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Potential for biodegradation of crude oil by *Pseudomonas aeruginosa* strains and analysis of residual oil by Gas Chromatography – Mass Spectrometry

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ABSTRACT
The potential biodegradation of crude oil was assessed by 50 strains of *Pseudomonas aeruginosa* cultured in a basal mineral medium using crude oil as a sole carbon source. The strains are isolated from both environmental and hospital samples (non-contaminated crude oil sites). After 28 days of incubation, more than 60% of crude oil was degraded and further converted into accumulated cell biomass. Therefore, the use of the bacterial consortium increases the percentage of biodegradation up to 67 %. The analysis of residual crude oil by Gas chromatography-mass spectrometry (GC-MS) confirms the results that show that *Pseudomonas aeruginosa* could be effective in the biodegradation of crude oil.

1. Introduction:

Petroleum hydrocarbons are essential resources that drive not only industrial processes but also daily activities integral to modern life. However, the multifaceted nature of petroleum also designates it as a significant environmental concern, as it stands as a major contributor to pollution **[1]**. With its intricate composition, petroleum possesses the capacity to induce a diverse array of toxic effects, ranging from acute lethality to sub-lethal chronic toxicity. These effects are contingent upon a multitude of factors, including the degree of exposure, dosage, and the specific organism under consideration **[2]**.

Extended exposure to high concentrations of petroleum can potentially lead to the development of severe health conditions, such as liver or kidney diseases. Furthermore, there exists a plausible risk of bone marrow damage and an elevated susceptibility to cancer in cases where prolonged contact is established **[3]**. Simultaneously, various components intrinsic to petroleum are capable of accumulating within susceptible aquatic organisms. This bioaccumulation phenomenon subsequently facilitates the transfer of these harmful agents through trophic levels, disseminating the ecological impact of petroleum pollution **[4-5]**.

One of the critical requisites for effective oil spill bioremediation is the presence of microorganisms equipped with specific metabolic capabilities. These microorganisms play a pivotal role in breaking down petroleum hydrocarbons, thereby aiding in the restoration of contaminated environments **[6]**.

Conventional methods for managing oily wastewater, including strategies like containment and collection via floating booms, as well as adsorption using both natural and synthetic materials, have limitations in terms of achieving thorough degradation of crude oil **[7]**. These traditional approaches often fall short in completely eradicating the hazardous constituents of petroleum from the environment. This has prompted the exploration of alternative strategies that leverage the inherent capabilities of microorganisms to naturally eliminate pollutants.

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Microbial bioremediation, as a means of harnessing the inherent potential of microorganisms for the degradation of hydrocarbons, has garnered significant attention. These microorganisms possess the remarkable ability to break down complex hydrocarbon molecules into simpler and less harmful compounds. As such, they offer a promising solution to the challenge posed by petroleum pollution. Furthermore, microbial bioremediation is characterized by its autonomy and widespread prevalence in diverse environments **[8-9]**.

In light of these considerations, our study embarks on a comprehensive evaluation of the biodegradation capabilities of crude oil. Specifically, we focus on the role of *Pseudomonas aeruginosa*, a bacterium renowned for its hydrocarbon-degrading prowess. To ensure a comprehensive investigation, we have isolated *Pseudomonas aeruginosa* strains from non-contaminated sites, both in pure culture and within a mixed bacterial consortium. This approach allows us to ascertain the bacterium's efficacy in degrading crude oil, both in isolation and in collaboration with other microorganisms. By utilizing *Pseudomonas aeruginosa* in our study, we aim to shed light on its potential as a powerful tool in the battle against petroleum pollution. The evaluation of its performance in both pure culture and mixed consortium settings provides valuable insights into its capacity to thrive and effectively degrade hydrocarbons under varying conditions. Furthermore, the inclusion of a mixed bacterial consortium mirrors the complex interactions that occur within natural ecosystems, offering a more holistic understanding of the potential of microbial bioremediation.

2. Materials and methods:

2.1. Biodegradation Assays:

Crude oil-degrading bacteria were cultivated under aerobic conditions in mineral salt media (MSM) using Arabian Light crude oil as the sole carbon source. The MSM composition (g/L) comprised KH_2PO_4: 0.68, $MgSO_4.7H_2O$: 0.35, Na_2HPO_4: 1.7, $CaCl_2$: 0.02, NH_4NO_3: 1, and $FeSO_4$: 0.004, supplemented with trace elements (0.01% final concentration) containing $CuSO_4$: 0.05, H_3BO_4: 0.1, $MnSO_4$: 0.1, $ZnSO_4$: 0.1, Na_2MoO_4: 0.1, and $CoCl_2$: 0.1 g/L. The pH was adjusted to 7, and the medium was enriched with 0.5% filter-sterilized crude oil (v/v).

