PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol 9(3): 574–583
https://doi.org/10.14719/pst.1503

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

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

Kanchan Taskar & Sarika Gupta*

Department of Bioscience and Biotechnology, Banasthali Vidyapith, Banasthali, Tonk-304 022, India

*Email: sarika.ashish@gmail.com

OPEN ACCESS

ARTICLE HISTORY

Received: 23 September 2021
Accepted: 23 March 2022

Available online
Version 1.0: 01 May 2022
Version 2.0: 01 July 2022

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, etc. See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Taskar K, Gupta S. Exploiting bacterial isolates for diesel degrading potential under *in vitro* conditions. Plant Science Today. 2022; 9(3): 574–583. https://doi.org/10.14719/pst.1503

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 (Fourier Transform Infrared) and HPLC (High Performance Liquid Chromatography) analysis. During microbiological analysis hydrocarbon degrading bacteria were screened. FTIR spectral analysis indicated the presence of the functional group's alkanes and aromatic ringed compounds; 43% to 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 up to 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 up scaling.

Keywords

oil spillage, hydrocarbon degradation, FTIR analysis, HPLC analysis

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. (1). Hydrocarbons are the most used primary and fuel of energy resources that can be either organic or inorganic forms in all the strata of nature. Petro-carbon

based energy-products have been elevated day by day resulting in soil-water pollution (2). 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 (3).

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 (4). 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 (5). Contaminated soil with hydrocarbons causes wide harm of limited system, as the build-up of contaminants in plant tissue and organisms may well cause mutations or death in different event life forms (6). 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 (7). 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 (8). The Polycyclic aromatic hydrocarbons (PAHs) and dispersed biological carcinogenic pollutants from commonly removal of many persistent form pollutants from soil and water by their phyto-remediation metabolism (9, 10). To better understand the plant behaviour 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

Four soil-samples (n=4) were collected from motor garage and automobile repair workshop in from Bagru region of western Rajasthan (district is located between 26°49'0''North and75°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 2 mm 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 (11). 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 method (12, 13).

Fig. 1. Photograph showing contaminated soil samples (bagru); **(a)** Soil samples collection; **(b)** Solid-liquid extraction.

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 (14, 15). Ten gram 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 (6, 8, 15).

Fourier Transform Infrared [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 400-4000 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 **s**pectroscopy analysis result explains the variations in physical as well as chemical properties and indicates the presence of different peaks to identify the functional group along with bond strength of hydrocarbon in the soil samples (6, 13, 16, 17).

High-performance liquid chromatography [HPLC] Analysis

The samples were further subject to High-performance liquid chromatography [HPLC] (Shimadzu LC-10 ATVP Binary Gradient System) analysis of hydrocarbon peaks were carried out by using C-18 um 250 mm 4.6 mm and flow rate was se 1 ml min., solvent ratio of (methanol: water) (45:55) at ambient temperature. Hydrocarbon load was elucidated with respect to the control respectively [Carbon tetra chloride (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) was dissolved in methanol: water solvent (45:55) system at 25 ℃ specific temperatures. For separation C18 column detector with 5 µm × 250 mm × 4.6 mm was used with 2.5 µl 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 (8, 15).

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 (14, 17, 18). 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 (13, 19). Primary and secondary screenings (17, 18, 20) of hydrocarbon degrading microbes were performed. Soil suspension was made by mixing with 1 g 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 hrs. 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 hrs 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 (2, 13, 14, 18). Primary screening, soil suspension was inoculated in Bushnell Hass broth supplemented with 1% filter sterilized diesel incubated on orbital shaker incubator for 24 hrs. Secondary screening was performed by Liquid-liquid extraction or the gravitational analysis method by (20) to evaluate % 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 were 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 % 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 dataset were examined respectively.

Bacterial Growth Kinetic Study

In vitro optimization of bacteria was performed as growth kinetics vs. hydrocarbon degradation with respect to control up to 72 hrs of incubation period using UV visible spectrophotometric analysis [Elico BL 198 Bio spectrophotometer] by taking the absorbance at 600 nm. The media was supplemented with 100 ppm, 300 ppm, 500 ppm and 1000 ppm 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 ±SD, n = 3 from an independent experiment. A one-way ANOVA using SPPSS software between the means of 2 groups were evaluated and p-value < 0.05 were considered as the analysis of significant statistically.

Results

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.

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).

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.

Fourier Transform Infrared [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).

