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Isolation and characterization of hydrocarbon degrading bacteria from oil contaminated Mangrove sediment of Cochin, Kerala, India

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Abstract

Petroleum hydrocarbons pose a significant issue because they are commonly found in the environment and can adversely affect various living organisms, including humans. Utilizing biodegradation techniques to restore soil contaminated by crude oil can be an effective approach. The study explored the existence of native microorganisms in mangrove soil sediments polluted with crude oil and examined their potential to break down engine oil and diesel *in vitro*. From 15 soil samples contaminated with oil along Marine Drive in Cochin, thirteen bacterial strains were isolated. Bacterial identification was done by 16S r RNA sequencing in addition to morphological and biochemical methods. GC–MS was conducted for hydrocarbon degradation analysis. The findings disclosed that the local bacterial isolates possessed a significant ability to degrade crude oil hydrocarbon fractions. The research gives astute observations on the significant capabilities of *Klebsiella variicola* RUK1 and *Bacillus fleuxs* RUK3 (*Priestia flexa* RUK3) for breaking down crude oil hydrocarbons, signifying that they could be developed for use as effective agents in bioremediation for oil clean up.

Key words : - Bioremediation, Crude oil, Soil microorganisms, Optical density, Gas Chromatography.

Oil spills and hydrocarbon pollution continue to be significant environmental challenges today, primarily due to the operations of associated industries, particularly

the petrochemical sector. During industrial operations, oil spills are often released into aquatic environments, leading to significant issues for the ecosystem[8]. Hydrocarbons cause cancer, genetic mutations and pose a major threat to animal population including mankind[10]. Mangrove forests are highly susceptible to oil pollution because they are seen in coastal belts and estuaries[14]. Microorganisms associated with mangrove ecosystem had the potential to degrade the organic contents including oil hydrocarbons[1]. The indigenous bacteria of the ecosystem voraciously feed on the carbon content of the oil and disintegrate it[8].

The diverse catalytic enzymes present in these microbes are responsible for their effective oil degrading capacity. An association of various functional and indigenous bacteria will be more effective to achieve optimal ecological cleaning property. Previous studies prove that bioaugmentation and biostimulation procedures will helps to pick up the efficacy of bioremediation efforts. *Acinetobacter, Dietzia, Mycobacterium, Pseudomonas, Staphylococcus, Enterobactor, Pandoraea, Streptobacillus, Arthrobacter Streptococcus*, *Achromobacter* and *Rhodococcus* bacteria have the capacity to breakdown the petroleum hydrocarbons[9]. Biosurfactants are amphipathic metabolites that produced by specific microorganisms. Biosurfactant reduces surface tension (ST) and critical micelle dilution (CMD) both in hydrocarbon mixtures and water-based solutions, which helps in the formation of micro-emulsions through micelle creation, allowing hydrocarbons to dissolve in water or vice versa[2].

Traditional methods for clean up, such as the use of chemicals or manual containment and removal, typically lead to quick outcomes. Bioremediation is more efficient, cost effective and also eco friendly[6]. Optimal conditions including pH levels, soil makeup nutrient supply, temperature, oxygen levels moisture and RED-OX potential should be maintained for the successful bioremediation. These factors are essential for the targeted organism or biological process to enable essential[4]. The current research investigated the ability of native bacteria to break down petroleum hydrocarbon found in the mangrove soil sediment Marine Drive, Cochin, Kerala. The study also aimed to enhance the growth conditions of isolates by examining various factors such as carbon sources, salinity, pH, and nitrogen sources[9]. Gas chromatography-mass spectrometry (GC–MS) can be used to analysis hydrocarbon fractions and capability to break down engine oil and diesel by the isolates can be evaluated.

Geographical location and residue sampling :

The study area is situated at Marine Drive in Cochin of Central Kerala, India. The exact place of the site lies between 9°58'48"N, 76°16'30"E (figure 1). Soil samples were collected from five different locations at a depth of 0-10 cm on the beach front, following vertical cross sections. Samples were inserted into sterilized polyethylene bags. The polyethylene bags were stored in a container filled with ice for transportation to the lab, where they were subsequently kept at a temperature of 4 °C[7].

