RESEARCH ARTICLE

Degradation of Petroleum Sludge in Soil by Bacterial-Fungal Co-Culture in Presence of Organic and Inorganic Stimulants

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Abstract

A microcosmic study was carried out for degradation of petroleum sludge [4% (w/w) in soil] in presence of constructed microbial consortium of three bacterial strains i.e., *Pseudomonas* sp. BP10, *Acinetobacter* sp. PSM11 and *Rhodococcus* sp. NJ2 and two fungal strains *Panicillium oxalicum* PS10 and *Curvularia verruculosa* PS8, isolated from different petroleum hydrocarbon contaminated sites) supplemented with vermicompost and inorganic fertilizer as biostimulants. After six months of incubation, the maximum degradation of TPH from petroleum sludge was recorded as high as 80% in the presence of combination of inorganic and organic fertilizer and microbial consortium while only 33% degradation was attributed by native organisms and abiotic factors. Enhancement (%) in degradation rate of TPH due to addition of vermicompost, inorganic fertilizer and microbial consortium in separation and combination was recorded as 57%, 13%, 35% and 139%, respectively. Besides the enhancement in specific growth rate of soil microbes due to addition of nutrient, bioaugmentation of this constructed microbial consortium also boost the total bacterial and fungal strains present in petroleum sludge contaminated soil. Catabolic enzymes played an important role in degradation and maximum induction of enzymes likes catechol 1, 2 dioxygenase, catechol 2, 3 dioxygenase, catalase, laccase and dehydrogenase activity were recorded 223.89 µ mol g^{-1}, 323.83 µ mol g^{-1}, 0.714 µ mol H_2O_2 g^{-1}, 0.623 µ mol g^{-1} and 3.4 µg g^{-1} h^{-1}, respectively.

Keywords: Petroleum sludge; Bacteria, Fungi, Vermicompost, Degradative enzymes.

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Introduction

Petroleum oil is not only a major source of energy to sustain a modern society, but also a source of serious contaminant when released into the environment. This includes accidental discharge of oil spill through bursting of oil supply pipeline, refining, production, exploration, leakage from tankers and adsorption of combustion products onto the soil. Besides, the upstream operations including extraction, transportation or storing of petroleum oil and downstream operation i.e., refining process of crude oil generate large amount of petroleum sludge from petroleum industries (Dara and Sarah, 2003). The safe disposal of petroleum oil sludge is still a big issue for oil refineries. In year 2011-12, Indian oil refineries produced around 47,000 MT of sludge (Kumar and Mohan, 2013). However, oily sludge varies in composition, it basically comprise water, sediment, light hydrocarbons, asphaltene and heavy metals (Islam, 2015). Hydrocarbons present in the form of saturated or unsaturated aliphatic and aromatic compounds like resin, wax, tar, etc. and among these, Polyaromatic hydrocarbons (PAHs), are considered as a major environmental threat because of their high toxicity, mutagenicity and carcinogenicity.

The oily sludge contaminated soils usually create nutrient deficiency, inhibit seed germination, and cause restricted growth or mortality of plants on contact (Al-Mutairi *et al*., 2008). Due to its high viscosity, oily sludge components can be fixed in soil pores, adsorbed onto the surface of soil mineral constituents, or form a thin cover on the soil surface (Trofimov and Rozanova, 2003).

Among several cleanup techniques available to remove petroleum hydrocarbons from the soil, bioremediation processes are gaining importance due to their simplicity, high efficiency and cost-effectiveness as compared to other techniques (Khashayar and Mahsa, 2010). Besides, there are few factors which check the biodegradation process like low solubility of hydrocarbons, deficiency in the ratio carbon, nitrogen and phosphorous (C:N:P) of soil etc. Hence, bioremediation of contaminants can be further stimulated by two methods, bioaugmentation and/or biostimulation. The use of native micro biota from the oil petroleum contaminated soil is of great interest as these microorganisms develop the ability to degrade petroleum hydrocarbons and are also more adapted to the particular soil environment than non-indigenous commercial microbial inocula (Silva *et al*., 2009). Many bacterial and fungal species have already been reported for degradation of petroleum hydrocarbons (de Carvalho *et al*., 2005; Farhadian *et al*., 2008; Haritash and Kaushik 2009; Al-Jawhari *et al*., 2014). In case of fungi, lignolytic fungi have been extensively studied, because lignolytic system consists of three main enzyme groups with lignin peroxidase, manganese peroxidase, phenoloxidases (lacases and tyrosinases), and H_2O_2-producing enzymes. In spite of single microbes, co-culture of bacteria and fungi may be applied for the fast degradation of petroleum hydrocarbons (Boonchan *et al*., 2000). Further, addition of biostimulants in the form of nutrients, such as N, P, K, S, and Cu, were found to have increased dissipation of petroleum hydrocarbons from the soil (Fernández-Luqueño *et al*., 2009; Ofoegbu *et al*. 2015) by stimulating the growth of microorganism that takes part in the mineralization of petroleum sludge (Yu *et al*., 2005).

The purpose of this microcosmic study was to evaluate the degradation of petroleum sludge by using microbial consortium, which is the combination of three native petroleum hydrocarbon

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degrading bacteria and two fungal strains isolated from different petroleum hydrocarbon contaminated sites. Besides, changes in soil pH, viable colony count of total and bioaugmented soil fungal and bacterial strain, nutrients (available phosphorus, potassium and nitrogen), soil protein and activities of degradative enzymes like catechol 1, 2 dioxygenase (C12O), catechol 2, 3 dioxygenase (C23O), catalase and laccase and dehydrogenase were also observed to find out their correlation with the degradation of petroleum sludge during 6 months incubation.

Material and Methodology

Microbial consortium formation

Three bacterial strains (*Pseudomonas* sp. BP10, *Acinetobacter* sp. PSM11 and *Rhodococcus* sp. NJ2) isolated from petroleum hydrocarbon contaminated soil/ sludge from Barauni Oil Refinery, Begusarai, Bihar (India), Mathura Oil Refinery, Mathura, U.P. (India) and procured from North East Institute of Science and Technology, Jorhart, Assam (India), respectively, were selected for the microbial consortium based on their degradation ability of petroleum hydrocarbons as reported by Kumari *et al*. (2012), Kumar *et al*. (2011). While fungal strains, i.e., PS8 and PS10 were isolated from crude oil contaminated soil from Barauni Oil Refinery, Begusarai, Bihar (India) and selected for consortium based on the degradation capability of crude oil (data not shown). Thus, consortiums of these potent hydrocarbon degrading strains were used for bioaugmentation in microcosmic study to accelerate the degradation of petroleum sludge.

Identification of fungal strains

For molecular identification of the fungal strains PS8 and PS10, conidia from each fungus were inoculated on liquid media culture (potato dextrose broth), and incubated at 27 ± 2°C with continous shaking (200 rpm) for 3-5 days. Approximately, 200 mg fresh mycelial biomass were grinded in motar-pestle with liquid nitrogen and incubated with 750 µl lysis buffer **(**100 mM Tris-HCl (pH 9.5), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB) at 74°C for 60 minutes followed by centrifugation at 12,000 rpm for 15 minutes. An equal volume of chloroform: isoamylalcohol (24:1) was added to pellet cell debris and again centrifuged at 10,000 rpm for 10 minutes. The DNA was precipitated by adding equal volume of isopropanol that was mixed gently by inverting microcentrifuge tubes and then centrifuged the content at 10,000 rpm for 10 minutes. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in TE buffer. A volume of 1.5 µl of RNAse A (20 mg ml^{-1}) was added and incubated at 37°C for 30 minutes to degrade RNA in the sample. The DNA quality was checked on 1% agarose gel in TBE buffer and quantified spectrophotometrically using Nano Drop ND 1000 (Nanodrop Technologies, DE, USA).