For the experiments, individual bacterial cultures and mixed consortia, derived from overnight log-phase growth, were transferred to 250 mL conical flasks containing 100 mL of sterile mineral salts medium supplemented with 0.5% crude oil (v/v). The study was conducted in triplicate, with non-inoculated flasks serving as controls. All flasks were incubated at 30°C and 150 rpm on a shaker (Lab-line, Environ shaker - USA) for specified time intervals: 14, 21, and 28 days.

2.2. Extraction and analysis of residual oil :

Residual concentrations of crude oil from both cultures and control samples were determined through a liquid-liquid extraction process. This method involved the addition of a double volume of chloroform (70 ml x 2) to each sample using separating funnels. The extracted solution was then treated with 2g of anhydrous sodium sulfate to eliminate any moisture. Subsequently, the chloroform phase containing the residual hydrocarbons was carefully decanted and allowed to air dry. Upon evaporation of chloroform, the remaining residual oil was quantified. The percentage of biodegradation was assessed using the equation developed by Fusey and Oudot (1976) **[10]**, which is described below:

$$Biodegradation\ Percentage = \frac{(pi - pev) - pr}{pi - pev} \times 100$$

With:

- *Pi: Quantity of initial crude oil.*
- *Pev : Quantity of crude oil evaporated.*
- *Pr : Quantity of residual crude oil.*

2.3. Gas Chromatography Analysis:

After the completion of the 28-day incubation period, the residual crude oil was extracted and subsequently quantified using gas chromatography coupled with mass spectrometry (GC-MS). The extraction process was performed chromatographically. The extracted oil was analyzed spectrophotometrically at the National Scientific Research Center in Rabat, Morocco. The Gas Chromatography/Mass Spectrometry (GC-MS) analysis was conducted utilizing a Hewlett-Packard gas chromatographer (HP 6890) coupled with a mass spectrometer (HP 5973). The fragmentation was achieved through electron impact at 70 eV. The chromatographic separation employed an HP-5MS column (30 m x 0.25 mm, film thickness: 0.25 µm). The carrier gas utilized was helium with a constant flow rate of 1.5 ml/min. The injection mode was set to split (split ratio: 1/70, flow rate: 112 ml/min). The column temperature was programmed to increase from 50 to 200 °C at a heating rate of 4 °C/min for a duration of 5 minutes. In the chromatographic analysis, the residual oil was appropriately diluted in methanol at a ratio of 1:20 (v/v).

3. Results and discussion:

After each incubation period (14, 21, and 28 days), we assessed the degradation of crude oil by the isolated *P. aeruginosa* strains using the previously described equation. The results were presented in the form of two histograms. One histogram displayed the biodegradation of crude oil by environmental strains, while the other depicted the biodegradation by hospital strains (Figures 1 and 2).

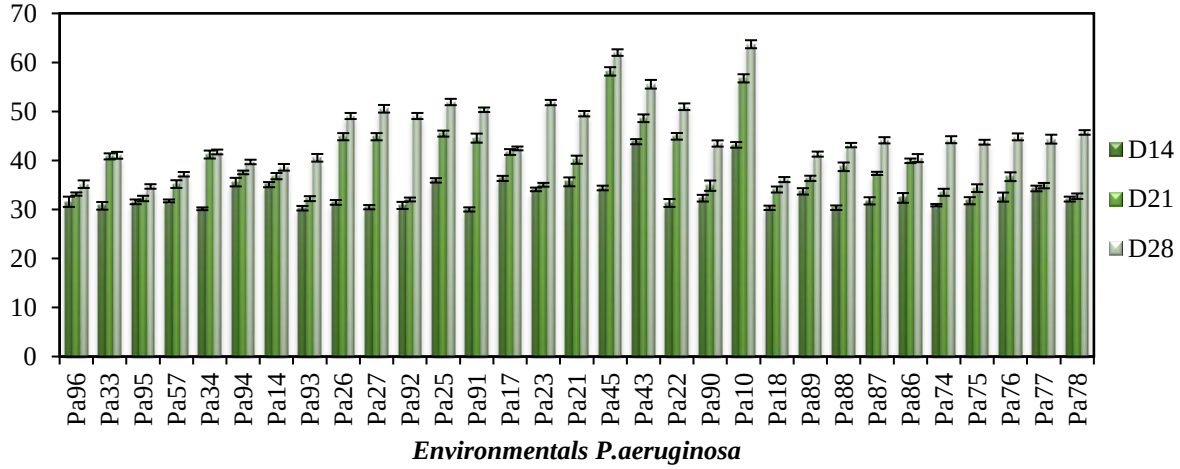

Figure 1. Biodegradation potential of crude oil by environmental *P. aeruginosa* strains.

