# Comparative Remediation of N-Alkanes Compounds: Assessing the Efficacy of Pseudomonas aeruginosa (AHJ8) and Acinetobacter lwoffii (AHJ6)

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***Abstract***- The objective of this work was to investigate the bacterial association to remediate diesel oil. We reported the biotic and abiotic contributions to difference in diesel oil degradation efficacy between two bacterial strain Pseudomonas auerigonsa (AHJ8) and Acinetobacter lwoffii (AHJ6) isolated from diesel oil contaminated soil and garden soil. We characterized the physical variations in bioremediation efficacy for diesel oil. Diesel oil and them constitutes as sole carbon sources, quantify the components which they degrade. Initially isolation was carried the detection of hydroxyl metabolite catechol during degradation of diesel oil. We further estimated the degradation efficacy between the two strains, also studies the biotic and abiotic parameters. On the basis of these analysis, we concluded that AHJ8 strain has higher degradation efficacy. This strain degrades the 75%n alkanes,11% n -paraffin, and 13% aromatic compounds during 15 days periods. Comparatively, AHJ6 has 97%degradation efficacy during 15 days. These observations with GC-MS data confirm that these isolates have rapid diesel oil remediation. These strains were thereby suggestive for application in diesel oil biodegradations under all investigated nutrition and environmental conditions in polluted soil.

***Key words: Bioremediation, n-alkanes, n-paraffins, aromatic compounds, hydroxyl metabolite catechol, HPLC, GCMS.***

## I. INTRODUCTION

Diesel oil is a primary and essential energy source, and this energy source is used in different fields, including industry, transport, and daily human activities. Anthropogenic contamination is a form of pollution, produced directly by human activities such as the burning of this petroleum oil. This causes environmental deterioration. Discharges of petroleum hydrocarbons often occur due to petroleum production, storage, and transportation, refining, and processing, as well as spills, result of blowout accidents during oilfield development, leakage from oil pipelines and storage tanks, oil tanker and tanker leakage accidents, oil well waxing, and during overhauls of refineries and petrochemical production equipment (Chaerun et al., 2004; Chen et al., 2015; Wang C. et al., 2018). The biodegradation technique for diesel is actuated by many factors like contents of diesel fuels (32).Diesel is a composite mixture of hydrocarbon with carbon no. of $C_{10}$-$C_{22}$.For diesel different 70 compounds detected 8% of mass fraction was aromatic hydrocarbons 9 % was saturated alkanes and 2% was alcohol acid and Olefins 33%.In aromatic hydrocarbon half were monocyclic aromatic hydrocarbons, while other half were Naphthalene, anthracene and Phenanthrene for saturated alkanes 69% were $C_{13}$- $C_{12}$, straight alkanes ,15% were isomers and 6% were naphthenic hydrocarbon( 34). their biodegradation varied depending on the carbon number (35) branch no.(36) including the biotoxicity of aromatic hydrocarbons.

In this study, we reported the isolation, characterization detection and efficacy of the diesel oil-degrading organisms like the *Pseudomonas auerigonsa (*AHJ8) obtained from petroleum oil-containing soil from the petrol pump and *Acinetobacter lwoffii* (AHJ6) from garden soil. Using them to biodegrade diesel oil, we focused to analyse both biotic and abiotic integral factors that contribute to the performance of two strains to degrade diesel oil containing N alkanes and other aromatic hydrocarbons.

## II. IDENTIFY, RESEARCH AND COLLECT IDEA

It's the foremost preliminary step for proceeding with any research work writing. While doing this go through a complete thought process of your Journal subject and research for it's viability by following means:

1) Read already published work in the same field.
2) Goggling on the topic of your research work.
3) Attend conferences, workshops and symposiums on the same fields or on related counterparts.
4) Understand the scientific terms and jargon related to your research work.

