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A study on Feasibility of Bioremediation of Crude Oil contaminated soil from Kalol with Indigenous Mixed Culture

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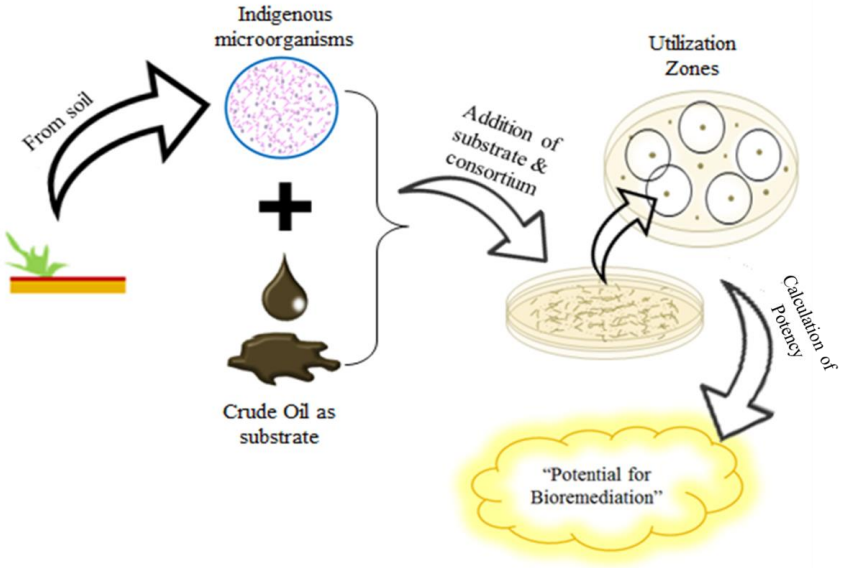
Graphical Abstract



***Abstract*-- Bioremediation is a technique for treating various contaminants with application of microorganisms and provision of their kinetics renders implementation of biochemical reactions based on their rates of decomposition. The present study aims to test the feasibility of bioremediation of crude oil contaminated soil from agricultural land in Kalol area of Ahmedabad district, Gujarat, India. The experiment comprises five batches with descending levels of contamination that was measured in Total Petroleum Hydrocarbon percentage (TPH%); initial contamination present in both batch A and B was 11.7% TPH whereas, batch C, D and E was contaminated with 7.3%, 7.24% and 2.3% TPH respectively. The indigenous mixed culture was prepared from the collected soil sample and used to remediate batch A, C and E whereas an unknown culture provided by OTBL (ONGC TERI Biotech Limited) as reference was applied to batch B and C. The kinetics of indigenous mixed culture revealed a pattern of diauxic growth upon observation which gives suitability to Verlhurst growth model. The degradation of crude oil was estimated using solvent extraction at regular intervals of time that showed 40% to 70% in degradation range amongst the batches. The rate of degradation was implied using integral method resulting in first order kinetics. Other characteristics of indigenous mixed culture such as morphology and substrate utilization zones were observed using standard determination methods. The variation**

in physical and chemical properties of soil such as pH, ORP, conductivity, colour, TPH were observed on prior and latter basis of bioremediation.

Key words - Bioremediation, Biodegradation, Crude oil contamination, Total Petroleum Hydrocarbon, First order kinetics, Verlhurst model, Indigenous mixed culture, Substrate utilization zones.

I. INTRODUCTION

Crude oil is source of energy for the sustenance of most human requirements which is obtained from the earth's crust via drilling, suction and extraction. The contamination of fertile land occurs due to anthropogenic activities involved in extraction and processing of crude oil which involves oil spill while recovery from an artificial lift or natural pressure, after decommissioning of the oil recovery plant, transportation, oil spill disasters over water, etc. The proper remediation treatment given to the source of contamination holds the possibility of reclamation. The method of reclamation involves physical, chemical and biological techniques. The physical methods of reclamation involves capping (Perelo, 2010), grouting, electro-kinetic (Yeung and Gu, 2011), grouting, thermal desorption, soil washing, vapour stripping, incineration, stabilization, thermal treatment whereas chemical method includes treatments such as chemical immobilization, solvent extraction (Silva et al., 2005), oxidation, photo degradation, actinide chelators and others (Ezeji et al., 2007; Pavel and Gavrilescu, 2008). The biological method viz mycoremediation, phytoremediation, land farming (Mohan et al., 2006), rhizoremediation and bacterial remediation are described by Thapa et al. (1970). It is observed through literature that the biological methods are the most reliable and environment advantageous hence acquired considerable significance. Bioremediation is an effective strategy for detoxifying organic contaminants from soil, water, etc. with application of microorganisms found in nature hence, it can be stated as a technique that uses non-pathogenic environment friendly microorganisms to remediate contaminated source. It gives an economic and eco-friendly way of sustaining clean energy with little manipulation and enhancement in natural mechanism of degradation and deterioration. The treatment methods involving of microbial application consists bio-stimulation, bio-augmentation, bio-sparging and bio-venting which ensures the sustenance of required concentrations of microbial population (Thapa et al. 1970, Mohan et al. 2006, Perelo 2010, Sharma 2012) for necessary decomposition reactions to occur. Mixture of different microbial isolates i.e. consortium has been found to be more efficient in hydrocarbon degradation because of its synergetic actions rather than isolated species of similar microorganisms.

