

Original Research Article

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Bioremediation of Poly Aromatic Hydrocarbons (PAHs) and Crude Oil by Fungal Consortium from West Coast of Karnataka, India

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ABSTRACT

The present study primarily aims at developing an effective fungal consortium for the bioremediation of Poly Aromatic Hydrocarbons (PAHs) and crude oil. The hydrocarbon utilization capabilities of the potent isolated strains were studied for test hydrocarbons like benzene, toluene, naphthalene, paraffin and crude oil as model hydrocarbons for a period of 10 days. Degradation process was assessed by measuring percentage of TPH in residual oil, biological oxygen demand (BOD), chemical oxygen demand (COD), total plate count, UV spectrophotometric analysis and GC-MS studies. The study revealed that, among 45 isolated strains from PAHs/crude oil contaminated soil; four strains showed maximum degradation of crude oil & PAHs. 18s rRNA sequencing was used to identify the strains and, was identified as *Aspergillus terreus*, *Aspergillus aculeatus*, *Scedosporium boydii* and *Aspergillus sp.* The degradation process for this consortium was further optimized using Response Surface Method (RSM). The fungal isolates were also screened for biosurfactant production, Immobilization of the isolates on sodium alginate beads will be used as model of bioremediation studies.

Keywords

Bioremediation,
Fungi,
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PAHs,
Crude oil, GC-MS.

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Introduction

Increase in industrialization and other derogatory anthropogenic activities have led to elevated releases of dangerous compounds. Prevalent contaminants include petroleum hydrocarbons (PHCs), polycyclic aromatic hydrocarbons (PAHs), halogenated hydrocarbons, pesticides, solvents, metals, and salt. Polycyclic aromatic hydrocarbons (PAHs) are some of the most toxic pollutants that have been accumulated in the environment by natural and anthropogenic

activities (Obahiagbon *et al.*, 2014). They are mainly produced from incomplete combustion of organic materials, fossil fuels, spillage of petroleum products and various industrial activities (Dasaria *et al.*, 2014). PAHs have been identified as hazardous chemicals by different State and Central Pollution Control Boards, because of their toxic, carcinogenic and mutagenic effects on living body (Bisht *et al.*, 2015; Igiehon *et al.*, 2015; Balaji *et al.*, 2013; Sayara *et al.*, 2011).

The hydrocarbon fuels contain an excessive quantity of PAHs, utilization of which causes abundant distribution and retention of the same in the ecosphere (Antizar-Ladislao *et al.*, 2006; Weissenfiels *et al.*, 1992; Manilal and Alexander, 1991). These PAHs are considered as the most frequent organic pollutant of environment and are classified as hazardous wastes due to their cytotoxic, mutagenic and carcinogenic effects on human (Rahman, 2002; Margesin, 2003). TPH in the environment are toxic to both animals and humans and can affect the liver, lungs, kidneys and nervous system, leading to cancer as well as having immunological and reproductive effects (Rushton *et al.*, 2007). Hence strategies to detoxify the PAHs is the need of the day. PAHs pollution can be removed using other conventional techniques viz., chemical and physical method, where these methods are expensive and normally require the addition of more chemicals, energy or the removal of large areas of polluted land, which can be damaging to the surrounding ecosystem (Ravikrishna *et al.*, 1998).

Bioremediation is the use of microorganisms that are capable of utilizing the contaminant hydrocarbons as energy sources, thus leading to their degradation and clean up in the environment (Karimi *et al.*, 2015; Vanishree *et al.*, 2014; Hadibarata *et al.*, 2009). In this process microorganisms convert hazardous pollutants into less toxic compounds. Biodegradation involves mineralization of organic constituents to the soluble inorganic compounds or to transform organic constituents to other soluble organic compounds (Kumar *et al.*, 2015, Chandran and Das, 2011). Bioremediation is said to be efficient, inexpensive and environmentally safe cleanup for waste. Although many species of bacteria and algae have been found to be efficient in degradation of low molecular

weight hydrocarbons, for degradation of high molecular weight hydrocarbons, fungal species are preferred (Potin *et al.*, 2004). This is because; the use of fungi is economical since they grow on inexpensive substrates like forest and agricultural wastes (George-okafor *et al.*, 2009). Also, fungi have the ability to produce many extracellular enzymes that can degrade a range of hydrocarbons (Vanishree *et al.*, 2014). Hence, development of bioremediation tool which could be; cost effective, environmental friendly, highly efficient will help in the amelioration of PAHs in aquatic and terrestrial habitat.