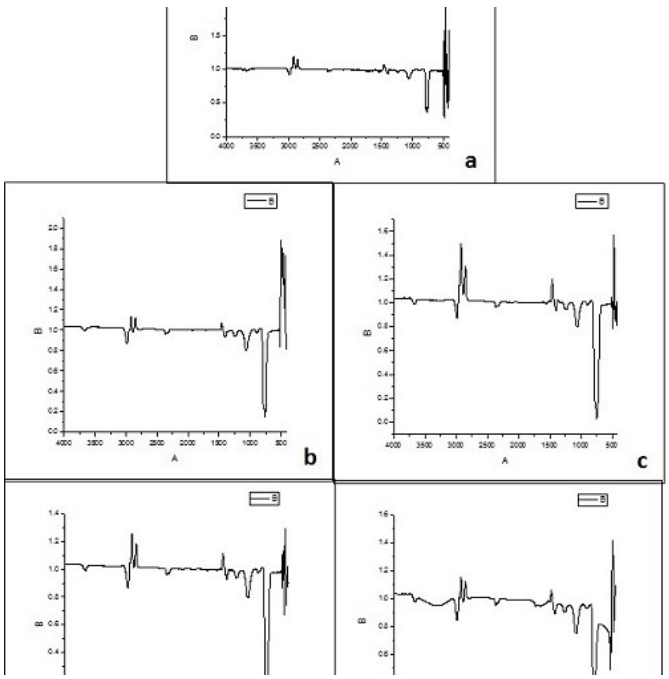

Fig. 2. FTIR spectra of the soil samples after solid liquid extraction with respect to their **(a)** control, **(b)** sample 12, **(c)** sample 13, **(d)** sample 14, (e) sample 15.

High-performance liquid chromatography [HPLC] Analysis

In HPLC analysis, exhibits shift in retention-time and peak-area indicating the quantity of hydrocarbon within the soil sample and indicated the presence of hydrocarbon content with respect to their control. HPLC analysis showed presence of more peaks analysed 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 4 samples were heavily contaminated with hydrocarbon with common functional groups

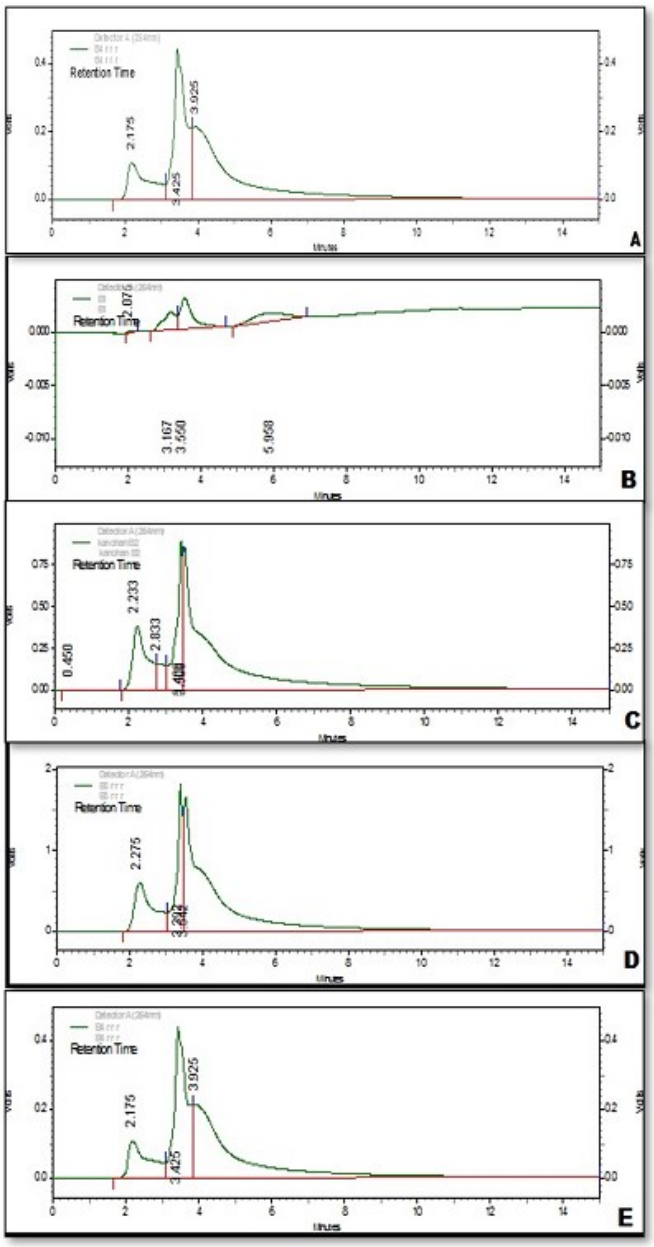

Fig. 3. HPLC spectra of the soil samples after solid-liquid extraction with respect to their **(a)** control, **(b)** sample 12, **(c)** sample 13, **(d)** sample 14, **(e)** sample 15.

and will serve as an excellent source for screening of bacterial isolates with potent hydrocarbon degrading capabilities.

