Exploiting bacterial isolates for diesel degrading potential under *in vitro* conditions

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Research Article

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Abstract

Hydrocarbon contaminated oil-spilled areas and oil-products have caused serious harm with increasing attention for development, implementation and removal of these contaminants. Bacterial diversity on succession at the petroleum hydrocarbon contaminated environment can give answer the problem. Such lands have serious problems as totally barren or with rare plantation. Bacteria can thereby be exploited for the mitigation of hydrocarbon to enhance the nutrient availability for vegetation. Present study involves collection of soil samples heavily-contaminated with hydrocarbon from Bagru, Rajasthan. Samples were analysed by solid liquid extraction method followed by FTIR and HPLC analysis. During microbiological analysis hydrocarbon degrading bacteria were screened. FTIR spectral analysis indicated the presence of the functional groups alkanes and aromatic ringed compounds; 43–69% hydrocarbon content recorded by HPLC analysis of all the soil samples respectively. From the soil samples six gram-positive and four gram-negative bacterial isolates were explored possessing hydrocarbon degrading capacities in the range 47.04–87.31% and 10.12–95.24% respectively. Growth kinetic studies revealed the degradation upto 1000 ppm diesel in 3 days under in vitro conditions. These bacteria can further be exploited for diesel degradation and will certainly propose a possible solution to the prevailing issue for its biodegradation in ex situ conditions after upscaling.

Highlights

- The article provides a comprehensive account of diesel degrading bacterial isolates under in-vitro conditions.
- It aims to correlate the diesel stress and their degrading capabilities by screened bacteria.
- Paper highlights the analysis of the degradative profile by HPLC and FTIR spectral scan to confirm the efficacy of bacteria under diesel stressed condition.

Introduction

The sustainability of microbial population globally is primarily because of plantation prevailing in that area. Different factors determine the type of vegetation in any area as soil-profile is the most significant one. During past few decades, oil spillage and seepage of hydrocarbons in soil and water bodies affect the food chain along with adversely affecting the soil-quality through reduced nutrients uptake along with water-retention capabilities. But various disasters such as petroleum oil-spillage, burns, accidental spillage, etc. create a have that disrupts the entire agricultural-belt around the area. Nowadays, globally people and ecologists are facing with the challenge of overcoming the effects of contaminated water; soil etc. Joshi and Walia (1996). Hydrocarbons are the most commonly used primary and fuel of energy resources that can be either organic and inorganic forms in all the strata of nature. Petro-carbon based energy-products has been elevated day by day resulting in soil-water pollution Kamble et al. (2011). Petro-based products are naturally composed of hydrocarbon and non-hydrocarbon complex compounds which are very harmful to the organism, environment impacting flora and fauna. To protect from existing

impurities of oil spill sites, there is a need to support improvement in contaminant reduction technologies Smith and Hansch (1973). Contaminated soil by petro-carbons regions and their derivatives are severe problem across the ecosphere. Out of the different methods the bio-augmentation and reclamation methods involving microbes into the polluted soil and water are extensively used for clean-up of contaminated sites Chang et al. (2011). These compounds also constitute a wide range of toxic chemical as many of them are carcinogens cause hazard to human health. Microbes that degrade the components of hydrocarbons are isolated from different areas, mostly from polluted sites Adebuseye et al. (2007). Contaminated soil with hydro-carbons causes wide harm of limited system, as the buildup of contaminants in plant tissue and organisms may well cause mutations or death in different event life forms Alvarez and Vogel (1991). Normally, saturated hydrocarbons are straight-chain and are most susceptible to degradation processes, whereas alkanes branched chains are less susceptible to bacterial occurrence. Aromatic compound is more problematic to degrade the susceptibility increases as the decreases number of alicyclic or aromatic compounds rings Das and Chandran (2011). Bioremediation processes are employs microbes that are proficient in oil-degrading toxic contaminants that can further be explored for the reclamation of oil spill sites Bragg (1994). The (Polycyclic aromatic hydrocarbons) PAHs and dispersed biological carcinogenic pollutants from commonly removal of many persistent forms pollutants from soil and water by their phyto-remediation metabolism Muratova (2003); Mukherji et al. (2002). To better understand the plant behavior i.e: physiological, microbiological, biochemical and molecular responses to PAHs in vegetation is the need of present scenario. Present study aims to isolate and screen the bacterial isolates possessing hydrocarbon degrading potential for the same four soil sample heavily contaminated with hydrocarbon was used and these samples were characterized using FTIR and HPLC analysis. Primary and secondary screening of microbial isolates possessing degradative potentials were focussed to be explored for the reclamation of vegetation in oil contaminated site respectively.

