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Targeting the potential hydrocarbon degrading bacteria from crude oil exploration well Hydrocarbon degrading bacteria from oil well

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ABSTRACT

Crude oil has been exploited for many decades as a prime energy source, providing a wide range of petroleum hydrocarbons. Their extensive use has led to environmental contamination by expressing their toxic effects on abiotic and biotic factors. It includes serious health effects such as biomagnification of TPH in marine organisms and damage to genetic, immunological, and endocrine systems in humans. During oil exploration drilling, bentonite slurry mixed with petroleum hydrocarbon is often left untreated at the drilling site. This slurry having multiple fractions of unknown petroleum hydrocarbons can cause a significant environmental issue. Bioremediation of petroleum hydrocarbon using indigenous bacterial flora of oil exploration wells can be a potential tool for restoring environmental sustainability. The present study focuses on the isolation of the indigenous bacteria from a 400-meter-deep soil layer as a part of drilling fluid in MSM and N-Agar media. Isolates were selected for their primary screening tests, including Tributyrin agar assay, Oil displacement method and Drop collapse method. A gravimetric test assessed their potential to degrade diesel in situ. Isolate A3 showed 35.9% degradation of diesel in 7 days.

Keywords: *Bioremediation, Bacteria, Crude oil, Diesel, Microbial degradation, Petroleum Hydrocarbon*

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INTRODUCTION

As energy needs increase, crude oil demand has risen to the top. Crude oil contains various petroleum fractions and has been exploited for various purposes. Crude oil has multiple valuable hydrocarbons and their need has been constantly increased. As a result, extensive crude oil exploration is needed, and this activity harbours the potential danger of causing environmental contamination (1). Additionally, crude oil processing involving processes such as transportation, refining, production and storage can also induce anthropogenic calamity due to accidental releases (2). When crude oil is released into the environment, their various fractions start reacting with the components of their respective ecosystems. Over time, they produce different intermediates or end-products depending upon their reaction. This phenomenon can be explained as a part of 'weathering'. A process of chemically and biologically induced changes in polluting petroleum hydrocarbons, called "weathering", can play a pivotal role in the remediation, which is dominated by microbial degradation (3). The weathering of petroleum hydrocarbons in the environment

encompasses physical (dispersion), physiochemical (evaporation, dissolution, sorption), chemical (photo-oxidation, auto-oxidation), and biological (plant and microbial hydrocarbon catabolism) impacts (4). It has been observed that the quantity of LMW (low molecular weight) in weathered contamination was substantially lower than the amount of HMW (high molecular weight) because LMW has a higher degradation rate than HMW (5). Petroleum hydrocarbons are hazardous in the short and long term. They have the potential to cause genetic and immunotoxic consequences (6). These effects include short-term effects such as nausea, skin irritation, and inflammation and long-term effects such as decreased immunity, neurological disorders and lung and stomach cancers in humans. Numerous volatile and hydrophobic aromatics, including benzene, indene, naphthalene, styrene, toluene, and xylene isomers, inhibited seed germination in some species. Natural weathering of a chemical from Petroleum hydrocarbons might result in by-products that are more phytotoxic and can cause plant stress (7). According to research on the accidental and purposeful discharge of gasoline and fuel oils into the aquatic environment, aquatic organisms can bioaccumulate specific TPH components, notably PAHs (8). Several alternative strategies have typically been utilized to eliminate pollution. Physical and chemical approaches such as membrane filtration, soil washing, adsorption, electrokinetics, thermal, oxidation, and photocatalytic treatments are examples of these techniques (9). Physical and chemical approaches for petroleum hydrocarbon pollution treatment are relatively costly and ecologically unsustainable compared to bioremediation (10). Bioremediation is a cost-effective and operationally advantageous clean-up approach that aims to accelerate the naturally occurring biodegradation of pollutants (11). Numerous strategies have been developed for the remediation of oil-contaminated sites; one of the best techniques for restoring soil is the introduction of bacteria capable of significantly reducing harmful compounds in a bioremediation process (12). Because leaks or spills frequently contain a variety of hydrocarbon molecules, their susceptibility to microbial degradation varies as follows: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (13). This study primarily focuses on the degradation of diesel. Diesel is composed of 75% aliphatic hydrocarbons ($C_{10}H_{20}$-$C_{15}H_{28}$) and 25% aromatic hydrocarbons (14). Using native bacteria obtained from an oil exploration well. Drilling was carried out 400 meters deep within the soil, and a slurry made of bentonite and drilling fluid was extracted. The major advantage of using native bacteria is their potential to utilize a wide range of hydrocarbons (15). With this hypothesis, the present work produced seven different isolates. These isolates were then screened by primary screening methods such as Tributyrin plate assay, drop collapse method and Oil spreading assay. Selected isolates were assessed for their degradation ability using gravimetric ability.