Materials and Methods

Sample collection

The PAHs/crude oil contaminated soil samples were collected from different localities of coastal Karnataka. The coordinates for the location from which the soil samples were collected are: 12.8700° N, 74.8800° E. About 10 grams of each sample was collected from a depth of 1-5 cm, transferred to a plastic container using sterile spatula, and tightly sealed and transported to the lab and further processed (Stephen *et al.*, 2014; Al-Nasrawi, 2012).

Isolation and maintenance of pure culture

To enhance the number of indigenous microorganisms capable of hydrocarbon degradation, enrichment procedure as described by Nwachukwu (2000) was followed. 1g of each of the soil samples were added to FSM taken in culture bottles containing 50 mL of media. 2% crude oil was used as carbon source. These bottles were incubated for a minimum of 10 days, at room temperature (Senthilkumaran *et al.*, 2015). Each of the soil samples were serially diluted and plated on Potato Dextrose Agar

(PDA) plates (Chaudhry *et al.*, 2012). After the incubation period, the growth of multiple colonies of fungi was observed. From these plates, non-repetitive single colonies were isolated onto fresh PDA slants for fungal isolation. These slants were stored at 4°C till further use.

Identification of fungal isolates

18S rRNA sequencing technique was employed to identify the potent fungal isolates. The fungal genomic DNA was isolated using the Insta Gen^e™ Matrix Genomic DNA isolation kit. Using 18S rRNA ITS region universal primers gene fragment was amplified using MJ Research PTC-225 Peltier Thermal Cycler. Sequencing reactions were performed using a ABI-PRISM BigDye[™] Terminator Cycle Sequencing Kits with AmpliTaq DNA Polymerase (FS enzyme) (Applied Biosystems). The 18S rRNA sequence was blast using NCBI blast similarity search tool. Finally, the program PhyML 3.0aLRT was used for Phylogenetic analysis.

Preparation of Fungal Consortium

Fungal consortium was prepared according to the method followed by Rahman 2002, with minor modifications. The spore suspensions of each potent isolates were prepared separately by scraping the spores aseptically from the pre-grown mass culture on PDA.

To make the consortium, the each prepared fungal spore suspension was mixed thoroughly in equal volumes and inoculated onto 250 mL shake flasks containing 100 ml FSM and incubated at 25 °C at 200 rpm for 12 hours. The prepared spore suspension of fungal consortium was used as inoculum for further degradation of crude oil and PAHs.

Preliminary degradation studies

The method of Youseff *et al.*, (2010) was adopted for the biodegradation studies. Fungal spore suspension was prepared and inoculated into FSM broth incorporated with sterile 2 % crude oil and redox indicator 2 % DCPIP. The uninoculated flask serves as control. The flasks were incubated at 30° C with constant shaking at a 180 rpm/min for 10 days. The aliquots in the flasks were monitored daily for color change (from deep blue to colorless).

PAHs and Crude Oil Utilization Studies

Each of the potential fungal strains obtained from the preliminary degradation studies were inoculated to full strength media containing 2 % different PAHs viz., benzene, toluene, naphthalene, paraffin and crude oil collected from ONGC as model hydrocarbons.

The flasks were incubated at 30 °C in an orbital shaker at 180 rpm for 10 days. The PAHs and crude oil utilization by the fungal isolates and consortium was confirmed by % TPH analysis, total plate count, BOD, COD level using the standard protocol and by UV spectrophotometric studies at regular intervals of time (0th, 2nd, 4th, 6th, 8th and 10th day) respectively.

Assessment of crude oil and PAHs Degradation by fungal isolates

Biomass estimation

The change in the yield/biomass of the each fungal strains and consortium was estimated gravimetrically. The flask weight was taken before and after incubation period at regular intervals of incubation period.

Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) analysis

In order to assess the rate of degradation and PAHs utilization by the isolate PM-1, the BOD & COD analysis were performed by following standard methods (APHA, 2001 & IS-3025).