Materials And Methods

Sample Source for the Study Plant

Sample collection: Four soil-samples (n = 4) were collected from motor garage and automobile repair shops in from Bagru region of western Rajasthan (district is located between 26°49'0"North and 75°33'0" East) as sample S12-Black; S13-Brown; S14-Brown; S15-Black were collected for the study conduct from soil heavily contaminated with hydrocarbons selected as sampling site (Fig. 1a). Samples were collected in a sterile container and were carried to the laboratory in icebox. All collected samples were air dried in laboratory followed by sieving through 2mm mesh size grid and store at 4°C. Hydrocarbon degradation process is a cost-effective technology among the in-situ technologies and extraction has gained efficacy and popularity for volatile and semi-volatile removal of organic compound (Azubuike et al. 2016). Bacterial isolates were screened from these samples capable of degrading hydrocarbon. Physiological characterization of soil samples was performed *i.e*; sub-angular blocky, granular and crump, prismatic columnar and platy form Lin and Ng (1997) method.

Solid Liquid Extraction of the Soil Samples for Determining Hydrocarbon Load of the Samples

Isolation of hydrocarbon from soil samples for determining hydrocarbon load was executed by solid-liquid extraction method Dixit et al. (2018); Panda et al. (2013); Patil et al. (2012); Bisnoi et al. (2009); Marquez-Rocha et al. (2001); Chang (1998).

10 g of soil was mixed with 20 ml of carbon tetrachloride (CCl_4) and the solution was placed in a separating funnel was shaken vigorously for 5–10 min and allowed to settle hold for overnight. The liquid was separated out and the moisture was removed by filtering through whatman no. 1 filter paper already coated with anhydrous sodium sulphate used to remove extra moisture and precipitation. The liquid phase was collected in 50 ml conical flask and used for further analysis of hydrocarbon degradation Patil et al. (2012).

FTIR Analysis

Fourier Transform Infrared [FTIR] Spectrophotometer (ALPHA-T) spectra through (Shimadzu Bruker IR solution) revealed a spectrum in the mid range IR region of $400\text{--}4000\text{ cm}^{-1}$ with 16 scan speed of the solid-liquid extract of the soil sample indicating the presence of the functional group with respect to control. The 1:1 ratio of Carbon tetrachloride (CCl_4) and Hydrocarbon (Diesel) serves as the reference (Fig. 1b). IR spectroscopy analysis result explains the variations in physical as well as chemical properties and also indicates the presence of different peaks to identify the functional group along with bond strength of hydrocarbon in the soil samples Dixit et al. (2018); Patil et al. (2012).

HPLC Analysis

The samples were further subject to High-performance liquid chromatography [HPLC] (Shimadzu LC-10 ATVP Binary Gradient System) analysis of hydrocarbon peaks was carried out by using C-18um 250 mm 4.6 mm and flow rate was set 1 mL min., solvent ratio of (methanol: water) (45:55) at ambient temperature. hydrocarbon load was elucidated with respect to the control respectively [Carbon tetrachloride (CCl_4): Hydrocarbon (Diesel) in 1:1 ratio serves as positive control]. For HPLC analysis, hydrocarbon extracted was considered as samples. Mobile phase (Samples aliquots) were dissolved in methanol: water solvent (45:55) system at 25°C specific temperatures. For separation C18 column detector with $5\mu\text{m} \times 250\text{ mm} \times 4.6\text{ mm}$ was used with $2.5\text{ }\mu\text{L}$ of injection volume. The sample exhibits shift in retention time along with peak area depicting the fraction of hydrocarbon within the soil sample with respect to their control Patil et al. (2012).