The ITS region was amplified by using two fungal specific pair of primers ITS1f (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4r (5′-TCCTCCGCTTTTGATATGC-3′); and ITS5f (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4r (5′-TCCTCCGCTTATTGATATGC-3′) (targeted to the conserved regions in the 3′end of the 18S rRNA gene (small subunit, SSU) and the 5′end of the 28S rRNA gene (large subunit, LSU), respectively. The PCR amplification was carried out in 50µl reaction volume containing 50ng DNA, 2.5 mM $MgCl_2$, 1X *Taq* polymerase buffer, 400 µM dNTPs, 10pmoles primers and 1U *Taq* DNA polymerase. The PCR thermal cycle was programmed as follows: initial denaturation at 95°C for 5 minutes; 38 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 45 seconds and extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes. The amplified products were separated on 1.3% agarose gel in TBE buffer pre stained with 0.1% ethidium bromide and bands were purified by using Sure Extract PCR Clean-up (Genetix, Biotech Asia Pvt. Ltd). The sequencing of PCR product was carried out using ABI 3730xl capillary sequencer (Applied Biosystems, CA, USA) in both ways with forward and reverse primers. The resultant sequences of forward and reverse primers were subjected to their multiple sequence alignment using Clustal_W software (version 1.83). Finally, the consensus sequences generated from the alignment converted into FASTA format and searched for the most homologous sequences and compared with the sequences at NCBI.

Experimental design

Five plots (1 m x 1 m x 20 cm) were prepared in NBRI garden and each plot was filled with 100 kg soil supplemented with 4 kg of petroleum sludge. These plots were treated with different amendments as shown in Table 1. Soil of each plot was thoroughly mixed and soil moisture in all the plots was maintained up to 60-70% of water holding capacity. The sites were covered with thick polythene cover to prevent photo volatilization. Thick polyethylene was also applied beneath the soil to present the petroleum hydrocarbon leaching. Soil samples were collected from each plot on the monthly basis up to 6 months starting from zero day and preserved at 4°C till all the parameters were to be studied.

Extraction and analysis of soil TPH and their fractions (Alkane, Aromatic, NSO and asphaltenes)

To determine the residual amount of total petroleum hydrocarbon (TPH), 10 g of soil s ample was put in a porous cellulose thimble (25 x 70 mm) and placed in a soxhlet extractor. The soil was consecutively extracted with equal volumes of hexane, dichloromethane, methanol and chloroform (100 ml each). Extracted solutions were pooled in pre weighed beakers. Solvents were evaporated under fume hood and total petroleum hydrocarbons were estimated through the gravimetric method. Addition of *n*-pentane separated out the asphaltene portion as undissolved fraction while dissolved fraction was filtered with syringe filter. This dissolved fraction was loaded on silica gel column. The different fractions of petroleum hydrocarbons, i.e., alkane, aromatic and group of heterocyclic

Table 1: Amendments applied to different treatment.

	Amendments				
Plots	*Soil (Kg)*	*Oily sludge (Kg)*	*Vermicompost (Kg)*	*NPK (60 g NH_4SO_4 + 3.67 g K_2HPO_4)*	*Microbial consortium*
Control	100	4	-	-	-
Tr1	100	4	10	-	-
Tr2	100	4	10	Yes	-
Tr3	100	4	10	-	Yes
Tr4	100	4	10	Yes	Yes

hydrocarbon containing nitrogen, sulphur and oxygen (NSO-PAC) were eluted by using different organic solvents; hexane, benzene and methanol + chloroform, respectively and determined through gravimetric method. Alkane and aromatic compounds were analyzed using a gas chromatograph flame ionization detector (Kumari *et al*., 2012).

Biodegradation Kinetics

Biodegradation of TPH over the time period was calculated in percentage degradation (Eq. 1) while rate constant of degradation of TPH and time required to degrade half of the given concentration of TPH were calculated by using equation 2 and 3, respectively for the first order kinetic. Efficiency of bioaugmentation and biostimulants was calculated by using equation 4 (Ofoegbu *et al*., 2015).

$$\text{Degradation (\%)} = (TPH_0 - TPH_t)/ TPH_0 \times 100 \quad (1)$$

$$k = (\ln TPH_0 - \ln TPH_t)/t \quad (2)$$

$$t_{½} = 0.6932/k \quad (3)$$

B.E. = (% D in TPH of biostimulant treated - % D in TPH of biostimulant untreated) / % D in TPH of biostimulant untreated) x 100 (4)

Where: TPH_0 and TPH_t are the initial and residual total petroleum hydrocarbon, k is rate constant, t is the time of incubation and % D is degradation in %.

Viable count of soil microbes and bioaugmented microbial strain

Growth of total soil bacteria and fungi was measured in the term of CFU g^{-1} of soil by serial dilution and colony counting on nutrient agar (NA) and potato dextrose agar (PDA) plates, respectively separately after the incubation for 24-48 h at 37°C and 30°C, respectively in incubator.

For analysing the status of bioaugmented bacterial strains in the plots (Tr3 and Tr4), their viable counts were counted on NA plate supplemented with antibiotics to the concentration where native soil bacteria could not grow. Bacterial strain BP10 were tagged with vancomycin at concentration 250 µg ml^{-1} while NJ2 and PSM11 that are morphologically different in colour were tagged with amphicillin for 100 µg ml^{-1}. In case of fungi, bioaugmented strains were recognized with their morphological structure. Specific growth rate of total and bioaugmented microbial strains was calculated through the following equation (5).

$$\mu = \frac{dX}{Xo.dt} \quad (5)$$

Where: µ = Specific growth rate, dX = change in viable counting of microbes, Xo = viable counting at the time of incubation and dt= time interval.

Soil pH

The changes in pH of the soil extract (soil : water ratio; 1:5) was measured using a pH meter (Orion EA940) after the calibration by using standard buffer solutions of pH= 7.0, 4.0 and 10.01.

Soil protein

Total soil protein was estimated by using the method of Rahman *et al*. (2007). Ten ml of distilled water was mixed with 1 g of soil and vortexed for 5 min. After settling of the soil particles, 1 ml supernatant without any soil particle was centrifuged at 13000 rpm for 10 min. Supernatant was discarded and 1 ml of 3 N NaOH solution was added to the pellet and boiled for 3 min. After cooling at room temperature, 1 ml of 1 M H_3PO_4 solution was added. Sample thus prepared was used for both protein estimation and enzyme assays. Protein was determined by following the method of Lowry *et al*. (1951) using BSA as standard.

Soil enzymes assay

Catalase activity

Enzyme was extracted from 0.25 g of soil by adding 2 ml extraction buffer (containing 0.1 M potassium phosphate buffer (pH 7.0) and 1 mM EDTA with pinch of polyvinyl pyrrolidone) and collecting the supernatant after centrifugation at 20,000 x g for 15 min at 4°C. The reaction mixture consisted of 1.2 ml phosphate buffer, 0.2 ml H_2O_2 (30 mM) and 0.1 ml of enzyme extract. Catalase activity was assayed by recording a decrease in absorbance at 240 nm occurring due to disappearance of peroxide (Beers and Sizer, 1952).

Laccase activity

To extract laccase enzyme, 5 ml of 0.1 M potassium phosphate buffer (pH 6.5) was added to 5 g soil samples and then centrifuged at 14,000 x g for 10 min. The specific activity of laccase was assayed based on oxidation of syringaldazine by monitoring an increase in absorbance at 530 nm (e = 65 mM^{-1} cm^{-1}) through spectrophotometrically. Reaction mixture (3 ml) contained 2.2 ml phosphate buffer (pH 6.5), 300 µl of 0.216 mM syringaldazine in absolute methanol and 500 µl enzyme extract.

Dehydrogenase activity

For the assay of dehydrogenase activity, 2 ml of 0.1M Tris buffer (pH 7.4) and 2 ml of 1% triphenyl tetra zolium chloride (prepared in 0.1 M Tris buffer, pH 7.4) solution were added to 2 g of soil sample and incubated at 180 rpm for 24-h in an orbital shaker. The product of hydrolytic reaction, i.e., triphenyl formazan, was extracted in 4 ml of methanol by centrifugation at 4°C and 3000 x g for 10 min. Formation of formazan was determined by measuring the absorbance at 485 nm as described by Stevenson (1956).