Total Petroleum Hydrocarbon (% TPH) analysis

Residual oil was extracted by adding hexane to equal volume of broth culture and shaking thoroughly as described by Obayori *et al.*, 2009 after removing the aqueous phase with separating funnel. The residual oil concentration of the after treatment and before treatment using the fungal isolates and consortium was determined, % of Total Petroleum Hydrocarbon (TPH) by gravimetrically and results were expressed as percentages of respective controls (Kumar & Manjunatha, 2015).

Analysis of PAHs/ crude oil degradation through GC-MS

The extracted residual crude oil/PAHs were used to detect the components left after the degradation study using the method employed by Pasumarthi *et al.*, 2013 with slight modifications. The mixture was stirred vigorously on a magnetic stirrer for 15 min and centrifuged at 2000 g for 5 min. The lower organic phase was collected carefully using a micropipette and was condensed at 28 rpm and 40 °C until the hexane was evaporated. The dried crude oil sediment was collected by adding 1 ml of hexane and then dehydrated in a column with 2 g anhydrous Na₂SO₄. The oil content in the sample was determined by gravimetric analysis by using 0.5 ml of the eluent and carefully evaporated until dry. An aliquot

(10 mg) of the eluent was cleaned by passing it through 2 g of Al₂O₃ (5 % w/w deactivated), concentrated and exchanged with hexane (1.0 ml) by gentle solvent evaporation under a stream of nitrogen gas. The GC-MS analysis was performed using a 7890A gas chromatograph (Agilent Technologies), fitted with a HP-5 MS (30 m _0.25 mm i. d., 0.25 lm film) capillary column (J&W Scientific, Folsom, CA USA) coupled to a 5975C MS spectrometer equipped with a triple axis detector (Agilent Technologies). The initial column temperature was held for 1 min at 60 °C, followed by an initial ramp up rate of 15 °C per minute to 150 °C and then 6 °C per minute to a final temperature of 320 °C, as previously reported (Jimenez *et al.*, 2012). Data were acquired in the full screen mode from 510 to 490 units with 5 min of solvent delay and processed by the Agilent Chemstation software.

Results and Discussion

Isolation and identification of isolates

From the soil samples, total of 45 fungal strains were isolated and checked for the crude oil/PAHs utilization ability. Among the obtained isolates 4 best fungal strains (K-08, K-12, K-25 & K-43) were selected for the present study, The isolates were identified as, *Aspergillus sp.*, *Aspergillus terreus*, *Aspergillus aculeatus* and *Scedosporium boydii* respectively using 18s r RNA sequencing (Table-1 & Fig.1)

Performance of the isolated fungal strains to utilize PAHs & crude oil

The obtained isolates were checked for the utilization capability of PAHs & crude oil as sole carbon source using DCPIP method, where out of 45 fungal isolates, 15 fungal strains were selected for further degradation

studies based on their ability to produce a color change in the media from blue to colorless.

The selected 15 isolates were further subjected for the degradation of Naphthalene, Benzene, Toluene, Liquid Paraffin and Engine oil and Crude oil as model poly aromatic hydrocarbons. From the degradation studies, four best performers; *Aspergillus sp.* (K-08), *Aspergillus terreus* (K-12), *Aspergillus aculeatus* (K-25) and *Scedosporium boydii* (K-43) were selected based on their degradation capability, ability to produce surfactant and faster degradation in full strength media at 10th day of incubation.

Based on their co-existing nature and biodegradation performance, the four strains were clubbed together to make the consortium and used for degradation studies, which showed the effective degradation by reducing the time taken by individual strains by 10 days to 6 days and degrade most of the toxic metabolites of PAHs and crude oil.

Assessment of rate of degradation

After incubation, the degradation performance by both individual and consortium was assessed using following methods,

Visual observation: The presence of the crude oil/PAHs in the test as well as in control sample was visualized. The change in the nature (emulsification) of the crude oil/PAHs was recorded at regular intervals of time. Where out of 15 tested fungi sample, 4 strains viz., K-08, K-12, K-25 and K-43 shows the, richer emulsification due to the enzyme activity and presence of biosurfactants (Figure-2a, 2b &2c). After the incubation period, the biomass was increased significantly, indicating the

affinity of the test organism to use hydrocarbon as sole carbon/energy source.

