

Isolation of Potential Hydrocarbon-degrading Microorganisms to Enhance Biodegradation in Hydrocarbon-contaminated Soils

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Abstract

Oil contamination is a global concern. Bacteria that use petroleum oil and hydrocarbons for growth, nourishment, and metabolic processes are more prevalent in oil-contaminated environments. The bacteria isolated in this study were characterized using morphological and biochemical tests. Gram-positive rod-shaped isolates were identified morphologically at 37°C and pH 7.0, under ideal growth conditions. Our results demonstrated the ability of colony-07 to degrade crude oil. Through physicochemical optimization, biochemical analysis, and oil degradation studies, potential isolates of colony-07 were screened and demonstrated an exceptional ability to remove oil contamination. In our study, colony-07 showed 63.6% degradation efficacy towards crude oil, and 16S rRNA sequence analysis showed that the isolates shared a close relationship with *Bacillus aerius* strain 24 K and *Bacillus altitudinis* strain 41KF2b. These findings imply that colony-07 could potentially be used for bioremediation purposes to clean crude oil-polluted areas.

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INTRODUCTION

Petroleum hydrocarbons are valuable energy sources and raw materials in various industries. Because appropriate alternatives are currently unavailable, the rising demand for petroleum products in everyday life may lead to scarcity and increased expenses.^{1,2} Priority pollutants include persistent petroleum hydrocarbons (PHCs), which are persistent. Petroleum hydrocarbon contamination is caused by anthropogenic activities such as industrial and municipal runoff, effluent discharge, offshore and onshore petroleum industry operations, and accidental discharge during transportation. Pollution harms ecosystems and poses direct and indirect health risks to all life forms on Earth.³ Petroleum hydrocarbons include carbon, oxygen, hydrogen, nitrogen, and sulfur. Colloid, asphaltene, saturated hydrocarbons, and aromatic hydrocarbons are the four main components of TPH that are dangerous and slow for soil bacteria to break down. Petroleum-contaminated soils

often have poor nutrient levels, porosity, and microbial populations, which restrict the effectiveness of microbial decomposition.⁴ Many traditional engineering-based physicochemical decontamination techniques such as soil washing and chemical inactivation (using hydrogen peroxide and/or potassium permanganate as chemical oxidants to mineralize non-aqueous contaminants such as petroleum) are available; however, they are expensive because of the need to excavate and transport significant quantities of contaminated materials for ex situ treatment.^{5,6} Owing to their high costs, alternative technologies based on biological remediation using microorganisms have emerged, limiting the use of more conventional methods. Through the use of microorganisms, bioremediation is a novel approach that converts harmful organic pollutants into harmless substances like CO₂, CH₄, H₂O biomass without having an unfavourable effect on the environment.⁷ The major factors in bioremediation are the quantity of microbes, soil nutrients,

and the O₂ status. The contaminated areas are rich in local microorganisms that participate in biodegradation.

Recent studies have elucidated the existence of over 79 genera of bacteria capable of metabolizing the hydrocarbons present in petroleum.⁸ Oil-rich environments, such as spill sites and reservoirs, harbor a substantial population of bacteria capable of hydrocarbon degradation, as demonstrated by numerous studies. The abundance and distribution of these microorganisms are directly correlated with the specific types of petroleum hydrocarbons present and the environmental factors that influence them.⁹ Research has demonstrated that multiple bacterial species play crucial roles in the degradation and remediation of petroleum hydrocarbons. These included *Flavobacterium spp.*¹⁰ *Acinetobacterium calcoaceticum*, *Citrobacter freundii* (MRC3),¹¹ and *Pseudomonas aeruginosa*.¹² These microorganisms have been identified as crucial components for hydrocarbon degradation and remediation. The aim of the present study was to identify microbial species capable of biodegrading crude oil in contaminated soil environments.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected from different sites in Lucknow: the Palka Petrol Pump, Paikramau, Rajeev Petrol Pump, Basha, and from a motor mechanic shop near Integral University, Lucknow. Soil samples were collected in sterile polybags at depth of 2–2.5 inches beneath the surface. The extracted soil exhibited black coloration. Furthermore, a reference sample was obtained from Shakeel's Mechanic Shop, located in proximity to Integral University. This sample comprised two-month-old used engine oil that exhibited brownish-black coloration. The complete set of specimens was subsequently conveyed to the research facility for comprehensive analytical examination.