Catechol dioxygenases

Catechol 1, 2 dioxygenase (C12O) was assayed using the colorimetric procedures through the detection of the product *cis, cis* muconate (pH = 7.0, k_{max} = 260 nm; e = 25,600 $\mu mol^{-1}cm^{-1}$) (Hegeman, 1966). The reaction mixture (1 ml) contained 8.7 µM sodium phosphate buffer (pH = 7.0), 1 µmol EDTA, 0.1 µM catechol and soil protein extract in a final volume of 1 ml. An increase in absorbance at 260 nm was recorded that is due to accumulation of *cis, cis* muconic acid. Amount of enzyme catalyzing the oxidation of 1 nmol of substrate per min represents one unit of enzyme activity.

Catechol 2, 3 dioxygenase (C23O) occurs with concomitant formation of 2-hydroxy muconic semialdehyde (pH = 7.5) exhibiting increase in absorbance at 375 nm (e = 33,400 $mol^{-1}cm^{-1}$). Reaction mixture of 1 ml contained 48 µM sodium phosphate buffer (pH = 7.5), 0.1 µM catechol and soil protein extract. The reaction mixture was heated for 10 min at 60°C before analysis of enzyme activity (Klecka and Gibson, 1981).

Estimation of available NPK in soil

The available nitrogen was measured using the Kjeldahl method as specified by Black (1965). Air- dried and sieved (mesh sieve of 6 mm of pore size) soil sample (0.25 g) was taken in micro Kjeldahl unit. All the reagents (2.5% of NaOH, 1% Boric acid, 50% Bromocresol in methanol and 0.32% potassium permagnet) were placed in the unit and run the process to obtain green color solution. The green

solution was the boric acid which absorbed ammonia gas released from the soil. This solution was titrated with 0.1 M HCl solution till the color of solution turned pink.

Available phosphorus was determined by Olsen's method. For this purpose, 50 ml $NaHCO_3$ (pH 8.5) and a pinch of charcoal were added to 2.5 g of soil sample in 100 ml conical flask. Flasks were incubated in orbital shaker at 150 rpm for 30 min at room temperature. Solutions were filtered through the Whatman filter paper (No. 41). Ten ml of filtrates with 10 ml of distilled water was acidified with using 2.5 M H_2SO_4 till the disappearance of yellow colour of added p-nitrophenol indicator. After addition of 8 ml Murphy-redy color solution (250 ml 2.5 M Sulphuric acid + 75 ml Ammonium molybdate (40 g l^{-1}) + 50 ml Ascorbic acid (52.8 g l^{-1}) + 25 ml Antimony Potassium Tartrate (2.908 g l^{-1}) + 100 ml distilled water for 500 ml preparation), volume was made up to 50 ml. Amount of available phosphorus was measured at 730 nm after 15 min of reaction time by using UV-VIS spectrophotometer (Perkin Elmer Lambda 35).

Available potassium was determined using a flame photometer (Systronix-128) after the extraction of soil samples (0.25 g) in 20 ml of 2% ammonium-acetate solution for 30 min with shaking. Extracted sample was filtered and make up to a final volume of 20 ml with 2% ammonium acetate solution.

Results and Discussion

Microbial consortium and identification of strains of consortium

Microbial consortium had the combination of 5 microbial strains in which three were bacterial strains and two strains were from fungi. Bacterial strains were already identified as *Pseudomonas* sp. BP10, *Acinetobacter* sp. PSM11 and *Rhodococcus* sp. NJ2 and their 16S rDNA gene sequence have been submitted to NCBI (Kumar *et al*., 2011; Kumari *et al*., 2012). Their accession numbers are KC493413, KC493414 and KR155187, respectively. Based on the gene sequence of isolated fungal strains PS8 and PS10, these strains were identified as *Curvularia verruculosa* and *Panicillium oxalicum* having NCBI accession no. KX398948 and KX398949, respectively. There strains were isolated from different sites and proved their capability to mineralize petroleum hydrocarbons with faster rate.

The best approach for selecting competent microbes should be based on the prior knowledge of the microbial communities inhabiting the target site (Thompson *et al*., 2005). In spite of single microbial strain, bioaugmentation by microbial consortium is always preferred rather than a single strain because the interaction among consortium members may also be necessary for initial steps in conversion and later for the transformation or mineralization of compounds (Kenelly *et al*., 2002). Few researchers applied the co-culture of bacterial and fungal strains for the degradation of petroleum hydrocarbon but especially for poly aromatic hydrocarbons (Chávez-Gómez *et al*., 2003; Kim and Lee, 2007).

Fungal genera especially *Aspergillus, Fusarium, Penicllium* etc. are widely studied for the degradation of different petroleum hydrocarbons (Opasols and Adewoye, 2010; Al-Jawhari *et al*., 2014) but least has been reported for *Curvularia* sp. in the field of petroleum hydrocarbon degradation (Hashem, 2007; Juckpech *et al*., 2012).

Degradation of TPH

When sludge contaminated soils were treated by constructed microbial consortium in field condition with different amendments like NPK and/ or vermicompost, highest degradation (80%) of TPH of petroleum sludge was observed in Tr4 followed by Tr3 (70%), Tr2 (59%) and Tr1 (52%) and least was in control (33%) after 6 months of incubation (Fig. 1). Degradation of TPH was initially very slow

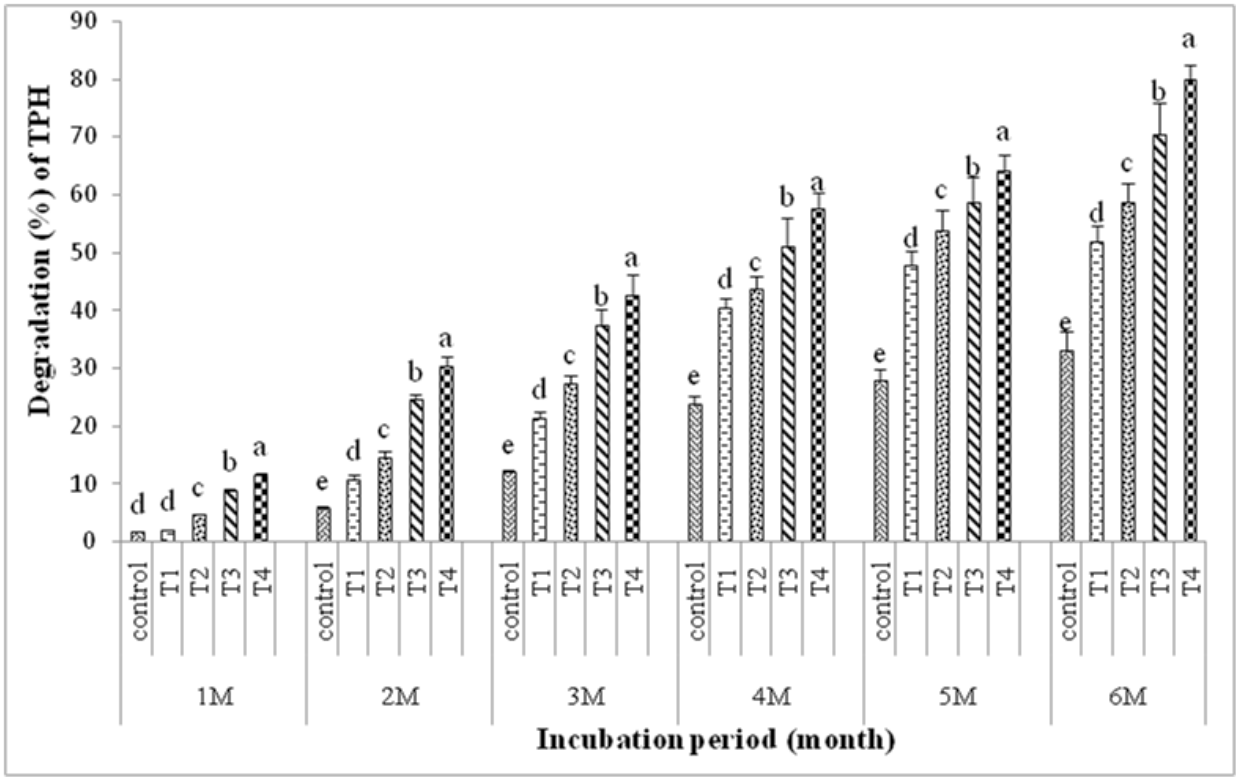

Fig. 1: Degradation of total petroleum hydrocarbon during 6 month.