Accumulation of the fungal mat was observed under treatment with PAH's which is in correlation with and biomass estimated gravimetrically. It was observed that, degradation capacity of the individual strains was less than 70% for all test hydrocarbons by 4 isolates.

However the consortium showed more than 70% degradation for all test hydrocarbons. Degradation percentage was seen to be greater than that of individual performance for 6 days. 67.58 %, 76.83 %, 75 %, 61.5 % and 72 % of Crude oil, Benzene, Toluene, Naphthalene and Paraffin were degraded respectively by consortium culture in 6 days.

BOD & COD analysis

The results showed that, BOD and COD levels decrease as the incubation time increases from day 6 to day 10. This is because these organisms utilizes hydrocarbon as the energy source, and as incubation time increases, the hydrocarbon level decreases, thus reducing the available carbon source. The BOD and COD values for the consortium were found to be 315.4 mg/mL and 143.5 mg/MI respectively at the 10th day of incubation. With correspondence to the BOD and COD values, the total plate count also increases with respect to the time duration.

Estimation of % TPH for the residual PAHs & crude oil

The percent of TPH was analysed gravimetrically, at two days intervals of time, where the residual PAHs were estimated. The consortium shows up to 90 % of the PAHs within 10th days of incubation (Fig.3).

Table.1 Sequence-based Identification of best fungal isolates

No.	Isolation Code	closely related fungal isolate	Identity	Coverage
1	K-08	<i>Aspergillus Sp.</i> KM401402.1	94 %	1131/1880
2	K-12	<i>Aspergillus terreus</i> KF554499.1	100%	1002/1427
3	K-25	<i>Aspergillus aculeatus</i> KJ862074.1	96%	1059/2484
4	K-43	<i>Scedosporium boydii</i> KM461120.1	98%	1059/1059

Table.2 GC-MS analysis: Assessment of biodegradation of Crude oil by fungal consortium after 10th day of incubation reveals degradation of major hydrocarbon moieties

Molecular formula	Control		Consortium	
	RT	% Area	RT	% Area
C ₉ H ₁₈	2.83	5.047		
C ₉ H ₁₆ O	5.45	1.672		
C ₁₀ H ₁₆ O	6.2	6.79		
C ₁₀ H ₁₈	6.83	3.731		
C ₁₁ H ₁₆	7.67	2.753		
C ₁₁ H ₂₀	8.75	2.963	8.8	0.728
C ₁₁ H ₁₈ O	10.58	3.887		
C ₁₃ H ₂₄	11.83	2.649	11.5	1.975
C ₁₄ H ₁₆	13.43	3.634		
C ₁₄ H ₂₈	14.02	5.148	14.3	3.763
C ₁₄ H ₃₀	16.13	2.044		
C ₁₆ H ₁₈	16.83	6.158	17.02	6.184
C ₁₅ H ₃₀ O	18.53	3.770		
C ₁₆ H ₃₀ O	19.92	1.892	19.57	7.684
C ₁₇ H ₃₆	21.43	0.930		
C ₁₈ H ₃₈	22.32	2.614	22	9.085
C ₁₉ H ₃₈	23.97	4.222	24.37	14.497
C ₂₀ H ₄₂	25.9	2.382		
C ₁₈ H ₃₆ O ₂	26.93	2.961	26.53	12.437
C ₁₉ H ₄₀	27.72	4.194		
C ₁₉ H ₃₂ O ₂	28.58	1.524		
C ₂₀ H ₄₂	29.7	5.387	30.6	7.786
C ₂₁ H ₄₄	31.6	6.475	32.52	6.610
C ₂₂ H ₄₆	33.38	5.020	34.32	5.725
C ₂₃ H ₄₈	35.07	3.421		
C ₂₄ H ₅₀	36.7	1.832		
C ₂₅ H ₅₂	38.32	1.164	39.32	2.584
C ₂₆ H ₅₄	39.85	0.556	40.85	1.755
C ₂₇ H ₅₆	41.35	0.633		
C ₂₈ H ₅₈	42.87	0.718		
C ₂₉ H ₆₀	44.6	0.556	45.22	0.669
C ₃₀ H ₆₂	46.73	0.446	46.58	0.551
C ₃₅ H ₇₀ O	49.22	0.335		
C ₃₆ H ₇₄ O ₂	52.67	0.779		

Fig.1 Based on sequence homology and phylogenetic analysis, the fungal culture (a) K-08 was found to be *Aspergillus* sp., (b) K-12 as *Aspergillus terreus* (c) K-25 as *Aspergillus aculeatus* (d) K-43 as *Scedosporium boydii*.

