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Behavioral Responses to Hydrocarbon Stress in Marine Bacteria Isolated from Arzew Harbor in Northwestern Algeria

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Abstract

Marine hydrocarbon pollution has emerged as a pressing concern, imperiling the ocean's abundant resources and disrupting the crucial services rendered by marine ecosystems. The presence of hydrocarbons in seawater poses a significant challenge to microorganisms, prompting adaptive modifications in bacterial behavior to thrive in these adverse conditions. A comprehensive investigation into marine bacteria's potential to utilize and break down hydrocarbons was conducted, focusing on strains isolated from Arzew harbor. These strains underwent rigorous phenotypic and biochemical characterization. Their ability to form biofilms in the presence of varying concentrations of sodium chloride, crude oil, and kerosene was meticulously assessed. Hydrophobicity and chemotactic responses of these strains were studied in media supplemented with crude oil and kerosene. Notably, the results unveiled that eight of these strains exhibited a profile akin to *Pseudomonas sp.* While one strain resembled *Aeromonas sp.,* all strains exhibited varying degrees of biofilm-forming capacity, ranging from low to high. A predominantly hydrophobic phenotype was observed among these strains. The majority of the bacteria displayed a noteworthy chemotactic attraction to crude oil. Strain S7 emerged as the most promising, displaying remarkable proficiency in handling the studied hydrocarbons, closely followed by strain S16. It's worth emphasizing that robust outcomes were associated with emulsification potential and chemotaxis towards both hydrocarbons, coupled with bacterial cell surface hydrophobicity. These findings illuminate the potential of these strains for enhancing water quality in hydrocarbon-contaminated environments.

Keywords: *Hydrocarbons, Biofilm, Emulsifying Activity, Hydrophobicity, Chemotaxis.*

1. Introduction

Microbial communities, inhabiting marine environments, include different groups of bacteria, archaea, fungi, protists, and viruses (Fuhrman *et al*., 2015). These microorganisms play a crucial role in the recycling of elements, taking the example of carbon, nitrogen, and sulfide cycling. These processes contribute to the insurance of nutrients in the marine environment and the regulation of global climate. It is important to note that the structure and function of microbial communities allow us to better understand their role in the marine environment (Jégousse *et al*., 2022). These organisms can be phototrophs or chemotrophs as primary producers, as well as heterotrophs that represent the secondary producers. Recently, several studies have focused on molecular analysis to determine the organisms present in a given site and their spatiotemporal distribution. The data,generated by phylogenetic studies, is based on a limited number of genes citing 16S and 18S rRNA. Scientists have confirmed that these tools are insufficient to understand and assess environmental functions and community ecology. New tools are being developed to allow us to much understand about these functions. These tools are brief omics analysis which includes metagenomic, metatranscriptomic, metaproteomic, and metabolomic analyses (Fuhrman *et al*., 2015).

The analysis of total microbial communities is considered a rigorous criterion for seawater quality. It enhances sustainable development. As already noted, it is crucial to evaluate any change in the structure of microbial communities because of their contribution to biogeochemical cycles and the biodegradation of pollutants. Various parameters can influence community structures especially the gradient of inorganic substrates (N, P), season, adjacent habitat, depth, oxygen, protist predation, pressure, salinity, algal dominance, particulate organic carbon, human disturbance, and sand mining (Nimnoi and Pongsilp, 2020). It is known that oil pollution is an environmental concern in marine ecosystems. This is related to oil extraction and transportation activities, shipping, urban runoff, and industrial discharges. The hydrophobic nature of these compounds allows them to accumulate in sediments that act as a sink. Therefore, high concentrations can be accumulated in sediments with the potential to be quite harmful to the health of marine ecosystems. This nature makes bioremediation encouraging biotechnology to reduce the adverse effects associated with

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this type of pollution while avoiding negative implications such as the use of dispersants or oil burning (Lozada *et al*., 2014; Perdigão *et al*., 2021). The use of indigenous microorganisms can improve the efficiency of biodegradation due to their adaptation to the environment. Therefore, several studies have recommended the importance of using indigenous microbial communities for the bioremediation of petroleum hydrocarbons in marine ecosystems (Perdigão *et al*., 2021). It highlights that Polycyclic Aromatic Hydrocarbons (PAHs), resulting from pyrolytic processes and mainly anthropogenic activities, are considered priority pollutants due to their carcinogenic and mutagenic properties (Imarhiagbe and Obayagbona, 2029). Microbial degradation plays a key role in remedying environmental contamination caused by petroleum spills. Numerous strains of bacteria and fungi have showcased their remarkable capability to selectively break down distinct fractions of petroleum compounds, signifying their important role in restoring affected ecosystems (Olalemi et al., 2021).

