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EVALUATION OF 4T ENGINE OIL DEGRADATION POTENTIAL OF *STUTZERIMONAS BALEARICA* STRAIN IMB16-157 ISOLATED FROM PETROLEUM-CONTAMINATED SOIL OF THAR DESERT OF RAJASTHAN

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Abstract
Microbial degradation of 4T engine oil by *Stutzerimonas balearica strain* IMB16-157 is explored, revealing significant alterations in hydrocarbon composition based on carbon chain length and area percentage reduction. *Stutzerimonas balearica* is isolated from the petroleum-contaminated soil of the Thar desert of Rajasthan. The original engine oil contained short (C_6-C_{10}) and long-chain hydrocarbons (C_{30}-C_{40}) as prominent constituents with notable area percentages. Exposure to *Stutzerimonas* resulted in a reduction in the area percentage of short-chain hydrocarbons like Hexane, 2-chloro- (C_6), with the emergence of chlorinated derivatives, indicating microbial activity. It has degraded 4T engine oil up to 78.6 % after 21 days of incubation. These results emphasize the dynamic nature of microbial degradation, offering insights into its potential for bioremediation and resource management in hydrocarbon-rich environments.
Keywords: *Stutzerimonas balearica,* contaminated, bioremediation, Hydrocarbon.

1. Introduction

The petroleum sector has contributed significantly to the world economy, but as is customary, it also negatively affects our ecosystem. The pollution that results from the refining, storing, and transport of petroleum products is a global issue (Yusuf et al. 2021). As magnificently big industrial complexes, petroleum refineries are linked to sizable chemical processing and production facilities by inland and outlying pipes that carry a variety of fluid streams. Since these pipelines are underground, they occasionally traverse water bodies as well as agricultural land. There is a significant risk of inadvertent spilling while moving oil from the point of production to different consumption sites (Zhu et al. 2022). Accidental spillage has a significant impact on the marine ecosystem's food web and food chain.

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According to Rehman et al. (2022), soil is crucial in solving global challenges like the production of food and fiber, freshwater availability, biodiversity preservation, greenhouse gas mitigation, energy sustainability, and ecosystem service provision. Soil resources need to be enhanced and protected in order to tackle these worldwide concerns. Since soil conditions are very dynamic, anthropogenic factors and climate change both have the potential to hasten soil transformation. We collected contaminated soil samples for this investigation from the Thar Desert, Rajasthan, India.

The fertility of the soil, which was severely damaged with PAHs, can be restored using certain technologies. One natural method for removing hazardous chemicals from the environment is bioremediation, which relies on microorganisms (Amran et al. 2022). Petroleum hydrocarbons can cause contaminated places, which can be cleaned up by microbial species. According to Mastropetros et al. (2022), microorganisms frequently transform these chemical molecules into energy, cell mass, and biological waste materials.

The goal of the current experiment was to locate and identify the microorganisms from the desert soil responsible for decomposing petroleum hydrocarbons. The main goal of the inquiry is to evaluate each microbial isolate's biodegradation capacity.

2. Material and Methods

Soil Collection

The soil sample was collected from automobile workshop sites located in the Thar desert of Rajasthan. The samples were dug from a trowel from 0 to 20 cm below the topsoil surface. In order to remove plants or other waste residues the soil gets air dried and sieved through 200 mesh sieves. The soil samples were then stored in sample storage bags and kept in the refrigerator till further analysis.

Isolation and Screening of hydrocarbon utilizing bacterial strain

The enrichment culture technique was used to isolate petroleum hydrocarbon-degrading microbes. Bushnell Haas Media was used for the isolation of pure culture and 1% (v/v) 4T engine oil was used as a carbon and energy source (Bushnell and Haas 1941). For screening, 1 gm of contaminated soil samples was suspended and vortexed in 10 ml of sterile distilled water. After that 1ml of the solution was taken out and used as inoculum for the isolation of oil-degrading microorganisms. 100ml of BHM broth medium was transferred to each flask and sterilized. The flask was kept in a rotary shaker at 150 rpm and 28 ºC for 7 days. After one week of incubation, 10ml of sample from primary enrichment was transferred to a fresh BHM broth. Unless otherwise stated, after 2nd enrichment 1 ml of medium was plated after appropriate dilution on BHM agar plate and incubated at 28 ºC. After 48-hour incubation, pure colonies were isolated using the streak plate method. All isolated microorganisms were stored at 4 ºC for further use.