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. 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 ($3.1x10^{-08}$) 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.

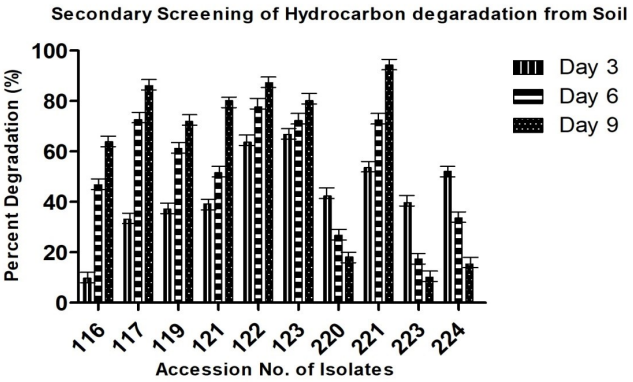

Fig. 5. Liquid-liquid extraction, secondary screening of hydrocarbon degradation from soil samples for determining hydrocarbon load (gram positive and negative).

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

Fig. 4. Showing Liquid-liquid extraction **(A)** Determination of hydrocarbon degradation 3-, 6- and 9-days experiment **(B)** Gravimetric analysis (gram positive and negative).

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).

Discussion

Oil contamination in soil has been recognized as a key factor for degrading of microbial hydrocarbon growth reduction of bacteria and the effect being more distinct at higher level of soil pollution. Another study reported that the micro-organisms are primary degrades of petroleum-hydrocarbons within a contaminated-ecosystem (6, 13, 21). 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 focuses on the screening of bacte-