Remarkably, hydrocarbon degrading bacteria can exhibit chemotactic behavior as a signaling syste in order to make these compounds accessible (AlKaabi *et al*., 2020). Moreover, the chemotaxis allows bacteria to migrate to favorable environments or to move away from unfavorable environments (Wang et *al*., 2022). This process in Escherichia coli, Pseudomonas aeruginosa, Pseudomonas putida, and Rhodococcus erythropolis, has demonstrated their ability to detect and adapt to chemical changes in their environment (Liang et al., 2021). *P.* aeruginosa with a polar flagellum has demonstrated a directed mobility that allows it to accumulate at the hydrocarbon-water interface. The monitoring of the unicellular mobility near the hydrocarbon-water interface revealed four types of movements. The first type is a visitor that visits the interface and then leaves. Other bacteria have proved their ability to stay at the interface for at least 60s by passive diffusion, otherwise, they are known as “diffusive”. Some bacteria swim in circle perpendicular trajectories “pirouettes” or flat “loops” by locating themselves at the hydrocarbon-water interface (Conrad, 2020).

Bioremediation represents an environmentally-friendly waste management approach, harnessing the power of living organisms to efficiently eliminate or render harmless pollutants, present within a tainted location (Mercy et *al*., 2019). The efficiency of biodegradation relies on the interactions between the bacterial cells and the hydrocarbon, as does the hydrophobicity of the cell surface (AlKaabi et al., 2020). The hydrophobicity of bacterial cells is considered to be an important factor in controlling bacterial growth and adhesion to surfaces (Meliani and Bensoltane, 2014). It should be noted that microbial adhesion to hydrocarbons is one of the methods to determine the affinity of bacteria for dispersed hydrocarbons (Conrad, 2020). Many microorganisms can produce biosurfactants. These products increase the interaction between hydrocarbons and microbial cells, bioavailability which subsequently improves the bioremediation of hydrocarbons in polluted sites (Shahaliyan et al., 2015). The production of biosurfactants is associated with the emulsification potential of strains. It was also described that an effective emulsification of hydrocarbons and the stabilization of emulsion in marine environment, represent a crucial requirement for hydrocarbon remediation (Gong et al., 2015). Our study signifies a novel research endeavor, focused on observing and understanding the responses of native bacteria in the Arzew region when exposed to hydrocarbon stress. Two main characteristics of the indigenous marine bacteria are assessed: the cells’ surface hydrophobicity and their emulsification potential. The profiling of communal traits such as biofilm formation and chemotaxis of native marine bacteria improves potent data related to the future application of these strains for environmental purposes.

2. Material and methods

2.1. Sampling and bacterial isolation

Arzew harbor is located on the eastern side of Cape Carbon in Arzew Bay, approximately 350 kilometers west of Algeria and 500 kilometers to the east of the Strait of Gibraltar (35°51’38.196 N 0°18’26.1864 2W) (Fig.1). Its main activity is the exportation of hydrocarbons (Bensari et al., 2020). Water sampling was performed during early winters in December 2020 and December 2021 from the border and depth of 20 cm. One millimeter of each sample was transferred into a flask, containing 100 ml of Bushnell and Haas Broth at pH = 7. Flasks were kept in incubator at 30 °C under shaking for 48 to 72h. A 100µl of each bacterial suspension was transferred to BH agar plates. Colonies were purified and stored at 4°C in fresh BH medium supplemented with 20% of sterile glycerol (Borah *et al*., 2019).

Figure 1. Map of Arzew harbor of Oran
(Map Data© 2022, Google Maps.)

2.2. Morphological and biochemical characterization

All isolates were identified by studying their morphology and biochemical profile. The morphological study of the colony concerns its shape, color, diameter, and contour. These characteristics were observed on Pseudomonas F agar plates after incubation at 35 ± 2°C for 24h. Bacterial identification is based on Gram staining, catalase, and oxidase test by using commercial disks (Sigma Aldrich), and biochemical and carbohydrates fermentation profiles were studied by using API 20NE (Biomerieux: API (Analytical Profile Index) is a standardized system for the identification of non-fastidious, non-enteric Gram-negative rods, containing 20 miniature of biochemical tests (combines 8 conventional tests, 12 assimilation tests, and a database) multitests. All strains were

conserved in LB with glycerol 30 per cent at -80°C (Celik *et al*., 2008).

2.3. Biofilm formation

Biofilm formation evaluation was performed using the microplate method. Bushnell and Haas Broth were used to fill transparent 96-well polystyrene microplates. Each well was inoculated with a pre-culture prepared with the studied strains, and the control wells were filled with sterile BH broth. The microplates were then incubated at 37°C for 24h. After incubation, the microplates were emptied of their contents and then rinsed three times with phosphate buffer (PBS). The microplates were inverted to dry slightly and then stained with 0.5% (W/V) crystal violet solution. After 15 min. of staining, the dye excess was removed, and the wells were rinsed three times with sterile bi-distilled water. The microplates were dried for 15 min. at room temperature. The crystal violet, retained by adhered cells to the polystyrene surface, is recovered by 95% ethanol, and it was determined by reading the absorbance at the wavelength (ʎ) = 595 nm by Eliza reader (YSENMED) (Stephanovic et al., 2007; Wijesinghe et al., 2019).