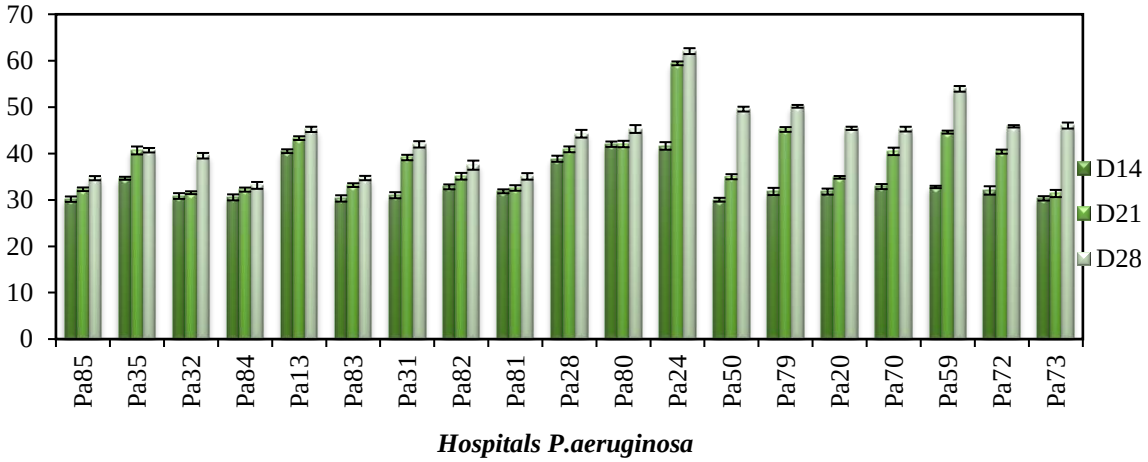

Figure 2. Biodegradation potential of crude oil by hospitals *P. aeruginosa* strains.

Initially, crude oil was observed as a large stretch covering the aqueous medium or attached to the glass wall. Over time, this large stretch or attachment of petroleum gradually transformed into dispersed oil droplets within the medium **[11]**. The results indicate that out of the 50 tested *P. aeruginosa* strains, 11 strains exhibited a crude oil biodegradation potential ranging between approximately 50% and 60%, 27 strains demonstrated potential within the range of 40% to 50%, and 12 strains showed a biodegradation potential between 30% and 40% (Figures 1 and 2). Moreover, a majority of the tested strains displayed a biodegradation percentage falling between 40% and 50%, with only 3 strains exhibiting a potential slightly exceeding 60%. The minimum and maximum values recorded for biodegradation potential or percentage are summarized in Table 1.

Table 1. Minimum and maximum values of the biodegradation potentials of crude oil of 50 *P. aeruginosa* strains tested.

Biodegradation potential (%)/ Days		**D14**	**D21**	**D28**
Environmentals strains	*Minimum values*	30.01 (Pa 91)	32.04 (Pa 92)	34.69 (Pa 95)
	Maximum values	43.83 (Pa 43)	58.16 (Pa 45)	63.71(Pa 10)
Hospitals strains	*Minimum values*	30.02 (Pa 50)	31.37 (Pa 73)	33.11(Pa 84)
	Maximum values	42.19 (Pa 80)	59.42 (Pa 24)	62.05 (Pa 24)

The data presented in Table 1 reveals that the minimum values for crude oil biodegradation potential are approximately 30% after a 14-day incubation period. Interestingly, for the same duration of incubation (14 days), the maximum values surpass 42% (Figure 2), highlighting distinct kinetics of crude oil biodegradation among the strains. Furthermore, the recorded maximum biodegradation potential values exhibit considerable heterogeneity, ranging from 42% to approximately 63%, indicative of varying capabilities in utilizing specific components of crude oil.

Comparatively, the obtained biodegradation potential values are notably higher than those reported in the literature. A comparative analysis of crude oil biodegradation conducted by Adelaja *et al.* (2014) **[12]** utilizing *Pseudomonas aeruginosa, Aspergillus terreus,* and *Candida petrolium* demonstrated that only *Pseudomonas aeruginosa* strains exhibited a substantial percentage of biodegradation. Additionally, Guo-liang *et al.* (2005) **[13]** observed consumption of 58% to over 60% of the initial crude oil concentration (0.7 g/L) after an 8-day incubation. Microbial biodegradation of crude oil entails the decomposition of oil components, transforming them into other organic compounds **[14].** In essence, crude oil biodegradation involves the conversion of chemical compounds by microorganisms into energy, biomass, and organic products. Given its effective utilization of hydrocarbons, *Pseudomonas aeruginosa* has garnered significant attention for its role in crude oil biodegradation **[15]**. The outcomes of biodegradation from both the mixed cultures and individual strains are consolidated in Table 2.