## I. II. MATERIAL AND METHOD

### 1. 16s sequencing and phylogenetic analysis

(A.H. Jobanpurta V.R. Kothari, Monitoring and analysis of petrol biodegradation using Chromatography method 2014 Asian Journal of Microbial.Biotech.env. Sc. Vol.17, No. (1)2015:259-263). The diesel degrading isolates were identified by 16S rDNA sequencing from NCCS Pune, Maharashtra, India. Molecular characterization was based on 16S ribosomal DNA (rDNA) sequencing. The BLAST programs from the National Center for Biotechnology information server were used for similarity searches. The phylogenetic analysis with the evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987; Felsenstein, 1985). The optimal tree with the sum of branch length = 14.91121887. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) has been shown next to the branches (Tamura, 2004). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, 2011) and were in the units of the number of base substitutions per site. The analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1323 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Vander Meer et al., 1992).

### 2. Whole cell fatty acid Methyl Ester analysis

Chromatography [GC] was carried out using the Sherlock Microbial Identification System [MIDI, Inc. Newark, DE, USA]. The bacterial cultures were grown in triplicate on trypticase soya agar for 24 hours at 28$^0$C and fatty acid derivatives were separated from cell wall by saponification and extracted with hexane and methyl tertiary butyl ether. Extracts were analyzed on a GC 689 [Agilent Technologies, USA] fitted with Ultra 2 phenyl methyl silicone fused silica capillary column 25× 0.2mm [Agilent Technologies, USA], using hydrogen as the carrier gas, nitrogen as the “make up” gas and air to support the flame. GC oven was programmed from 170 to 270$^0$C at 5$^0$C rise per minute with a 2-minutes holding at 300$^0$C. Fatty acids were identified and quantified by comparison of retention time and peak area obtained and compared with the fatty acid standards. Qualitative and quantitative differences in fatty acid profiles were used to compute the distance for each strain relative to other strains in the Sherlock bacterial fatty acid ITSA1 aerobe reference library. This part of the work was accomplished Disha chemicals Ahmedabad.

### 3. Detection of hydroxy metabolite Catechol by HPLC and TLC

Catechol is intermediatory product obtained by degradation of aromatic compounds by microorganisms (Collins and Daugulis, 1997). The metabolic rate of the substrate was assessed by quantification of the key hydroxyl metabolite catechol that accumulates in aqueous spent medium. Detection of catechol has been done by 4- amino antipyrine reagent (Michaud et al ., 2004). The yellow colour of the reagent turns reddish brown at pH 10 in presence of catechol with absorbance maxima of 510 nm. The acidified spent medium was extracted three times with diethyl ether (1:3 volumes). The extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue obtained was dissolved in methanol and further characterized by HPLC (Shimadzu LC8A, Japan) equipped with a 250 x 4.6 mm C18 Phenomenex column. The elution scheme was methanol-water (70:30) with the flow rate of 1 mL minute-1. TLC 25 aluminium sheets (Merck TLC Silica gel 60F25K Germany) 5×10 cm was used for separation after the extraction of catechol.

### FTIR Analysis

The functional groups present in degradation pattern of substrate diesel oil were determined by FTIR. After the required time for degradation by the two isolates (*Acinetobacter lwoffii (AHJ6)* and *Pseudomonas auerigonsa (AHJ8)*) the medium was extracted with hexane. The residue was subjected to FTIR analysis using FTIR spectrophotometer Perkin Elmer Spectrum Version 10.03.06 spectrum. The two spectra were recorded in 4000/cm to 650/cm range.

### 4. Gas Chromatography analysis

### 5.1 Sample preparation

For Gas chromatographic analysis samples were prepared according to the methods of Michaud *et al*., (2004). After the incubation period, 5 ml of the cultures were extracted with two 20 ml volumes of n-hexane as a solvent by using separating funnels to remove cellular material. The residues were transferred to tarred vials and the volume of each extract was adjusted to 100 ml by adding n-hexane. The vials were kept at 4$^0$C until gas chromatographic analysis.