The evaluation of biofilm formation is performed by comparing values of the optical density of the bacterial film against the optical density of negative controls (sterile media). Biofilm formation ability is expressed using the threshold value (DO_C). The threshold value (DO_C) is defined as the mean of the negative controls plus three times the standard deviation of the negative controls (Diaz *et al*., 2016).

2.3.1. The Effects of salinity on biofilm formation

In order to test the effect of salinity on the bacterial adhesion, different concentrations of NaCl were tested (0, 0.3M, 0.5M, 0.8, 1M, 2M, and 3M). 96-well polystyrene microplate was incubated at 37°C for 24h. After incubation, the optical density was determined by an Eliza reader at ʎ= 595 nm.

2.3.2. The Effects of hydrocarbons on biofilm formation

Various concentrations of the crude oil and kerosene have been tested in order to evaluate their effect on the biofilm formation. In this goal, seven concentrations were used (0, 0.5%, 1%, 1.5%, 2%, 3%, and 4%). It should be noted that the same conditions were kept. After incubation, the optical density was read at ʎ= 595 nm by Eliza reader.

2.4. Bacterial chemotaxis toward Hydrocarbons

Chemotactic responses of marine strains towards hydrocarbons were detected by the capillary method as previously described (Gordillo *et al*., 2007; Shu *et al*., 2018). First, Eppendorfs were used as a chamber to introduce a volume equal to 200 µl of the bacterial suspension (OD=2.5) into the chemotactic buffer. A needle of 2 cm in length was used as a capillary and attached to a syringe containing 200 µl of the tested substances. After 90 minutes of incubation at room temperature, the syringe was removed from the bacterial suspension, and its content was diluted and spread on Pseudomonas F agar, the incubation was performed at 37°C for 24h. After incubation, the developed colonies were counted and expressed as $CFU.ml^{-1}$(CFU: Colony-Forming Unit).

2.5. Bacterial adhesion to hydrocarbons (BATH)

The bacterial adhesion to hydrocarbons assay serves to evaluate the hydrophobicity of bacterial cell suspension (Rosenberg et al., 1980). Briefly, the bacteria were grown in Lauria Bertani broth at 30 °C. They reach their exponential phase overnight (OD600 = 0.6). A volume of 2 ml of bacterial suspension was suspended in an eppendorf tube and centrifuged at 1500×g. The supernatant was removed and the bacteria were resuspended in 2 ml of fresh motility buffer. This operation was repeated three times before transferring the bacterial suspension to round bottom test tubes. Then, 15 µl of the tested hydrocarbon was added and a thin layer of oil formed which covers the bacterial suspension. After 2 minutes of agitation, the mixtures were left 15 minutes to settle for phase separation. A volume of 1 ml of aqueous phase was transferred to cuvette for optical density reading at 600 nm (Chao *et al*., 2014; May *et al*., 2019).

$$RH = (OD_{initial} - OD_{residual})/OD_{initial}) \times 100\%$$

RH: Relative hydrophobicity

OD initial: Optical density of the preculture before adding the hydrocarbons

OD residual: Optical density of the preculture after adding the hydrocarbons

2.6. Hydrocarbon emulsifying activity

To determine the emulsifying activity of bacterial strains, a tube containing an overnight culture inoculated previously in LB (Lauria Bertani) broth. An equivalent volume of crude oil and kerosene were introduced under vortexing for 3 min. at a maximum speed. A tube containing sterile broth with an equivalent volume of the hydrophobic phase was used as a negative control. Incubation was performed at 37°C for 24 h. After incubation, the emulsification index was calculated according to the following formula (Cooper *et al*., 1987).

$$E24 = (He / Ht) \times 100$$

He: height of emulsion

Ht: total height of the mixture

2.7. Statistical analysis

All experiments were performed in triplicates. The results were analyzed by one-way ANOVA ($P<0.05$). All data were presented as the average and standard deviation (SD) The results were followed up with Bonferroni test ($P<0.05$) groupings. The descriptive statistics, Principal Component Analysis (PCA), Hierarchical Cluster analysis (HCA), and correlation were executed. All statistical treatments were undertaken using STAISTICA software (version 10).