Isolation of Bacteria from Oil Contaminated Soil

Bacterial isolation was performed using serial dilutions. 50 mL solution of normal saline (0.85% NaCl) was prepared, and 50 mL was transferred to a test tube labeled first. From this, 4.5 mL was distributed into test tubes labeled 10⁻¹–10⁻⁶. 0.5 mL aliquot of the solution was transferred to a 10⁻¹ tube. Next, 0.5 g of soil was dissolved in the first tube, and serial dilutions were performed by transferring 0.5 mL from one test tube to the next. Finally, 50 µL of the inoculum from each serially diluted tube was spread on nutrient agar (NA) plates, which were incubated at 37°C for 24 h in an inverted position.¹³

Purification and Colony Morphology Study of Obtained Mixed Culture

Bacterial colonies grown on agar plates were analyzed for morphology, which provides key information for organism identification. Key criteria included colony shape (circular, rhizoidal, spindle, filamentous, punctiform, irregular), margin

(entire, discrete, curled, lobate, filamentous), elevation (flat, raised, convex, pulvinate, umbonate), texture (shiny, dull, smooth, wrinkled, rough, granular, mucoid), opacity (transparent or opaque), and pigmentation (yellow, off-white, white). These characteristics help to distinguish between different bacterial colonies. These physical characteristics were studied to identify them. Purification of the mixed culture ensured that single colonies grew from individual bacterial cells, which helped obtain a pure culture for further screening. In this procedure, 120 mL of NA was prepared, autoclaved, poured into sterile petri plates, and incubated at 37°C for 24 h.¹⁴

Screening of Purified Cultures for Oil Degradation Potential

The screening for oil-degrading bacteria was performed using the pour-plate method. NA medium (120 mL) was mixed with 20 mL of crude oil and poured into sterile petri dishes after autoclaving. Eight bacterial colonies, labeled Colony 1 through Colony 8, were streaked onto the plates. The plates were incubated at 37°C for 72 hours.¹⁵

Biochemical Characteristics of Purified Isolates

Biochemical tests, including catalase, indole, methyl red, starch hydrolysis, and gram staining, were conducted following standard procedures to identify unknown bacterial cultures. The experiments were conducted in accordance with the protocols outlined in Bergey's Manual of Systematic Bacteriology, which provides guidelines for identifying microorganisms based on staining characteristics and biochemical behaviour.¹⁶

Optimization of physicochemical factor for optimum growth of isolate colony 07

Effect of Different Carbon Source Concentration

Nutrient broth (NB) media was prepared in three flasks with varying concentrations of NaCl as the carbon source: 5 g/L (Flask-1), 3 g/L (Flask-2), and 7 g/L (Flask-3). All flasks were adjusted to the optimum pH, autoclaved, and supplemented with 5 mL of used engine oil. Pure culture Colony-07 was inoculated into each flask, and one flask was used as a control without inoculation. The flasks were incubated in a shaker at 37°C for 48 h. After incubation, absorbance was measured at 620 nm.