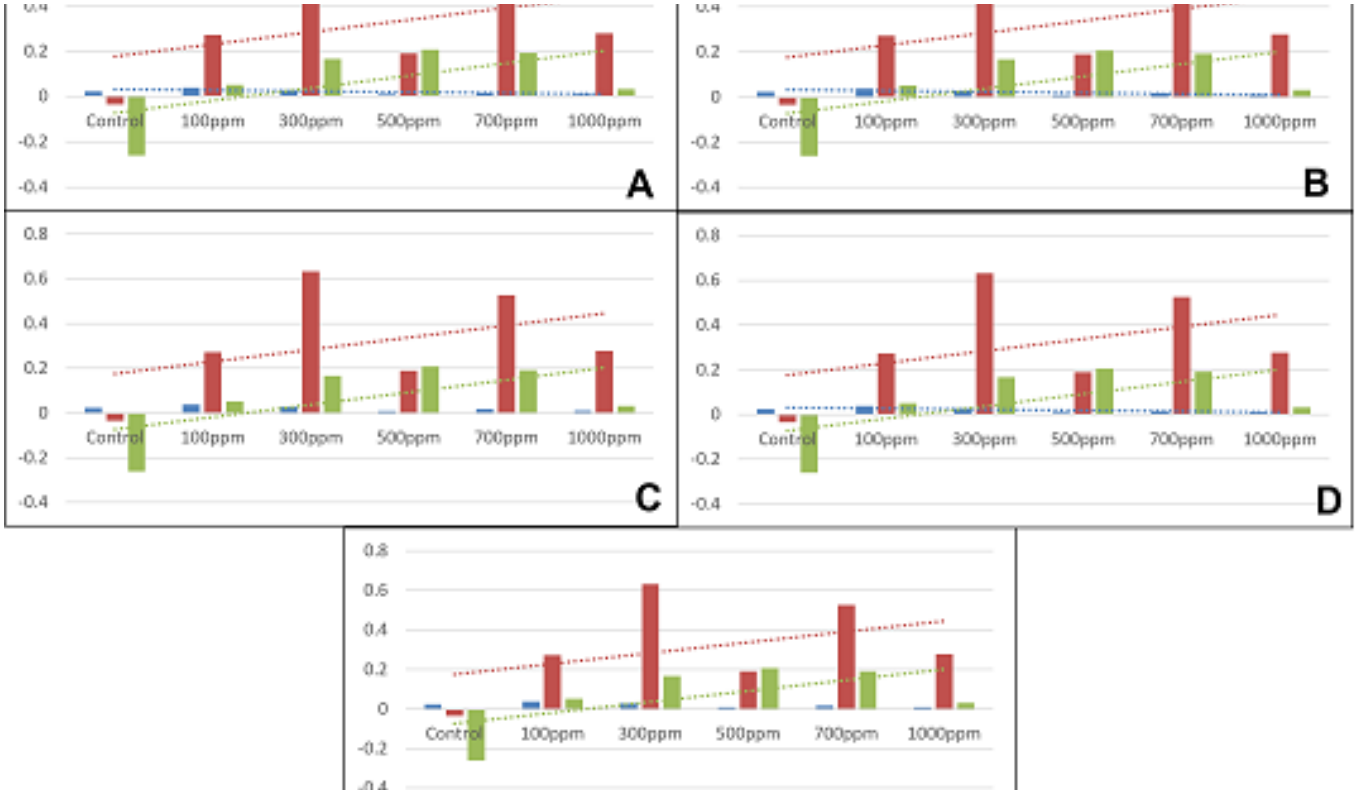

Fig. 6. Showing *in vitro* optimization hydrocarbon effect on growth kinetics vs. degradation (**A**. 222, **B**.221, **C**.223, **D**.122, **E**.117).

ria possessing hydrocarbon degrading potential (22). 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 (23). Another study suggested that the use of pure cultures that provides practical advantage over the use of bacterial consortia or diversity by eradicating the ambiguity associated with constantly variation microbial diversification in case of community exploitation (24). Biodegradation of hydrocarbons through natural microflora allows for the bioconversion 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 (10, 20, 25–27). Most of bioremediation studies suggested using pure culture of the bacteria in a natural environment that remains unknown substantially (28).

Physiological Characterization of Soil Samples

The current experiment demonstrates that the concentration of diesel and physiological characterization of production are directly related to each other. 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 (10). Oil-products not only lead 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 (29).

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

Present investigation shows that the presence of microorganisms significantly analysed by the methods for the biodegradation and detoxification of diesel oil. 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 were exploited (30, 32). Generally, hydrocarbons were ranked in the gradation of reducing in the susceptibility to biodegradation 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 (4, 13, 32).

Fourier Transform Infrared [FTIR] Analysis

Additionally, in the current experiment indicate the presence of various functional groups and bond strengths in the samples subjected to analysis, another 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 (13, 33). According to the analysis, the absence of characteristic peaks in the region of 1610-2042

in extracted samples indicated absence of nitro compound linkage.

High-performance liquid chromatography [HPLC] Analysis

Evaluation of hydrocarbon load was recorded and analysed by the HPLC spectral scan of the soil samples, similar reports were indicated by research as, these analyses 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., *Penicillin* 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 biodegradation of diesel-oil validating the water-soluble derivatives of naphthalene as major-pollutants (15). The similar results were 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 (13, 34).

Microbiological Characterization of Hydrocarbon Degrading Microbes

The screening of the potential diesel degrading bacterial isolates showed that the studies strongly suggest that environmental conditions of the contaminated sites and their significant function as an integral role in the diesel-degrader at hydrocarbon polluted site (35). In nature, biodegradation of complex hydrocarbons needs the co-operation of more than one species detected in petroleum -contaminated soil (36, 37). A study strongly demonstrates that each strain has their specific mechanism in the hydrocarbon transformation process (24, 30, 31, 38). Microbes possess enzyme-systems to degrade as well as utilize diesel-oil as a source of carbon and energy (39, 40, 41). Biostimulation is regarded as an 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 (42). Study documented the capabilities of microbes to use hydrocarbons in oil-contaminated environments (27, 43, 44). Microbial degradation process aids to the removal of spilled oil from the environment after critical mitigation of large volume of oil through physiochemical methodology (13, 45).

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, Toulene, Xylene) as Benzene was maximally degraded followed by Toluene by a Gram-positive bacteria (46). 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 (47). A report indicated that of seventy-three aerobic bacteria were able to degrade petroleum- hydrocarbon (48). The crude-oil fraction with fewer amounts of saturated-hydrocarbons exhibits more resistance to microbial-degradation in comparison to the crude-oil fraction with more amounts of saturated-hydrocarbons (49). 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, (22, 48) also reported the crude oil degradation by *Bacillus* sp. and *Pseudomonas* sp. (21, 27) from oil contaminated area by gravimetric analysis for determinate the degrading capacity by anaerobic degradation as well as aerobic environments (26). Mixed hydrocarbons were not degraded better (25) whereas, the (51) 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 are the foundation for clean-up technologies for environment (13, 28, 52). Elaborative studies are needed to clarify the taxonomic position of the microflora for their biodegradation potential for other PAH's (polycyclic aromatic hydrocarbons) (53). 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 (10). Environmental microbes with the potential to degrade crude-oil are ubiquitously distributed in soil as well as marine environments (54).

