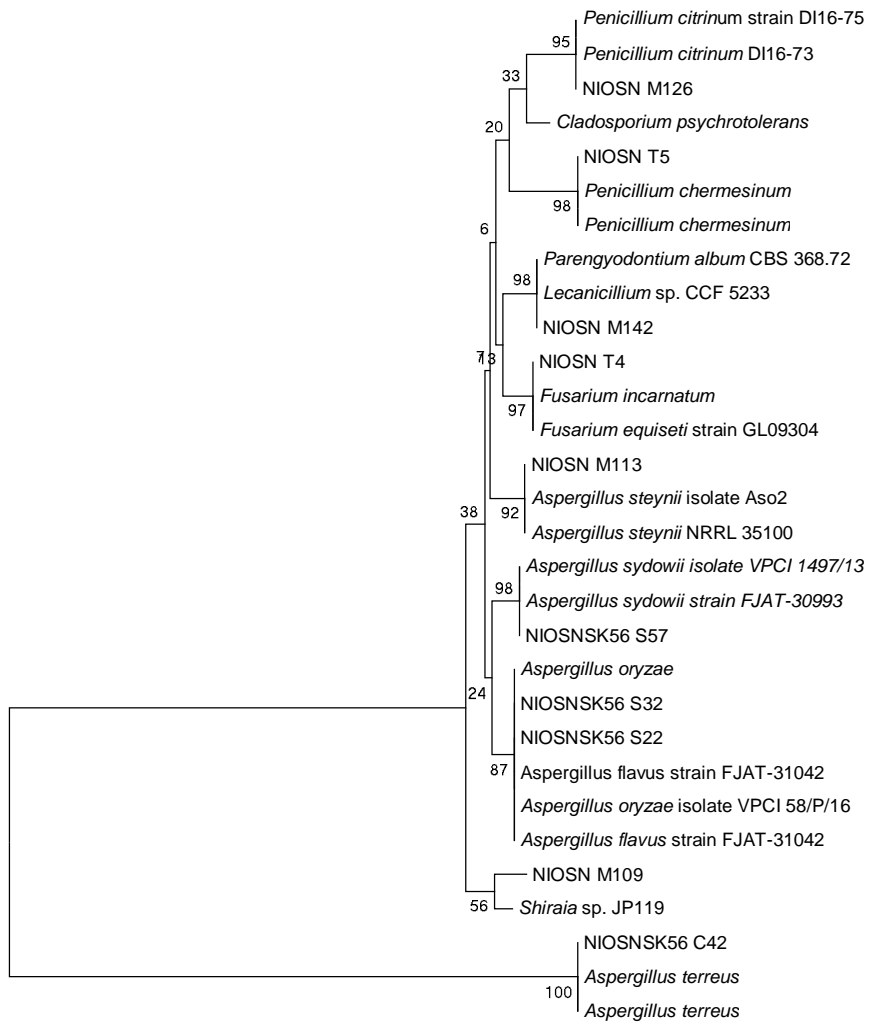
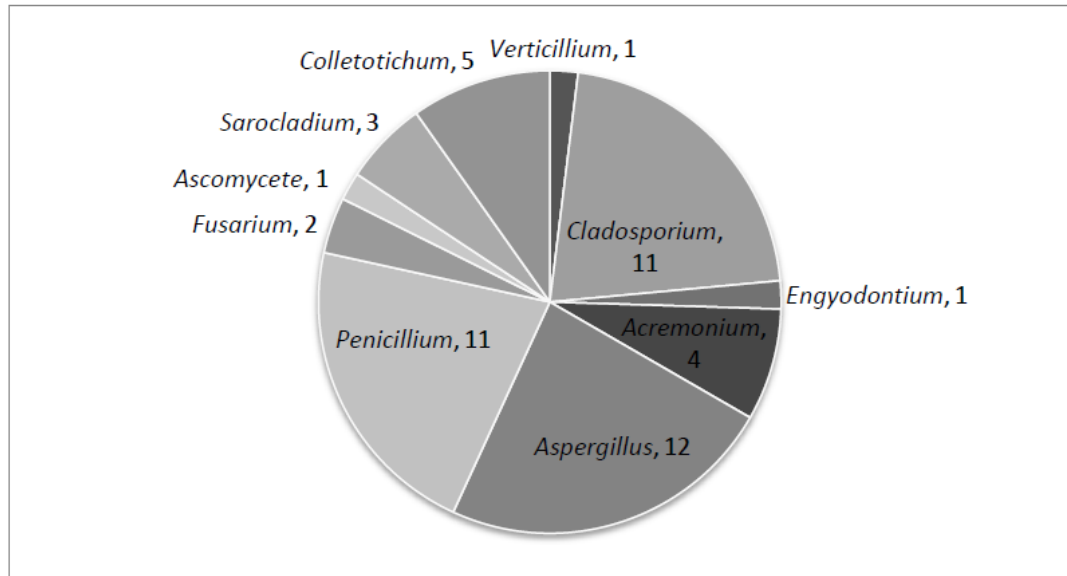