(2-12%) in all treatment. It gradually increased with increasing the incubation period (Fig. 1). In this experiment, addition of vermicompost contributed 19% enhancement (Tr1 Vs control) in degradation of TPH while NPK contributed 7% (Tr2 Vs Tr1) and microbial consortium contributed 18-21% (Tr3 Vs Tr1 & Tr4 Vs Tr2). Total cumulative effect in degradation of petroleum sludge due to additional treatments was 47% (Tr4 Vs control) which was approximately 2.4 fold of natural attenuation (control). F values through ANOVA between the treatments and days were determined 31.6 and 80.1 that also confirmed the significance differences in TPH degradation at the level of $p< 0.01$ due to application of different bioaugmentation and biostimulation in this microcosmic study. Further post-ANOVA analysis i.e., LSD showed that application of bioaugmentation significant degradation of TPH from first month and maintained till 6th month of incubation. Natural Biodegradation rate constant was recorded 0.55 m^{-1} when it was not supported by additional microbes or stimulants while by applying the vermicompost, vermicompost + NPK, vermicompost +NPK and vermicompost +NPK + microbial consortium, it was 0.086 m^{-1}, 0.098 m^{-1}, 0.117 m^{-1} and 0.133 m^{-1}, respectively (Table 2).

In present study, three things had been added to stimulate the TPH degradation process, i. e., organic compost (vermicompost), inorganic fertilizer (NPK) and microbial combination (MC). Addition of each plays an important role to boost the degradation process. Based on the calculation for efficiency of augmentation (Table 2), vermicompost contributed more in degradation of TPH, followed by microbial combination and NPK. Apart from augmentation of potent microbial combination, heterotrophic bacteria are ubiquitous in nature as their presence and degradation activity may be noticed in control. Vermicompost also had the heterotrophic bacteria and it also maintained the required ratio of C:N:P needed to support the microbial growth that's why its addition contributed more in enhancement of petroleum hydrocarbon degradation. Thus, biostimulation supports the growth of native microbes and enhances the degradation of TPH, *in situ*. According to Machın-Ramırez *et al*. (2008) biostimulation treatment was more effective than bioaugmentation treatment for TPH degradation. Vyas and Dave (2010) reported optimum concentration of N (1%), P (0.5%) and K (0.01%) for degradation enhancement. Many researchers reported benefit of a mixture of more than one organic waste in the biodegradation of petroleum hydrocarbons in soil (Agbor *et al*., 2012; Nduka *et al*., 2012) while Ofoegbu *et al*. (2015) found the combination inorganic and organic fertilizer (cow dung) had more biostimulation efficiency than using these singly. Similarly in our investigation, a combination of inorganic and organic fertilizers boosted the degradation process more as compared to their use in the isolation. Addition of fertilizer improves the soil quality by reducing the bulk density, moisture content and oxygen diffusion.

In this study, bioaugmentation with biostimulation was found more effective to boost the degradation process than biostimulation alone as TPH reduction was observed 80% in Tr4 (bioaugmentation with biostimulation) than 59% in Tr2 (biostimulation only). The findings suggest that nutritional limitation or presence of low indigenous population of hydrocarbon degrading bacteria play an important role in the bioremediation of oily sludge contaminated soil.

Degradation of different fractions of TPH

Total petroleum hydrocarbons were fractionated into four fractions, i.e., Alkane, Aromatic, NSO, Asphaltene. Asphaltene is highly complex and recalcitrant fraction that degraded least (Fig. 2) while highest degraded fraction was alkane followed by aromatic and NSO. Maximum degradation of alkane (91%), aromatic (86%), NSO (79%) and asphaltene (67%) were found in Tr4. Degradation of aliphatic compounds occurred faster than aromatic compounds in our microcosmic study. They observed 92% of TPH reduction after 345 days in which alkane, aromatic and NSO with asphaltene fractions were degraded by 94.2%, 91.1% and 85.2%, respectively. The microbial degradation of aromatic hydrocarbons is hampered for mainly three reasons; firstly, the high activation energy is needed to chemically attack aromatic rings (Boll *et al*., 2002), secondly by the PAH toxicity to bacteria (Loibner *et al*., 2004; Jonker *et al*., 2006), and thirdly because aromatic hydrocarbons have high tendency to sorb on hydrophobic surfaces.

Viable count of total and bioaugmented microbes

Native bacterial count was initially found 4.5 x 10^5 CFU (g^{-1} of soil) in soil while their counting increased up to 12-13 x 10^5 CFU g^{-1} of soil

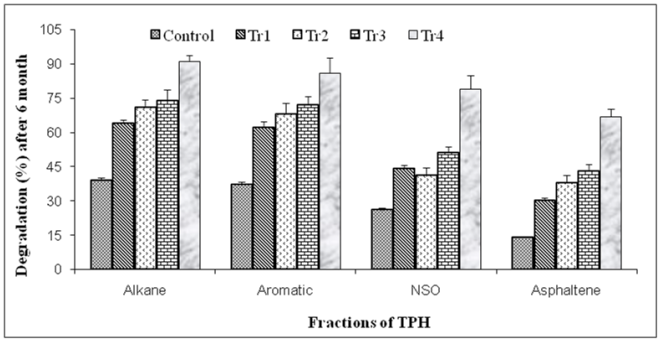

Fig. 2: Degradation of different fraction of TPH after 6 months in each treatment.

Table 2: Degradation Kinetics of TPH for different treatments.

Treatments	*Augmentation*	*TPH degradation (%)*	*K (per month)*	$t_{½}$	*Efficiency of Augmentation (%)*
Control	-	33.2 ± 2.5	0.055	12.53	-
Tr1	Vermicompost	52 ± 1.8	0.086	7.99	Vermicompost (Tr1 vs control) 56.77
Tr2	NPK	58.8 ± 3.1	0.098	7.07	NPK (Tr2 vs control) 12.99
Tr3	Vermicompost +BC	70.3 ± 2.7	0.12	5.91	Microbial combination (Tr3 vs Tr1) 35.13
Tr4	Vermicompost +NPK+MC	80 ± 4.2	0.13	5.20	Vermicompost +NPK+MC (Tr4 vs Control) 141.02

by adding 10% of vermicompost (Fig. 3a). After bioaugmentation of microbial consortium, bacterial count reached up to 32.6 x 10^5 CFU g^{-1} of soil. In all the treatments, the bacterial count gradually increased and reached to maximum to 4th month of incubation and then showed declination. Maximum bacterial count was observed in Tr4 (1.12 x 10^8 CFU g^{-1} of soil), followed by Tr3 (9.7 x 10^7), Tr2 (3.7 x 10^7), Tr1 (2.2 x 10^8) and least was recorded in control (3.2 x 10^6) after 4 month of incubation.

Similarly, fungal growth was also found highest in Tr4 treatment. Maximum growth was recorded after 4 month of incubation in the following sequence; Tr4 (1.02 x 10^7 CFU g^{-1} of soil) > Tr3 (8.3 x 10^6 CFU g^{-1} of soil) > Tr2 (5.4 x 10^6 CFU g^{-1} of soil) > Tr1 (3.5 x 10^5 CFU g^{-1} of soil) > control (5 x 10^5 CFU g^{-1} of soil) as shown in Fig. 3b. This result showed 32.6 and 25.2 fold enhancement in fungal count due to supplementation of vermicompost and NPK, respectively. The result showed that 35 and 20 fold enhancement of soil bacterial and fungal count due to bioaugmentation and biostimulation.