Effect of Different of Nitrogen Source Concentration

NB media were prepared in five flasks by varying the nitrogen source concentration with ammonium chloride at 2 g/L (Flask-1), 4 g/L (Flask-2), and 6 g/L (Flask-3). All flasks were adjusted to the optimum pH, autoclaved, and supplemented with 5 mL of used engine oil. Pure culture Colony-07 was inoculated into each flask, and one flask was used as a control without inoculation. The flasks were incubated in a shaker at 37°C for 24 h. After incubation, absorbance was measured at 620 nm.¹⁵

Determination of Oil Degradation Potential by Shaker Flask Method

Screening was performed in minimal salt medium (NB medium supplemented with 5% crude oil), with a limiting amount of carbon source in the form of glucose. Mineral salt medium (MSM) and NB (100 mL) were prepared and supplemented with various concentrations of carbon and nitrogen sources, respectively. The cultures were incubated in a shaker at 37°C and 180 rpm for 24 hours. After incubation, fresh MSM and NB were prepared as controls and the optical density (OD) at 620 nm was measured. OD measurements were also taken on the 1st, 2nd, 3rd, 4th, 5th, and 6th day.¹³

Calculation of Oil Degradation Percentage of Colony-07

NB (100 mL) was prepared, autoclaved, and supplemented with 5 mL of engine oil. The medium was inoculated with 1 mL of Colony-07 culture and incubated in a shaker incubator at 37°C for five days. After the incubation period, the remaining oil was extracted from the flask, and its volume was measured. The percentage of oil degradation was calculated using a standard formula, providing an assessment of the oil-degrading efficiency of Colony-07.

The percentage of oil degradation was calculated using the following formula:

$$\% \text{ Oil degradation} = \frac{(\text{Volume of oil on zero day} - \text{Volume of oil on 5th day})}{\text{Volume of oil on zero day}} \times 100$$

16S-rRNA sequencing for identification of pure culture colony-07

To meet our sequencing needs, we selected Biokart India Pvt. Ltd. as the service provider. Subsequently, the culture plate

was transferred to a facility inside a container maintained at 4°C.¹³

RESULTS AND DISCUSSION

Isolation of Microorganism from Oil Contaminated Soil Sample

Oil-contaminated soil was found to be the optimal inoculum source for isolating crude oil-degrading bacteria.¹⁴ Samples were collected from the contaminated soil at several locations, serially diluted, and transferred to nutrient agar plates (figure. 1 (1)). Bacterial colonies were grown on medium after 24 hour. of incubation at 37°C. At pH 7.0, the isolates showed the highest growth rate. The ideal temperature for the growth of the isolated bacteria was 37°C. The aim of the optimization process was to determine the ideal growth conditions for the isolates to maximize bacterial growth at the lowest possible cost for use in contaminated sites. Colonies were separated based on their morphological characteristics using the streak and spread-plate methods (Table 1). The isolates included in this study were screened, and further tests were performed to determine their ability to break down the oil.

Purification of Obtained Mix Culture

Purification of the mixed culture ensures that single colonies grow from individual bacterial cells, which helps to obtain a pure culture for further screening.¹⁴ Successive dilution and nutrient agar plating allowed for the growth of well-isolated colonies during a 24-hour incubation period after the first enrichment. To ensure the isolation of specific bacterial strains, the colonies were successfully purified by streaking on fresh