Fig. 4



Supplementary Table. The percent degradation obtained from gas chromatographic analysis for the individual components of the n-alkane fraction of crude oil as shown by the ten fungal isolates

n-alkane compound	Percent degradation [#]									
	NIOSN-SK56S32	NIOSN-SK56S22	NIOSN-SK56S57	NIOSN-SK56C42	NIOSN-T4	NIOSN-T5	NIOSN-M109	NIOSN-M126	NIOSN-M142	NIOSN-M113
C30	83.15	100.00	91.06	82.97	88.29	97.12	95.08	99.97	88.91	91.41
C29	83.34	98.07	91.33	82.83	88.80	90.32	95.21	99.99	89.21	91.70
C28	83.53	99.88	91.08	82.59	99.01	90.67	95.26	99.90	89.37	91.36
C27	83.72	99.97	91.16	82.43	89.96	91.05	95.39	99.99	89.78	91.28
C26	84.11	99.84	91.26	98.51	90.35	91.29	95.48	99.99	90.03	91.24
C25	84.00	97.84	90.99	82.09	99.13	91.14	95.41	99.98	89.91	90.88
C24	91.09	99.98	98.46	98.52	99.99	97.69	95.69	99.99	98.32	98.56
C23	85.62	98.92	91.77	83.82	93.24	92.10	95.67	99.83	91.46	91.44
C22	86.97	99.77	93.79	87.35	99.34	93.84	95.40	99.97	93.37	93.15
C21	88.23	99.99	99.98	86.43	95.37	93.14	95.78	99.99	98.46	92.06
C20	88.06	99.75	93.30	86.60	99.63	93.32	95.79	99.95	93.50	92.45
C19	96.73	98.55	98.03	97.55	98.43	86.10	58.34	98.23	77.08	98.76
C18	87.86	96.18	92.77	87.77	93.93	93.82	90.06	94.56	94.06	93.58
C17	84.80	91.72	92.79	88.23	88.48	94.07	96.51	86.50	90.92	93.74
C16	83.50	94.70	90.59	85.57	92.81	92.84	96.26	92.05	95.43	92.69
C15	88.78	99.83	93.00	93.35	93.22	91.98	95.00	99.83	95.72	95.50
C14	88.86	99.92	92.36	88.60	94.36	92.78	95.45	99.16	96.06	93.00
C13	87.26	98.88	96.57	97.56	98.13	97.72	45.48	99.47	97.50	98.43
C12	*	99.40	87.19	61.04	99.19	62.55	*	96.28	89.67	94.48
C11	55.45	95.68	86.42	82.76	93.36	75.91	92.25	88.15	94.19	88.41
C10	72.74	94.92	89.31	71.53	92.18	85.69	89.02	96.88	95.81	95.98
C9	22.72	86.94	90.05	89.71	81.61	60.67	81.86	90.39	96.26	96.57
C8	60.09	83.02	91.92	95.11	89.21	88.99	95.87	52.47	51.78	98.01

* indicates there was an increase in concentration of C₁₂ after Gas chromatographic analysis.

The values denoted are average of biological and technical triplicates.

Supplementary Figure Chromatograms obtained following Gas Chromatography analysis for (a) C₇–C₃₀ n-alkane standards obtained from Sigma Chemicals, India. (b) crude oil from the untreated control flask (c) – (l) crude oil treated with: (c) isolate NIOSN-M109 (d) isolate NIOSN-M113 (e) isolate NIOSN-M126 (f) isolate NIOSN-M142 (g) isolate NIOSN-SK56S22 (h) isolate NIOSN-SK56S32 (i) isolate NIOSN-SK56S57 (j) isolate NIOSN-SK56C42 (k) isolate NIOSN-T4 (l) isolate NIOSN-T5.