Bacterial Growth Kinetic Study

Consequently, the bacterial progression with the utilisation of carbons and energy sources as diesel significantly degraded by the population, similar records were reported by another study (26). Moreover, soil contaminated with petroleum hydrocarbons should be kept properly to optimize the remediation of crude oil by microbes (54, 55). 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 (55). A study demonstrated that the bacterial consortium (BC) of *Acinetobacter, Flavobacterium*, *Pseudomonas* and *Serratia* as hydrocarbon-utilizers (27, 56). Samples were incubated in orbital shaking incubator at 37 °C at 125 rpm 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 (14) and by *Bacillus* as 30-40% (42). Another study reported that the *Bacillus* spp. is most tolerant in high hydrocarbon concentration in soil because of resistant endospores and could be effective in clearing of the oil-spills (5). In a study, pyrene was degraded with *P. putida* (up to 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 (16). Some studies reported that the inoculation had positive/marginal/no effects on rate of oil biodegradation. Whereas various factors must be taken into considered before *in situ* bioremediation, 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 (13, 56). 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 (57).

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. Current study is the first one to report the bacterial culture utilization for the diesel degradation with the efficacy as high as 94 %. Since the hydrocarbon contamination is recalcitrant, persistent and non-biodegradable in nature, therefore it is a challenge to be handled by conventional approaches. The screened bacterial isolated potentially degrade the diesel contamination up to 2.5 % concentration in soil by weight. This study will certainly give a possible solution to the prevailing issue and helps in the reconstruction of nutrient pool in affected sites. The identification and understanding the role of isolates in influencing the effectiveness of *in situ* degradation process is yet to be explored.

Acknowledgements

The authors are grateful to Professor Ina Aditya Shastri, Vice-Chancellor, Banasthali Vidyapith, Rajasthan for providing research facilities. The authors also give thanks to Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India for providing financial assistance in conducting our research work.

Authors 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.

Compliance with ethical standards

Conflict of interest: The author declare that they have no conflict of interests.

Ethical issues: None

References

1. Joshi B, Walia S. PCR amplification of catechol 2,3-dioxygenase gene sequences from naturally occurring hydrocarbon degrading bacteria isolated from petroleum hydrocarbon contaminated groundwater. FEMS Microbiology Ecology. 1996;19:5-.15 https://doi.org/10.1111/j.1574-6941.1996.tb00193.x
2. Kamble PN, Gaikwad VB, Kuchekar SR. Der chemica Sinica 2011;2:229-34.
3. Kebede G, Tafese T, Abda EM, Kamaraj M, Assefa F. Factors influencing the bacterial bioremediation of hydrocarbon contaminants in the soil: mechanisms and impacts. Journal of Chemistry. 2021;(15). https://doi.org/10.1155/2021/9823362
4. Chang LK, Ibrahim D, Omar IC. A laboratory scale bioremediation of Tapis crude oil contaminated soil by bioaugmentation of *Acinetobacter baumannii* T30C. African Journal of Microbiology Research. 2011;5:2609-15. https://doi.org/10.5897/AJMR11.185
5. Adebusoye SA, Ilori MO, Amund OO, Teniola OD, Olatope SO. Microbial degradation of petroleum hydrocarbons in a polluted tropical stream. World Journal of Microbiology and Biotechnology. 2007;23(8):1149-59. https://doi.org/10.1007/s11274-007-9345-3
6. Dixit H, Lowry M, Mohsin U, Moond M, Kumar S, Chokriwal A, Gupta S. Screening and identification of diesel oil degrading bacterial isolates from petroleum contaminated soil of Barmer. Int Journal of Recent Scientific Research. 2018;6(1):34-40.
7. Das N, Chandran P. Microbial degradation of petroleum hydrocarbon contaminants. Biotechnology Research International. 2011;13:1-13.https://doi.org/10.4061/2011/941810
8. Bishnoi K, Sain U, Kumar R, Singh R, Bishnoi NR. Distribution and biodegradartion of polycyclic aromatic hydrocarnbons in contaminnnated sites of Hisar. Indian Journal of Experimental Biology. 2009;47:210-17.
9. Muratova AY, Turkovskaya OV, Hübner T, Kuschk P. Studies of the efficacy of alfalfa and reed in the phytoremediation of hydrocarbon-polluted soil. Applied Biochemistry and Microbiology. 2003;39(6):599-605. https://doi.org/10.1023/A:1026238720268
10. Mukherji S, Vijay A. Critical issues in bioremediation of oil and tar contaminated sites, In: Proceedings of the International Conference on Advances in Civil Engineering. 2002; 507-16.
11. Azubuike CC, Chikere CB and Okpokwasili GC. Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. World Journal of Microbioly and Biotechnology. 2016;32(11):1-18. https://doi.org/10.1007/s11274-016-2137-x
12. Lin X, Ng TT. A three-dimensional discrete element model using arrays of ellipsoids. Geotechnique. 1997;47(2):319-29. https://doi.org/10.1680/geot.1997.47.2.319
13. Lima SD, Oliveira AF, Golin R, Lopes VC, Caixeta DS, Lima ZM, Morais EB. Isolation and characterization of hydrocarbon-degrading bacteria from gas station leaking-contaminated groundwater in the Southern Amazon, Brazil. Brazilian Journal of Biology. 2019;5(80):354-61. https://doi.org/10.1590/1519-6984.208611
14. Panda SK, Kar RN, Panda CR. Isolation and identification of petroleum hydrocarbon degrading microorganisms from oil contaminated environment. International Journal of Environ-