Degradation can be aerobic as well as anaerobic in process which depend on the difference in the reaction pathway and their products. Biodegradation refers to the breakdown of organic compounds by microorganisms into carbon dioxide and water or methane. Inorganic compounds are not biodegraded, but they can be bio-transformed, into lesser or no toxic compounds compared to their original form (Thapa et al. 1970). The anaerobic degradation will give its end products as methane, carbon dioxide and little amounts of other gasses and bio-nutrient rich slurry whereas aerobic degradation will result into carbon dioxide, water and microorganism nutrient rich substrate (Sims et al. 1990). Wilson and Jones (1993) studied the mechanism of reaction that takes place in biodegradation process as oxidation-reduction reaction that shows degrading microorganisms produce enzymes called oxygenases which act as catalysts for biochemical reactions. Their study stated that fungi produces mono-oxygenases whereas bacteria produces di-oxygenases that binds with substrate to give dioxyethanes and is further oxidised to cis-dihydrodiols followed by dihydroxy products giving catechol, protocatechuic, and gentisic acids. This oxidation-reduction reaction is responsible for ring cleavage, holds the main rate limiting step for the degradation of complex hydrocarbons present in crude oil. The oxidation reaction forms the basis for products at ortho, para or meta position which results into succinic, fumaric, pyruvic, and acetic acids and acetaldehyde that are utilised as energy for cell-protein synthesis by microorganisms with the production of water and carbon dioxide (Wilson and Jones 1993, Bamforth and Singleton 2005, Das and Chandran 2011). Factors affecting microorganisms are pH, temperature, moisture, toxic chemicals, nutrients, surfactants, concentration of contamination (Sims et al. 1990, Wilson and Jones 1993, Ezeji et al. 2007, Das and Chandran 2011).

Fulekar (2017) reported degradation of complex hydrocarbon contamination from soil using indigenously derived microorganism latter identified as *Pseudomonas aeruginosa*. *Pseudomonas fluorescens* and *Bacillus subtilis* isolated from Bonny river, Forcados river and Chanomi Creek areas of operation of Shell Petroleum Development Company (Nig.) Ltd. (SPDC) were reported to be effective for crude oil degradation. The biodegradation is calibrated with decomposition rate profiles over microbial populations in (Colony Forming Units) CFU/ml on logarithmic scale as observed by Ekundayo and Obire (1987). Latter on Karamalidis *et al.* (2010) showed PAH (Poly Aromatic Hydrocarbons) present in crude oil was degraded by 79% and 89% after 191 days of treatment at laboratory scale using three approach of bioaugmentation and biostimulation with indigenous microorganisms and bioaugmentation with isolated *Pseudomonas aeruginosa* and thirdly repeating the second approach with inoculation of cells of *P. aeruginosa* in the presence of starch showing that bioagumentation with indigenous microorganisms achieved better and highest results compared to isolated cells. The study involving the effect of bioremediation by Wang *et al.* (1990) using *Salmonella typhimurium* on diesel oil spill having PAH contamination of 60 mg/g manifested degradation in 12 weeks and reduction in toxicity after 20 weeks. Application of *Pseudomonas sp.* with rice husk and ploughed at oil contaminated site in Jianghan Oil field, China became instantiate for in-situ bioremediation that gave results in 95% of complex hydrocarbon removal in Xu et al. (2016) and 89% of degradation of crude oil by

actinomycete-Nocardia group on ex-situ bioremediation at Serbia oil refinery was also reported in Milic et al. (2009).

There are many theories and examinations in literature that supports the degradation potential of indigenous microorganisms for crude oil contamination. The effect of indigenous microorganisms on Hamada Crude Oil Tank Bottom Sludge (COTBS) where 49 isolates were detected and their potential capability to degrade crude oil was studied as 90% of isolates were capable of using aromatic compounds as substrate as suggested in Mansur et al. (2014). Examination carried out by Liu *et al.* (2010) involving in-situ bioremediation of crude oil contaminated soil with indigenous microbes at Shengli oil field, China that resulted into reduction in TPH by 58.2% after 360 days of operation. With addition of 10% to 20% of indigenous consortium in soil collected from industrial area of China, the degradation of PAH contamination in soil enhanced by 20% to 35% (Mao et al. 2012). Li *et al.* (2002) performed experiment for field scale remediation technology of crude oil contamination at Liaohe Oil Fields, China using compost comprising chicken excreta and agriculture waste along with indigenous consortium giving 57% degradation rate of crude oil showing their synergetic effectiveness. The development of novel microbial consortium consisting of highly thermophilic paraffin degrading bacteria PDS-10 by Biswas et al. (2010) concluded for down hole tubulars that microbial treatment is also capable of improving quality of oil other than biodegradation because certain bacterial isolates are better at improving flow characteristics and hence used for enhancing oil recovery (Al-Sayegh et al., 2016).

The present work carries an objective of studying the biodegradation potential of unidentified indigenous mixed culture derived from crude oil contaminated soil and its impact of application upon the same soil matrix. The changes in physical and chemical parameters such as pH, moisture, conductivity, oxidation potential, hydrocarbon remains were examined and also kinetics are determined for microbial growth as well as decomposition of crude oil.

II. MATERIAL AND METHODS

Collection of Raw Material

The provision of crude oil contaminated soil sample was availed from Oil and Natural Gas Corporation, Gujarat, India from abandoned agricultural site with identified well number 104, Kalol of ONGC Kalol Asset (Refer figure 1). The collected raw material was in need of preliminary treatment because of excessive heterogeneous contamination and stickiness. Hence, the soil was initially cleaned by removing grits, immersed plastic wires and other non-degradable substances. It was crushed and air dried under ambient atmosphere and sunlight for 12 days prior to raw material soil analysis. For preparation of inoculum for indigenous mixed culture, land sediments and extremely crude oil contaminated soil samples were collected from five sides of well number 292 of Kalol.[1]

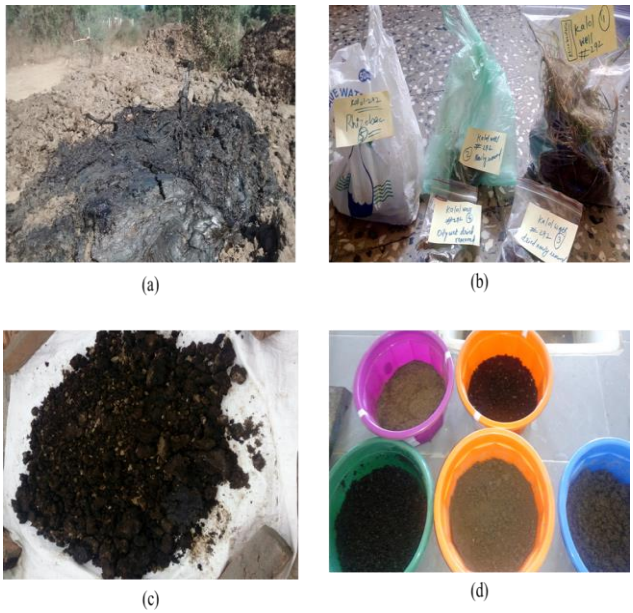


Figure 1: (a) Contaminated site at ONGC Kalol well no. 292; (b) Sample inoculates collected from five different zones of well no. 292, Kalol for preparation of inoculum; (c) Raw material provided from Kalol well no. 104 containing crude oil contaminated soil; (d) Experimental batch set-up for in-situ bioremediation.