Table 2. Biodegradation potentials of crude oil mixed cultures

Strains	Biodegradation potentiel (%)
Pa10+Pa45	66.87
Pa10+Pa45+Pa24	67.05
Pa10	63.71
Pa45	62.00
Pa24	62.05

Legend: Pa10 and Pa 45 are environmental strains; Pa 24: Hospital strain.

The obtained results indicate a noteworthy enhancement in the biodegradation potential of crude oil when a mixed bacterial culture is utilized compared to a pure culture. This observation suggests that the presence of diverse strains within the mixed culture allows for the metabolism of a wider range of substrates. The mixed cultures employed in our study comprised *P. aeruginosa* strains that exhibited significant biodegradation percentages, surpassing 60%, after a 28-day incubation period. This phenomenon is consistent with findings from various authors who have demonstrated that the biodegradation of oil is more effective in the presence of a consortium of bacterial species rather than a single species **[16-17]**. Notably, cocultivation of *Pseudomonas aeruginosa* with a microbial consortium may lead to an enhanced bioavailability of pollutants, thereby promoting the biodegradation of crude oil, owing to the production of rhamnolipids by *Pseudomonas aeruginosa* **[18]**.

Crude oil is composed of a complex array of hydrocarbons and related compounds, primarily saturated alkanes, cycloparaffin rings, aromatic and polynuclear compounds (PAHs), resins, and asphaltenes **[19]**. However, it has been reported that no single microbial species possesses the enzymatic capability to metabolize more than a few classes of compounds commonly found in crude oil **[20]**. Hence, the degradation of crude oil necessitates a consortium composed of diverse bacterial species.

Indeed, the utilization of a bacterial consortium offers distinct advantages, particularly in scenarios where pollutant toxicity is substantial or when appropriate microorganisms are lacking in terms of both quantity and quality **[21]**. The confirmation of crude oil biodegradation was further substantiated through the identification of residual crude oil compositions using gas chromatography-mass spectrometry (GC-MS) (Figures 3, 4, and 5). The initial profile (Figure 3) serves as the control, displaying the crude oil compositions in the non-inoculated flask following 28 days of biodegradation. In contrast, the second and third profiles (Figures 4 and 5) depict the crude oil compositions after a 28-day incubation in the inoculated flasks. A striking observation is the disappearance of most peaks seen in the first profile, evident in the second and third profiles. This phenomenon signifies a substantial rate of crude oil utilization by *Pseudomonas aeruginosa*, thereby reinforcing the biodegradation process **[22]**.

Based on the gas chromatographic profiles, it is evident that *Pseudomonas aeruginosa* Pa10 and the consortium (Pa10 + Pa45) exhibited substantial degradation of numerous oil fractions. Specifically, after a 28-day incubation in a medium containing crude oil, these strains demonstrated a marked reduction in peaks with a retention time around 50 minutes. Interestingly, in the case of the consortium profiles, a slight decrease in peak intensity was observed, aligning well with existing literature findings. This trend reaffirms that the degradation potential of the bacterial consortium was indeed influencing the observed changes. The congruence between the results of biodegradation potential and the outcomes from the GC-MS analysis further solidifies the accuracy and reliability of our findings. This notion finds support in previous research, where GC-MS analyses indicated a substantial reduction in the relative abundance of peaks for isolated bacterial organisms exhibiting varying degrees of oil degradation potential, in comparison to the control.

In a broader context, our results unveiled a comprehensive degradation or transformation of numerous oil compounds, resulting in their conversion into less complex entities. This is a promising indication of the efficacy of *Pseudomonas aeruginosa* Pa10 and the consortium (Pa10 + Pa45) in driving the biodegradation process, ultimately contributing to the breakdown and alteration of crude oil components. Such outcomes hold significant implications for potential applications in bioremediation strategies aimed at mitigating the environmental impact of crude oil contamination.