The biofilm formation capacity was expressed using cut-off values (ODC). ODC value can be defined as the mean of negative controls (ODnc) plus three standard deviations (SDs) (Diaz *et al*., 2016). Strains were classified into the following categories: $ODc < OD \leq 2 \times ODc$: weak biofilm producer, $2 \times ODc < OD \leq 4 \times ODc$: moderate biofilm producer, and $OD > 4 \times ODc$: strong biofilm producer.

3. Results

3.1. Isolates characterization

A total of 9 strains were isolated and subjected to conventional phenotypical and physiological tests. As the phenotypical study, all strains are characterized on the basis of Gram staining and cultural aspect of colonies on agar medium. However, the physiological study consists in the catalase and oxidase test. The biochemical profile was determined by a classical gallery, API 20NE.

According to the obtained results on Pseudomonas F agar, 8 strains have proved a similar aspect with yellow-green pigments (S5, S6, S7, S8, S9, S11, S16 and S25), and one strain with beige colonies (S30). All strains are Gram negative rods, aerobics, oxidase and catalase positive. They grow well at 42°C, and none has the capacity to grow at 4°C (Fig. 2).

The resulting biochemical profiles were interpreted using the API-web software (bioMérieux). The similarity index of eight isolates was found to be 99.98% for Pseudomonas taxa and only S30 strain with 99.92% as a similarity index for *Aeromonas* taxa. The phenotypical and biochemical traits are summarized in Table 1.

Table 1. Phenotypical and biochemical characteristics of marine strains.

	S5	S6	S7	S8	S9	S11	S16	S25	S30
Gram	-	-	-	-	-	-	-	-	-
Oxydase	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Pigment	+	+	+	+	+	+	+	+	-
Growth at 4°C	-	-	-	-	-	-	-	-	-
Growth at 42°C	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	-	+	-	-
Citratase	+	+	+	+	+	+	+	+	+
ONPG	+	+	+	+	+	+	+	+	+
ADH	+	+	+	+	+	+	+	+	+
LDC	+	+	+	+	+	+	+	+	+
ODC	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-
Indole	-	-	-	+	-	-	-	-	-
Gelatinase	+	+	+	+	+	+	+	+	+
VP	-	-	-	-	-	-	-	-	-
NO_3	+	+	+	+	+	+	+	+	+
TRP	-	-	-	-	-	-	-	-	+
GLU	-	-	-	+	-	-	-	-	+
ESC	-	-	-	+	-	-	-	-	+
PNPG	-	-	-	+	-	-	-	-	+
GLUa	+	+	+	+	+	+	+	+	+
ARAa	-	-	-	-	-	-	-	-	+
MNEa	-	-	-	-	-	-	-	-	+
MANa	+	+	+	+	+	+	+	+	+
NAGa	+	+	+	+	+	+	+	+	+
MALa	-	-	-	-	-	-	-	-	+
GNTa	+	+	+	+	+	+	+	+	+
CAPa	+	+	+	+	+	+	+	+	+
ADIa	+	+	+	+	+	+	+	+	-
MLTa	+	+	+	+	+	+	+	+	+
CITa	+	+	+	+	+	+	+	+	-
PACa	-	-	-	-	-	-	-	-	-

+ : Positive - : Negative

3.2. Biofilm formation

The evaluation of biofilm formation on microplate presents a method of quantification based on the demonstration of cell adhesion to a polystyrene surface. All strains were tested for their ability to form biofilms on polystyrene microplate as mentioned above. This ability was tested by varying the concentration of sodium chloride, crude oil, and kerosene. After incubation, the wells were stained with crystal violet to visualize the bacterial adhesion. The categorization of strains (weak, moderate, or high biofilm-forming) was based on the comparison between the optical densities, read at 595 nm with the critical optical density (OD_c) (Fig.3).

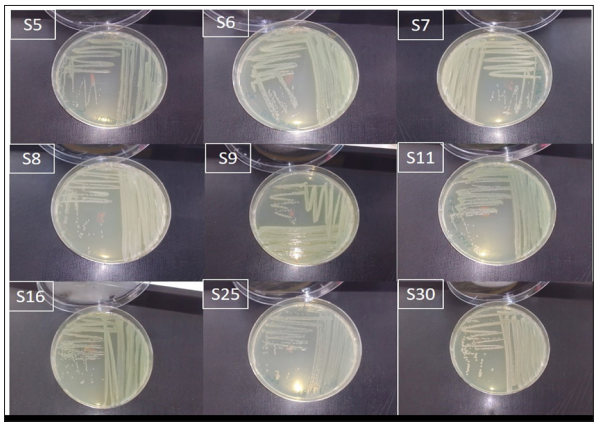

Figure 2. Cultural aspect of marine isolates on Lauria Bertani agar.