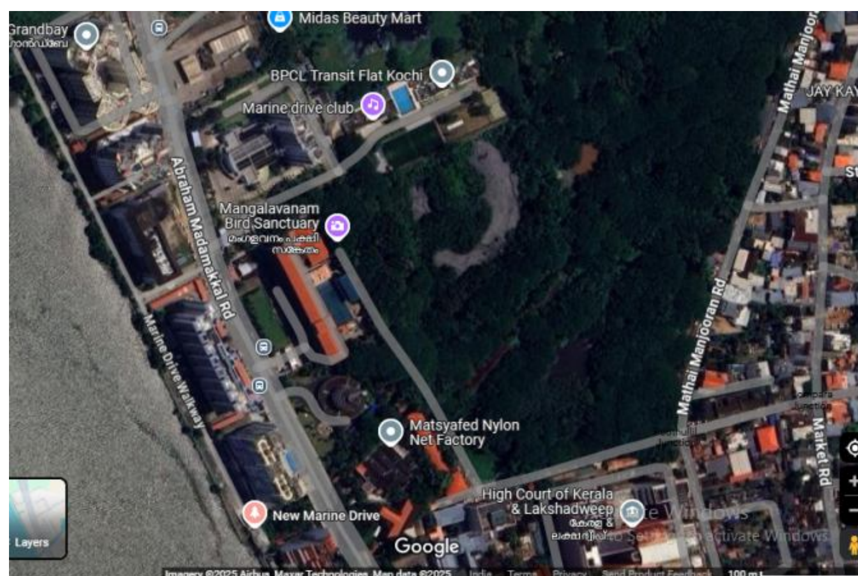


Figure 1. Map showing the location of Marine drive

Isolation and enrichment of oil degrading bacteria

Isolation of oil degrading bacteria, 1g from each hydrocarbon-contaminated soil sample was introduced into two sets of Bushnell Haas (BH) agar media that included 2% (v/v) engine oil and diesel as carbon sources for enrichment. The medium underwent autoclaving at a temperature of 121 °C for 15 minutes, and after it cooled down, a 0.1% (v/v) solution of micronutrients was aseptically incorporated. This micronutrient solution consisted of 5.00 g L1 of $FeSO_4 \cdot 7H_2O$, 0.39 g L1 of $CuSO_4 \cdot 5H_2O$, 0.25 g L1 of $Co(NO_3)_2 \cdot 6H_2O$ 0.17 g L1 of $Na_2B_4O_7 \cdot 10H_2O$, 1.54 g L1 of $MnSO_4 \cdot H_2O$ and 10.95 g L1 of $ZnSO_4 \cdot 7H_2O$. The pH of the medium was set to 7.0 ± 0.2. The conical flasks rotating at 150 rpm were incubated at 35°C for a period of 7 days. After a week, 1 ml of inoculum was transferred into 100 ml of fresh Minimal salt medium. The medium was nurtured again for a week under the same conditions to reduce the presence of unwanted microbial populations. After one week, 1 ml of the medium was subjected to serial dilution. Subsequently, 100 µl from the diluted samples, ranging from 10^{-4} to 10^{-6}, was spread to nutrient agar culture medium, which was then incubated for 24 hours at 35°C. Structurally distinct bacterial culture were selected and individually inoculated onto nutrient and BH agar plates to isolate uncontaminated cultures of the bacterial samples. The isolated 87 bacteria were kept on a slanted nutrient agar medium.

Extraction of effective Hydrocarbon degrading Bacterial cultures :

Bacteria capable of efficiently degrading oil were chosen based on their growth

conditions, enriched with both diesel and engine oil. For the screening process, a 5 ml sample stock culture from each organism was introduced into 500 ml Erlenmeyer flasks with 100 ml of sterilised BH broth that was enriched with 2% volume per volume engine oil and diesel. Kept the flasks in a shaking incubator, at a temperature of 35°C and a speed of 200 rpm for 7 days. A collection of flasks containing identical culture media was kept under the similar parameters. Proliferation of the organism in the culture was assessed on the 0 and 7th days by recording the OD value at 600 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800). The strains demonstrating the highest performance were selected for subsequent experiments.

Evaluating Biosurfactant Production :

Drop Collapse Test :

Efficient hydrocarbon-degrading isolates E01 and D1 were chosen and tested for biosurfactant activity. The test was conducted employing crude oil as the carbon source, following a modified method based on the work of John *et al.*,[6]. A portion of acellular supernatant was placed amidst separate samples of engine oil and diesel on a sterile microscopic slide, following the procedure established by Zeena *et al.*,[19]. After one minute, the drop was examined visually. The presence of destabilization of oil by the cell-free broth indicated a positive outcome.

Emulsification Stability Assay (E24) :

The Emulsification Stability Assay (E24) was performed following the procedure outlined by John *et al.*,[5]. A 2 ml quantity of oil was mixed with an equal volume of a cellular supernatant, stirred thoroughly for two minutes using an electronic vortex mixer and then kept idle for 24 hours before taking measurements. The emulsion height was measured and recorded. Calculation of the E24 index was performed in milli meter divided by the total height of the liquid column (in mm), multiplied by 100.

Identification of the most potent oil degrading microbial isolates :

Morphological and Biochemical characterization :

The characteristics outlined were identified following the guidelines of Bergey's bacterial identification manual. Tests like Gram stain, motility analysis, amylolytic activity, indole testing, hydrogen sulfide (H_2S) production, as well as catalase and oxidase activity was carried out. Further characterised by oxidation, fermentation, nitrate reduction, and growth along with carbohydrate acidification.