mental Sciences. 2013;3(5):1314-21. https://doi.org/10.6088/ijes.2013030500001

15. Patil TD, Pawar S, Kamble PN, Thakare SV. Bioremediation of complex hydrocarbons using microbial consortium isolated from diesel oil polluted soil. Pelagia Research Library. 2012; 3 (4):953-58.

16. Marquez-Rocha FJ, Hernandez-Rodriguez V, Lamela MT. Biodegradation of diesel oil in soil by a microbial consortium. Water, Air Soil Pollution. 2001; 128:313-20. https://doi.org/10.1023/A:1010392821353

17. Bekele GK, Gebrie SA, Mekonen E, Fida TT, Woldesemayat AA, Abda EM, Tafesse M, Assefa F. Isolation and characterization of diesel-degrading bacteria from hydrocarbon-contaminated sites, flower farms and soda lakes. International Journal of Microbiology. 2020;(2). https://doi.org/10.21203/rs.3.rs-119479/v1

18. Bôto ML, Magalhães C, Perdigão R, Alexandrino DA, Fernandes JP, Bernabeu AM, Ramos S, Carvalho MF, Semedo M, LaRoche J, Almeida CM. Harnessing the potential of native microbial communities for bioremediation of oil spills in the Iberian Peninsula NW coast. Frontiers in Microbiology. 2021;(12):879. https://doi.org/10.3389/fmicb.2021.633659

19. Dixit H, Lowry M, Mohsin U, Moond M, Kumar S, Chokriwal A, Gupta S. Growth optimization and comparative analysis of diesel oil degradation potential of *Bacillus* sp. Isolated from petroleum contaminated Soil of Barmer, Rajasthan. Journal of Pharmaceutical, Chemical and Biological Sciences, 2018;9(3B):24730-37. http://dx.doi.org/10.24327/ijrsr.2018.0903.1717

20. Ganesh A, Lin J. Diesel degradation and biosurfactant production by Gram-positive isolates. African Jornal of Biotechnology, 2009;8(21):5847-54. https://doi.org/10.5897/AJB09.811

21. Hossain MF, Akter MA, Sohan MS, Sultana N, Reza MA, Hoque KM. Bioremediation potential of hydrocarbon degrading bacteria: isolation, characterization and assessment. Saudi Journal of Biological Sciences. 2022;29(1):211-16. https://doi.org/10.1016/j.sjbs.2021.08.069

22. Jirasripongpun K. The characterization of oil degrading microorganisms from lubricating oil contaminated (scale) soil. Letters in Applied Microbiology. 2002;35:296-300. https://doi.org/10.1046/j.1472-765X.2002.01184.x

23. Bhadauria S. Characterization of agricultural soils irrigated with petroleum refinery effluents physico chemical analysis. Journal of Enviornmental Ploymer. 1997;4(4):295-302.