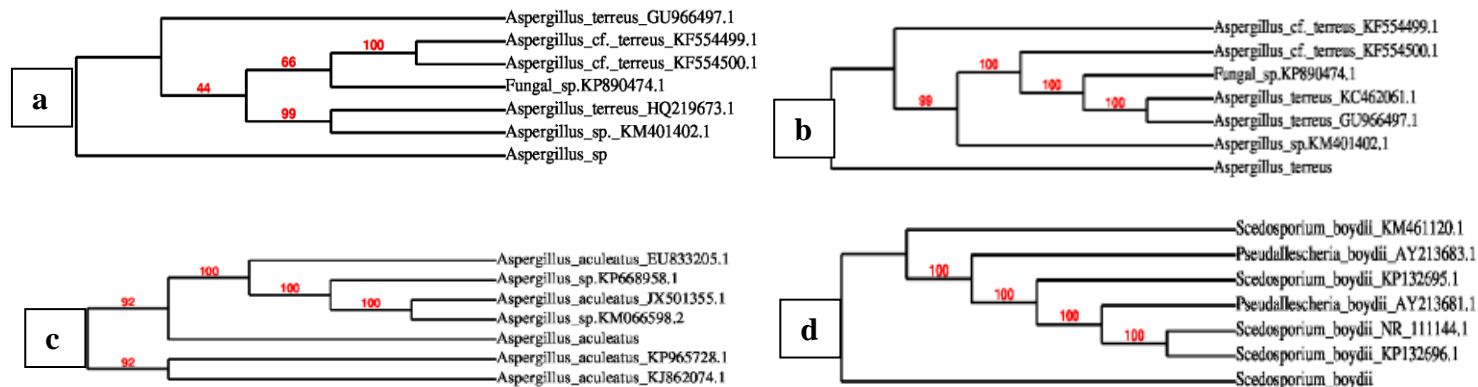


Fig.2a,b,c The Degradation of Crude oil and PAHs using fungal consortium.