Molecular identification by 16SrRNA *:*

The amplification of the 16S rRNA gene was conducted for strain identification via taxonomic characterisation. CTAB method was adopted for genomic DNA extraction from bacterial isolates and carried out molecular characterization through 16S rRNA. PCR was used to amplify16 S rRNA gene sequencing. The PCR reaction was performed in 25 µl volume, which included 2 mM MgCl2 (1 µl), a 10×amplification buffer consisting of 200 mM Tris and 500 milli molar potassium chloride, 2 mM of dNTP, 0.15 mM of primer, 1 unit (0.5

μl) of Thermus aquatics DNA polymerase and 2 μl of DNA template. Mad up the total volume 15 μl with milliQ water. An amplification protocol comprising 35 thermal cycles was executed using a Gene Amp 5700 DNA amplifier. The PCR thermal settings were retained at 94°C for 5 minutes, proceeded by 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 1.5 minutes, concluding with 72°C for 10 minutes and retained at 4°C. Then the amplified 16S rRNA gene sequences were characterized by V3.1 cycle analysing kit on an automated DNA sequencing system. To evaluate the resemblance of partial 16S rRNA gene codes to the parallel 16S rRNA genetic codes, similarity rankings from the ribosomal database repository and FASTA Nucleotide Database repository were utilized. The evolutionary relationship diagrams were generated using MEGA5 software.

Growth optimization :

Biodegradation experiments were carried out with 100 ml aliquots of nutrient broth that had been sterilized at 121 °C for 15 minutes in 250 ml Erlenmeyer flasks. A one ml aliquot from individual cultures of interest was incorporated into this broth. To optimize the growth conditions, the two isolates were cultivated in BH broth, with one batch containing engine oil and the other containing diesel. Different groups of boiling tubes were prepared, each containing 100 ml of broth and 2% oil. Each group varied in pH levels (2, 4, 6, 8, 10), sodium chloride concentrations (0.005g, 0.01g, 0.02g, 0.03g, 0.04g), and sources of nitrogen (Sodium nitrate, Ammonium nitrate, Ammonium chloride, Ammonium sulfate). Different concentrations of oil (1%, 5%, 10%, 15%, 20%, etc.) were introduced to another set of flasks. The tubes containing diesel were inoculated with isolate D1, while those with engine oil were treated with EO1, and all were incubated at room temperature for duration of 7 days. Different temperature (4ºC, 18ºC, 28ºC, 37ºC, 45ºC) is also selected as a parameter. The optical density (OD) of the tubes was measured on the second and seventh days of incubation at 600nm by UV Spectroscopy. The recorded OD values, which indicate the concentration of the cells, were organized into figures (2-11).

Biodegradation Screening and GCMS analysis :

The bacterial isolates, which had been cultured in BH Medium for five days, were transferred to a flask with 100 ml of BH broth. Different concentrations of engine oil and diesel were then introduced into two sets of flasks, each with three replicates. The flasks were incubated at a heat level of 31°C for one week in a laboratory shaker operating at 150 RPM. Then the contents of flask were subjected to centrifugation at 9,000 RPM for one hour to collect the cell precipitates. The resulting filtrate was then re suspended in a 1:1 ratio with hexane and evaporated to dryness at 80°C using a water bath. The dried filtrate, which represents the residue of hydrocarbons, was subsequently analyzed using a gas chromatograph (model: Shimadzu Japan, 2014).

The deterioration percentage of crude oil was determined using gravimetric analysis, calculated as follows:

$$\text{Deterioration percentage of crude oil} = \frac{\text{Pre-treatment weight} - \text{Post-treatment weight of crude oil}}{(\text{Pre-treatment weight of crude oil})} \times 100$$

It was then assessed against the initial amount of hydrocarbons present the control funnel. GC was utilized to assess the quantity of left over hexadecane present in the growth medium. The microbial degradation was measured based on TRACE gas chromatography analysis using 99.9% hexadecane as external standard equipped with a flame ionisation detector and OV5 capillary column. The cultures taken at the beginning and after 48 hours were subjected to centrifugation (Sigma 6-15) at 10,000 rpm for 10 minutes at a temperature of 4 °C. The clear liquid above precipitate is then extracted using dichloromethane in 1:3 volume by volume ratio ensuring vigorous shaking for 60 seconds.

Hydrogen served as the carrier gas, with injection and detection temperatures set at 220 °C and 280 °C, respectively. A volume of one microlitre of each supernatant was injected in split mode, and measurements commenced at 100°C, increasing the temperature to 230 °C at a rate of 250°C per minute

Selection of the oil denaturing bacteria :

The initial screening involved selecting all isolates mainly for the use of engine oil and diesel in culture to remove autotrophic organisms and bacteria that utilize agar. At the end of the incubation period, the isolates exhibited turbidity. Out of this, 31 isolates were identified as capable of hydrocarbon degradation. A total of 31 isolates collected from the initial screening were evaluated their ability to utilize crude oil, the same media as in the first screening, and were incubated for 7 days. In the third screening, out of the 13 isolates, only 4 were able to multiply, with counts exceeding 10^6 CFU/ml.