MATERIAL AND METHODS

Sampling

The sample was provided by ONGC Bhavan, Ahmedabad. The sample was collected from a depth of 400 meters. When collected, the sample was in the liquid phase and consisted of a slurry of drilling fluid and bentonite. The sample was collected in a sterile jar, transported to the laboratory for examination, and stored at 4°C.

Enrichment and Isolation from sample

In a sterile 250 ml Erlenmeyer flask, 1 g of sample sludge and 1% diesel as carbon source was aseptically added to 100 ml sterile MSM medium. The MSM, having g/L 2.0 KH_2PO_4, 7.0 K_2HPO_4, 1.0 $(NH_4)_2SO_4$, 0.1 $MgSO_4 \cdot 7H_2O$, 0.5 $CaCl_2 \cdot 2H_2O$, 0.52 $MnSO_4 \cdot 7H_2O$ and 2.5 $FeSO_4 \cdot 7H_2O$, was used and the pH was adjusted to 7.0 ± 0.2 before autoclaving. The flask was kept in shaking condition on a rotary shaker at 37 °C and 150 rpm for seven days. 5 ml aliquot was then transferred to 100 ml freshly prepared sterile MSM broth containing 1% diesel. This process was further repeated two times. After completion of 3 consecutive transfers, the broth was stored for further study at 4 °C. 100 µl aliquot from the enriched broth was serially diluted from the 10^{-1} to 10^{-8} and was spread onto sterile N-Agar plates. N-Agar plates were incubated at 37 °C for 24 - 48 h. The selection of bacterial colonies was carried out based on colony morphology. Bacteria from the developed colonies were then further subjected to Gram's staining and biochemical characterization.

Preservation of bacterial isolates

Pure and representative colonies were transferred to nutrient agar slants for preservation. The isolated strains were preserved in a 25% v/v glycerol solution at -20°C. Nutrient agar slants were also stored at 4 °C for routine usage. All the isolates were maintained regularly by subculturing.

Screening of isolates

Phenotypically distinct colonies were screened by primary screening methods such as Tributyrin agar method, Drop collapse method and Oil spread method.

Primary Screening

1. Tributyrin agar plate assay

Tributyrin Agar plate assay was used to evaluate isolated bacterial strains for their lipase activity. The experiment used a Tributyrin agar medium containing 1.0 % (v/v) olive oil. Strains were streaked on tributyrin agar plates and incubated for 24 hours at 37°C. The hydrolysis was detected by the formation of a clear zone surrounding the colony.

2. Drop Collapse Method

A drop of oil was placed on a transparent glass slide to test the bacterial synthesis of biosurfactants. As suggested by Ali *et al.* (16), the broth was centrifuged for 20 minutes at 5000 rpm, and the supernatant was decanted in a pre-sterile tube. A single drop of cell-free supernatant was put on an oil drop, and observations were made after one minute. When the structure of the drop changed to flat, the test was considered positive and similarly, when no changes were observed in the drop structure, the results were considered negative.

3. Oil Displacement method

This test was carried out to assess the production of biosurfactants. Because biosurfactants can alter oil solubility, oil is displaced as a result of the surface tension of biosurfactants. The area of oil displacement is in proportion to the concentration of biosurfactants. The zone of clearance within the oil layer confirms the presence of biosurfactants. As described by Habib *et al.* (17), in a clear glass petri plate, 20 ml of sterile distilled water was added. 300 µl of oil sample was added to form a layer of oil above water. Addition of 300 µl cell-free supernatant was added at the center of the oil layer. Zone formation within the oil layer was interpreted as a positive test for biosurfactant production. Isolates showing the promising results were selected for further quantitative test.

Quantitative analysis of hydrocarbon degradation activity

Each of the three isolates were then proceeded for their application in degradation of diesel. 1% of inoculum of selected three isolates was added in flasks having 50 ml sterile Bushnell Hass broth, having g/L 0.2 $MgSO_4$, 0.02 $CaCl_2$, 1.0 KH_2PO_4, 1.0 K_2HPO_4, 1.0 NH_4NO_3, 0.05 $FeCl_3$, with added 2% of diesel. A negative control having 100 ml Bushnell Hass broth and 2% diesel was kept in the same conditions to compare the evaporative loss caused. The flasks were incubated on a rotary shaker at 150 rpm at 37 °C for seven days. The procedure was carried out in a set of triplicates to reduce personal and random errors. After seven days of incubation, 20 mL of n-hexane was added to the medium in a separating funnel. The mixture was mixed well and allowed to stand for 15-20 minutes to form separate layers. The upper layer consisting of n-hexane and diesel was taken in a pre-weighed beaker. This beaker was kept in a hot air oven at 65 °C to evaporate n-hexane. The residual oil amount was then measured by weighing the beaker. The amount of oil degraded in percentage was then measured by the following formula:

Weight of residual diesel: Weight of beaker having freshly extracted diesel – Weight of beaker after evaporation.