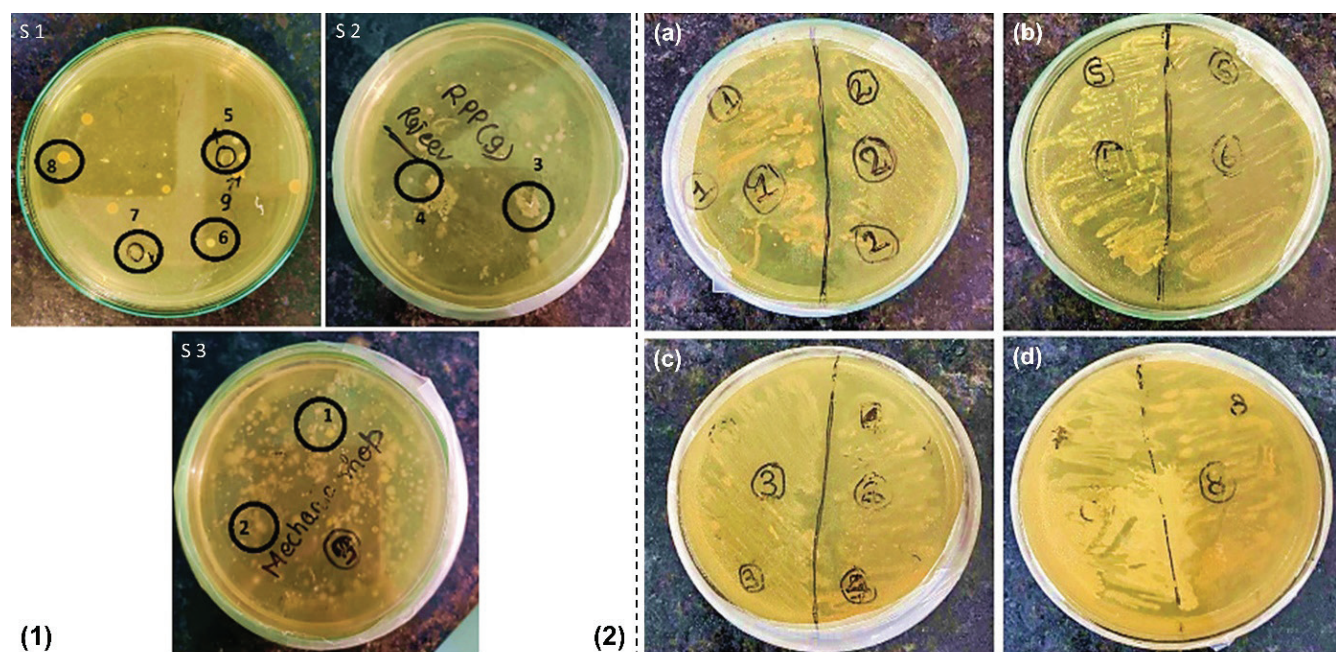
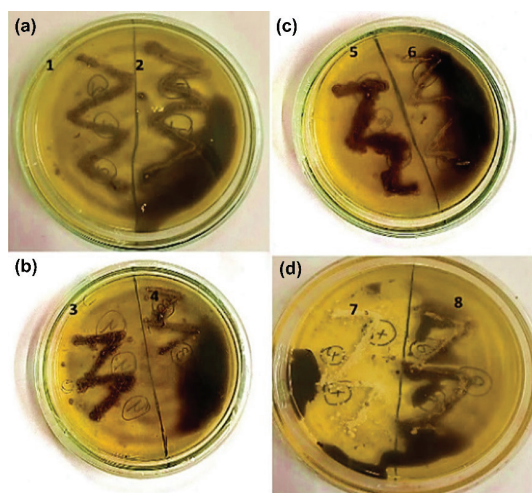


Figure 1: (1) Isolated colonies of three Samples, and (2) Pure culture plates.

Table 1: Colony morphology of selected cultures (Sample-1, 2, 3).

	Sample-1				Sample-2		Sample-3	
Morphology	Colony-05	Colony-06	Colony-07	Colony-08	Colony-04	Colony-03	Colony-02	Colony-01
Shape	Round	Oval	Round	Circular	Round	Discrete	Circular	Circular
Margin	Entire	Discrete	Entire	Entire	Entire	Braked	Entire	Entire
Colour	Light yellow	Off-white	Stone white	Off-white	Yellow	Off white	Orange	Off-white
Surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Opacity	Translucent	Translucent	Translucent	Translucent	Translucent	Translucent	Translucent	Translucent

**Figure 2:** Screening of isolated colonies for oil degradation potential. [Note: S1, S2, S3: Sample-1, Sample-2, Sample-3]

agar plates during additional subculturing, and pure cultures that underwent subsequent testing for biochemical activity (Figure. 1 (2)). Purified isolates, which are thought to be oil-degrading species, are now ready for further molecular characterization and functional tests to confirm their role in the process and to assess their potential.