### 4.2 GC analysis

For detection of petroleum hydrocarbon that is diesel oil a gas chromatography [GC] was performed using Shimadzu, GC model 2014 equipped with a capillary column TR-WAXMS [30 m X 0.25 mm internal diameter] and a flame ionization detector [FID]. The spectra obtained were compared with the spectra for standard diesel oil. One micro litre of diesel oil extract was injected through split injection port with a split ratio of 10:1 using 10 µl syringe [SGE analytical, Australia]. Helium was used as carrier gas with flow rate of 15 ml minute$^{-1}$. The column oven temperature was programmed from 280$^0$C for 40 minutes, increased at a rate of 8$^0$C per minutes to 360 $^0$C, and held at this temperature for 6 minutes. The temperature of injector and detector was 230 $^0$C and 240 $^0$C respectively.

### 5. GC-MS analysis

For identification of hydrocarbons present in diesel oil obtained from residual oil remaining in the broth, a coupled gas chromatography/mass spectrophotometry [GC-MS] was performed using Perkin Elmer gas chromatograph model Auto system XL with turbo mass GC+ equipped with an SGE forte GC capillary column BP20, [30 m X 0.250 µ internal diameter X 0.25 µ] [Australia] and mass spectrophotometer. The mass spectra obtained were compared with the National institute of standards and technology [NIST] mass spectra library. One µl of , diesel oil extract was injected by split injection with a split ratio of 10:1 using 10 µl syringe [SGE analytical, Australia]. Helium was used as carrier gas at a flow rate of 1 ml/min. The column oven temperature was programmed from 120$^0$C for 2 minutes, increased at a rate of 20$^0$C/min up to 230$^0$C, and further held at

this temperature for 10 minutes. The temperature of injector and detector was 225$^0$C and 230 $^0$C respectively.
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## II. Result and Discussion

1. 16s sequencing and phylogenetic analysis

The diesel degrading isolates were identified by 16S rDNA sequencing from NCCS Pune, Maharashtra India. These organisms also have ability to degrade petroleum oil. (A.H. Jobanpurta V.R. Kothari,2014). Phylogenetic tree based on 16S rRNA gene sequences drawn using the neighbour joining method with evolutionary distances computed using MEGA5 software showing the relationship of petroleum oil degrading organism with validity published sequences of related genera.

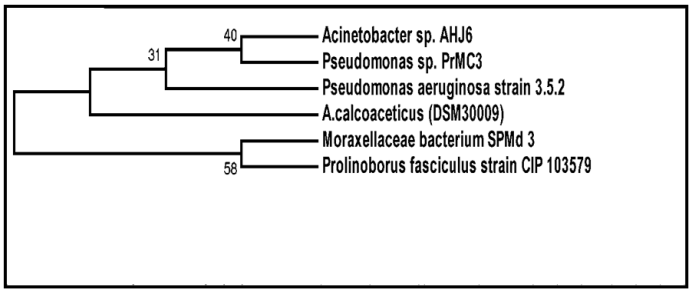


Fig. 1a: Phylogenic tree of Acinetobacter lwoffii

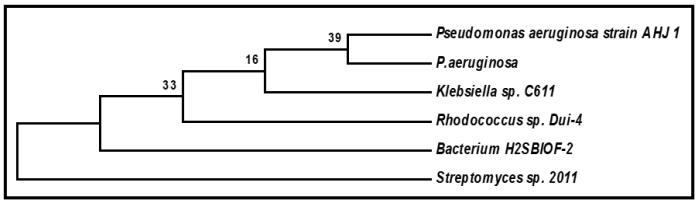


Fig. 1b: Phylogenic tree of Pseudomonas aeruginosa

2.Whole cell fatty acid Methyl Ester analysis

Fame profile used to analyse the principal components (PCA) and cluster analyses to identify similarities and differences among soil microbial communities. Strain Acinetobacter species exhibited presence of 13:0 iso, 13:0 anteiso, 14:0 iso, 14:0 anteiso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 iso, 17:0 anteiso. Pseudomonas species exhibited presence of 11:0 iso, 12:0 iso, 13:0 iso, 15:0 iso, 15:0 anteiso, 16:0 iso, 17:0 anteiso.