Amount of diesel utilization: Weight of diesel added in media – Weight of residual diesel obtained after evaporation.

$$\% \text{ diesel degradation} = \frac{\text{Amount of degraded diesel}}{\text{Amount of diesel added in media}} \times 100$$

RESULTS AND DISCUSSION

Bacteria are the principal degraders and most efficient organisms in the degradation of petroleum. Multiple bacteria have even been shown to thrive only on hydrocarbons. Based on the ability of bacteria to utilize hydrocarbons, they have been isolated from samples retrieved from a crude oil exploration well.

Isolation of Hydrocarbon degrading bacterial strains:

Bacteria present in the sample can utilize hydrocarbons as their sole energy source, mainly observed while they are grown in a hydrocarbon-rich environment. Culturable bacterial strains were isolated from the sample using an enrichment culture technique. It was carried out using MSM broth containing 1% diesel as the sole carbon source, as reported by Joy *et al.* (18). Mittal and Singh isolated eleven hydrocarbon-degrading bacteria from the Lingala oil field, ONGC (19). Similarly, five bacterial isolates from hydrocarbon-contaminated sites expressed hydrocarbon-degrading activity (20). Thirteen phenanthrene-degrading bacteria were isolated in a study carried out by Thomas *et al.* (21). Post enrichment, isolation was carried out on sterile N-Agar plates by serial dilution method. Seven dominant and distinct colonies were isolated from the sample. Isolated strains were preserved in 25% v/v glycerol solution at -20 °C and stored at 4 °C on N-Agar slants for routine use.

Macroscopic and microscopic examination:
Bacterial isolates were grown in a Nutrient Agar (NA) medium and incubated for 24 hours. The morphological characteristics of colonies were observed. 1000X magnification was used to examine the cell shape and Gram's characterization of each isolate under the microscope.

Biochemical characterization:
Biochemical techniques such as the Indole production test, Methyl red test, Vogus-Proskauer test, Citrate utilization test, Catalase test, and Gelatinase test were used to characterize the isolates. Tables 1.0 and 2.0 show the colony morphology, Gram staining and biochemical characterization.

Screening of Isolates:

Tributyrin agar plate assay: Tributyrin agar plate assay was carried out (22). This assay was carried out for all seven isolates. From seven isolates, five were showing positive results by exhibiting a zone of hydrolysis around the colony. The zone diameter (mm) was measured, and the results are as described in table 3.0. Similar study was also carried out by Balakrishnan *et al.* (23), where the same test was used as primary screening test.

Oil displacement test: This test was carried to detect potential biosurfactant production from isolates. Upon addition of cell free supernatant, a formation of zone considered as positive result and zone diameter was measured. For positive and negative control, SDS and uninoculated MSM were used respectively (23). The concentration of promising biosurfactant production is related to the degree of oil displacement. Four of the seven isolates, A2, A3, A5, and A6 had positive results. In the current investigation, Isolate A3 had a maximum zone diameter of 30 mm. In a similar study by Ibrahim *et al.* the isolates had a comparatively high zone diameter of 27-51 mm (24).
Table 3.0 summarises the findings of the current investigation.

Drop collapse test:
The drop collapse test is a rapid determinative test confirming surface-active compounds' presence or absence. The principle behind this method includes reducing the surface tension of oil droplet through biosurfactant activity. The unstable effect of the bacterial surfactant on oil droplets is tied to the surfactant's activity and its surface tension (25). In the present study, 4 out of 7 isolates showed positive results, as presented in Table 3.0. In a study by Kurniati *et al.* 13 out of 20 isolates showed positive results (26).

Table 1.0 – Colony morphology and Gram's staining characteristics of isolates

	A1	A2	A3	A4	A5	A6	A7
Size	Small	Small	Medium	Medium	Small	Medium	Big
Shape	Round	Round	Round	Round	Round	Circular	Circular
Margin	Entire	Entire	Undulate	Entire	Entire	Lobate	Undulate
Elevation	Raised	Raised	Slight raised	Flat	Convex	Raised	Convex
Texture	Smooth	Mucoid	Mucoid	Moist	Moist	Filamentous	Moist
Opacity	Translucent	Opaque	Opaque	Translucent	Opaque	Opaque	Opaque
Pigmentation	Yellowish white	White	Yellowish white	White	White	White	White
Gram's staining	Gram Positive rods	Gram Negative rods	Gram Negative rods	Gram Negative rods	Gram Positive short rods	Gram Negative rods	Gram positive rods