Preliminary Screening and Biodegradation Potential of strains

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The screening of biodegradation potential was performed using the DCPIP method (Montagnolli et al. 2015). The organisms were cultivated on Bushnell Hass Broth for 48 h and exposed to different concentrations of 4T engine oil in test tubes having a constant amount (0.5 g L-1) of DCPIP at pH 7. The experiments were performed in sealed-lid test tubes to sustain carbon dioxide saturation and block further aeration. The uninoculated tube serves as a control for the study. Test tubes were then incubated at 37 ºC at 180rpm in a digital shaking incubator. Continuous shaking ensured proper oxygen availability to maintain aerobic conditions. The reaction was observed visually till the end of incubation and also spectrophotometrically (600 nm) at an interval of 24 hrs using an Elico SL-159 model UV visible spectrophotometer. Active bacterial culture able to reduce DCPIP results colour less cell solution which ultimately indicates 100 % cell biodegrading activity.

Identification of Bacterial strain by 16S rRNA nucleotide sequencing

The DNA extraction process involved meticulously homogenizing the sample with extraction buffer, transferring the resulting homogenate to a 2 ml-microfuge tube, and precipitating the DNA-rich upper aqueous phase following the addition of Phenol: Chloroform: Isoamyl alcohol (25:24:1). A subsequent round of centrifugation and phase separation led to the DNA's precipitation using 3 M Sodium acetate pH 7.0 and Isopropanol. After a 15-minute incubation, the DNA pellet underwent careful washing, air-drying, and dissolution in TE buffer, ensuring its integrity. To eliminate RNA, DNAse-free RNAse A was introduced. Quantification indicated DNA concentrations, quantifying at 155 ng/µl. Subsequently, for targeted amplification of the 16S gene, a DNA amount of 155 ng from the extracted pool was coupled with 10 pM of each primer that 16S rDNA (SENSE) 5'TAGGGAGGAAAGGTGTGAA3' (Tm: 54.5 ºC) and 16S rDNA (ANTISENSE) 5'CTCTAGCTTGCCAGTCTT3' (Tm: 53.7 ºC). Employing a TAQ Master MIX comprising High-Fidelity DNA Polymerase, dNTPs, $MgCl_2$, and PCR Enzyme Buffer, PCR cycling encompassed initial denaturation for 3 minutes at 94 °C, followed by a set of 30 cycles involving denaturation for 1 minute at 94 °C, annealing for 1 minute 50 °C, and extension phases for 2 and 7 minutes at 72°C. The resultant PCR reaction mixture, composed of DNA, primers, dNTPs, Taq DNA Polymerase enzyme, and water, was primed for DNA sequencing. This subsequent sequencing process involved employing the Big Dye Terminator chemistry, with an ABI 3130 Genetic Analyzer and SeqScape software employed for analyzing the sequencing reactions. 1% of agarose gel electrophoresis was performed again with a loaded DNA ladder (100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1500, 2000, and 3000 base pairs Bangalore Genei, India) for the analysis of yield of amplified product. The amplified DNA bands were then visualized in a UV Trans illuminator and the photograph was taken into a gel documentation system. The amplified fragment was then sequenced into Applied BioSystems 3500 genetic analyser, USA. The sequence was subjected to BLAST at NCBI (www.ncbi.nlm.nih.gov/blast). The estimation of sequence composition and phylogenetic tree construction was done by using MEGA software version 6.0.