3.Detection of hydroxy metabolite Catechol by HPLC and TLC

It was mandatory to detect the most common intermediates which appear during the biodegradation of most of the hydrocarbons. With this motive at hand routine method for catechol detection was attempted. Catechol was detected using 2, 4 antipyrine reagent. A characteristic reddish colour was imparted to test samples by both the organisms Pseudomonas aeruginosa AHJ6 and Acinetobacter lwoffi AHJ8. This was taken as a confirmatory test for detection of catechol. The appearance of prominent reddish color can be visualized for the two organisms in figure 2a and 2b. The results were counter confirmed by HPLC where in after running standard catechol the intermediates obtained after biodegradation by the two isolates were also passed through the columns. The results of the three were comparable and the retention time showed similarity in all the three cases. Figure 2 depicts results for standard catechol while figure 2a and 2b showed presence of catechol in the degraded residue. Further detection of catechol was done by TLC. The metabolite exhibited identical Rf and Rt values on co-chromatography with standard catechol which comes to 0.18 and 20 minutes respectively, which corroborate with those of standard catechol.

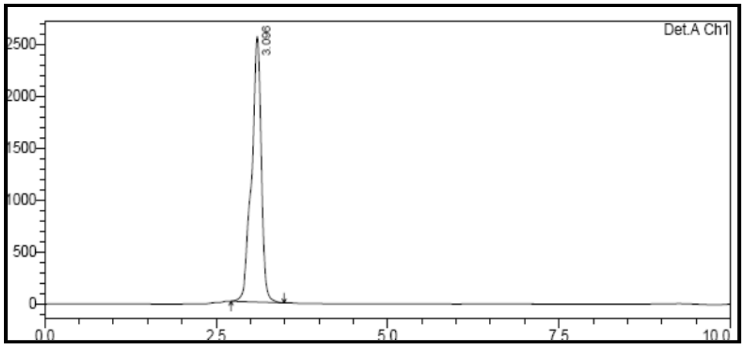


Fig. 2 Standard catechol

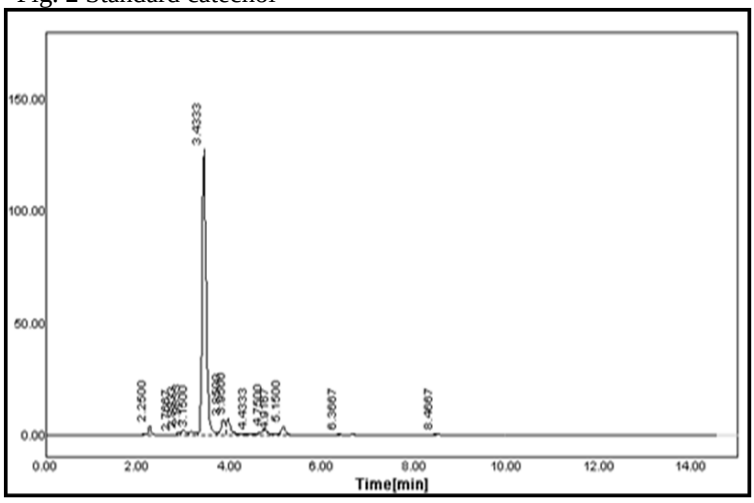


Fig. 2a HPLC of intermediate sample obtained during degradation of diesel oil by Pseudomonas aeruginosa

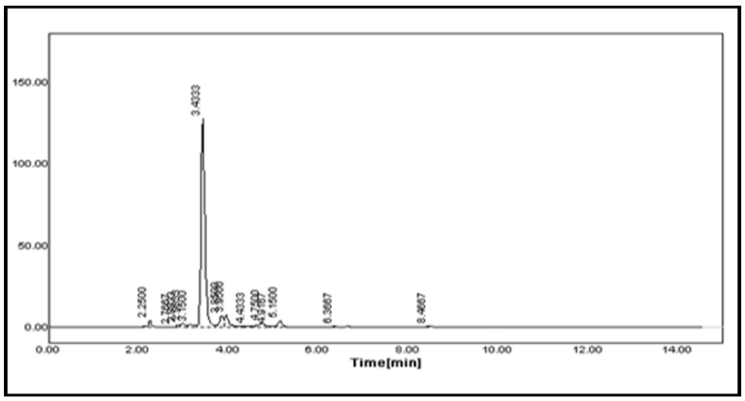

Fig.2b HPLC of intermediate sample obtained during degradation of diesel oil by Acinetobacter lwoffii (AHJ 8)