Identification of Potent Hydrocarbon Degrading Bacteria :

In the set of four isolates, growth was observed in the B H that contained oil. Of these, two bacterial cultures were identified could break down engine oil and diesel by using them as their only carbon and energy source. These cultures have been designated as E01 and D1, respectively, for further degradation studies. Two distinct isolates, initially referred to as E01 and D1, recognized as likely candidates for bioremediation purposes These isolates were cultivated in a medium where diesel and used engine oil served as their exclusive carbon sources for survival, growth, and reproduction. This suggests that these isolates can assimilate diesel and burned engine oil for nutrition, which allows them to degrade these substances effectively. Over the past several decades, numerous hazardous waste sites have emerged globally due to the build up of xenobiotics in the soil, water, and air. These xenobiotic substances contaminate the environment, particularly because many are persistent and resistant to breakdown. One notable example of this is contamination by petroleum hydrocarbons. Many microorganisms have already been extracted from two primary environments: soil and marine sources that are predominantly polluted with petroleum hydrocarbons[11]. In this experiment, diesel and engine oil are utilized as the exclusive carbon substrate for the bacteria that have been isolated to assess their degradation efficiency. Although these isolates can break down diesel and used engine oil, their effectiveness in degradation is influenced by various physical, chemical, and factors.

Screening for Biosurfactant production :

Drop collapse and Emulsion stability assay:

To evaluate the efficacy of E01 and D1 in producing biosurfactants, the supernatant obtained from preliminary demonstrated a remarkable emulsification index along with favorable drop collapse results. The findings presented in Table-1 illustrate the production of biosurfactants under optimal culture conditions at the 48-hour mark. The data indicates a rise in biosurfactant production as the incubation period extends. The maximum emulsification index values for E01 and D1 were recorded at 15.78% and 22.72%, respectively. Results of the study reveals that the organism has the potential for optimization, indicating its capability for biosurfactant production. Sharma *et al.*,[16] investigated the rhamnolipid biosurfactant and the breakdown of waste cooking oil by Pseudomonas aeruginosa in batch and fed-batch system with a peak biosurfactant yield of16 g/L.

Identification of the most potent crude oil degrading bacterial isolates :

Morphological studies :

Bacterial identification was conducted in part by analyzing their colour and the morphology of their colonies. Results were noted after a period of 48 hours for both spreading and streaking methods. Three colonies have been cultured separately and their characteristics noted. One colony was described as cream-coloured and convex, while another appeared white. According to Gram staining results, isolate E01 is gram-negative and D1 is gram-positive. The motility test indicated that E01 is non-motile, whereas D1 is motile.

Biochemical characterization :

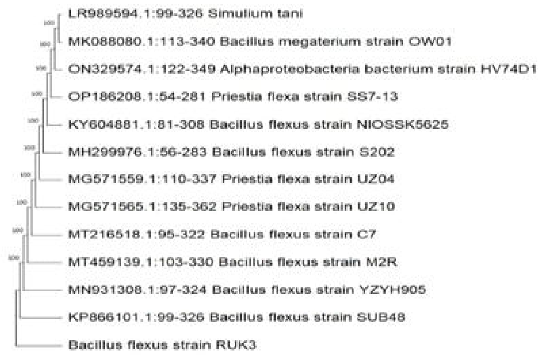
Biochemical analyses of the bacterial isolates were conducted as outlined in Bergey's Manual of Systematic Bacteriology[17]. Table 2 presents the biochemical test results for the three bacterial isolates.

Identification of isolates by using 16 S rRNA *:*

The analysis of bacterial sequences, specifically 16S rRNA that yielded positive PCR results, revealed that a Blast search on NCBI indicated significant similarity. The strains E01 and D1 were found to be most closely related to *Klebsiella variicola* RUK1, displaying a 99.86% identity, and *Bacillus fleuxs* RUK3 (*Priestia flexa* RUK3), showing a 96.49% identity, when compared to Gene Bank databases.

Dendrogram of oil degrading organism *Klebsiella variicola* (E02) from mangrove soil sediments of Cochin

Dendrogram of oil degrading organism *Bacillus flexus* (D1) from mangrove soil sediments of Cochin



Growth optimization :

The ideal conditions for growth were determined at a pH of 6 with salinity at 0.3%, 10% oil and ammonium nitrate were served as the carbon and nitrogen source respectively. The purpose of the optimization was to pinpoint the most advantageous growth conditions for the isolates. It was found that the growth rates of the isolate D1 diminished the concentration of diesel oil. Similarly, the growth rates of isolate E02 decreased the concentrations of burned engine oil. The isolates demonstrated a greater ability to degrade diesel oil compared engine oil. *Klebsiella variicola* RUK1 and *Bacillus fleuxs* RUK3 (*Priestia flexa* RUK3) are potential candidates for the bioremediation of sites contaminated with engine oil and diesel. In 2020, Agota and Silke observed that microorganisms could only degrade less than 4% of diesel oil during the biodegradation process. In contrast, the isolates are capable of breaking down a greater amount of diesel oil and used engine oil than indicated in that report. This research reveals a method for extracting oil-degrading bacteria from an oil-polluted location, highlighting that oil contaminated soil serves as the main source for isolating bacteria that break down petroleum. Present study strongly suggests that these isolates have significant potential for use as a bioremediation agent to clean oil from contaminated environments.