Screening of Isolates for Potential Degradation of Crude Oil

Oil-contaminated soils are the main sources of petroleum-degrading bacteria. Previous studies have revealed that various bacterial species are capable of oil degradation, and three bacterial isolates—*Flavobacterium sp.*, *A.calcoaceticum*, and *P. aeruginosa*—can use engine oil as a carbon source. They demonstrated that these isolates could utilize 80 and 90% of the used engine oil in a 4-week period. The optimal temperature for biodegradation is 30–37°C. This study suggests that these isolates can be used for in situ and ex situ oil bioremediation. The current investigation employed Nutrient agar plates supplemented with crude oil were used to screen for purified bacterial cultures.¹⁵ Purified bacterial colonies, labeled Colony-01 to Colony-08, were streaked onto oil-supplemented plates. In figure 3a, b, it was clear that colony-01, 2, 5, and 6 have no impact on crude oil; similarly, in figure 3c, there is no zone of inhibition for crude oil, but in figure 3d, Colony-07 has a clear zone of inhibition and great

impact on crude oil. This suggests that the isolate has a high ability to degrade crude oil. However, colony-08 also gives a zone of inhibition but is not as effective as colony-07, while other colonies do not have any degrading capability and zone of inhibition towards crude oil. With this screening, we found that only Colony-07 was capable of degradation and further experiments were performed on Colony-07.

Biochemical Characteristics of Purified Culture Isolates

All isolated bacterial strains underwent biochemical analysis following the procedures outlined in Bergey's Manual of Systematic Bacteriology.¹⁶ Biochemical tests were performed to identify eight colonies, and the results showed that these colonies were significantly different from each other. In addition, these eight colonies were catalase-positive, indicating that they were able to detoxify hydrogen peroxide and utilize citrate as the carbon source, whereas these colonies were indole and methyl red-negative. The results of biochemical analyses of the bacterial colonies are summarized in Table 2.

Optimization of Physicochemical Factor for Optimum Growth of Isolated Colony-07

Oil-degrading organisms that use hydrocarbons as their primary source of energy, carbon, and nitrogen are exceedingly diverse and widespread in nature.¹⁷ Physicochemical factors for optimum growth of the isolated Colony 07 strain were optimized by evaluating the effects of different carbon and nitrogen source concentrations on bacterial growth. Following incubation, spectrophotometric analysis indicated that Flask-1 (5 g/L NaCl) exhibited the highest absorbance, suggesting the optimal growth of Colony-07 at this NaCl concentration. Flask-2 (3 g/L) and Flask-3 (7 g/L) exhibited lower growth rates, indicating that the concentration of the carbon source significantly influenced the bacterial proliferation. Different concentrations of ammonium chloride as a nitrogen source also revealed that the highest growth occurred in Flask-1 (2 g/L), indicating that this concentration of nitrogen was the most favorable for the growth of Colony-07. Flasks with 4 and 6 g/L of nitrogen showed lower absorbance values, suggesting that an optimal balance of the nitrogen source is critical for bacterial growth (figure. 3 (1)).¹⁵