4. FTIR analysis

FTIR analysis spectra were considered for investigating the chemical profile of the sample which containing to predict the functional groups present in the diesel oil substrates. On the basis of the peaks obtainable most of the groups present in diesel oil was observed which included the common components -COOH, -CH3,-CH2. Depending upon these groups that is -COOH, -CH3,-CH2 are degraded by these isolates that is Acinetobacter lwoffii (AHJ8) and Pseudomonas aeruginosa (AHJ 6). FTIR chromatogram of standard diesel oil. Figure 3, 3aand 3b, of degraded diesel oil by Acinetobacter lwoffii (AHJ8) and Pseudomonas aeruginosa (AHJ6).

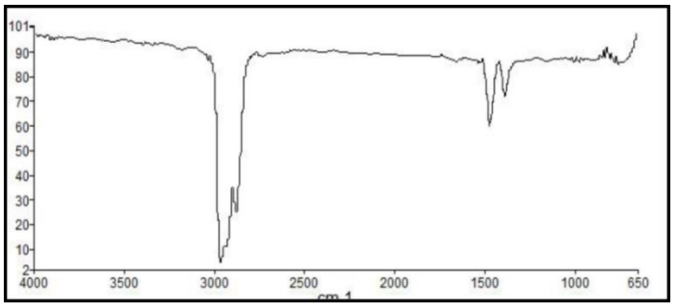


Fig.3 FTIR of the standard diesel oil

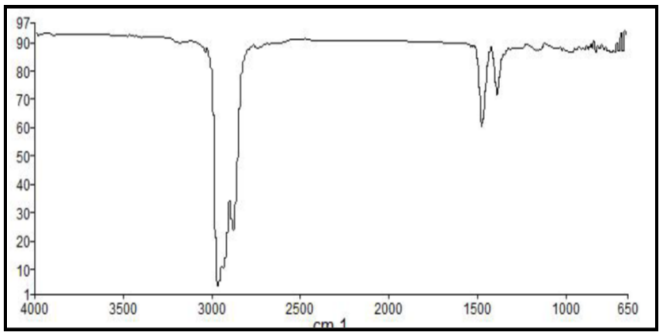


Fig.4a FTIR of diesel oil degraded by Acinetobacter lwoffii

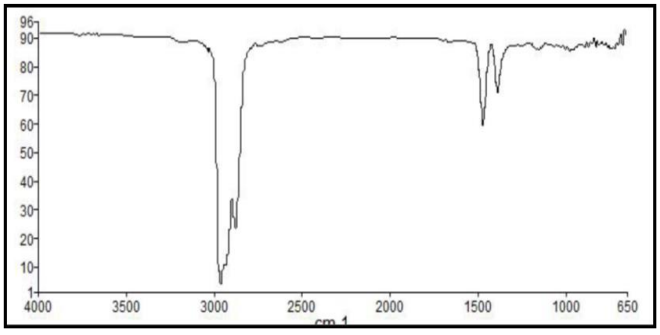


Fig.4b FTIR of diesel oil degraded by Pseudomonas aeruginosa

GC analysis

Quantification of residual substrate on biodegradation was done with a view to determine, the biodegradation efficiency (BE). The residual quantity of diesel oil was quantified, on the basis of retention time of the un-inoculated sample or the respective standards of the diesel oil.