Biodegradation testing and GC-MS assay:

Through a culture-dependent method,

Table-1. Morphological features of isolates E02 & D1 from Cochin mangrove soil sediments

Isolates	Morphological Characteristics	Grams staining	Shape	Motility	Percentage of Oil Removal	Emulsi-fication activity
E01	Cream coloured, convex colonies, 1-2 mm in size	Negative	Short Rod	Non Motile	Engine Oil 90%	15.78%
D1	White coloured, small circular colonies with smooth texture was obtained.	Positive	Long Rod	Motile	Diesel 30%	22.72%

Table-2. Biochemical features of isolates E02 & D1 from Cochin mangrove soil sediments

Characteristics	E01	D1
Indole Test	-	-
MR Test	+	+
VP Test	+	+
Citrate utilisation Test	+	+
TSI Test	Acidic slant/Acidic butt	Acidic slant/Acidic butt
Catalase Test	+	+
Oxidase Test	-	+
H2S	-	_
Starch Hydrolysis	-	+
Lactose fermentation	+	+
Urea Hydrolysis	+	+
Nitrate Reduction Test	+	+

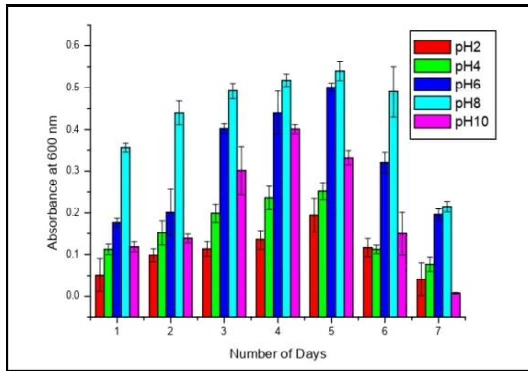


Figure 2. Effect of ph on growth of E01 isolate

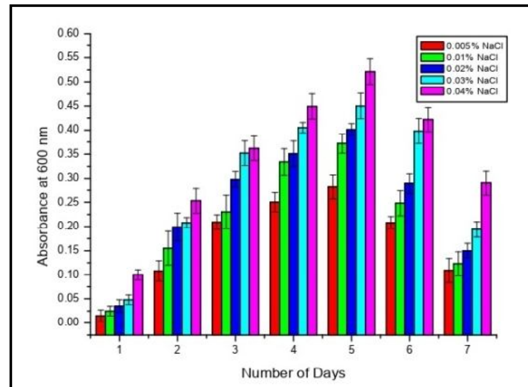


Figure 3. Effect of Salinity on growth of E01 isolate

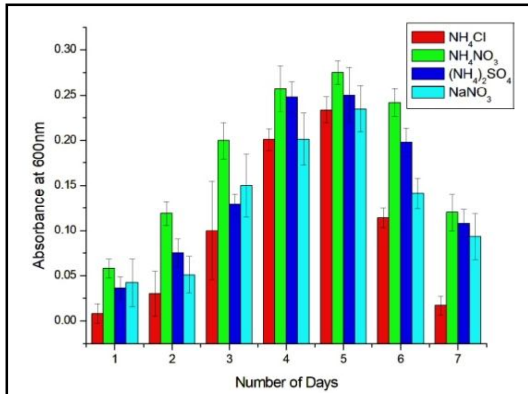


Figure 4. Effect of Nitrogen source on growth of E01 isolate