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Gas chromatography/Mass spectrometry analysis

Analysis of hydrocarbons present in 4T engine oil was carried out via GC/MS technique. 1 ml of extracted residual oil, after 7, 14, 21, 28, and 35 days of incubation was used for the analysis of Gas Chromatography and Mass Spectrometry (GC-MS) (Shimadzu QP-2010 Plus with Thermal desorption system TD20). Volatile compounds present in a mixture are separated by Gas Chromatography. The separated compounds can be identified and quantified through Mass spectrometry. The gas chromatograph equipped with a split–split less injector (split ratios of 50:1) was used for the GC-MS analysis. The oven temperature was initially at 40 °C and then programmed to 270 °C at a rate of 8 °C/min where it was held for 5 min. The temperatures of the injector, transfer line, and ionization source were all 250 °C. The electron impact ionization was tuned at 70 eV and Helium was used as carrier gas with an average linear velocity of 1.0 mL/min.

3. Results and Discussion

Isolation and Characterization of *Stutzerimonas balearica strain* IMB16-157 (M1)

The isolate grew well on Bushnell Hass medium with 1% (v/v) 4T engine oil. The pure culture of the microbe was obtained after successful enrichments to the medium. The isolate grew as it used the 4T engine oil as the sole source of carbon and energy. The microbial isolate is characterized morphologically based on its size, shape, opacity, and pigmentation. Microbe *Stutzerimonas balearica strain* IMB16-157 (M1) is found to be *a* rhizoid shape with a smooth texture. The opacity of the microbe was also been recorded to opaque with flat elevation and white pigmentation. 50µl of DNA stock was prepared for further molecular analysis which was then diluted after determining the DNA concentrations. Integrity checking of extracted genomic DNA was found to have high intensities of bands compared with the Lambda DNA marker. The presence of a highly resolved, high molecular weight band indicates a good quality of the DNA. The quantity of the DNA was determined by measuring the absorbance at 260nm using a spectrophotometer. 50ng/µl was considered as enough concentration for DNA amplification. The concentration of DNA strain was obtained to be 150ng/µl which is then diluted to attain the desired concentration by DNase RNase free water. Molecular sequencing of the conserved part of the 16S rRNA gene reveals the most potent 4T engine oil-degrading bacteria is *Stutzerimonas balearica strain* IMB16-157 which shows remarkable catabolic activity for petroleum hydrocarbons. Sequence similarities were found by aligning sequences through BLAST and identifying the closest strains from the NCBI database. The partial sequence of the 16S rRNA gene of identified bacterial strains has been deposited into the Gene bank (Figure 1).

3.2 Preliminary Screening and Biodegradation Potential of Strains

Stutzerimonas balearica showed decent decolorization activity. The ODs taken at 600nm from 0 to 48hrs with an interval of 24 hrs were observed shown in Figure 2. Growth strains were also determined by taking ODs from 7 to 35 days of incubation at 620 nm through the turbidometric method. The OD values are shown in Figure 3. The degradation efficacy of *Stutzerimonas balearica strain* IMB16-157 is 78.6% after 21 days of incubation (Figure 4).

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> Stutzerimonas balearica strain IMB16-157 16S ribosomal sequence partial sequence
AACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGACATT
CTGATTCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTAC
GATCGGTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCAC
GTGTGTAGCCCAGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCAGTCTCCTTAGAGTGCCCACCTTAACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCG
TTACGGGACTTAACCCAACATCTCACGACACGAGC

Figure 1. Nucleotide Sequence of *Stutzerimonas balearica strain* IMB16-157 submitted in NCBI.