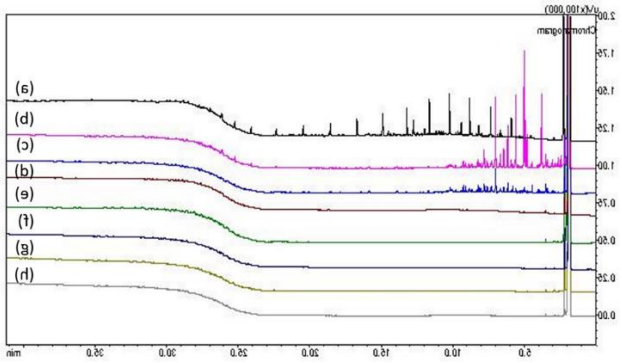


Fig 4a Chromatogram of broth supplemented with diesel oil and inoculated with Pseudomonas aeruginosa

The degradation pattern was measured in terms of percentage degradation. Extracted samples were monitored by GC analysis. Initially both the organisms Acinetobacter lwoffii (AHJ 8) and Pseudomonas aeruginosa (AHJ 6) did not exhibit any significant variation in the chromatograms. By the nineth day most of the components were resolved.

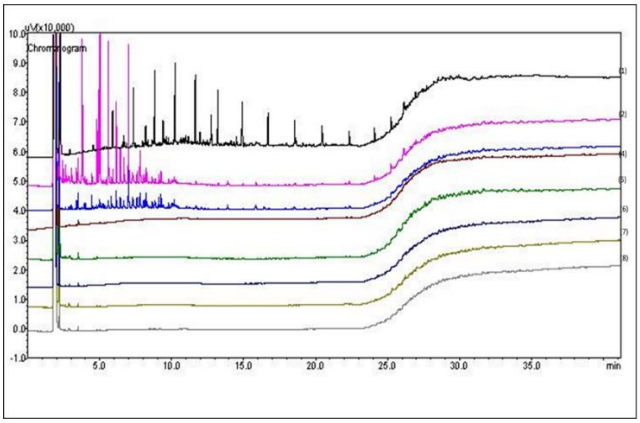


Fig 4b. Chromatogram of broth supplemented with diesel oil and inoculated with Acinetobacter lwoffii (AHJ 8)

2 GC –MS

The degradation patterns were studied on the basis of rate and the removal or decrease in the retention time of the peak. The biodegradation was interpreted on the GC chromatograms for residual petroleum oil that is, diesel oil. The peak identities were first confirmed by matching relative retention time (with respect to internal standard) in the chromatograms for residual diesel oil extracted at time zero. The diesel range of n-alkanes contributes to 75%, and the remaining 25% was composed of C6–C8 and

C28–C44. The resolved n-alkane peaks in the diesel chromatograms were found to be in the carbon number range C9–C26 and the peak eluting close to C-17 was identified as pristane. Out of 99 diesel range n-alkanes, only 7 were present in the abiotic control of Pseudomonas aeruginosa. On degradation of diesel by Acinetobacter lwoffii only ten diesel range n-alkanes were left. Based on such GC chromatograms, the sum of peak areas for diesel range resolved peaks (DRRP) between two retention time boundaries at 16.3 minutes and 26.1 minutes. Pseudomonas aeruginosa and Acinetobacter lwoffii exhibited two boundaries at 16.3 and 25.2 minutes. DRRP thus represents resolved peaks between C16 and C26. This measure comprised of two parts, DRRPn-alk representing the sum of n-alkanes and DRRPo representing the other compounds in this range, such as, branched alkanes. Figure 5 depicts the chromatogram of abiotic control of diesel oil. Figure 5a and figure 5b depict the chromatogram of biotic tests for both the organisms.