24. Ghazali FM, Rahman RNZA, Salleh AB, Basri M. Degradation of hydrocarbons in soil by microbial consortium. International Biodeterioration and Biodegradation. 2004;54:61-67. DOI: https://doi.org/10.1016/j.ibiod.2004.02.002

25. Lidderdale T. Demand, supply and price outlook for low-sulfur diesel fuel. In Energy Information Adminismtion 1993; Short term energy outlook annual supplement DOE-E0202 (93).

26. Perdigão R, Almeida CM, Magalhães C, Ramos S, Carolas AL, Ferreira BS, Carvalho MF, Mucha AP. Bioremediation of petroleum hydrocarbons in seawater: Prospects of using lyophilized native hydrocarbon-degrading bacteria. Microorganisms. 2021 Nov;9(11):2285. https://doi.org/10.3390/microorganisms9112285

27. Ejaz M, Zhao B, Wang X, Bashir S, Haider FU, Aslam Z, Khan MI, Shabaan M, Naveed M, Mustafa A. Isolation and characterization of oil-degrading Enterobacter sp. from naturally hydrocarbon-contaminated soils and their potential use against the bioremediation of crude oil. Applied Sciences. 2021 Jan;11(8):3504. https://doi.org/10.3390/app11083504

28. Harayama S, Kishira H, Kasai Y, Shutsubo K. Petroleum biodegradation in marine environments. Journal of Molecular Microbiology and Biotechnology. 1999;1(1):63-70.

29. Wyszkowskwa J, Kucharski J, Waldowska E. The influence of diesel oil contamination on soil enzyme activities. Polish Journal of Environmental Studies. 2002;48:58-62. https://doi.org/10.17221/4360-PSE

30. Scragg A. Environmental Biotechnology. 2nd edition, Oxford University Press. 2005;179-82.

31. Balba MT, Al-Awadhi N, Al-Daher R. Bioremediation of oilcontaminated soil: microbiological methods for feasibility assessment and field evaluation. Journal of Microbiological Methods. 1998;32:155–64. https://doi.org/10.1016/S0167-7012(98)00020-7

32. Atlas R, Bragg J. Bioremediation of marine oil spills: when and when not—the exxon valdez experience. Microbial Biotechnology. 2009;2(2):213–21. https://doi.org/10.1111/j.1751-7915.2008.00079.x

33. Liu H, Yang G, Jia H, Sun B. Crude oil degradation by a novel strain *Pseudomonas aeruginosa* AQNU-1 isolated from an oil-contaminated lake wetland. Processes. 2022 Feb 4;10(2):307. https://doi.org/10.3390/pr10020307

34. Shrivastava S, Gupta M. Study on diesel degradation by *Acinetobacter* species and its effect on germination of Medicago sativa. Asian Journal of Microbiol Biotechnology and Environmental Science. 2008;10(3):487-96.

35. Singh C, Lin J. African Isolation and characterization of diesel oil degrading indigenous microrganisms in Kwazulu-Natal, South. African Journal of Biotechnology. 2008;7(12):1927-32. https://doi.org/10.5897/AJB07.728

36. Kim JS, Crowley DE. Microbial Diversity in Natural Asphalts of the Rancho La Brea Tar Pits. Applied and Environmental Microbiology. 2007;73(14):4579-91. https://doi.org/10.1128/AEM.01372-06

37. Da Cunha CD, Rosado AS, Sebastián GV, Seldin L, Weid VDI. Oil biodegradation by *Bacillus* strains isolated from the rock of an oil reservoir located in a deep-water production basin in Brazil. Applied Microbiology and Biotechnology. 2006;73(4):949-59. https://doi.org/10.1007/s00253-006-0531-2

38. Cunliffe M, Kertesz MA. Effect of Sphingobium yanoikuyae B1 inoculation on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. Environmental Pollution. 2006;144(1):228-37. https://doi.org/10.1016/j.envpol.2005.12.026

39. Ezeji EU, Anyanwu BN, Onyeze GOC, Ibekwe VI. Studies on the utilization of petroleum hydrocarbon by micro organism isolated from oil contaminated soil. International Journal of Nature Applied Science. 2005;1(2):122-28. https://doi.org/10.4314/ijonas.v1i2.36073

40. Ijah UJJ, Antai SP. Degradation and Mineralization of crude oil by bacteria. Nigerian Journal of Biotechnology. 1998;5:79-86.

41. Antai SP, Mgbomo E. Pattern of Degradation of Bonny light crude oil by *Bacillus* spp. and *Pseudomonas* spp. isolated from oil spilled site. W.A. Journal of Biological and Applied Chemistry. 1993;38(1-4):16-20.