Microbiological Characterization of Hydrocarbon Degrading Microbes

Enumeration of bacterial population was performed by plating 0.1 ml of serially-diluted soil sample on nutrient agar. Diesel degrading bacterial strains were screened by streaking them over Bushnell Haas (BH) Agar supplemented with 1% (v/v) filter sterilized diesel as carbon source using the standard methods Panda et al. (2013); Marquez-Rocha et al. (2001); Chang (1998). The soil samples after physical and

analytical characterization were subjected to the microbiological analysis. In order to find out the microbial isolates possessing hydrocarbon degrading capabilities Dixit et al. (2018). Primary and secondary screenings Ganesh and Lin (2009); Marquez-Rocha et al. (2001); Chang (1998) of hydrocarbon degrading microbes were performed. Soil suspension was made by mixing with 1g soil in 10 ml Bushnell and Hass Broth (BH broth) conditioned with 1% (v/v) filter-sterilized diesel as the sole source of carbon.

Primary and Secondary Screening (Liquid-Liquid Extraction)

During the primary screening, after growing in suspension culture the culture was streaked on the petri plates to obtain the only hydrocarbon degrading microbes. The soil suspension was inoculated in the BH broth supplemented with 1% filter sterilized diesel and was incubated on orbital shaker incubator for 24 hours. Afterwards, the bacterial population was further streaked on chromogenic media as Mannitol salt agar with phenol red and sodium azide for gram-positive bacteria and for gram-negative bacteria MacConkey agar media containing crystal violet for selective enrichment was used. After 48 hours of incubation of pure single isolates, colonies were subjected to microbiological identification (cultural, microscopic and biochemical characterization) of all the gram positive and gram negative isolates. All the identified microbial isolates were transferred to agar slants and glycerol stocks for further use Dixit et al. (2018); Panda et al. (2013); Kamble et al. (2011); Chang (1998). Primary screening, soil suspension was inoculated in Bushnell Hass broth supplemented with 1% filter sterilized diesel incubated on orbital shaker incubator for 24 hours. Secondary screening was performed by Liquid-liquid extraction or the gravitational analysis method by Ganesh and Lin (2009) to evaluate percent hydrocarbon degradation potential of the bacterial isolates with respect to the baseline control (set as on 0 Day). For extraction of hydrocarbon, 20 ml of culture broth (BH Broth) was mixed with 20 ml petroleum ether: acetone (1:1) solvents in a gravimetric funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, resulted in three layers appeared. Top layer was a mixture of (oil, petroleum ether and acetone), (clumping cells) make a middle layer and the bottom aqueous layer contains (acetone, water and biosurfactant in soluble form). The lower two layers were spread out while upper top layer containing petroleum ether mixed with hydrocarbon and acetone was taken in a preweighed clean beaker. The extracted oil was passed through anhydrous sodium sulphate (1%) to remove all moisture. The petroleum ether and acetone was evaporated on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tarred beaker. The percentage of hydrocarbon degraded was calculated as -

Weight of Residual Hydrocarbon Degradation = (Weight of beaker containing extracted samples – Weight of empty beaker)

Amount of hydrocarbon degraded = Weight of hydrocarbon added – Weight of residual hydrocarbon

% degradation of hydrocarbon = Amount of hydrocarbon degraded media x 100

Hydrocarbon degradation was determined on 3, 6 and 9 days of incubation period were conducted in 3 sets (triplicates) and data-set were examined respectively.

In vitro-optimization of bacteria was performed as growth kinetics vs hydrocarbon degradation with respect to control up to 72 hours of incubation period using UV visible spectrophotometric analysis [Elico BL 198 Bio spectrophotometer] by taking the absorbance at 600nm. The media was supplemented with 100ppm, 300ppm, 500ppm and 1000ppm concentration of filter sterilized diesel in BH broth.

Statistical Analysis

All the experiments were performed in triplicates and the experiments were repeated three times to detect the reproducibility of results. The results are presented as means $\pm$ SD, $n = 3$ from an independent experiment. A one-way ANOVA using SPSS software between the means of 2 groups were evaluated and p -value < 0.05 were considered as the analysis of significant statistically.