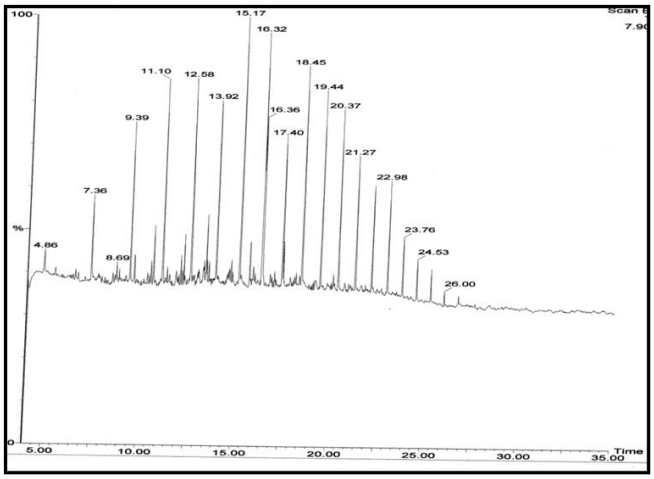


Fig 1. Standard of diesel oil

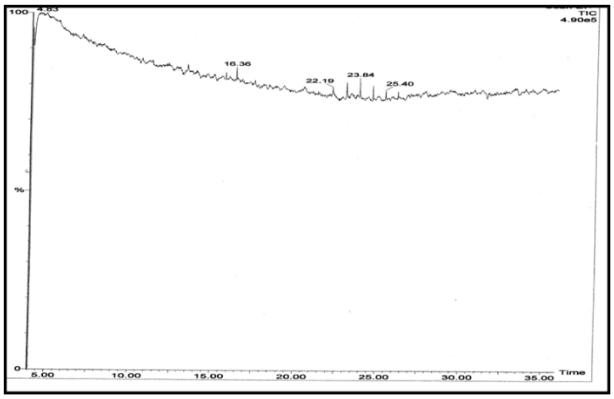


Fig. 1a Chromatogram of broth supplemented with diesel oil and inoculated with Pseudomonas aeruginosa

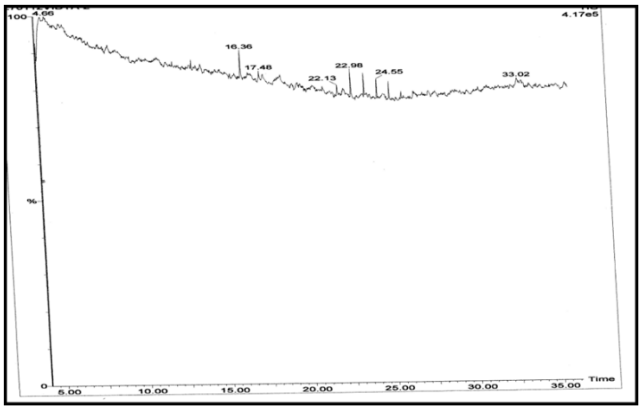


Fig 1b. Chromatogram of broth supplemented with diesel oil and inoculated with Acinetobacter lwoffii.

## III. CONCLUSION

The comparative remediation of N-alkanes by Pseudomonas aeruginosa (AHJ8) and Acinetobacter lwoffii (AHJ6) showed promising results. Both bacteria demonstrated the ability to degrade N-alkanes, which are common environmental contaminants found in petroleum hydrocarbons. However, some differences were observed in their remediation capabilities. Pseudomonas aeruginosa (AHJ8) exhibited higher degradation efficiency and faster degradation rates compared to Acinetobacter lwoffii (AHJ6). This could be attributed to the specific metabolic pathways and enzymes possessed by Pseudomonas aeruginosa, which are well-suited for the degradation of N-alkanes. On the other hand, Acinetobacter lwoffii showed slower degradation rates, possibly due to differences in its metabolic capabilities or environmental conditions required for optimal growth and degradation.

Both bacteria also demonstrated varying substrate preferences, with Pseudomonas aeruginosa showing higher preference for longer-chain N-alkanes, while Acinetobacter lwoffii preferred shorter-chain N-alkanes. This suggests that the selection of bacterial strains for N-alkane remediation should be based on the specific target contaminants and environmental conditions. Furthermore, the degradation of N-alkanes by both bacteria was influenced by factors such as temperature, pH, and nutrient availability. Optimization of these factors could potentially enhance the overall degradation efficiency of N-alkanes by both bacteria. Overall, the comparative remediation study of N-alkanes by Pseudomonas aeruginosa (AHJ8) and Acinetobacter lwoffii (AHJ6) highlights their potential for bioremediation of N-alkane-contaminated environments. Further research is warranted to better understand the mechanisms and optimize the conditions for effective N-alkane degradation by these bacteria, and to evaluate their performance under field conditions.

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