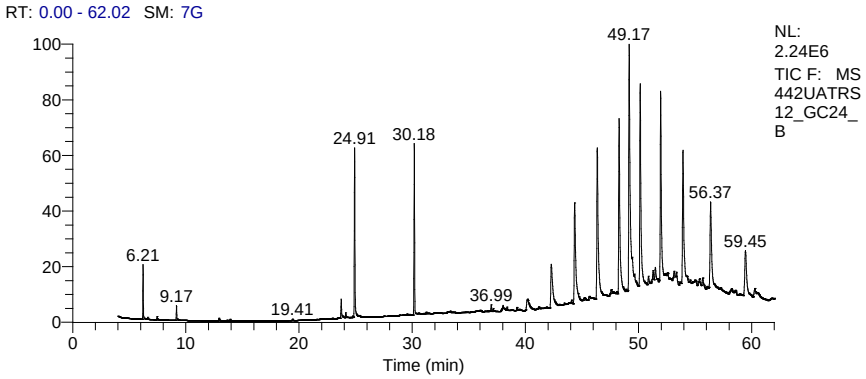

Figure 3. Gas chromatographic-mass spectrometry analysis of untreated crude oil after 28 days of incubation.

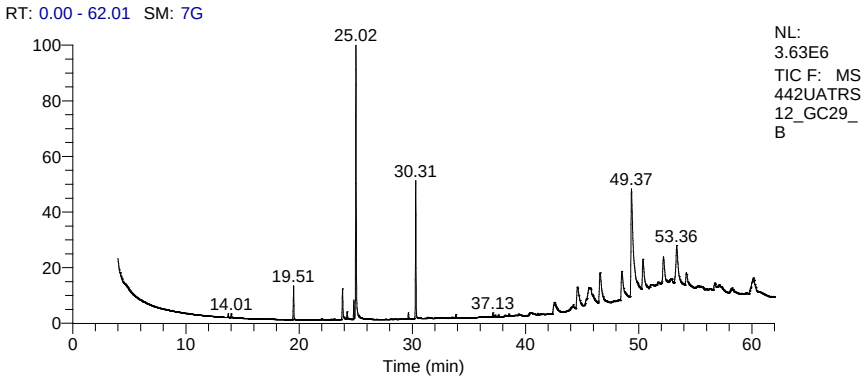

Figure 4. Gas chromatographic-mass spectrometry analysis of residual crude oil after 28 days of incubation with *Pseudomonas aeruginosa* (Pa10).

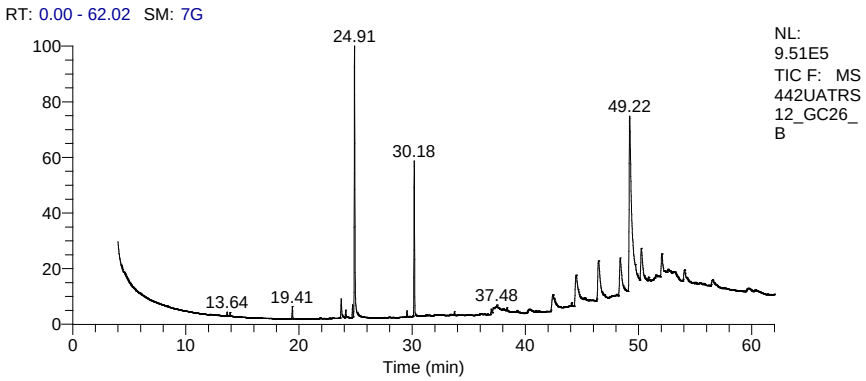

Figure 5. Gas chromatographic-mass spectrometry analysis of residual crude oil after 28 days of incubation with *Pseudomonas aeruginosa* Pa10+Pa45.

4. Conclusion:

In summary, our study has revealed a compelling contrast in biodegradation capabilities between individual bacterial strains and their cooperative consortium. This disparity can be attributed to the distinct characteristics of hydrocarbon mixtures, encompassing variations in volatility, solubility, and susceptibility to degradation. Furthermore, the intricate process of biodegradation necessitates a specific ensemble of enzymes that may not be inherently present within a solitary organism. Consequently, the amalgamated efforts of a mixed bacterial culture have demonstrated remarkable efficacy, achieving a

noteworthy degradation rate of up to 67.05% for the investigated crude oil under incubation conditions of 30°C over a 28-day period. These findings illuminate the synergistic potential inherent in a consortium approach, capitalizing on the collective strengths of constituent strains to address the intricate challenges posed by crude oil pollution. This holistic strategy taps into the complementary attributes of individual bacterial strains, offering a dynamic toolset for bioremediation endeavors in diverse environmental scenarios. As such, our study provides valuable insights into the capacity of *Pseudomonas aeruginosa* isolated strains as a promising avenue for mitigating the impact of crude oil contamination. The observed potential for bioremediation, whether deployed singularly or within a consortium framework, highlights a versatile and adaptable approach that holds promise for the restoration and preservation of crude oil-affected sites.

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