Soil Analysis

The primary analysis of physical characteristics of obtained crude oil contaminated soil was carried out using standard methods from International System of Classification as per Indian Society of Soil Science standards. Sieve analysis was carried out with available mesh size of BSS mesh number 150, 44, 30, 16 for obtaining coarse sand, medium sand, fine layer sand and silt (DIRD, Pune, 2009). Whatman filter paper no. 42 with pore size 2.5µm was used to separate very high fines/clay from bulk. Bulk Density of sample was estimated by weighing bottle method that is calculated from weight to volume ratio of sample. The colour of sample soil was directly observed as brown or as dark chocolate. Soil pH and the oxidation-Reduction potential (ORP) were measured with Syntronics (µpH) system 361 (µ-controller) ORP indicator of 0.1 mV resolution. Positive ORP is an indication of tendency of the soil to undergo oxidation, confirming the decomposition of toxicants. Electrical conductivity of soil sample was measured with Chemline CL-120, 0 to 200µS/cm with cell constant 1.05 and cell range 20µS/cm to 200µS/cm after immersion of electrode in soil solution prepared from soil-water mixture in ratio 1:2. The measured conductivity is converted to decisiemens dS/m (Carter and Gregorich, 2008). The obtained results are shown in Table 2.

Moisture Analysis

[1] Picture of collection of oily soil sample and contaminated sediments from well no. 292 Kalol is given in supplementary file.

For the estimation of initial moisture content, 10 g soil sample was dried in the oven at 60 ± 5˚C and dry mass was recorded upto constant value was achieved ensuring maximum evaporation loss. The percentage moisture was calculated from ratio of evaporated mass of water by measured amount of soil sample. Water holding capacity of soil was determined by wetting 10 g of sample soil upto surface level and oven-dried. The evaporated moisture accounts from water holding capacity was nearly 31% which proves for an average soil. Total Dissolved Solids was estimated by pipetting 60ml of soil suspension solution to Whatman filter paper no.42 with pore size 2.5µm and weighing the filtrate. The difference in mass of filtrate from initial mass of soil sample was calculated. Moisture analysis data are given in Table 1.

TABLE 1
Moisture Analysis of Contaminated Soil Sample

Characteristic Moisture Property	Moisture %
Moisture	22.5 %
Water holding capacity	30.67 %
Total Dissolved Solids	0.17 %

Estimation of TPH

TPH (Total Petroleum Hydrocarbon) present in collected soil sample was estimated using solvent extraction followed by gravimetric analysis at regular intervals of time. 10 g of soil each from experimental batches were given solvent wash from 60 ml FINAR GC grade 99% sulphur/thiophene free Toluene wash and filtered via BOROSIL 500 ml separating funnel as per gravity separation method and washed three to four times with distilled water to remove any contaminants present in oil. The solvent-crude oil mixture was collected from bottom and separated using heat using their difference in volatile property. The extracted oil was then desiccated, weighed and measured. The degradation% was calculated as given in equation 1 (John and Okpokwasili 2012, Prakash *et al*. 2014).

$$\text{Degradation\%} = \left[\frac{TPH_{Initial} - TPH_{Interval}}{TPH_{Initial}} \times 100\right] \quad \ldots\ldots\ldots\ldots(1)$$

Preparation of Culture

The MSM (Mineral Salt Medium) was prepared with 250 ml distilled water containing 0.2g of $MgSO_4 \cdot 7H_2O$, 0.2g of $CaCl_2$, 1.0g of KH_2PO_4, 1.0g of K_2HPO_4, 1.0 NH_4Cl, and 0.05g of $FeCl_3$ (John and Okpokwasili 2012; Parthipan et al. 2017; Fulekar 2017) using 500 ml Erlenmeyer flask and autoclaved at 121˚C, 15 psi for 20 minutes. The autoclaved medium was inoculated with 0.5 grams of five samples each collected from Kalol-well number 292 and with 1 ml of extracted crude oil from experimental batches. The prepared flasks were incubated for a period of 1 week under room temperature (30 ± 2.0^{0}C) with continuous stir in orbital shaker at 150 $\pm$ 5 rpm.

Experimental Setup

The in-situ bioremediation experimental setup was arranged in five batches. Batch A and B containing 11.7% raw amount of initial TPH whereas batch C and D was adjusted to 7.3% and 7.24% TPH respectively by addition of fresh clean soil with an estimated volumetric ratio of 60:40. Batch E was prepared analogous to C and D with addition of more fresh clean soil with calculated ratio of 80:20 providing further reduction in contamination level to 2.32%. The prepared soil in every batch was weighed to 1 kg and mixed with 10% w/v indigenous mixed culture and tilled. The batches were kept under sunlight with ambient atmospheric conditions during day time and under 100 watts bulb during night. Batch A was treated with indigenous mixed culture, batch B was treated with unknown reference culture provided by OTBL and degradation in TPH was estimated for every 10 days of intervals. Batch C was treated with OTBL reference culture whereas; batch D and E was treated with indigenous mixed culture. The concentration of TPH was measured at regular intervals of 15 days for batch C, D and E.