Specific growth rate of bacterial strains was found significantly highest in Tr4 (0.28 d^{-1}), followed by Tr3 (0.239 d^{-1}), Tr2 (0.236 d^{-1}) and Tr1 (0.206 d^{-1}) whereas least was recorded in control (0.05 d^{-1}) based on the growth till 4th month. Similarly, specific growth rate of fungi was found highest in Tr4 (0.179 d^{-1}), followed by Tr2 (0.152 d^{-1}), Tr3 (0.15 d^{-1}), Tr1 (0.113 d^{-1}) and least was in control (0.095 d^{-1}). The result indicates the bacterial biomass enhanced in sludge contaminated soil with comparative higher rate in presence of nutrient supplements in the form of vermicompost and NPK. Besides the addition of biostimulants, bioaugmented microbes also supported to boost the specific growth rate of total soil microbes when rate of Tr3 and Tr4 were compared to rate of Tr1 and Tr2, respectively. Specific growth rate of bioaugmented bacterial and fungal strains was also analyzed for Tr3 and Tr4 (Fig. 4). Specific growth rate for all the bioaugmented species was higher in Tr4 than Tr3 while among bioaugmented species, highest specific growth rate was of BP10 followed by PS10, NJ2, PSM11 and PS8 in both treatments.

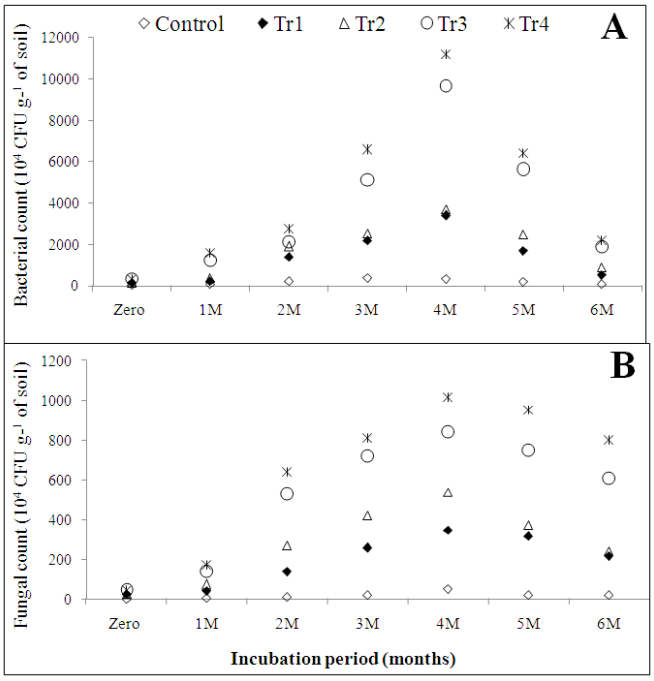

Fig. 3: Viable count of total bacterial (A) and fungal (B) strains in different treatments.

Indigenous bacteria in the soil can degrade a wide range of target constituents of the oily sludge, but their population and efficiency both are affected when toxic contaminant is present at high concentration (Olivera *et al*., 1997). Adaptation process in microbes, which occurs as a result of an increase in the hydrocarbon oxidizing potential of the microbial community, allows the development of microbial population with the ability to degrade petroleum hydrocarbon (Macleod and Semple, 2006). High porosity of soil allows greater hydrocarbon degradation upon biostimulation by nutrient addition and aeration (Chang *et al*., 2013). Addition of vermicompost reduced the bulk density of soil and thus increased the porosity of soil which allowed diffusion of air in soil particle and also increased water holding capacity. This condition supports the growth of microbes which stimulate the degradation process and reduce the toxicity. Liu *et al*. (2010) observed a sharp increase in EC_{50} of the soils after bioremediation of oily sludge contaminated soil, indicating that bioremediation reduced the toxicity of the soil. However, addition of NPK must be adequate as their excessive addition may be toxic for the microbial growth (Kenelly *et al*., 2002; Vyas and Dave, 2010).

Soil protein

Soil protein represent different kinds of degradative enzymes that are directly involved in the mineralization of petroleum hydrocarbons presents in soil amended with petroleum sludge. Addition of vermicompost increased the microbial count to soil and that's why soil protein of Tr1 (0.76 mg g^{-1} of soil) and Tr2 (0.69 mg g^{-1} of soil) was found enhanced compared to control (0.39 mg g^{-1} of soil). Further, soil protein in Tr3 (1.39 mg g^{-1} of soil) and Tr4 (1.44 mg g^{-1} of soil) was recorded higher than Tr1 and Tr2 due to augmentation of microbial combination at the time of inoculation (Fig. 5). Soil protein gradually increased up to 4 month of incubation

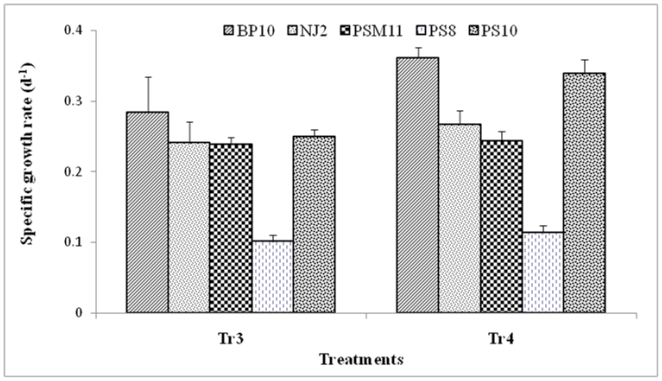

Fig. 4: Specific growth rate of bioaugmented bacterial and fungal strains.

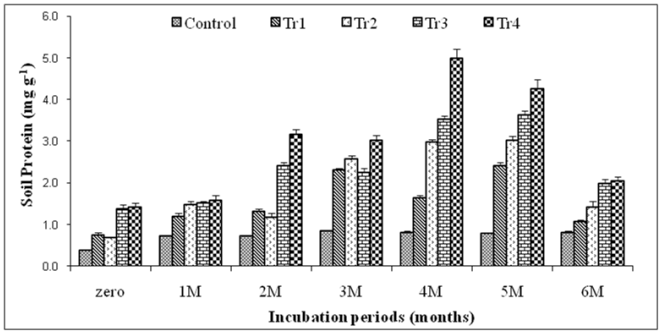

Fig. 5: Changes in soil protein in different treatment during sludge degradation.

period and then started to decline with microbial count decline in soil. Highest protein induction was recorded for Tr4 i.e., 5 mg g^{-1} of soil for 5th month.

Soil protein correspond the induction of protein by microbes being utilized for their metabolic and catabolic activity. It included various degradative enzymes that help in the degradation of petroleum hydrocarbons in oil sludge contaminated soil.

Soil pH

pH of soil used in this experiment was slightly alkaline (7.57) that reduced to 7.23 and 7.01 after addition of vermicompost without and with NPK, respectively. Soil pH gradually decreased with increase in incubation period up to 2 months and then started increasing. After 2 months, the soil pH was recorded to 5.94 in Tr2 which became alkaline again after 6 months (Fig. 6). Soil pH might be linked to decomposition of petroleum hydrocarbons and formation of acidic or alkaline intermediates of oil sludge during decomposition. Soil pH is one of the factors which effect the rate of microbial degradation process because different microbes have different optimum pH to grow that sustains the degradation of petroleum hydrocarbons (Jain *et al*., 2011). Mostly pH range between 6.5-8.0 is considered as optimum for hydrocarbon degradation (Maletić *et al*., 2013).

Change in micronutrient (available NPK)

Available nitrogen, phosphorus and potassium (NPK) of soil amended with sludge at the time of microbial inoculation were 168.1, 138.2 and 0.5 mg kg^{-1}, respectively. Addition of vermicompost enhanced the NPK content of soil up to 224.3, 152.2 and 1.28 mg kg^{-1}, respectively while further addition of inorganic fertilizer increased NPK content up to 284.7, 179.2 and 1.36 mg kg^{-1}, respectively (Table 3). After 6 months of incubation, the NPK content of soil continued decrease in each treatment, but still found higher than control. Addition of chemical NPK in soil amended with vermicompost assisted soil microbes to multiply and this directly supported biodegradation of oily sludge.