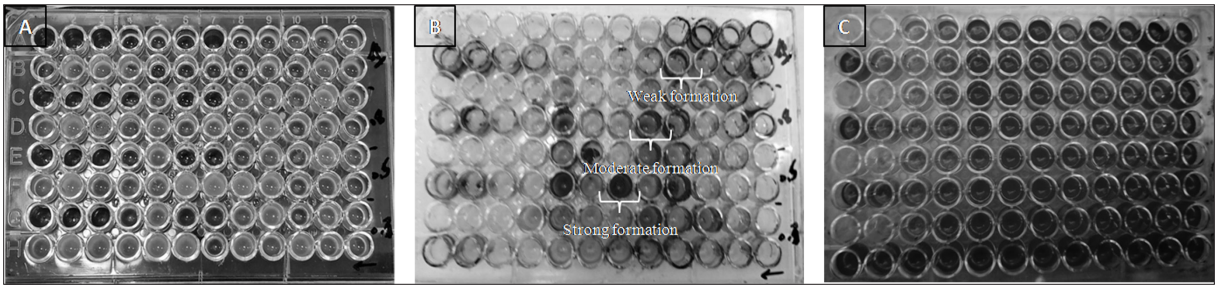

Figure 3. Results of biofilm formation demonstrating the ability of marine strains, to adhere to polystyrene microplate. **A:** Before coloration with Crystal violet; **B:** After crystal violet Removal and drying; **C:** Crystal violet recovery by ethanol

3.2.1. Effect of salinity on bacterial communities

In the absence of sodium chloride stress, most strains display a moderate level of adherence to the polystyrene surface. However, only one strain (S16) exhibited strong biofilm-forming ability, as evidenced by its significantly higher optical density value ($P<0.05$), indicating substantial adherence. Conversely, this same strain demonstrated robust biofilm formation at varying concentrations, ranging from 0.5M to 2M of sodium chloride. In other words, these concentrations amplified bacterial adhesion to the polystyrene surface. Bacterial adhesion predominantly ranged from weak to moderate across concentrations fluctuating from 0.3M to 1M. Interestingly, 88.89% of the strains exhibited moderate adhesion at a concentration of 3M. However, this particular concentration had an adverse effect on bacterial adhesion, except for the S16 strain, which maintained strong biofilm formation under the same conditions. Most of the tested concentrations moderately enhance the biofilm-forming capacity of marine isolates, possibly due to their halophilic traits. However, this observation doesn't hold true for the 2M concentration, as it significantly strengthens bacterial adhesion. Consequently, most strains can now be classified as strong biofilm formers. The most robust biofilm formation was evident in S6 (0.906) at the 2M concentration and S16 (0.963) at the 3M concentration ($P<0.05$) (see Fig. 4).

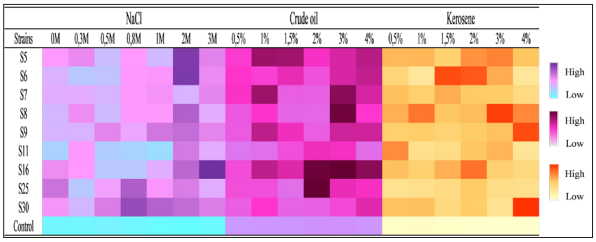

Figure 4. Bacterial ability to form biofilm at different concentrations of sodium chloride; crude oil and kerosene ($p<0.05$).

3.3.2. Effect of hydrocarbons on bacterial communities

The impact of hydrocarbons on the biofilm-forming capability of bacterial isolates was studied by varying the concentrations of crude oil and kerosene. A range of concentrations, spanning from 0.5% to 4%, was selected for investigation. In the case of crude oil, marine strains have generally demonstrated a moderate capacity to create biofilms when the concentrations range from 0.5% to 2%. However, when the concentrations increase to 3% and 4%, there is a noteworthy enhancement in biofilm formation in 55.55% of our strains ($P<0.05$). This concentration enhanced biofilm formation can be attributed to the marine strains' ability to degrade and utilize hydrocarbons as a carbon source.

However, the introduction of both 0.5% and 1.5% concentrations of kerosene exerts a detrimental impact on biofilm formation. This adverse effect is primarily attributed to the fact that a significant portion of strains within the population exhibits weak adhesion properties. Conversely, when exposed to concentrations of 1%, 2%, 3%, and 4% of kerosene, these strains display a noticeable shift towards moderate biofilm formation, as depicted in Figure 2. These findings suggest that the marine strain may not be adept at utilizing kerosene as a carbon source, possibly due to its toxic nature.

3.3. Bacterial chemotaxis toward hydrocarbons

All strains underwent assessment for their chemotactic responses across various sodium chloride concentrations. Remarkably, all of our strains exhibited positive chemotaxis responses to both crude oil and kerosene. Particularly intriguing was the chemotactic behavior displayed by the S7 strain, with a concentration of 980.104 CFU.ml^{-1} showing a notable response to crude oil and 396.104 CFU.ml^{-1} towards kerosene. The majority of strains demonstrated a moderate level of chemotaxis. When comparing bacterial chemotaxis to the tested hydrocarbons, it became evident that the most robust cell concentrations were observed in response to crude oil. Notably, crude oil significantly enhanced the chemotactic behavior of the S7 strain ($P<0.05$), while no statistically significant differences were observed for the majority of strains (refer to Fig. 5).