Analysis of Indigenous Mixed Culture

The presence of indigenous microorganisms was confirmed through observation of their morphology using gram staining test and zone of substrate utilization. The 7.5 cm x 2.4 cm glass slide was rinsed with alcohol and dried. The gram-staining was performed by washing dried glass slide containing smeared consortium with crystal violet stain followed by iodine then alcohol and saffarine. Stained slides were observed under compound microscope. Both gram positive and negative microbes such as gram-positive cocci, rod and spiral shaped and gram negative small rod shape cells were observed as shown in Figure 2.

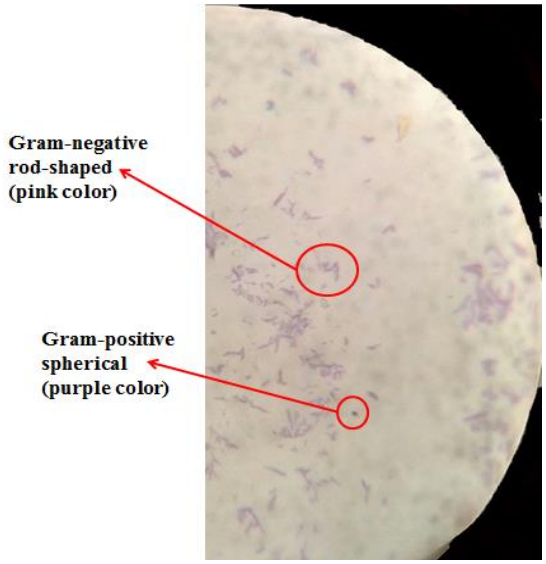


Figure 2: View of presence of rod-shaped and spherical microorganism under compound microscope.

The effective degradation of crude oil was confirmed with the examination of substrate utilization giving zones of

clearance (Farjadfard et al., 2012; Shahaby and El-din, 2017). Agar plates prepared from 2.8 gm N-agar medium and 1.5 gm agar-agar in 100 ml distilled water was autoclaved at 121˚C, 15 psi for 20 min. Two plates were prepared with the inoculation of indigenous mixed culture having serial dilution of 10^{-5} and 10^{-4} which were supplemented with extracted crude oil as substrate using aseptic technique. The plates were incubated at 30 ± 2.0^0C. The plates were observed for growth of microorganisms after 24 and 48 hours of incubation (Ekundayo and Obire 1987, Mulani et al. 2017). The zone of utilization was estimated from the area cleared by the individual colonies of microorganisms. By measuring the zone of clearance diameter, the area of the zone was calculated as, area of clearance zone = $\pi D^2/4$, where D is diameter (cm), and C_n = Type of microbial colonies with zone of clearance (Refer to Table 3).

Microbial Growth Estimation

The growth of microorganisms in the prepared indigenous mixed culture was observed in terms of colony counts. Required serial dilution of 10^{-5} was prepared from indigenous inoculates and 0.1 ml of prepared dilutions was spread upon the prepared agar plates. The inoculated plates were observed for growth after 24 hours to next 264 hours. The growth was observed for microbial colonies in terms of colony forming unit/ml (CFU/ml) (Ekundayo and Obire 1987, Raza et al. 2010, Mulani et al. 2017) increase per day.

III. Results and Discussion

The characterization of soil as given in table 2, was done for preliminary estimation of soil properties before carrying out the main experimental work and due to the presence of sand more than coarse particles estimated from sieve analysis proves its suitability for vegetation and water retention ability. The water holding capacity of soil is low due to the presence of crude oil as contamination which will eventually improve with oil degradation. The water holding capacity increases with increase in silt. The positive ORP of soil was near to the range of water - is indication of the ability to carry out oxidation reaction for decomposition of toxics into non-toxics. The electrical conductivity is the estimation of the ionic salts present in the soil holding significance about soil fertility. The Electrical Conductivity was between 0 dS/m to 1 dS/m which reveals that the sample belongs to good soil and suitable for agriculture (refer table 2) (Carter and Gregorich, 2008; DIRD, Pune, 2009). The pH is an important factor that affects the microbial activity. Some microbes survive in acidic pH and some in alkaline pH. Most of the microbes that serve for oil retardation are not prone to acidic pH or alkaline pH. They also require suitable pH condition to survive. This signifies pH as an important characteristic that can be manipulated upto some extent which not only increases the growth but also enhances the microbial activity. The initial pH of soil sample measured as 7.5 was same for all and is considered suitable pH condition for the survival of microorganisms. The final pH measured at the end of experiment for batch A and B, C, D was 7.5 and 9.0 respectively and for E it was 8.0. Hence the optimum pH found at which bioremediation takes place was between 8 to 9 pH. The range of pH measured at the end of the experiment stands in range of about 7.5 to 9 for all the batches.

TABLE 2
Initial Characteristics of Contaminated Soil Sample

Properties	Values
Coarse sand in wt%	24.59
Medium sand in wt%	45.39
Fine sand in wt%	16.01
Silt in wt%	12.74
Clay in wt%	1.27
Bulk density in kg/L	1.433
pH	7.5
ORP in mV	+135
Conductivity in dS/m	0.2
TPH %	11.7

Degradation Potential Calibration

The gradual degradation of crude oil from the soil is shown by the zone of utilization deducing that the consortium used has tendency to degrade crude oil from soil (Farjadfard et al., 2012). Viable counts from the inoculated plates were performed; the development of fungi on supplemented crude oil substrate specifies an option for mycoremediation. The fungal colonies were observed to be different in size, colour and appearance. The black colour fungi gave rapid growth and bigger zone of oil clearance than other blue, grey, white and yellow fungi (Figure 3). The hazy growth of fungi hides bacterial colonies due to their vast expansion.