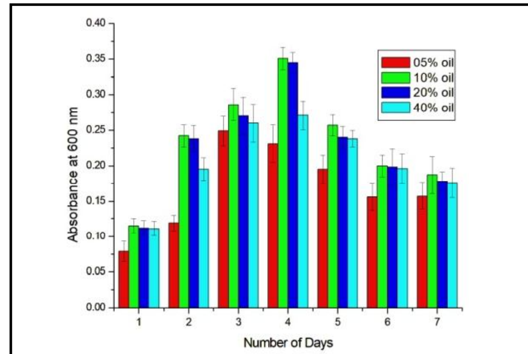


Figure 5. Effect of Carbon source on growth of E01 isolate

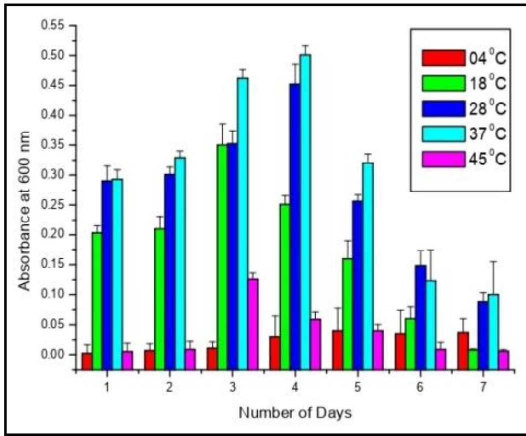


Figure 6. Effect of Temperature on growth of E01

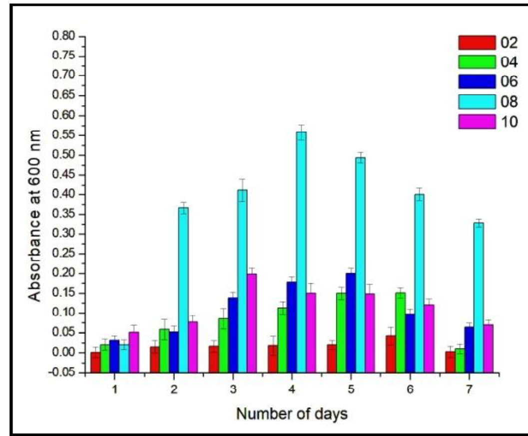


Figure 7. Effect of pH on growth of D1

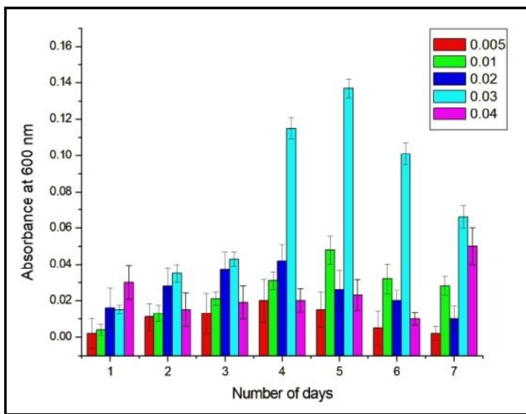


Figure 8. Effect of Salinity on growth of D1

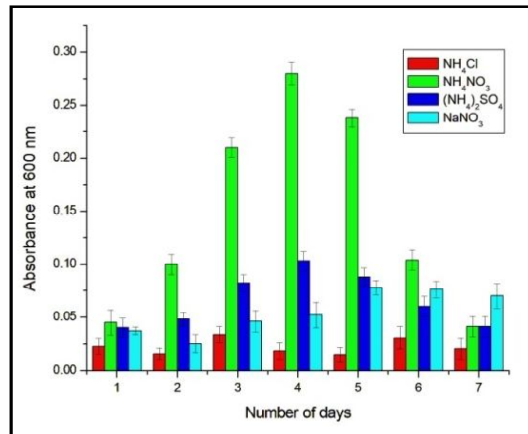


Figure 9. Effect of Nitrogen source on growth of D1

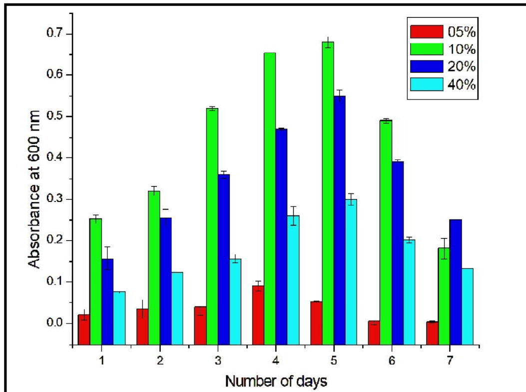


Figure 10. Effect of Carbon source on growth of D1

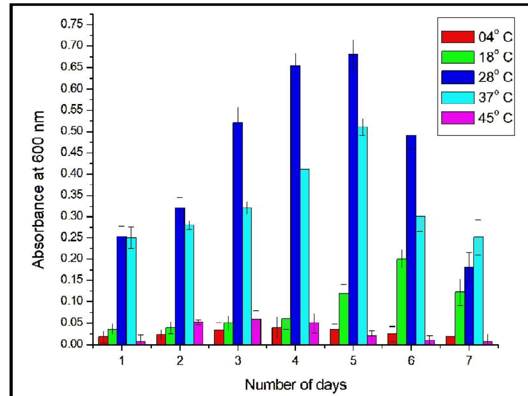


Figure 11. Effect of Temperature on growth

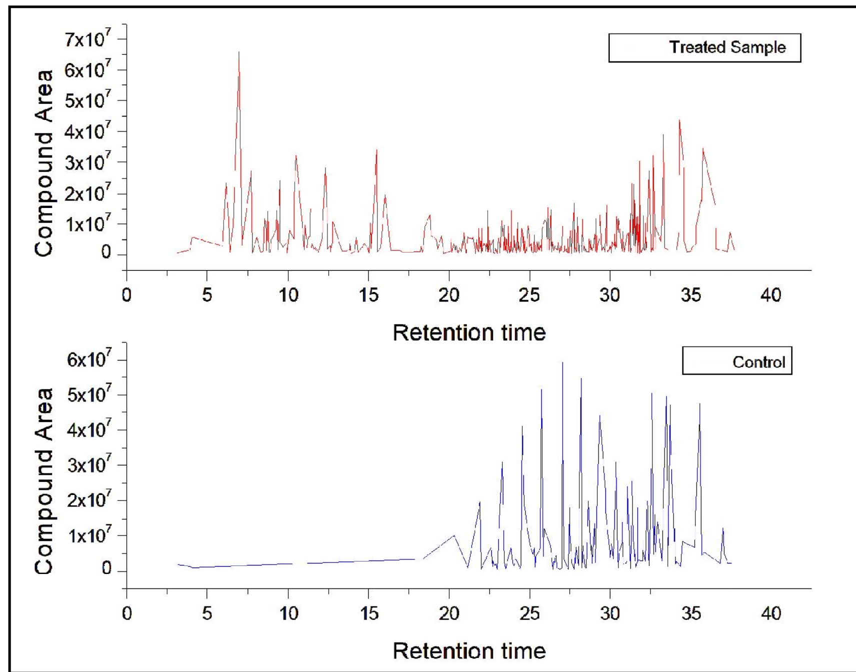


Figure 12. GC MS of Engine oil sample

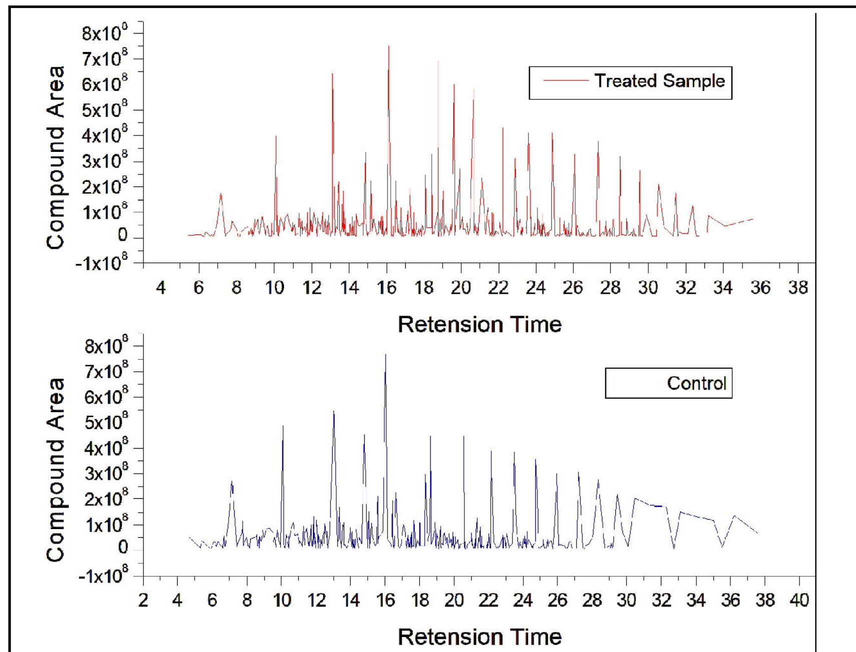


Figure 13. GC MS of Diesel control sample

Table-3. Metabolites detected after biodegradation of Diesel by *Bacillus flexus*

Component RT	Compound Name	Formula	Match Factor
12.3098	Naphthalene	$C_{10}H_8$	92.3
12.7601	Benzene, pentamethyl	$C_{11}H_{16}$	84.9
14.2348	1H-Indene 2,3 dihydro 4,7 dimethyl	$C_{11}H_{14}$	92.3
15.5050	Naphthalene,2-methyl	$C_{11}H_{10}$	93.6