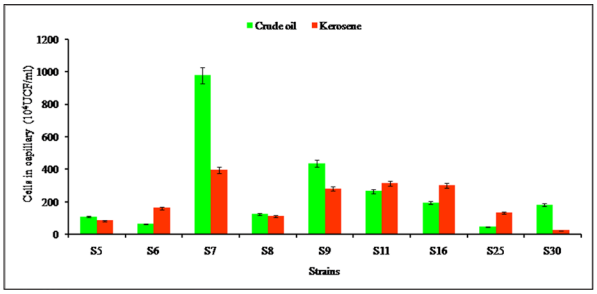

Figure 5. Cells accumulation in capillary, in presence of crude oil and kerosene in after 24h of incubation at 37°C. Data represent means ± SD of three experiments ($p<0.05$).

3.4. Bacterial cell surface hydrophobicity

To investigate the surface cell hydrophobicity concerning crude oil and kerosene, a comparison was made between the initial optical density and the residual optical density. The outcomes of these cell hydrophobicity assessments are illustrated in Figure 6. A standout observation emerges with the S11 strain, showcasing a remarkable 61.33% hydrophobicity towards crude oil, and the S16 strain displaying a significant

59.33% hydrophobicity towards kerosene. Evidently, the presence of crude oil substantially amplifies surface cell hydrophobicity across the majority of marine isolates.

Regarding kerosene, the majority of marine isolates exhibited a moderate level of hydrophobicity, with the notable exception of the S16 strain, which displayed a particularly intriguing degree of hydrophobicity (as illustrated in Figure 4). When comparing the hydrophobicity of these strains across the tested hydrocarbons, it becomes apparent that they demonstrated relatively similar hydrophobic characteristics. However, it is worth highlighting that this trait appears to be particularly pronounced in response to crude oil.

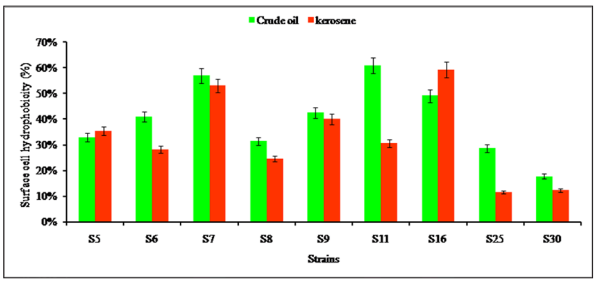

Figure 6. Bacterial cell hydrophobicity of marine strains toward crude oil and kerosene, after 24h of incubation at 37°C. Data represent means ± SD of three experiments ($p<0.05$).

3.5. Emulsifying activity

The emulsification index served as a quantifiable measure for evaluating the capacity of the studied strains to emulsify hydrocarbons. Interestingly, nine strains exhibited emulsifying potential, with nine strains demonstrating emulsifying activities against crude oil within a range of 29.27% to 59.79%. A lower emulsifying potential was observed in 22.22% of the strains. In the case of kerosene, all strains exhibited emulsification indices varying between 17.38% and 58.19%, as depicted in Figure 7.

An interesting emulsifying capability was observed in 44.44% of the tested strains. Among these, the most noteworthy performance was exhibited by the S25 strain, showcasing an impressive 59.25% emulsification index when interacting with crude oil. In contrast, both the S7 and S16 strains displayed comparable emulsification indices of 58.19% when exposed to kerosene. The emulsification indices of the S5 and S8 strains were also quite similar. It is striking that there was a notably higher emulsifying potential for crude oil, compared to kerosene within this study.

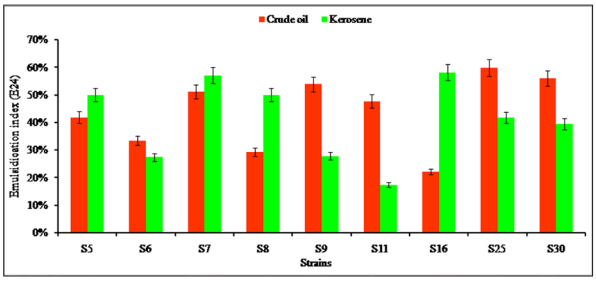

Figure 7. Emulsification index of marine strains, toward crude oil and kerosene after 24h of incubation at 37°C. Data represent means ± SD of three experiments ($p<0.05$).