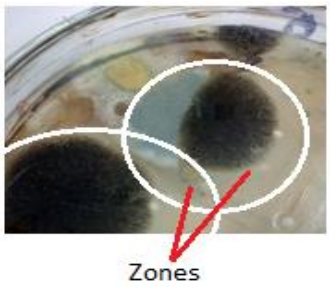


Figure 3: Zone of Clearance by Fungi

Crude oil was applied on the plates as substrate which was utilized by microorganisms as energy source showing visible clearance zones in plates after 48 hours of observation (Ekundayo and Obire 1987, Shahaby and El-din 2017). The circular area cleared upon utilization of extracted crude oil as substrate was measured as recorded in Table 3.

TABLE 3
Area of Zones of Clearance for Plate 1 and Plate 2

Colony type	Colour of Microbial colonies	Diameter (cm)	Radius (cm)	Area of zone (cm^2)
Zones from plate with dilution 10^{-5} (plate 1)				
C_1	Red	1.5	0.75	1.766
C_2	Other	0.8	0.4	0.502
C_3	Other	0.8	0.4	0.502
C_4	Other	1.25	0.625	1.226
C_5	Red	1.0	0.5	0.785
C_6	Red	2.2	1.1	3.799
C_7	Red	2.5	1.25	4.906
C_8	Other	1.0	0.5	0.785
C_9	Other	0.5	0.25	0.196
C_{10}	Other	0.2	0.1	0.031
Zones from plate with dilution 10^{-4} (plate 2)				
C_1	Red	1.2	0.6	1.1304
C_2	Red	1.5	0.75	1.766
C_3	Red	0.8	0.4	0.502
C_4	Other	0.8	0.4	0.502
C_5	Other	1.0	0.5	0.785
C_6	Red	1.7	0.85	2.2686
C_7	Red	1.5	0.75	1.766
C_8	Red	1.6	0.8	2.0096
C_9	Red	1.3	0.65	1.3266
C_{10}	Red	1.1	0.55	0.9498
C_{11}	Red	1.5	0.75	1.766
C_{12}	Red	1.5	0.75	1.766

The plate containing 10^{-5} dilution showed larger zones by red coloured pigmented microbial colonies compared to other microbial colonies as shown in table 3. From the area measured, it can be deduced that the red coloured colonies C_6 and C_7 gave highest utilization zones than other colonies in plate of dilution 10^{-4} whereas, C_6 and C_8 showed highest utilization zones in plate of dilution 10^{-5}. Here, the numbers of red coloured microbial colonies were more than other colonies. The comparison of the data obtained from two plates as shown in figure 4 shows that highest zone of clearance obtained amongst red coloured microbial colonies was from plate with dilution 10^{-5} which has an area of about 4.91 cm^2 and the next highest clearance zone was exhibited by an area of 3.8 cm^2.

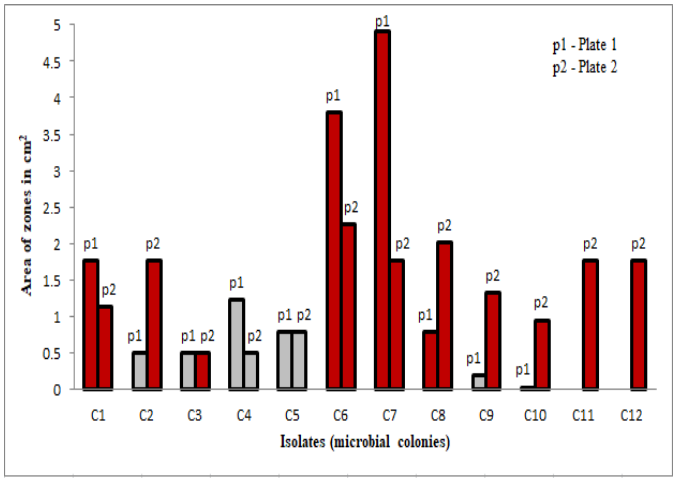


Figure 4: Comparison of clearance zones measured for red coloured colonies with respect to other colonies observed from two plates.

The 47 unidentified isolates from red coloured colonies of plate 1 with dilution 10^{-5} were observed. Due to crowded growth in plate 2 with dilution 10^{-4}, exact determination of total number of colonies was not possible. Approximate value was beyond 200.

Microbial Growth Estimation

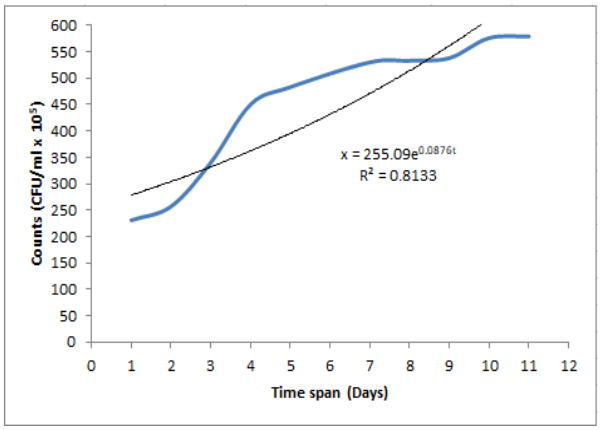
The growth of colonies upon agar plates with applied dilution of indigenous mixed culture were observed for 10 to 11 days and total visible microbial colonies were counted each day as given in graphical representation of figure 5(a). Malthusian law is used to derive relationship for growth rate (Bailey and Ollis, 1986; Horowitz et al., 1975; Najafpour, 2015),

$$\mu x = r_x = \frac{dx}{dt} \quad \text{.....(2)}$$

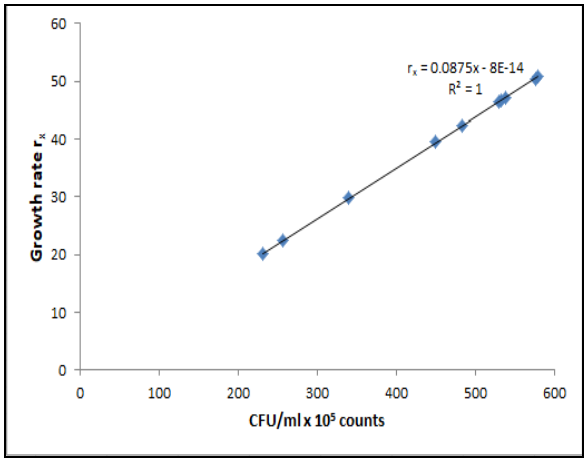
The integrated simplified form of equation 2 gives;

$$x = x_0\, e^{\mu t} \quad \text{.....(3)}$$

Where x is increase in number of colony counts multiplied to its dilution, μ is growth constant and r_x is rate of growth.