16.0227	Naphthalene,2-methyl	$C_{11}H_{10}$	93.8
18.8026	Naphthalene 1,4 dimethyl	$C_{12}H_{12}$	96.0
20.2786	1,1 Biphynyl 4 methyl	$C_{13}H_{12}$	92.7
20.3474	Pentadecane	$C_{15}H_{32}$	91.9
20.3475	Dodecane 2 6 1 1 trimethyl	$C_{15}H_{32}$	91.8
20.5183	4 4 Dimethyl biPhenyl	$C_{14}H_{14}$	90.0
21.6403	Succinic acid 3Methyl but	$C_{22}H_{24}O_4$	90.0
27.9683	Phenanthrene 25 dimethyl	$C_{16}H_{14}$	91.3
27.6674	Dip tolylacetylene	$C_{16}H_4$	90.5

Table-4. Metabolites detected after biodegradation of Engine oil by *Klebsiella variicola*

Component-RT	CompoundName	Formula	Match Factor
10.5760	Benzene,1,2,4,5 tetramethyl-	$C_{10}H_{14}$	94.9
6.9420	Benzene, 1,2,4- trimethyl-	C_9H_{12}	94.7
31.4862	Tetracosane	$C_{24}H_{50}$	92.5
30.5549	Tricosane	$C_{23}H_{48}$	92.3
13.1131	Dodecane	$C_{12}H_{26}$	91.8
10.7287	Trans-4a- Methyl deca hydro naphthalene	$C_{11}H_{20}$	91.5
32.3620	Pentacosane	$C_{25}H_{52}$	91.4
18.6662	Naphthalene,1,5- dimethyl-	$C_{12}H_{12}$	91.1
8.5453	Benzene, 1methyl- 4propyl	$C_{10}H_{14}$	91.0
10.0982	Undecane	$C_{11}H_{24}$	90.3
7.1584	Decane	$C_{10}H_{22}$	90.1
33.1949	Hexacosane	$C_{26}H_{54}$	90.0