3.6. Principal component analysis (PCA)

Correlation circle plots serve as a valuable tool within Principal Component Analysis (PCA) to unveil relationships among variables (as illustrated in Fig. 8). Our examination of these variables unveiled two principal components, where PC1 accounted for a substantial 47.73% of the total variance while PC2 contributed significantly at 28.45%. The inherent correlation between two variables is visually apparent through the angular orientation of their vectors. When the angle is acute, it signifies a positive correlation. An obtuse angle indicates a negative correlation, and a right angle implies a null correlation. Notably, the correlation circle closely envelops the chemotaxis towards kerosene (EK), rendering it exceptionally well-represented on the map.

The narrow angle between Chemotaxis towards kerosene (CK) and Hydrophobicity towards crude oil (HC) signifies a robust correlation, as indicated by a correlation coefficient of 0.932478. This same strong correlation is evident between Chemotaxis towards crude oil (CC) and both Chemotaxis towards kerosene (CK) (r= 0.745503) and Hydrophobicity towards crude oil (HC) (r= 0.636069). Additionally, the angle formed between the vectors representing Hydrophobicity towards kerosene (HK) and the Emulsification index towards kerosene (EK) reveals a positive correlation (r = 0.493149). Conversely, the Emulsification index towards crude oil appears largely independent of the other parameters.

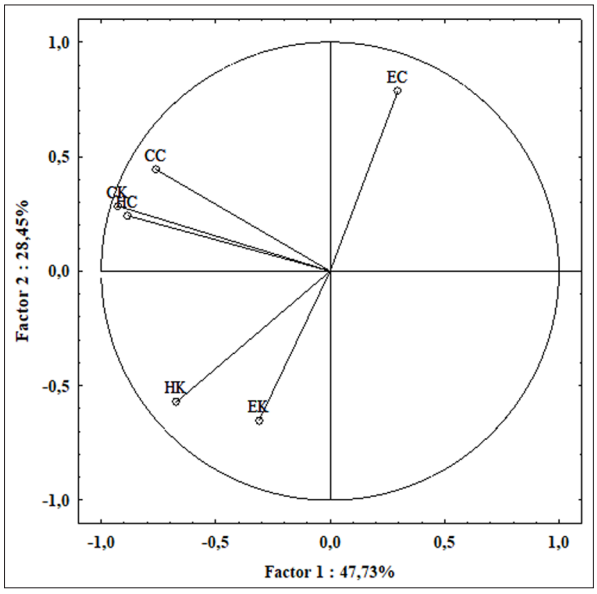

Figure 8. Correlation circle plot for PCA between the studied variable. EC: Emulsification index towards crude oil; EK: Emulsification index towards Kerosene; HC: Hydrophobicity towards crude oil HK: Hydrophobicity towards Kerosene;
CC: Chemotaxis towards crude oil; CK: Chemotaxis towards Kerosene.

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The narrow angle between Chemotaxis towards kerosene (CK) and Hydrophobicity towards crude oil (HC) signifies a robust correlation, as indicated by a correlation coefficient of 0.932478. This same strong correlation is evident between Chemotaxis towards crude oil (CC) and both Chemotaxis towards kerosene (CK) (r= 0.745503) and Hydrophobicity towards crude oil (HC) (r= 0.636069). Additionally, the angle formed between the vectors representing Hydrophobicity towards kerosene (HK) and the Emulsification index towards kerosene (EK) reveals a positive correlation (r = 0.493149). Conversely, the Emulsification index towards crude oil appears largely independent of the other parameters.

3.7. Hierarchical cluster analysis (HCA)

The analysis of bacterial behaviors concerning crude oil and kerosene reveals a classification of marine strains into three distinct clusters: Cluster 1 (comprising strains S5, S8, S6, S25, S30), Cluster 2 (with strains S9, S11, S16), and Cluster 3 (represented by strain S7). Within each cluster, there is a remarkable similarity in various traits. This hierarchical clustered heatmap illustrates that these behaviors can be categorized as low, medium, or high, as shown in Figure 9.

Notably, S7 emerges as the most significant strain, demonstrating compelling results in its interactions with the studied hydrocarbons, closely followed by the S16 strains These strong results are equally associated with emulsification activity (44.44%) and chemotaxis toward both hydrocarbons (44.44%), with bacterial cell surface hydrophobicity contributing at 22.22%.

The analysis of the variables shows a clear division of our parameters into two distinct clusters. The first cluster comprises chemotaxis towards hydrocarbons, specifically crude oil and kerosene, while the second cluster encompasses hydrophobicity and emulsification related to hydrocarbons. Notably, our study reveals a high correlation in chemotactic behaviors between crude oil and kerosene. Consequently, our strains exhibit correlated responses to both hydrocarbons, primarily due to variations in chemotactic outcomes from other variables. Furthermore, their emulsification and hydrophobicity towards hydrocarbons demonstrate relatively close similarities. These findings are strongly supported by the correlation circle plot (Fig. 6).