(a)



(b)

Figure 5. (a) Growth of microorganisms present in indigenous mixed culture observed for 10-11 days in CFU/ml/day; (b) Relationship between rate of growth and colony counts in CFU/ml in days for indigenous consortium.

According to equation 2, the value of μ is predicted as 0.0876 from slope as shown in Figure 5(a) (Horowitz et al., 1975). The unstructured growth in figure 5(a) was seen in the early stages of development of microbes before reaching stationary phase. This unstructured growth pattern manifests diauxic growth where multiple carbon sources were present as substrate (Najafpour, 2015). This exhibits their shift in carbon substrates within the stages of growth due to multiple carbon compounds present; microorganisms tend to use the compounds with lower complexity first to higher complexity in hydrocarbon chains. Hence, Verlhurst model is used which defines the relationship between growth rate and biomass concentration. Using Verlhurst Model equation (Bailey and Ollis 1986; Najafpour 2015) for the specific growth we get,

$$\mu = \mu_{max}\left[1 - \frac{x}{x_m}\right] \quad \text{.....(4)}$$

The above equation for μ_{max} can be written as,

$$\mu_{max} = \frac{\mu}{\left[1-\frac{x}{x_m}\right]} \quad \text{.....(5)}$$

Where, x is CFU/ml x 10^5 per day, x_m is maximum CFU/ml x 10^5 per day, and μ is growth coefficient. Combining equation 2 and equation 4 and extracting values of x_m from figure 5(a), we get;

$$r_x = \mu_{max} x^2 \left[\frac{1}{x} - \frac{1}{579\times10^5}\right] \quad \text{.....(6)}$$

Values of μ_{max} is calculated based on equation 5 and estimated average is 2.41 which can be interpreted with equation 6 as,

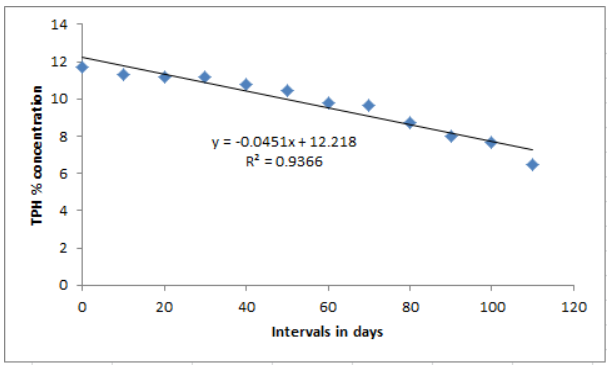
$$r_x = 2.41x^2 \left[\frac{1}{x} - \frac{1}{579\times10^5}\right] \quad \text{.....(7)}$$

The above equation holds for the specified indigenous microbial counts. The relationship between rate of growth and counts of microbial colonies in mixed culture is linear as shown in Figure 5(b).

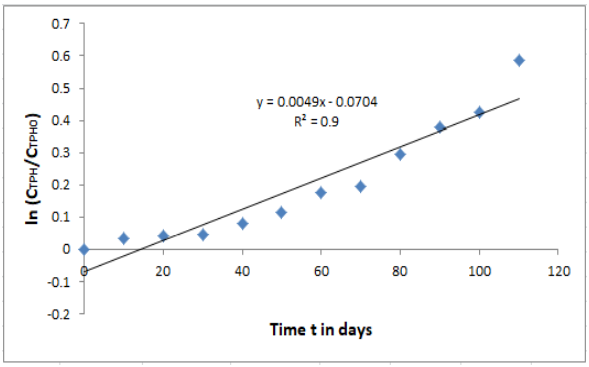
Degradation Kinetics

Bioremediation is a process of breaking down of molecules that are organic, toxic, oil, or any other compound. This process is generally expressed by the term 'degradation'. The contaminated soil was bifurcated into five batch experiments for which kinetics are studied (Abbassi and Shquirat, 2008; Yudono et al., 2010). The integral method $C_{TPH} = C_{TPH,0}\, e^{-kt}$ is used for determining rate expression for degradation in TPH concentration. The degradation percentage was estimated as given in equation 1 (Prakash et al., 2014; Zahed et al., 2011).