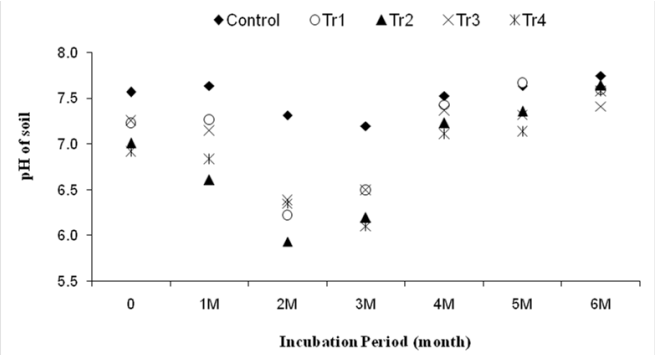

Fig. 6: Changes in soil pH in different treatment during sludge degradation.

The degradation rate of oil hydrocarbons is often limited by lack of nutrients (N, P) and anoxia in the soil (Jørgensen *et al*., 2000). Since microorganisms require both nitrogen and phosphorus for their growth and multiplication, their availability is critical for hydrocarbon degradation. Liu *et al*. (2010) found in an *in situ* experiment that the addition of 5% manure increased significantly the concentration of nutrients in soil, i.e., hydrolyzable N, available P, and available K, which might stimulate the growth of hydrocarbon-degrading microorganisms.

A reduction in phosphorus level could be possible due to oxidation of free phosphorus in the soil to phosphates, as hydrocarbons can act as electron acceptors or oxidizing agents in the presence of oxygen (Vincent *et al*., 2011). Besides fertilizer should be added gradually to the impacted zone in the soil in order to avoid excessively high pH and high concentrations of nitrogen that might be toxic to the soil microbes (Prince *et al*., 2002). Trindade *et al*. (2005) suggested that the optimum ratio for C:N:P should be 100:1.25:1 for bioremediation of petroleum hydrocarbons. Addition of nutrients, agricultural by-products and molasses along with bacterial inoculation has been reported to enhance the degradation process of hydrocarbons in general (Olivera *et al*., 1997).

Enzyme Induction

Dehydrogenase activities were observed higher during the initial incubation period in all treatment while maximum was in Tr4, i.e., 3.4 mg TPF $g^{-1}h^{-1}$. Maximum induction of this enzyme except Tr2 in all treatment was found for 2nd month (Fig. 7A). Dehydrogenase enzyme is one of the important enzyme among all enzyme group of soil as it occurs in all microbes (Moeskops *et al*., 2010). These enzyme oxides petroleum products through hydrogen transfer from organic substrate to inorganic acceptor (Zhang *et al*., 2010). The presence of petroleum product like diesel oil or fuel oil stimulates the soil dehydrogenase activities (Kaczyńska *et al*., 2015). They also observed that stimulation of this enzyme by augmentation of compost accelerate biodegradation of petroleum derived contaminants.

In case of catalase, increasing trend of induction of this enzyme was recorded till 4th month that is correspondence with the biomass of soil microbes (Fig. 7B). Highest induction was in Tr4 i.e., 0.722 m mol H_2O_2 g^{-1} of soil. Enhanced activity of catalase was also recorded in case of biodegradation of petroleum product by various authors (Ugochukwu *et al*., 2008; Mohsenzadeh *et al*., 2012) while Achuba and Okoh (2014) recorded higher activity of catalase in case of diesel and engine oil contaminated soil than petrol and kerosene.

In case of laccase enzyme, induction was reported highest for 1st month in all treatment and no specific trend was observed during incubation period (Fig. 7C). Highest induction was in Tr4 (0.83 mmol g^{-1}) followed by Tr3 (0.82 mmol g^{-1}), Tr1 (0.56 mmol g^{-1}) and Tr2 (0.48 mmol g^{-1}) while least was recorded for control (0.17 mmol g^{-1}). Laccase enzyme is mostly reported during fungal biodegradation of petroleum hydrocarbons (Balaji *et al*., 2014; Ameen *et al*., 2016)

Table 3: Change in level of available NPK in soil during degradation of crude oil.

	Avail. Nitrogen (mg kg^{-1})		*Avail. Phosphorus (mg kg^{-1})*		*Avail. Potassium (mg kg^{-1})*	
Treatment	*0 day*	*6th Month*	*0 day*	*6th Month*	*0 day*	*6th Month*
Control	168.1 ± 3.5	68.9 ± 4.5	138.2 ± 4.6	78.3 ± 8.1	0.51 ± 0.01	0.4 ± 0.01
Tr1	224.1 ± 4.2	140.1 ± 3.1	152.2 ± 2.3	75.6 ± 3.2	1.28 ± 0.21	0.31 ± 0.02
Tr2	280.2 ± 3.6	168.1 ± 5.6	171.2 ± 3.5	80.5 ± 2.3	1.24 ± 0.13	0.38 ± 0.03
Tr3	224.3 ± 2.6	131.4 ± 3.5	141.8 ± 8.7	71.6 ± 3.2	1.03 ± 0.09	0.36 ± 0.01
Tr4	284.7 ± 3.9	121.7 ± 4.3	179 ± 7.3	80.4 ± 3.4	1.36 ± 0.11	0.34 ± 0.02

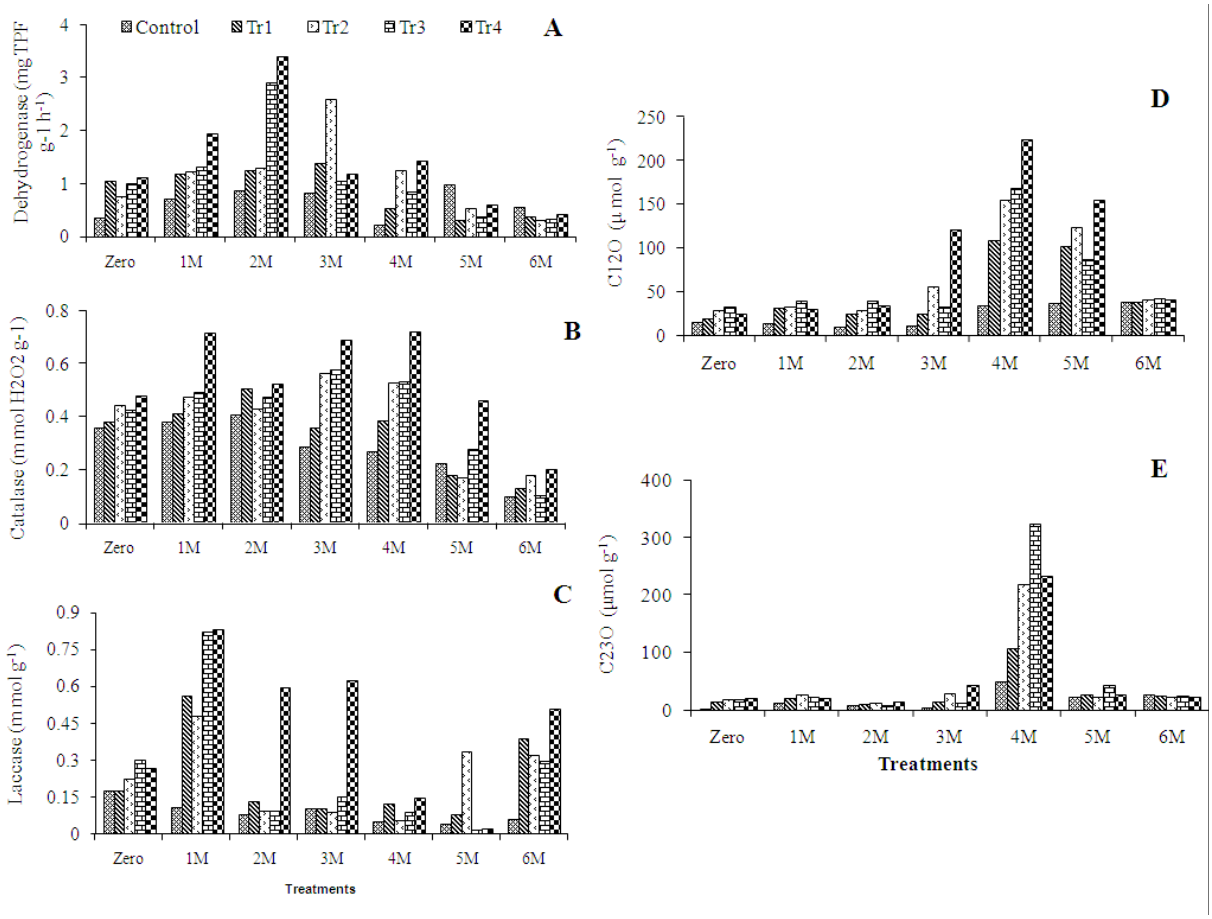

Fig. 7: Soil Enzymes induction in different treatment during sludge degradation.

but few bacterial species are also known as to their capability to induce this enzyme (Sharma *et al*., 2007).