42. Molina-Barahona L, Rodriguez-Vazquez R, Hernandez-Velasco M, Vega-Jarquin C, Zapata-Perez O, Mendoza-Cantu A, Albores A. Diesel removal from contaminated soils by biostimulation and supplementation with crop residues. Applied Soil Ecology. 2004;27:165-75. https://doi.org/10.1016/j.apsoil.2004.04.002

43. Obuekwe CO, Al-Jadi ZK, Al-Saleh ES. Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. International Biodeterioration and Biodegradation. 2009;63:273-79. https://doi.org/10.1016/j.ibiod.2008.10.004

44. Zakaria NN, Gomez-Fuentes C, Abdul Khalil K, Convey P, Roslee AF, Zulkharnain A, Sabri S, Shaharuddin NA, Cárdenas L, Ahmad SA. Statistical optimisation of diesel biodegradation at low temperatures by an Antarctic marine bacterial consortium isolated

from non-contaminated seawater. Microorganisms. 2021 Jun;9 (6):1213. https://doi.org/10.3390/microorganisms9061213

45. Ljah UJJ, Okang CN. Petroleum degrading capabilities of bacteria isolated from soil. W.A. Journal of Biological and Applied Chemistry. 1993;38:9-15.

46. Jayanthi R, Hemashenpagam N. Isolation and identification of petroleum hydrocarbon degrading bacteria from oil contaminated soil samples. International Journal of Novel Trends in Pharmaceutical Sciences. 2015; 5(3):102-06. https://doi.org/10.6088/ijes.2013030500001

47. Penet SR, Marchal AC, Bertonicini VF, Monot F. Characterisation of biodegration capacities of environmental microflorae for diesel oil by comprehensive two dimensional gas chromatography. Biodegradation. 2006;17:577. https://doi.org/10.1007/s10532-005-9028-4

48. Chosson P, Lanau C, Connan J, Dessort D. Biodegradation of refractory hydrocarbons biomarkers from petroleum under laboratory conditions. Nature. 1991;351:640-42. https://doi.org/10.1038/351640a0

49. Amund OO, Akaqngbou TS. Micribial degradation of four Nigerian crude oils in an estuarine microsm. Letters in Applied Microbiology. 1993;16:118-21. https://doi.org/10.1111/j.1472-765X.1993.tb01374.x

50. Subathra MK, Immanuel G, Suresh AH. Isolation and identificationof hydrocarbon degrading bacteria from Ennore Creek. Bioinformation. 2013;9(3):150. https://dx.doi.org/10.6026%2F97320630009150

51. Caetano MO, Kieling AG, Raimondi RL, Gomes LP, Schneider IA. Ecotoxicity tests with *Allium cepa* to determine the efficiency of rice husk ash in the treatment of groundwater contaminated with benzene, toluene, ethylbenzene and xylene. Environmental Science and Pollution Research. 2018;25(13):12849-58. https://doi.org/10.1007/s11356-018-1512-6

52. Watanabe K, Hino S, Onodera K, Kajie S, Takahashi N. Diversity in kinematics of bacterial phenoloxygenating activity. Journal of Fermentation and Bioengineering. 1996;81:562-65. http://dx.doi.org/10.5897/AJB09.1501

53. Braddock JF, Ruth ML, Catterall PH, Walworth JL, McCarthy KA. Enhancement and Inhibition of Microbial Activity in Hydrocarbon-Contaminated Arctic Soils: Implications for Nutrient-Amended Bioremediation. Environmental Science and Technology. 1997;31:2078. https://doi.org/10.1021/es960904d

54. Venkateswaran K, Harayama S. Sequential enrichment of microbial populations exhibiting enhanced biodegradation of crude oil. Canadian Journal of Microbiology. 1995;41:767-75. https://doi.org/10.1139/m95-106

55. Annweiller E, Richnow HH, Grams C. Naphthalene degradation and incorporation of naphthalene derived carbon into biomass by the thermophile bacillus therleovorans. Applied and Environmental Microbiology. 2000;66(2):518-23. https://doi.org/10.1128/AEM.66.2.518-523.2000

56. Rosenberg E. The hydrocarbon–oxidizing bacteria. In: A. Balows et al. (Editor), The prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. Springer Verlag, Heidelberg, Germany. 1992;446–59.

57. De I, Gupta S. Isolation and characterization of plant synergistic bacteria capable of degrading xenobiotics from oil spillage sites. 3 Biotech. 2016 Dec;6(2):1-5.

§§§