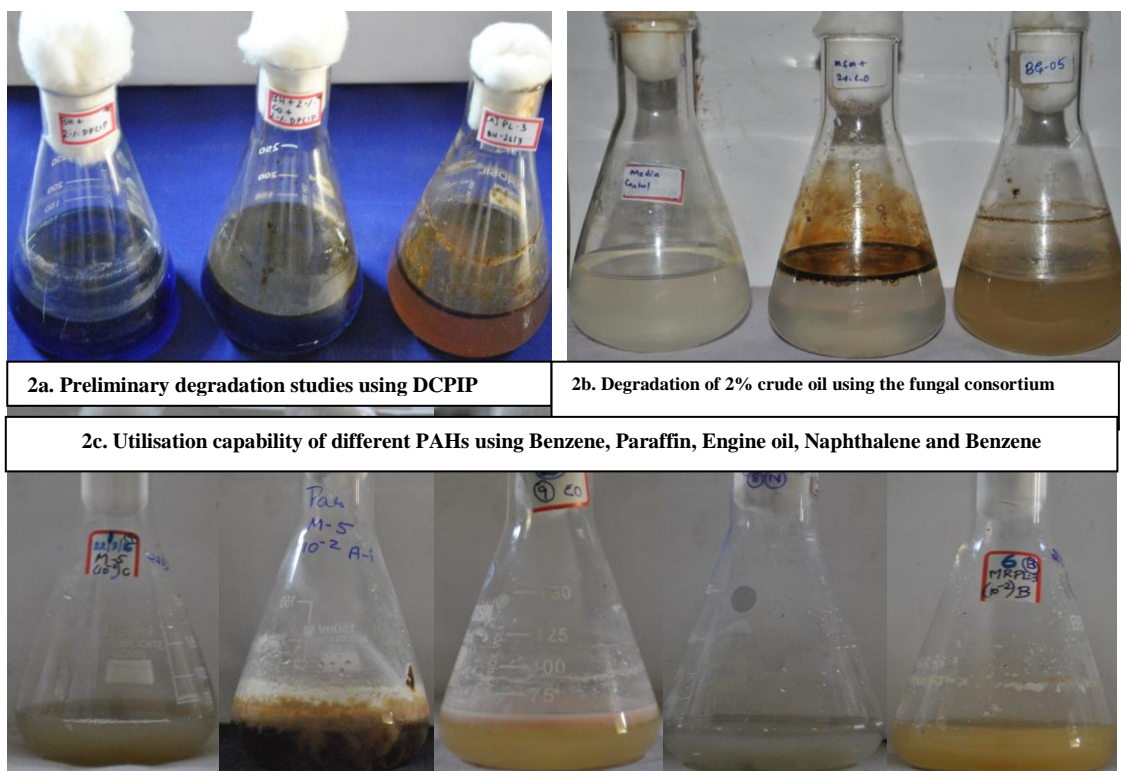
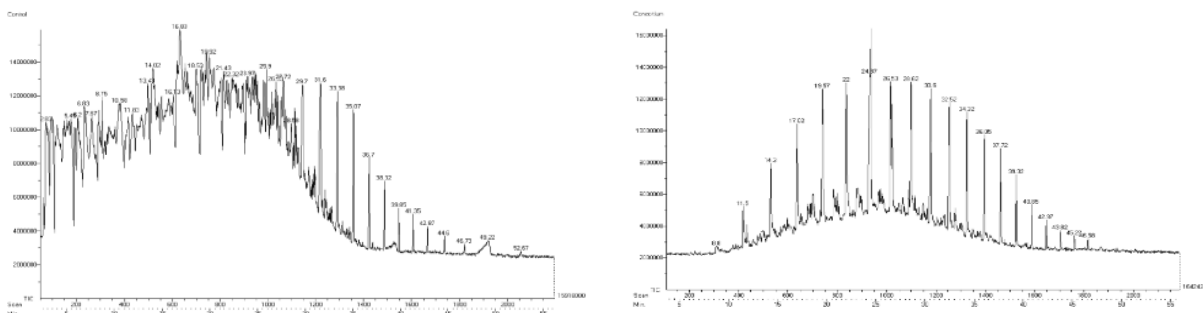


Fig.3 % of TPH for residual components left after 10th day.



Fig.4 GC-MS showing different hydrocarbon contents of crude oil (Control) and after the treatment with consortium after 10th day.



GCMS studies

From the GCMS analysis, it was observed that, all the 4 potent fungal strains are capable of degrading around 13 components of Crude oil completely. Most of the species show stable degradation of lower fractions i.e. C₉ to C₁₁ compounds, with the exception of K-43 strain. After that, there is a varying degree of degradation exhibited by the species. But all the species show the degradation of the highest fraction i.e. C₃₆H₇₄O₂ which was present in the control. It may be due to the degradation of hydrocarbons by the production of biosurfactants or dehydrogenase enzyme production, that assist in the total

degradation of various components of crude oil fraction (Fig. 4 & Table-2). Baker and Herson (1994) reported that degradation of hydrocarbons with long chains from C 18 to C25 proceeds only slowly. In this context isolation of a bacterium capable of degrading complex hydrocarbon is highly useful in the bioremediation of hydrocarbon polluted areas. This result indicated that branched alkanes, alkene, and alkylnaphthalenes were thoroughly degraded. However, linear alkanes chains, hopanes, and steranes were still remaining in the soil. In addition, the longer *n*-alkane chains were degraded with relatively higher rate, leaving the shorter part of the *n*-alkane as the residual fraction at the end of the

bioremediation test. Moreover, it was observed that the concentration of residual *n*-alkane (short chains) decreased as the inoculation volume increased.

In conclusion, the present study reveals the potency of the fungal isolates from different localities of west coast of Karnataka, to degrade PAHs and crude oil components. The prepared consortium was having the capability of utilizing the components of the crude oil and converts into less toxic components. Further off the optimization of process parameters, biosurfactant screening and immobilization studies need to undertake to enhance the degradation in both *ex-situ* and *in-situ* environment using the developed consortium.

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