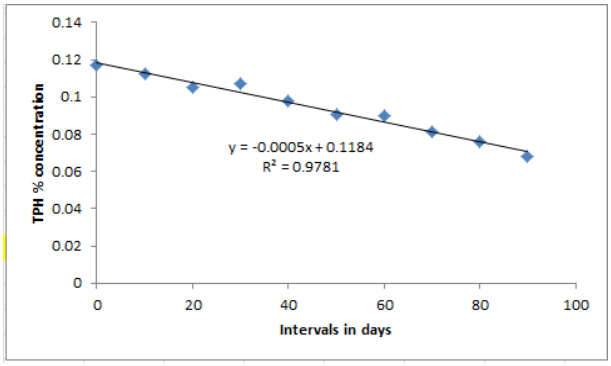
Batch A was treated with indigenous consortium with an initial TPH concentration of 11.7%. The reduction in concentration of TPH measured in every 10 days of interval. Batch A has shown degradation percentage of 44.44% in 110 days as seen from graph in figure 6(a) where concentration of TPH % is plotted against time t in days. The logarithmic ratio of initial and final concentrations ($C_{TPH}/C_{TPH,0}$) of TPH is plotted against intervals of time in days as represented in figure 6(b) which gives value of rate constant *k* as 0.005 day^{-1} for first order with regression 0.94 achieved. Batch B has similar TPH concentration of 11.7% initially and was treated with unknown reference culture provided by OTBL, degradation was observed at regular intervals of 10 days simultaneous to batch A. The TPH% degraded in batch B measured as 41.97% in 90 days compared to batch A as shown in figure 6(c). This forms the basis for comparison of degradation rate of indigenous versus unknown reference culture with same level of contamination present in soil. The kinetics of degradation in batch B from figure 6(d) shows that batch B obeys first order reaction kinetics with 0.006 day^{-1} *k*. The relationship was linear between contamination concentration and its degradation analogous to isotherm presented by Vincent et al. (2011) for all five batches. Other two batches C and D which were contain reduced contamination levels with initial TPH% of 7.3% and 7.24% respectively. Batch C was applied with unknown reference culture of OTBL whereas batch D was treated with developed indigenous mixed culture. The degradation of crude oil contamination for these two batches was estimated in the span of every 15 days simultaneously. The concentration of TPH in batch C reduced to 2.3% and in batch D 1.7% from their initial TPH concentration level. This shows the percentage degradation achieved for batch C and D was 68.49% and 76.52% in 75 days respectively as shown by graph in figure 6(c) and 6(e). The slope from figure 6(f) determines first order reaction with 0.0142 day^{-1} *k* for batch C whereas, the graphical representation of kinetics from figure 6(h) gives first order with rate constant *k* as 0.021 day^{-1} for batch D.


TPH % concentration
y = -0.0451x + 12.218
R² = 0.9366
Intervals in days

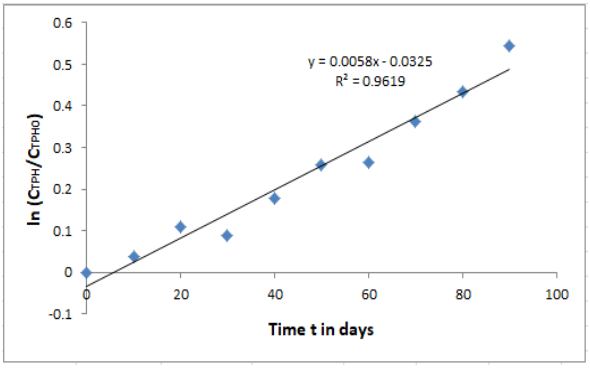
(a)


ln (CTPH/CTPHO)
y = 0.0049x - 0.0704
R² = 0.9
Time t in days

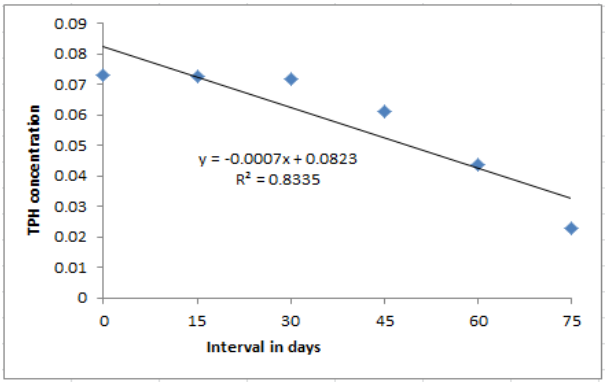
(b)


TPH % concentration
y = -0.0005x + 0.1184
R² = 0.9781
Intervals in days

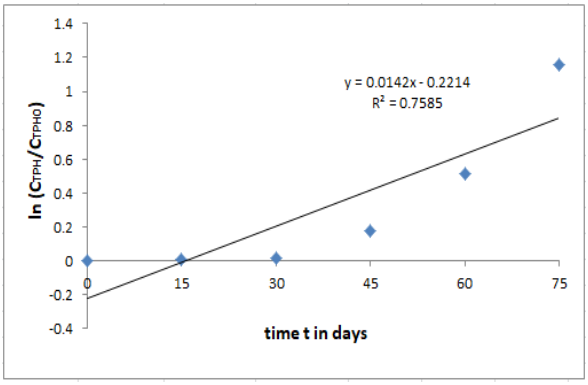
(c)


ln (CTPH/CTPHO)
y = 0.0058x - 0.0325
R² = 0.9619
Time t in days

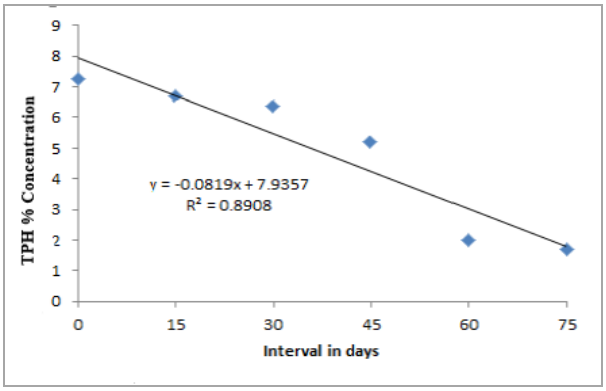
(d)


TPH concentration
y = -0.0007x + 0.0823
R² = 0.8335
Interval in days

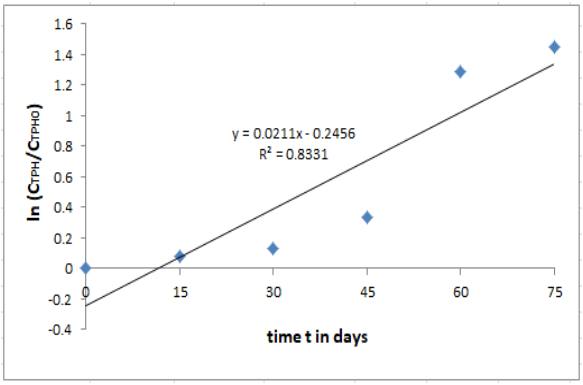
(e)


ln (CTPH/CTPHO)
y = 0.0142x - 0.2214
R² = 0.7585
time t in days

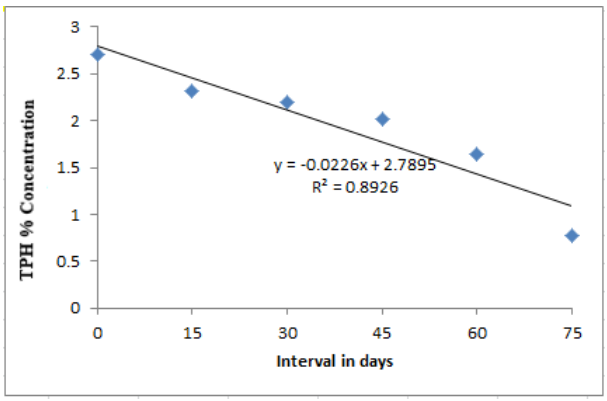
(f)