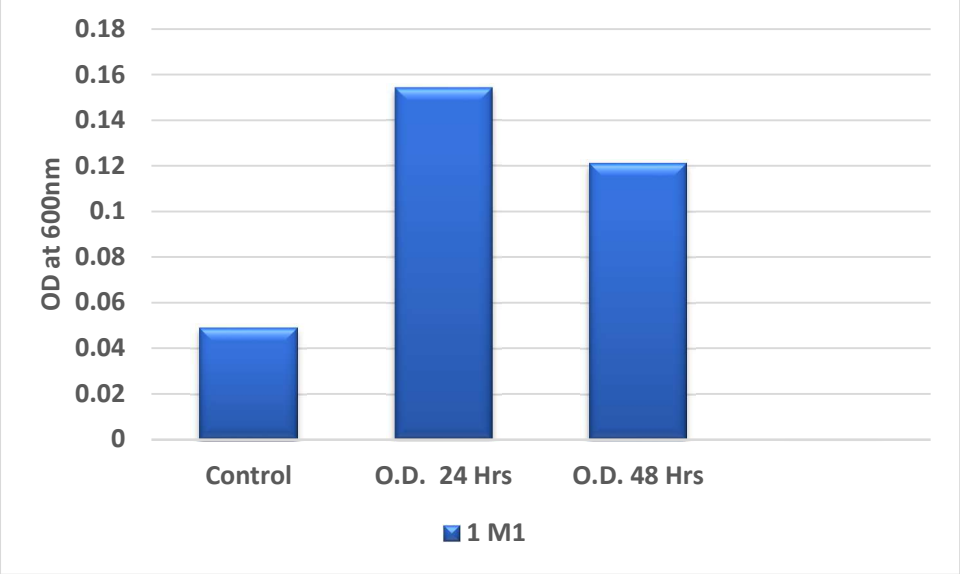

Figure 2. Showing microbial Growth at 620nm in DCPIP assay for isolates *Stutzerimonas balearica* (M1)

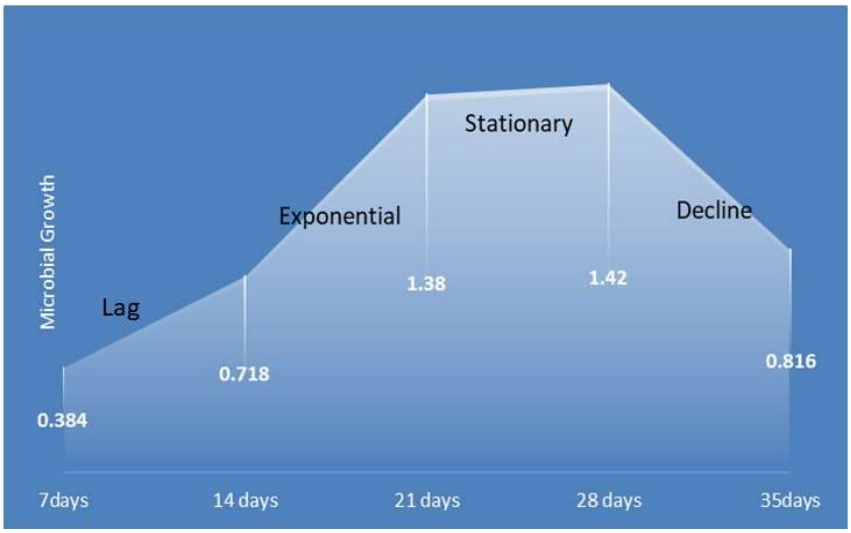

Figure 3. Showing Microbial Growth of isolates *Stutzerimonas balearica* (M1) from 7 to 35 days of incubation at 620nm