Gravimetric analysis:
Based on the results obtained from screening tests, three isolates, namely A3, A5, and A6, were selected for further study. Bacteria use hydrocarbons for nourishment, growth, and biomass synthesis. The amount of diesel utilized within seven days was measured gravimetrically, and the decrease in diesel volume was noted. A control flask with sterile BHMS broth with 2% diesel was kept to assess the evaporative loss caused during seven days at the same condition. In the present study, isolates A3, A5, and A6 were subjected to 2% diesel concentration, which reflected positively in 35.9%, 24.57%, and 8.6% diesel degradation, respectively. The results were further optimized statistically by calculating the mean degradation and standard error. The results are displayed in Table 4.0 and Figure 1.0. According to Panda *et al.* the maximum diesel degradation detected after 15 days of incubation was 53% when cultivated with 0.5% diesel concentration in BHMS medium (27).

Table 2.0 - Biochemical characterization of isolates

Test	A1	A2	A3	A4	A5	A6	A7
Methyl Red test	Positive	Negative	Negative	Positive	Negative	Negative	Negative
Vogus-Proskauer test	Negative	Negative	Negative	Negative	Negative	Negative	Positive
Indole production test	Positive	Negative	Negative	Positive	Negative	Negative	Negative
Citrate utilization test	Negative	Positive	Positive	Negative	Negative	Positive	Positive
Gelatin hydrolysis test	Positive	Negative	Positive	Negative	Negative	Positive	Positive
Catalase test	Positive	Positive	Positive	Positive	Positive	Positive	Positive

Table 3.0 – Results of Oil displacement test and Drop collapse assay

	A1	A2	A3	A4	A5	A6	A7
Tributyrin Agar Assay	-	+	+	+	+	+	-
Zone of oil displaced (mm)	-	12	30	-	21	19	-
Drop collapse Test	-	-	+	-	+	+	-

(+) Positive; (-) Negative

Table 4.0 – Results of Gravimetric analysis

Sample	Weight of empty beaker (g)	Weight of Beaker (Hexane + Oil) (g)	Weight of Beaker containing Oil (g)	Amount of Residual Oil (g)	Amount of Oil Degraded (g)	% of Degradation	Mean	Standard error	Actual degradation (observed degradation – loss by
A3	48.231	61.581	48.906	0.675	0.324	36.6%	39.33%	2.80	35.93±2.80%
	44.329	58.716	44.955	0.626	0.373	42.2%			
	49.634	56.912	50.328	0.694	0.347	39.2%			
A5	46.724	57.632	47.504	0.780	0.219	24.8%	27.97%	2.76	24.57±2.76%
	47.861	55.481	48.602	0.741	0.258	29.2%			
	49.027	58.357	49.762	0.735	0.264	29.9%			
A6	47.272	60.365	48.169	0.897	0.102	11.6%	12.00%	2.12	8.6±2.12%
	49.977	62.248	50.850	0.873	0.126	14.3%			
	46.144	58.934	47.054	0.910	0.089	10.1%			

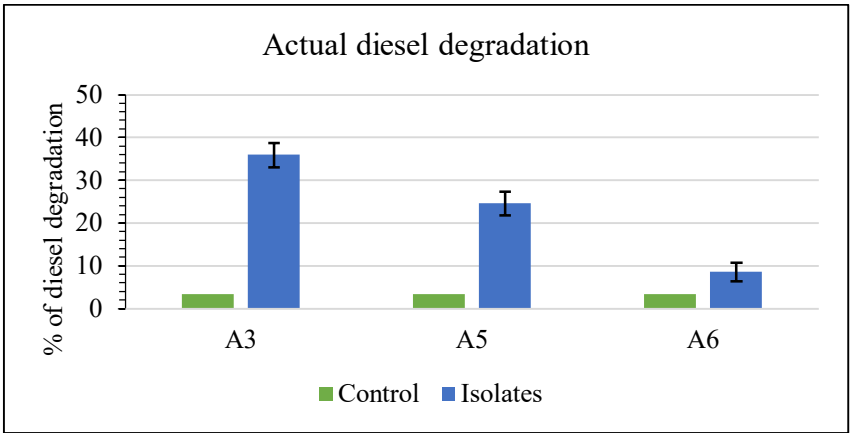

Figure 1.0 – Actual diesel degradation by isolates

CONCLUSION

According to the present study, three potent bacterial isolates are expressing potential hydrocarbon degradation activity out of several different bacteria isolated from a 400-meter-deep soil layer. Moreover, the ability of these isolates to adapt to a hydrocarbon-containing environment can be relatively higher than other bacteria. Upon application of three distinct isolates to BHMS media with 2% diesel for seven days at 37 °C with shaking conditions of 150 rpm, a maximum of 35.93% diesel degradation is observed.

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Conflict of Interest

The authors state that the publishing of this work does not include any conflicts of interest.

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