TPH % Concentration
y = -0.0819x + 7.9357
R² = 0.8908
Interval in days

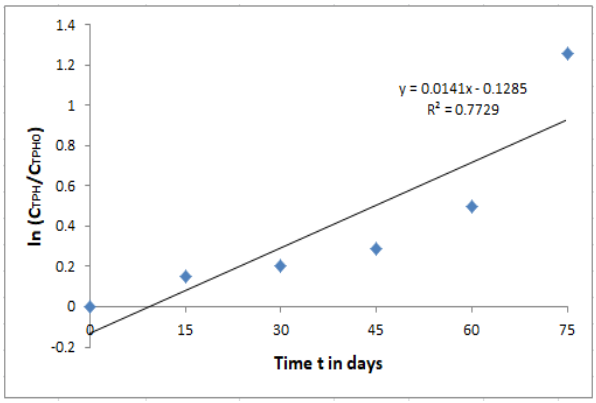
(g)


ln (CTPH/CTPHO)
y = 0.0211x - 0.2456
R² = 0.8331
time t in days

(h)



(i)



(j)

Figure 6. (Left) TPH% reduction over time for all batches; (Right) Degradation kinetics for all batches; (a) Graph showing TPH% degradation in time t days for Batch A; (b) Graph showing degradation kinetics for Batch A; (c) Graph showing TPH% degradation in time t days for Batch B; (d) Graph showing degradation kinetics for Batch B; (e) Graph showing TPH% degradation in time t days for Batch C; (f) Graph showing degradation kinetics for Batch C; (g) Graph showing TPH% degradation in time t days for Batch D; (h) Graph showing degradation kinetics for Batch D; (i) Graph showing TPH% degradation in time t days for Batch E; (j) Graph showing degradation kinetics for Batch E.

An increase in rate of degradation in batch D compared to batch A with same indigenous mixed culture derived from graphical representation could be explained as the possibility of interaction with beforehand available microbial population from the fresh soil that was mixed with the contaminated soil sample. The initial contamination level for batch E was 2.7% which was lower than other batches, as the ratio of fresh soil to contaminated soil varied by 80:20. The reduction in TPH% was measured as 1.93% from initial percentage which gives 71.48% degradation (refer figure 6(i)) in total 75 days. The degradation rate in batch E was faster compared to other batches due to very low level of initial contamination. Hence, this also proves for the effectiveness of the developed indigenous consortium at varying levels of contamination. The representation of linear relationship from figure 6(j) for first order kinetics of batch E with rate constant 0.014 day^{-1} *k*. Based on first order kinetics, derived for all batches reveals that developed indigenous mixed culture was effective in degrading contaminant at linear steady state (Admon et al., 2001). There was slight difference in degradation level between reference culture of OTBL and indigenous mixed culture for batch A. Reductions in TPH concentration for batch A 3.69% whereas for batch B was 4.91% from their initial concentration of 11.7% in 90 days. In case of batch C, the reduction in TPH was 5% and batch D, it was 5.54% comparatively. The degradation% observed with varied levels of contamination shows that degradation varies with time for different levels of contamination. Bioremediation is an environment friendly and cost-effective method for soil clean-up (Azubuike et al., 2016; Sharma, 2012). As there are no toxic chemicals involved in this technology, the after bioremediation effects will be nutrient rich, fertile soil.

IV. Conclusion

The provided crude oil contaminated soil was analysed for its physical and chemical characteristics. Oxidizing tendency from ORP signifies for good agricultural soil that can hold sustainable microbial life. The change in pH was observed from 7.5 to 9.0 after bioremediation. The observation from area of clearance zone manifests the ability of indigenous mixed culture to undergo decomposition of crude oil; also 47 unidentified isolates in visible red coloured colonies occupying the majority of population gave wide zone of clearance. Growth of fungi was also observed to undergo crude oil degradation which reveals potential possibility for mycoremediation. The diauxic pattern of growth observed in indigenous mixed culture validates the use of Verlhurst growth model during initial stages of development giving specific growth constant μ as 0.086 and μ_{max} as 2.41. The degradation of batch A and B has achieved about 44.44% and 41.97% in 110 days and 90 days respectively. The degradation of crude oil concentration in batch C, D and E was estimated as 68.5%, 76.52% and 71.48% in 75 days respectively. All five batches obey first order kinetics of oil degradation with rate constant *k* ranging from 0.005 day^{-1} to 0.021 day^{-1}. With reference to unknown culture provided by OTBL, the indigenous mixed culture comply degradation much satisfactorily.

V. Acknowledgement

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VI. Declarations of Interest

- Ethics approval and consent to participate: Not applicable.
- Consent for publication: Not applicable.
- Availability of data and materials: Not Applicable.
- Competing Interests: No competing interests.
- Funding: Not applicable.

VII. List of Abbreviations

CFU – Colony forming Units

MSM – Mineral Salt Medium

ONGC – Oil and Natural Gas Corporation

ORP – Oxidation-Reduction Potential

OTBL – ONGC TERI Biotech Ltd.

PAH – Poly Aromatic Hydrocarbon

SPDC – Shell Petroleum Development Company

TERI – The Energy and Resources Institute

TPH – Total Petroleum Hydrocarbon

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