Results And Discussion

The present study involves the collection of soil samples heavily contaminated with hydrocarbon followed by its characterization physiologically and analytically by FTIR and HPLC analysis. Another study reported that the micro-organisms are primary degraders of petroleum-hydrocarbons within a contaminated-ecosystem Dixit et al. (2018); Leahy and Colwell (1991). Enrichments were conducted by a research group to screen isolates using crude-oil as sole carbon along with energy source for different micro-organisms from contaminated soil. Similar study focusses on the screening of bacteria possessing hydrocarbon degrading potential Jirasripongpun (2002). In a report the research focus on collection of agricultural soil irrigated with petroleum-refinery effluent, fifteen species of bacteria were identified having hydrocarbon-degrading capabilities Bhadauria (1997). Another study suggested that the use of pure cultures that provides practical advantage over the use of bacterial consortia or diversity by erradicating the ambiguity associated with constantly varying microbial diversification in case of community exploitation Ghazali (2004). Bio-degradation of hydrocarbons through natural microflora allows for the bio-conversion of hazardous compounds into relatively less or non-toxic forms that indicates as one of the primary-mechanisms through which petroleum, diesel and petro-based products were mitigated from the eco-environment cost-effectively Mukherji et al. (2002); Lidderdale (1993); Leahy and Colwell (1990); Floodgate (1984); Atlas et al. (1981). Most of bioremediation studies suggested using pure culture of the bacteria in a natural environment that remains unknown substantially Harayama (1999).

Physiological Characterization of Soil Samples

The study indicated sample S12 sample as sub-angular blocky, sample S13 as granular and crump, sample S14 as prismatic columnar and sample S15 as platy form (Fig. 1a). A similar report is suggested by a study as the soil-samples were collected from the garage because the capacity of the native bacterial population is to mineralize crude-oil hydrocarbon in oil-contaminated sites that was earlier confirmed by many researchers Mukherji et al. (2002). Oil-products not only leads to modification of

physicochemical properties but also affect the biological profile of the soil by contributing to the limitation of the productive-capabilities of crop-plantation Wyszokowska (2002).

Solid Liquid Extraction of the Soil Samples for Determining Hydrocarbon Load of the Samples

The soil samples were subjected to solid liquid extraction (Fig. 1b), the liquid fraction was filter sterilized with syringe filters further analysed through FTIR and HPLC analysis to evaluate the hydrocarbon functional-groups and load in the respective samples. Another study suggested that the cumulative amount of petroleum-hydrocarbons were indicated as TPH (Total Petroleum Hydrocarbons). Various analytical techniques such as gravimetric analysis and chromatography as gas chromatography (GC) exploited to determine TPH in soil and water. None of these techniques measures the entire-range of petroleum hydrocarbons. Whereas only the subsets of hydrocarbons are evaluated by different technologies depending on the extraction with respect to analytical-methods exploited Scragg (2005); Balba et al. (1998). Generally, hydrocarbons were ranked in the gradation of reducing in the susceptibility to bio-degradation in the order as n-alkanes followed by branched-alkanes > low molecular-weight aromatics > cyclic-alkanes. Whereas, the molecules with higher molecular-weight aromatics as well as polar-compounds being extremely recalcitrant. However, the composition of microbial population along with the abiotic-factors prevailing in an area also affects the order of biodegradation greatly Dixit et al. (2018); Chang et al. (2011); Atlas and Bragg (2009).

FTIR Analysis

Study conduct revealed a spectrum of the solid-liquid extract of the soil samples through gravitational apparatus with respect to the positive control (Fig. 1a,b). In FTIR spectroscopy analysis result explains the variations in physio-chemical properties of the soil samples. The FTIR analysis the characteristics peak was observed for the band presence of 2987, 2883, 1541, 479 at indicating of alkanes and aromatic rings compounds with medium weak multiple bonds indicating C-H and C-C bond stretch in all the samples. The characteristic peak was observed for alkanes and aromatic compounds in the sample aliquots in sample S12, S13, S14, S15 showed fingerprint pattern. Straight chain alkanes are easier to degrade than aromatic compounds whereas nitro compounds (N-O) were only present in blank sample that indicates the presence of amines, alkanes along with aromatic ring compounds (Fig. 2). A study reported that the diesel oil act as a medium distillate of petroleum possessing different proportions of branched-alkanes, n- alkanes, olefins with low concentration of aromatic polycyclic components Dixit et al. (2018); Baker and Herson (1999). According to the analysis, the absence of characteristic peaks in the region of 1610–2042 in extracted samples indicated absence of nitro compound linkage.