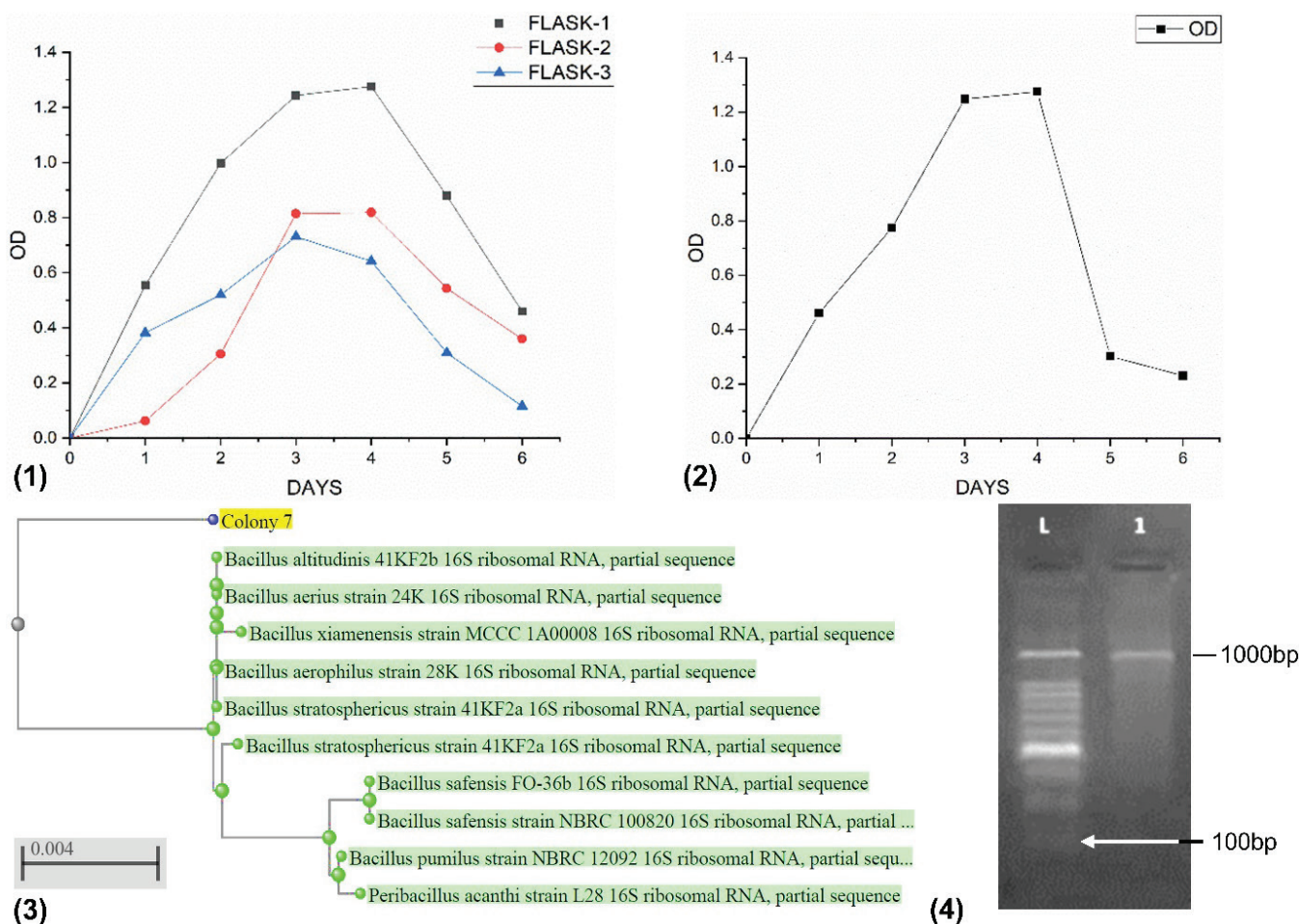


Figure 3: (1) Growth curve of purified culture isolate (colony-07) at different concentration of carbon and nitrogen source. **(2)** Growth curve and crude oil removal by colony-07. Optical density (OD₆₂₀) of colony-07 in MSM containing 5% crude oil. **(3)** Phylogenetic tree colony-07 strains based on 16S-rRNA sequences. **(4)** PCR product of olony-07.

Table 2: Biochemical analysis of purified culture isolates.

Tests	Colony-01	Colony-02	Colony-03	Colony-04	Colony-05	Colony-06	Colony-07	Colony-08
Catalase	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Indole	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Methyl red	Negative	Negative	Positive	Negative	Negative	Negative	Negative	Negative
Starch hydrolysis	Negative	Negative	Positive	Positive	Positive	Positive	Positive	Negative
Gram staining	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Negative

Screening of Oil Degradation Potential (in percent)