the effectiveness of hydrocarbon degradation by the isolated strains was assessed. E01 exhibited maximum biodegradation of engine oil (20% V/V), with 90% degradation rate (Table 1). In contrast, D1 demonstrated notable degradation of diesel (10% V/V), with a denaturation rate of 30% observed after a hatching period of 7 days. A culture-specific method was utilized to measure the hydrocarbon degradation efficacy of the identified strains. The Gas Chromatogram identified 43 chemical compounds in the referral sample; however, most of these components were remarkably absent in the denatured sample, as indicated by the lack of their respective peaks. This is robust evidence supporting the ability of *Klebsiella variicola* RUK1 to denature certain chemical components of crude oil. The microorganisms involved in oil biodegradation are frequently observed in aquatic and terrestrial ecosystems. These bacteria may be used in oil-based products as a carbon source of carbon due to their oil degrading capacity[18]. Present study intended to investigate the engine oil degrading potential of *Klebella variicola*. Qualitative analysis conducted using the Gas Chromatography techniques showed that several compounds were fully degraded, including 1,1 Biphenyl 4 methyl, Dodecane 2,6,1,1 trimethyl, Naphthalene, 1H-Indene 2,3 dihydro 4,7 dimethyl, Pentadecane, , Succinic acid 3-Methyl, Phenanthrene 25 dimethyl, 4,4 Dimethyl biphenyl and Diphenylacetylene, among others. The complex aromatic hydrocarbons served as a source of carbon for the growth and replication of microorganisms. The microorganisms gradually broke down each poly aromatic hydrocarbons to achieve absolute degradation (Table-3 and 4). Prior research assessed the bioremediation of crude oil using various bacterial isolates, including *Alcaligenes* sp. ASS-1, *Alcaligenes* sp. ASW-3, Pseudomonas *aeruginosa* isolate ASW-2, Exiguobacterium sp. ASW-1, and Bacillus sp. ASS-2, employing GC-MS methods[14] Analysis of crude oil degradation efficiency through GC-MS indicated that S*erratia proteamaculans* S1BD1 is the most effective strain, followed by *Alcaligenes* sp. OPKDS2 and *Rhodococcus erythropolis* OSDS1. Additionally, *Microbacterium* hydrocarbon oxydans showed high crude oil-degrading capability as exhibited by its high growth rate in crude oil-enriched medium[14].

Upon examining the chromatogram from the diesel degradation experiment using GCMS, Many peaks identified in the reference sample (Fig. 12 and 14). In contrast, when compared to the system inoculated with D1, many complex organic compounds, particularly those associated to peaks in the C9 to C23 range, were observed to have removed (Fig. 11 and 13). The molecular weights of the denatured products are detailed in Table 4. Previous research on hydrocarbon breakdown indicated that compounds within the C14 to C20 range were readily consumed and utilized by bacteria that degrade hydrocarbons as their primary carbon source[12]. Degraded metabolite identification was done based on NIST library matches. Upon evaluating the biodenaturation potential, *Bacillus flexus*, a marine isolate, achieved a complete degradation of 100% for compounds such as C_9H_20, C_2H_4 (Tricosane, Dodecane, Pentacosane, Undecane, Decane, and Hexacosane), as displayed in Table-4. The chromatogram revealed that many significant parent peaks completely vanished, with a noticeable reduction in intensity, thereby affirming the successful denaturation of the biosurfactant-generating EVR4. Poddar *et*

al.[13] studied hydrocarbon denaturation with a microbial assemblage consisting of *Enterobacter* sp., *Pantoea* sp., and *Klebsiella* spp., achieving a degradation rate of 4.05% per day, culminating in a total denaturation of 16.20% over four days. In contrast, our research demonstrated a higher degradation efficiency achieved in fewer days, highlighting the effectiveness of *Bacillus flexus*.

Klebsiella variicola RUK1 and *Bacillus fleuxs* RUK3 (*Priestia flexa* RUK3), when examined in laboratory, demonstrated the capacity to break down both engine oil and diesel by utilizing the oil components as source for growth and energy. Both isolates exhibited effective degradation capacity, making them potential bioremediation agents for oil-contaminated areas. This work is important since it offer valuable insights for utilising different bacteria as bioremediation agents. Further study is required to confirm the characteristics of the bio-emulsion produced by *Bacillus fleuxs* RUK3 (*Priestia flexa* RUK3), and *Klebsiella variicola* RUK1 and its impact on the ecosystem. Present study shows that the bacteria potent to degrade oil can be extracted from oil-polluted soil and can be used for microbial remediation purposes. The local oil-degrading bacteria extracted directly from oil-contaminated soil offers numerous benefits for the process of bioremediation. These bacteria of the locality are already adapted to the specific environment and the types of hydrocarbons they need to degrade, effectively leading to faster and more productive remediation. Results of the present study suggest that the isolated bacteria can be utilised for the biodegradation of oil contaminated soil and water.

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