HPLC Analysis

In HPLC analysis, exhibits shift in retention-time and peak-area indicating the quantity of hydrocarbon with in the soil sample and indicated the presence of hydrocarbon content with respect to their control. HPLC analysis showed presence of more peaks analyzed in control and less peaks in manner form were observed in extracted samples. Many components in sample showed shift retention time and peaks area

indicating changes in molecular weight and quantity of respective component present in control. During the HPLC analysis of the solid-liquid extract of the soil samples indicated the retention factor of the control as 0.97284. The samples exhibited a variable range of percent hydrocarbon in the range 43% in the sample S-12 to 69% in the sample S14 among all the collected soil samples respectively (Fig. 3). All the four samples were heavily contaminated with hydrocarbon with common functional groups and will serve as an excellent source for screening of bacterial isolates with potent hydrocarbon degrading capabilities. Similar reports were indicated by a research as, these analysis indicate that as these new techniques are viable at commercial practice for evaluating bioremediation. It will help to create a better cleaner environment. In another study report on biodegradation of diesel-oil in oil-polluted soil exhibited the presence of wide diversity of microbes. Hydrocarbon-degrading potential of fungi as *Aspergillus* sp., *Trichoderma* sp., *Penicillium* sp. and bacteria as *Acinetobacter* sp., *Bacillus* sp., *Micrococci* sp., *Pseudomonas* sp. *Streptomyces* sp. indicated hydrocarbon-degrading consortium of microbes. Evaluation of extracted diesel-oil was done using FTIR, HPLC, GCMS and ¹H NMR techniques. This also confirms the bio-degradation of diesel-oil validating the water soluble derivatives of naphthalene as major-pollutants Patil et al. (2012). The similar results was also reported as *Pseudomonas* sp., exhibited best growth along with high metabolic activity in broth culture and solid medium in presence of a wide range of crude-oil concentration Dixit et al. (2018); Shrivastava and Gupta (2008).

Microbiological Characterization of Hydrocarbon Degrading Microbes

A report suggests that the inoculation with the consortia does not exhibit a higher degradation potential in comparison to the individual microbial isolate. The studies strongly suggest that environmental conditions of the contaminated sites play an integral role in the diesel-degrader at hydrocarbon polluted site Singh and Lin (2008). In nature, bio-degradation of complex hydrocarbons needs the co-operation of more than one species detected in petroleum-contaminated soil Kim and Crowley (2007); Da Cunha et al. (2006). A study strongly demonstrates that each strain has their specific mechanism in the hydrocarbon transformation process Cunliffe and Kertesz (2006); Scragg (2005); Ghazali et al. (2004); Balba et al. (1998). Microbes possess enzyme-systems to degrade as well as utilize diesel-oil as a source of carbon and energy Ezeji et al. (2005); Ljah et al. (1998); Antai and Mgbomo (1993). Bio-stimulation is regarded as a utmost optimum remediation technique for the diesel removal in soil and it requires intrinsic degradation capacities of microflora along with the environmental parameters in the kinetics of the *in situ* procedure Molina-Barahona et al. (2004). Study documented the capabilities of microbes to use hydrocarbons in oil-contaminated environments Obuekwe et al. (2009); Atlas (1981); Atlas and Bartha (1972). Microbial degradation process aids to the removal of spilled oil from the environment after critical mitigation of large volume of oil through physiochemical methodology Dixit et al. (2018); Ljah and Okang (1993).

During the present study, in primary screening the isolates able to grow on BH agar were considered as hydrocarbon degraders and further subjected to secondary screening to evaluate the percent hydrocarbon degradation after 3, 6 and 9 days of incubation. Liquid-liquid extraction was performed to determine the hydrocarbon degrading capabilities. Six gram positive and four gram negative bacterial isolates were