Screening of microbial isolates for crude oil degradation is crucial for bioremediation, optimization of degradation rates, and reduction of environmental impact. Previous studies of six isolated strains shows the oil degradation rates, HDB-1, HDB-2, HDB-3, HDB-4, HDB-5, and HDB-6 were 45.76%, 40.52%, 37.88%, 43.29%, 54.01% and 49.48%, respectively.¹³ In this study, flask 1 supplemented with NH₄Cl (2 g/L) and NaCl (5 g/L) showed the best growth. Colony-07 pure culture isolates were used as the inoculants. Five millilitres of crude oil were then added to the flask and incubated in a shaker incubator at 37°C. On the initial day, colony-07 did not exhibit

a significant optical density (OD), potentially because of its adaptation to the novel environment. After two days, colony-07's OD levels significantly rose, indicating its ability to break down crude oil. After six consecutive days of OD at 620 nm, the crude oil was removed from the flask and its volume was measured. After six days, there was 1.82 mL of crude oil was left up from the initial volume of 5 mL. Overall, 63.6% of the oil was degraded (Figure. 3 (2)). Thus, the noteworthy results of our investigation indicate that degraded crude oil, which is not utilized further, builds up and pollutes the environment and can be eliminated by colony-07 from the planet. Therefore, colony-07 contained the highest number

Table 3: FASTA BLAST data retrieved from NCBI, the results of colony-07 strains based on 16S-rRNA sequences.

S. No.	Organism Name	Accession No.	% Match
1	<i>B. aerius</i> strain 24K 16S ribosomal RNA	NR_118439.1	98.21%
2	<i>B. altitudinis</i> 41KF2b 16S ribosomal RNA	NR_042337.1	98.21%
3	<i>B. aerophilus</i> strain 28K 16S ribosomal RNA	NR_042339.1	98.21%
4	<i>B. stratosphericus</i> strain 41KF2a 16S ribosomal RNA	NR_042336.1	98.21%
5	<i>B. xiamenensis</i> strain MCCC 1A00008 16S ribosomal RNA	NR_148244.1	98.14%
6	<i>B. stratosphericus</i> strain 41KF2a 16S ribosomal RNA	NR_118441.1	98.14%
7	<i>P. acanthi</i> strain L28 16S ribosomal RNA	NR_179899.1	97.95%
8	<i>B. safensis</i> FO-36b 16S ribosomal RNA	NR_041794.1	97.78%
9	<i>B. pumilus</i> strain NBRC 12092 16S ribosomal RNA	NR_112637.1	97.78%
10	<i>B. safensis</i> strain NBRC 100820 16S ribosomal RNA	NR_113945.1	97.78%

of oil-degrading bacteria found in nature. This study also demonstrated the isolation of these bacteria from the oil-contaminated sites. We strongly suggest the use of colony-07 isolates for bioremediation to remove crude oil from contaminated areas.

16S-rRNA Sequencing of Colony-07

16S rRNA sequencing is considered the best genetic procedure for identifying oil-degrading bacteria.¹⁸ Based on morphological, biochemical, and phylogenetic analyses, a BLASTN search revealed that microbes possess 98.21% identity with *B. aerius* strain 24 K and 98.21% identity with *B. altitudinis* 41KF2b. The sequences have been deposited in GenBank. The GenBank accession numbers are NR_118439.1 (for *B. aerius* strain 24 K) and NR_042337.1 (for *B. altitudinis* 41KF2b) (Table 3), and figure. 3 (3, 4) shows the constructed phylogenetic tree.

CONCLUSION

The aim of this study was to isolate and identify potential hydrocarbon-degrading microorganisms in oil-contaminated soils to enhance biodegradation. Soil samples were collected from various sites in Lucknow and bacteria were isolated using serial dilution and plating techniques. Morphological and biochemical tests were performed to characterize the isolates. Colony-07 showed exceptional crude oil degradation ability, with a degradation efficacy of 63.6% after six days of incubation. Physicochemical optimization revealed that Colony-07 exhibited optimal growth at 5 g/L NaCl and 2 g/L ammonium chloride concentrations. 16S rRNA sequence analysis identified Colony-07 as being closely related to *B. aerius* strain 24 K and *B. altitudinis* strain 41KF2b. These findings suggest that Colony-07 could potentially be used for the bioremediation of crude-oil-polluted areas.

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