Catechol is one of an intermediate product of microbial degradation of aromatic hydrocarbons that can be mineralized either by intradiol ortho-cleavage pathway or extradiol meta-cleavage pathway through the induction of catechol 1, 2 dioxygenase or catechol 2, 3 dioxygenase, respectively. In present study, catechol 1, 2 dioxygenase activity was found generally higher than catechol 2,3 dioxygenase, however for the 4th month activity of catechol 2, 3 activity was recorded higher than catechol 1,2 dioxygenase in almost all treatments (Fig. 7D-E). Hence, aromatic compounds present in crude oil were being degraded through both ortho or meta pathway. Activities of both enzymes were found maximum for 4th month. Maximum catechol 1, 2 dioxygenase and catechol 2, 3 dioxygenase activities were found in as Tr4 223.89 and 323.83 in Tr3, respectively.

Activity of catechol dioxygenase has been reported for both bacterial (Kumari *et al*., 2012) and fungal species (Vilimkova *et al*., 2009). Trend of enzyme activities was not exactly similar to degradation or biomass, however the highest activity was mostly reported for 4th month when biomass was also higher. It may be due to that degradation is not depending on any specific enzyme induction and there were various enzymes other than these five involved in mineralization process.

Conclusion

The present study clearly suggests that the application of Organic fertilizer, ie., vermicompost and in organic (NPK) with microbial consortia (bacterial strains; BP10, NJ2, and PSM11 in combination with fungal strains; PS8 and PS9) boosted the degradation of petroleum hydrocarbons (oily sludge). Bioaugmentation and biostimulation both enhanced the degradation of TPH by more than 3.5 fold as compared to control. Bioaugmented strains could successfully survive in the contaminated soil and released various extracellular enzymes which oxidized the complex, recalcitrant and hazardous hydrocarbons mixture to less toxic and low molecular weight compounds. Further, research in this direction can give a major boost to the development a microbial technology for cleaning of site contaminated with the organic pollutants.

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References

Achuba, F.I. and Okoh, P.N. 2014. Effect of petroleum products on soil catalase and dehydrogenase activities. *Open Journal of Soil Science* **4**: 399-406.

Agbor, R.B., Ekpo, I.A., Osuagwu, A.N., Udofia, U.U., Okpako, E.C. and Antai, S.P. 2012. Biostimulation of microbial degradation of crude oil polluted soil using cocoa pod husk and plantain peels. *Journal of Microbiology and Biotechnology Research* **2**: 464-469.

Al-Jawhari, I.F.H. 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. *Journal of Applied Environmental Microbiology* **2**(2): 46-52.

Al-Mutairi, N., Bufarsan, A. and Al-Rukaibi, F. 2008. Ecorisk evaluation and treatability potential of soils contaminated with petroleum hydrocarbon-based fuels. *Chemosphere* **74**: 142-148.

Ameen, F., Moslem, M., Hadi, S. and Al-Sabri, A.E. 2016. Biodegradation of diesel fuel hydrocarbons by mangrove fungi from Red Sea Coast of Saudi Arabia. *Saudi Journal of Biological Science* **23**(2): 211-218.

Balaji, V., Arulazhagan, P. and Ebenezer, P. 2014. Enzymatic bioremediation of polyaromatic hydrocarbons by fungal consortia enriched from petroleum contaminated soil and oil seeds. *Journal of Environmental Biology* 35: 521-529.

Beers, J.R.F. and Sizer, I.W. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *The Journal of Biological Chemistry* 195: 133-140.

Boll, M., Fuchs, G. and Heider, J. 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. *Current Opinion of Chemical Biology* **6**: 604-611.

Boonchan, S., Britz, M.L. and Stanley, G.A. 2000. Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Applied Environmental Microbiology* **66**(3): 1007-1019.

Chang,W., Akbari, A., Snelgrove, J., Frigon, D. and Ghoshal, S. 2013. Biodegradation of petroleum hydrocarbons in contaminated clayey soils from a sub-arctic site: The role of aggregate size and microstructure. *Chemosphere* **91**: 1620-1626.

Chávez-Gómez, B., Quintero, R., Esparza-Garcı́a, F., Mesta-Howard, A.M., Zavala, Dı́az, F.J., de la Serna, Hernández-Rodrı́guez, C.H., Gillén, T., Poggi-Varaldo, H.M., Barrera-Cortés, J. and Rodrı́guez-Vázquez, R. 2003. Removal of phenanthrene from soil by co-cultures of bacteria and fungi pregrown on sugarcane bagasse pith**.** *Bioresource Technology* **89**(2): 177-183.

Dara, O.R. and Sarah, C. 2003. Just Oil, The Distribution of Environmental and Social Impacts of Oil Production and Consumption. *Annual Review Environment Resource* **28**: 587-617.

de Carvalho, C.C.C.R. and da Fonseca, M.M.R. 2005. The remarkable *Rhodococcus erythropolis*. *Applied Microbiology and Biotechnology* **67**: 715-726.

Farhadian, M., Vachelard, C., Duchez, D. and Larroche, C. 2008. *In situ* bioremediation of monoaromatic pollutants in groundwater: a review. *Bioresource Technology* **99**: 5296-5308.

Fernández-Luqueño, F., Thalasso, F., Luna-Guido, M.L., Ceballos-Ramírez, J.M., Ordoñez-Ruíz, I.M. and Dendooven, L. 2009. Flocculant in wastewater affects dynamics of inorganic N and accelerates removal of phenanthrene and anthracene in soil. *Journal of Environmental Management* **90**(8): 2813-2818.

Haritash, A.K. and Kaushik, C.P. 2009. Biodegradation aspects of polycyclic aromatic hydrocarbons (PAHs): a review. *Journal of Hazardous Material* **169**: 1-15.

Hashem, A.R. 2007. Bioremediation of petroleum contaminated soils in the Persian gulf region a review. *Journal of Kuwait Science* **19**: 81-91.

Hegeman, G.D. 1966. Synthesis of the enzymes of the mandelate pathway by *Pseudomonas putida*. I. Synthesis of the enzymes in the wild type. *Journal of Bacteriology* **91**: 1140-1154.

Islam, B. 2015. Petroleum sludge, its treatment and disposal: a review. *International Journal of Chemical Science* **13**(4): 1584-1602.

Jain, P.K., Gupta, V.K., Guar, R.K., Lowry, M., Jaroli, D.P. and Chauhan, U.K. 2011. Bioremediation of petroleum contaminated soil and water. *Research Journal of Environmental Toxicology* **5**(1): 1-26.

Jonker, M.T.O., Brils, J.M., Sinke, A.J.C., Murk, A.J. and Koelmans, A.A. 2006. Weathering and toxicity of marine sediments contaminated with oils and polycyclic aromatic hydrocarbons. *Environmental Toxicology Chemistry* **25**: 1345-1353.

Jørgensen, K.S., Puustinen, J. and Suortti, A.M. 2000. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. *Environmental Pollution* **107**: 245-254.