explored possessing hydrocarbon degrading capacities as by gram positive (47.04–87.31%) and gram negative (10.12–95.24%) isolates (Table 1, Fig. 4,5). The degradative potential from 50–100% were kept as an inclusion criterion and was subjected for further studies but below 50% were kept in the exclusive criteria for further studies. The hydrocarbon degradation was found to be increasing with the days of incubation and 9-day incubation setup exhibit maximum degradation. The statistical analysis of revealed the P-value for gram-positive isolates (3.1×10^{-08}) and gram-negative isolates (0.060). Therefore, the data was found to be significant on performing one-way ANOVA with respect to the untreated control group. In another study, four potentials bacterial isolates were analysed by gravimetric analysis for estimating the biodegrading capabilities of hydrocarbon degraders. Another study suggests, *Bacillus cereus* degrade the maximum amount of BTX (Benzene, Toluene, Xylene) as Benzene was maximally degraded followed by Toluene by a Gram-positive bacteria Jayanthi and Hemashenpagam (2015). Another study indicated, microbes able of surviving on highly reduced organic components possess mechanisms to degrade the crude-oil components using it as source of carbon as well as nitrogen with different pattern of degradation by various enzymes Penet et al. (2006). A report indicated that of seventy three aerobic bacteria were able to degraded petroleum- hydrocarbon Chosson et al. (1991). The crude-oil fraction with less amount of saturated-hydrocarbons exhibit more resistance to microbial-degradation in comparison to the crude-oil fraction with more amount of saturated-hydrocarbons Amund and Akaqngbou (1993). Crude-oil blend contain 69.74% saturated-hydrocarbon, 22.05% aromatics, 5.65% residues and 2.56% asphaltenes account for the different rate of degradation of the oil. In south India, Subathra et al. (2013); Ghazali et al. (2004) also reported the crude oil degradation by *Bacillus* sp. and *Pseudomonas* sp. Atlas (1981); Leahy and Colwell (1990) from oil contaminated area by gravimetric analysis for determinate the degrading capacity by anaerobic degradation as well as aerobic environments Floodgate (1984). Mixed hydrocarbons were not degraded better Lidderdale (1993) whereas, the Caetano et al. (2018) has reported that the presence of benzene inhibited toluene, xylene degradation. Different aromatic compounds released into the environment via various human-activities metabolized by soil-bacteria and is the foundation for cleanup technologies for environment Dixit et al. (2018); Watanabe et al. (1996); Haryama et al. (1999). Elaborative studies are needed to clarify the taxonomic position of the microflora for their biodegradation potential for other PAH's (polycyclic aromatic hydrocarbons) Braddock et al. (1997). Bio-remediation process significantly affected by the presence of microbes in oil–water–soil multiphase environmental scenarios] along with the environmental factors as temperature, pH, nutrients and availability of electron-acceptor Mukherji and Vijay (2002). Environmental microbes with the potential to degrade crude-oil are ubiquitously distributed in soil as well as marine environments Venkateswaran and Haryama (1995).

Bacterial Growth Kinetic Study

Three gram negative (222,221,223) and two gram positive (122,117) bacteria were further taken for growth kinetic studies. It revealed that they can degrade the hydrocarbon content beyond 1000 ppm in a span of 3 days under *in vitro* conditions. Isolate no. 222 performed best followed by 221, 223 in gram negative isolates, on the other hand isolate no. 117 perform better than 122 among gram positive

isolates. These bacteria can further be exploited for the re-establishment of vegetation in the area heavily polluted with oil spillage or hydrocarbon contamination (Fig. 6). In another growth kinetic study with *Pseudomonas* sp. having high growth as compared than other test organisms and reported *Bacillus cereus* as most tolerant to withstand high levels of hydrocarbon in the soil due to the presence of endospore Annweiler et al. (2000). A study demonstrated that the bacterial consortium (BC) of *Acinetobacter*, *Flavobacterium*, *Pseudomonas* and *Serratia* as hydrocarbon-utilizers De and Gupta (2016); Atlas (1981). Samples were incubated in orbital shaking incubator at 37°C at 125rpm up to 15 days and the amount of petroleum-hydrocarbon degradation was estimated by gravimetric analysis at each 5 days interval. Gram negative bacillus bacteria was screened as *Pseudomonas* sp. with maximum 49.93% of diesel-oil degradation after 20 days incubation in 0.5% diesel-supplemented BHMS Panda et al. (2013) and by *Bacillus* as 30–40% Ijah et al. (1998). Another study reported that the *Bacillus* spp. are most tolerant in high hydrocarbon concentration in soil because of resistant endospores and could be effective in clearing of the oil-spills Adebuseye et al. (2007). In a study, pyrene was degraded with *P. putida* (upto 97.40%) and with *P. paucimobilis* (95.5%) after 42 days of incubation under optimum set of conditions, whereas, in un-optimized condition it was found to be 65.89%; 57.81% of phenanthrene and 59.80%; 52.07% of pyrene respectively Bisnoi et al. (2009). Some studies reported, that the inoculation had positive/marginal/no effects on rate of oil biodegradation. Whereas, various factors must be taken into consideration before *in situ* bio-remediation, it includes nature as well as concentration of contaminated oil in the prevalent climate condition such as sources of environment contamination and the nutrient content of soil-water along with pH of the contaminated-site Dixit et al. (2015); Rosenberg (1992). Furthermore research can be directed for exploring the participation of individual microbe in impacting the effectiveness of a microorganism association along with optimal degradation conditions under *in situ* conditions respectively De and Gupta (2016).