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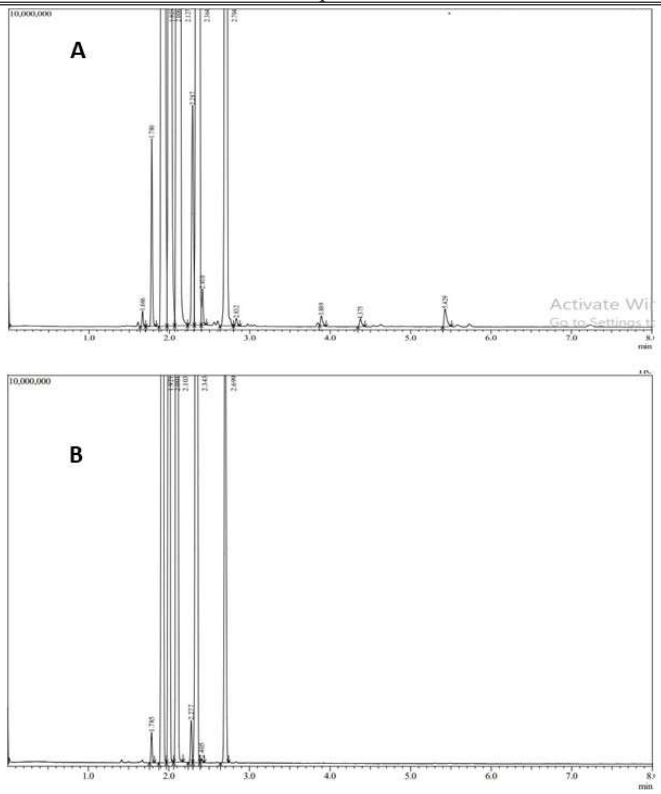

Figure 4. Showing GC-MS Chromatogram of A. 4T engine oil before degradation B. Degradation of 4T engine oil by strain *Stutzerimonas balearica* after 21 days.

The microbial strains were identified as *Stutzerimonas balearica strain* IMB16-157 through 16s RNA sequencing. *Stutzerimonas balearica strain* is a gram-negative, rod-shaped, belongs to the family *Pseudomonadaceae.* There are a number of other variants of these strains involved in the bioremediation of hydrocarbons as research previously published (Salvà-Serra et al. 2023; Bubpachat et al. 2018; Mistry et al. 2022).

In the present study, *Stutzerimonas balearica strain* IMB16-157 degraded 4T engine oil into compounds like butane, pentane,2,3 dimethyl, cyclopentane, etc. Similar results were obtained by Kumar et al. 2022. *Stutzerimonas balearica* was isolated from hydrocarbon-contaminated sediments. The study also found that *Stutzerimonas balearica* had a different degradation profile. It was more effective at degrading the light hydrocarbons in the oil. The examination of the biodegradation capacity of diverse microbial isolates using GC/MS has been shown to be a trustworthy technique. Previous investigations described by Li et al. (2022) provided evidence for this fact.

In the original 4T engine oil, short-chain hydrocarbons with short carbon chain lengths ranging from (C_6-C_{10}) and long carbon chain length (C_{30}-C_{40}) were present, representing a substantial area percentage in the GC-MS analysis. Notably, Butane, 2,2-dimethyl- (C_8) was among the prominent constituents.

Upon exposure to the *Stutzerimonas* strain, an alteration in the composition was observed. While short-chain hydrocarbons (C_6-C_7) remained the same, they exhibited a reduced area percentage.

The appearance of Hexane, 2-chloro- (C_6) indicated potential microbial activity on short-chain hydrocarbons, suggesting degradation and the formation of chlorinated derivatives. These observations suggest microbial degradation and modification of intermediate-chain hydrocarbons, resulting in the reduction of their area percentages.

Conclusion

The microbial degradation of 4T engine oil by *Stutzerimonas* represents a dynamic and transformative process, as evidenced by the changes in hydrocarbon composition based on carbon chain length and the associated reduction in area percentages. This research has provided valuable insights into the microbial degradation capabilities of these strains and the consequential alterations in the engine oil's chemical makeup.

This research also offers a deeper understanding of how microorganisms can modify engine oil composition. The reduction in area percentages and the emergence of new compounds highlight the potential utility of these microbial strains in bioremediation efforts aimed at hydrocarbon-rich environments. Further investigations are warranted to elucidate the specific mechanisms involved and to harness the potential of these strains for practical applications in environmental remediation and sustainable resource management. This study underscores the significance of microbiological processes in shaping the fate of hydrocarbon-based pollutants in our environment.

Conflict of interest

The authors declare that there is no conflict of interest.

Author's contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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