Juckpeck, K., Pinyakong, O. and Rerngsamran, P. 2012. Degradation of polycyclic aromatic hydrocarbons by newly isolated *Curvularia* sp. F18, *Lentinus* sp. S5, and *Phanerochaete* sp. T20. *Science Asia* **38**: 147-156.

Kaczyńska, G., Borowik, A. and Wyszkowska, J. 2015. Soil dehydrogenases as an indicator of contamination of the environment with petroleum products. *Water Air Soil Pollution* 226(11): 372.

Khashayar, T. and Mahsa, T. 2010. Bidegrdation potential of petroleum hydrocarbons by bacterial diversity in soil. *World Applied Science Journal* **8**(6): 750-755

Kim, J.D. and Lee, C.G. 2007. Microbial degradation of polycyclic aromatic hydrocarbons in soil by bacterium-fungus co-cultures. *Biotechnology Bioprocess Engineer* **12**: 410-416.

Klecka, G.M. and Gibson, D.T. 1981. Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. *Applied Environmental Microbiology* **41**(5): 1159-1165.

Kumar, B. and Mohan B. R. 2013. Petroleum refinery oil sludge: the quantitative and qualitative analysis. *Indian Stream Research Journal* **3**(10): 1-9.

Kumar, S., Upadhayay, S.K., Kumari, B., Tiwari, S., Singh, S.N. and Singh, P.K. 2011. *In vitro* degradation of fluoranthene by bacteria isolated from petroleum sludge. *Bioresource Technology* **102**: 3709-3715.

Kumari, B., Singh, S.N. and Singh, D.P. 2012. Characterization of two biosurfactant producing strains in crude oil degradation. *Process Biochemistry* **47**(12): 2463-2471.

Liu, Y.C., Li, L.Z., Wu, Y., Tian, W., Zhang, L.P., Xu, L., Shen, Q.R. and Shen, B. 2010. Isolation of alkane-degrading *Alcanivorax* sp. strain 2B5 and cloning of alkB gene. *Bioresouce Technology* **101**: 310-316.

Loibner, A.P., Szolar, O.H.J., Braun, R. and Hirmann, D. 2004. Toxicity testing of 16 priority polycyclic aromatic hydrocarbons using Lumistox. *Environmental Toxicology Chemistry* **23**: 557-64.

Lowry, O.H., Roserbrough, N.T., Farr, A.L. and Randall, R.J. 1951. Protein measurement with folin phenol reagent. *Journal of Biological Chemistry* **193**: 265-75.

Machin-Ramirez, C., Okoh, A.I., Morales, D.K., Mayolo-Deloisa, M., Quintero, R. and Trejo-Hernaindez, M. R. 2008. Slurry-phase biodegradation of weathered oily sludge waste. *Chemosphere* **70**: 737-744.

Macleod, C.J.A. and Semple, K.T. 2006. The influence of single and multiple applications of pyrene on the evolution of pyrene catabolism in soil. *Environmental Pollution* **139**: 455-460.

Maletić, S., Dalmacija, B. and Rončević, S. 2013. Petroleum hydrocarbon biodegradability in Soil - implications for bioremediation in hydrocarbon. http://www.intechopen.com/books/hydrocarbon

Moeskops, B., Buchan, D., Sleutel, S., Herawaty, L., Husen, E., Saraswati, R., Setyorini, D. and De Neve, S. 2010. Soil microbial communities and activities under intensive organic and conventional vegetable farming in West Java, Indonesia. *Applied Soil Ecological* 45: 112-120.

Mohsenzadeh, F., Rad, A.C. and Akbari, M. 2012. Evaluation of oil removal efficiency and enzymatic activity in some fungal strains for bioremediation of petroleum-polluted soils. *Iran Journal Environmental Health Science Engineer* **9**: 26.

Nduka, J.K., Umeh, L.N., Okerulu, I.O., Umedum, L.N. and Okoye, H.N. 2012. Utilization of different microbes in bioremediation of hydrocarbon contaminated soils stimulated with inorganic and organic fertilizers. *Journal of Petroleum Environmental Biotechnology* **3**(2): 1-9.

Ofoegbu, R.U., Yusuf, R.U., Momoh, O.L. and Nwaogazie, I.F. 2015. Bioremediation of crude oil contaminated soil using organic and inorganic fertilizers. *Journal of Petroleum Environmental Biotechnology* **6(**1): 1-6.

Opasols, A.O. and Adewoye, S.O. 2010. Assessment of degradability potential of *Penicillium oxalicum* on crude oil. *Advance Applied Science Research* **1**: 182-188.

Olivera, N.L., Esteves, J.L. and Commendatore, M.G. 1997. Alkane biodegradation by a microbial community from contaminated sediments in Patagonia, Argentina. *International Biodeterioration and Biodegradation* **40**: 75-79.

Prince, R. 2002. Bioremediation effectiveness: removing hydrocarbons while minimizing environmental impact. ExxonMobil Res. Eng. Hand-out at DOE/PERF Bioremediation Workshop. May 30.

Rahman, K.S.M., Rahman, T.J., Banat, I.M., Lord, R. and Street, G. 2007. Bioremediation of petroleum sludge using bacterial consortium

with biosurfactant. In: Singh, S.N., Tripathi, R.D. (Eds.), *Environmental Bioremediation Technologies,* Springer, Germany, pp. 391-408.

Sharma, P., Goel, R. and Capalash, N. 2007. Bacterial Laccase. *World Journal of Microbiology Biotechnology* **23**(6): 823-832.

Silva, I.S., dos Santos, E., da, C., de Menezes, C.R., de Faria, A.F., Franciscon, E., Grossman, M. and Durrant, L.R. 2009. Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia. *Bioresource Technology* **100**: 4669-4675.

Stevenson, I.L. 1956. Dehydrogenase activity in soils. *Candian Journal of Microbiology* **5**: 229-235.

Thompson, I.P., van der Gast, C.J., Ciric, L. and Singer, A.C. 2005. Bioaugmentation for bioremediation: the challenge of strain selection. *Environmental Microbiology* **7**: 909-915.

Trindade, P.V.O., Sobral, L.G., Rizzo, A.C.L., Leite, S.G.F. and Soriano, A.U. 2005. Bioremediation of a weathered and recently oil-contaminated soils from Brazil: A comparison study. *Chemosphere* **58**: 515-522.

Trofimov, S.Y. and Rozanova, M.S. 2003. Transformation of soil properties under the impact of oil pollution. *Eurasian Soil Science* **36**: S82-S87.

Ugochukwu, K.C., Agha, N.C. and Ogbulie, J.N. 2008. Lipase activities of microbial isolates from soil contaminated with crude oil after bioremediation. *African Journal of Biotechnology* **7**: 2881-2884.

Vilimkova, L., Jechova, J., Koubkova, Z., Paca, J. Jr., Kremlackova, V., Poljakova, J., Paca, J. and Stiborova, M. 2009. Isolation and partial characterization of catechol 1, 2-dioxygenase of phenol degrading yeast Candida tropicalis. *Neuro Endocrinology Letter* 30(1): 80-87.

Vincent, A.O., Felix, E., Weltime, M.O., Ize-iyamu, O.K. and Daniel, E.E. 2011. Microbial degradation and its kinetics on crude oil polluted soil. *Research Journal of Chemical Science* 1(6): 8-14.

Vyas, T.K. and Dave, B.P. 2010. Effect of addition of nitrogen, phosphorus and potassium fertilizers on biodegradation of crude oil by marine bacteria. *Indian Journal of Marine Science* **39**(1): 143-150.

Yu, K.S.H., Wong, A.H.Y., Yau, K.W.Y., Wong, Y.S. and Tam, N.F.Y. 2005. Natural attenuation, biostimulation and bioaugmentation on biodegradation of polycyclic aromatic hydrocarbons (PAHs) in mangrove sediments. *Marine Pollution and Bulletin* **51**: 1071-1077.

Zhang, N., He, X., Gao, Y., Li, Y., Wang, H., Ma, D., Zhang, R. and Yang, S. 2010. Pedogenic carbonate and soil dehydrogenase activity in response to soil organic matter in *Artemisia ordosica* community. *Pedosphere* 20: 229-235.