Table 1
Secondary screening of hydrocarbon degradation percent by bacterial isolates after 3, 6 and 9 days

	Accession No.	3 DAY	6 DAY	9 DAY
Gram positive bacteria	116	9.812 ± 0.077	46.753 ± 0.031	63.780 ± 0.027
	117	33.044 ± 0.041	72.582 ± 0.011*	86.1471 ± 0.016*
	119	37.085 ± 0.050	61.183 ± 0.018	71.861 ± 0.002
	121	39.249 ± 0.034	51.515 ± 0.006	80.086 ± 0.034
	122	63.636 ± 0.062*	77.633 ± 0.034*	87.301 ± 0.017*
	123	66.811 ± 0.022*	72.294 ± 0.007*	80.086 ± 0.008
Gram negative bacteria	220	57.589 ± 0.068	73.214 ± 0.028*	81.845 ± 0.006
	221	53.571 ± 0.065	72.470 ± 0.060*	94.196 ± 0.002*
	222	64.434 ± 0.057*	89.881 ± 0.013*	93.154 ± 0.006*
	223	60.267 ± 0.052*	82.738 ± 0.031*	89.881 ± 0.020*
* P < 0.05 as compared to control				

Conclusion

Microbes screened from the oil contaminated soil degraded the diesel and petro-based products under *in-vitro* condition. Bacterial populations were evaluated for the biodegradation possessing capabilities to utilize hydrocarbons as a sole source of carbon as well as energy. Bacterial isolates might be applied as for future reclamation and bioremediation processes in polluted soil-sites. Potentially isolates were identified the experimental design used as a definite tool for hydrocarbon degradation. In secondary screening (solid-liquid extraction), FTIR analysis of extracted samples showed absence of nitro compound linkage (N-O) as compared to hydrocarbon (Control group) whereas, in HPLC showed the various quantification individual component of the mixture for the determination of hydrocarbon load. Liquid-liquid extraction for relatively fast growing hydrocarbon degrading microbes as 6 gram positive (63.780 ± 0.027 to 87.301 ± 0.017) and 4 gram negative (81.845 ± 0.006 to 94.196 ± 0.002) isolates possessing hydrocarbon degrading capacities. Contaminated soils were analyzed for the presence of hydrocarbon pollutants. Result showed change in physical and chemical properties of soil. Microbial analysis of soil indicates presence of bacteria. This study will certainly give a possible solution to the prevailing issue and helps in the reconstruction of nutrient pool in those sites. The identification and understanding the role of isolates in influencing the effectiveness of *in situ* degradation process.

Declarations

Author Contributions This work was carried out in collaboration between both authors. Author SG provided guidance during the study conduct, designed the study, performed the statistical analysis and standardized the protocol. Author KT wrote the first draft of the manuscript, managed the analyses of the study through managing the literature searches. Both authors read and approved the final manuscript.

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Availability of data and materials Not applicable.

Ethics approval and consent to participate Not applicable.

Consent to publication The authors hereby submit the consent for the publication of the manuscript.

Completing interest The author declare that they have no completing interests.

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Figures

Figure 1

Photograph showing contaminated soil samples (bagaru); (a) Soil samples collection; (b) Solid-liquid extraction.

Figure 2

FTIR spectra of the soil samples after solid liquid extraction with respect to their (a) control, (b) sample 12, (c) sample 13, (d) sample 14, (e) sample 15

Figure 3

HPLC spectra of the soil samples after solid-liquid extraction with respect to their (a) control, (b) sample 12, (c) sample 13, (d) sample 14, (e) sample 15

Figure 4

Showing Liquid-liquid extraction (A) Determination of hydrocarbon degradation 3, 6 and 9 days experiment (B) Gravimetric analysis (gram positive and negative).

Secondary Screening of Hydrocarbon degradation from Soil

Figure 5

Liquid-liquid extraction, secondary screening of hydrocarbon degradation from soil samples for determining hydrocarbon load (gram positive and negative)

Figure 6

Figure showing in vitro-optimization hydrocarbon effect on growth kinetics vs degradation (A. 222, B.221, C.223, D.122, E.117)