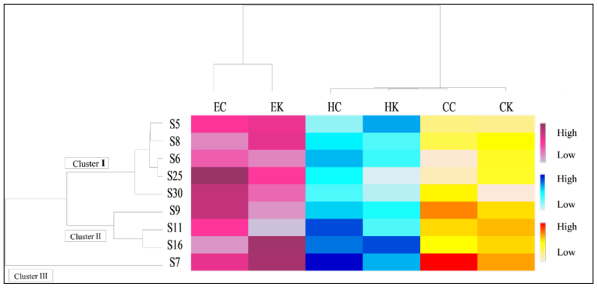

Figure 9. Correlation between the bacterial behaviors toward the tested hydrocarbons.

4. Discussion

This study aims to illuminate the intricate behaviors, exhibited by bacteria in response to varying levels of salinity and the presence of hydrocarbons within contaminated environments. Our primary objective is to unravel the specific bacterial traits that contribute to their superior adhesion and emulsifying capabilities in the context of hydrocarbons. Our results unequivocally establish that elevated salinity levels promote the formation of biofilms among marine strains. This phenomenon is in accord with prior research, as it can be attributed to the protective effects of biofilm structures against salt-induced stressors, as highlighted by Qurashi et al. (2012). Furthermore, A. hydrophila is shown to have an ability to form biofilms at lower concentrations of sodium chloride, as elucidated by Jahid et al. (2015). These findings reinforce the concept that biofilm formation bolsters bacterial resilience in the face of environmental challenges, particularly salinity. In highly saline environments, the formation of biofilms by halophilic bacteria emerges as a crucial strategy to counteract the detrimental effects of salinity. This was evident in a study on Halomonas stenophila HK30, an isolate from a saline wetland in Brikcha, Morocco, which exhibited a remarkable capacity to form biofilms even in the presence of a high salt concentration (5% w/v). Our exploration of the impact of salt stress on biofilm formation in Vibrio sp. B2, isolated from seawater, unveiled that these bacteria not only retained robust cell activity but also exhibited increased production of extracellular polymeric substances (EPS). This surge in EPS production is a key factor in the establishment of biofilms, as reported by Yin et al. (2019). Surprisingly, our research has unveiled fascinating findings, the presence of crude oil significantly amplifies the formation of biofilms within our examined bacterial strains. Notably, strains of Pseudomonas aeruginosa exhibit an extraordinary propensity for biofilm development when crude oil serves as their exclusive carbon source, as detailed in the works of Dasgupta et *al*. (2013) and Dutta and Singh (2016). In the context of crude oil reservoirs, the microbial biofilm communities feature a diverse array of genera, including Achromobacter, Arcobacter, Pseudomonas, and Bacillus, which have showcased their remarkable capacity to metabolize and degrade hydrocarbons, with a particular focus on aromatic hydrocarbons. In this intricate metabolic pathway, these microorganisms leverage various metabolites, such as enzymes and biosurfactants, to facilitate the degradation of hydrocarbons, as expounded upon by Elumalai et al. (2021). An aggregation of dense biomass at the oil-water interface was observed (Dasgupta et al., 2013). This phenomenon revealed a diverse range of biofilm-forming microorganisms in produced water within oilfields, which have been linked to the onset of biocorrosion in X80 steel (de Oliveira et *al*., 2021). As reported, the most frequently found bacteria contaminating kerosene include *Bacillus simplex*, *P. synxantha*, and *Sphingomonas zeae*. These microorganisms have displayed a notable capacity for biofilm formation, acting as primary colonizers in the initial stage of biofilm development (Chiciudean et al., 2019). A recent study delved into the structures and composition of microbial biofilms cultivated in kerosene-based fuels, utilizing Scanning Electron Microscopy (SEM). The SEM imaging unveiled a complex,

multilayered structure of these biofilms. Detailed analysis of the images identified various bacterial and fungi-like structures, highlighting the cells capability to produce exopolymeric substances. Additionally, it became evident that three-dimensional microbial aggregates were prominently present in both the biofilm and water samples (Krohn et al., 2021). It has been reported that kerosene exhibits a low level of toxicity, falling within the range of 2.41% to 3.09%, when present at a contamination level of 10% over a 24-hour period. This effect pertains to its impact on the logarithmic survival rates of hydrocarbonoclastic bacteria such as *Bacillus sp*, *Pseudomonas sp*, *Serratia sp*, and *Micrococcus sp*. The remarkable survival capabilities of these microorganisms can be attributed to their proficiency in hydrocarbon degradation, particularly kerosene, and their adeptness in utilizing it as a carbon and energy source for metabolic processes. Conversely, a different pattern emerges in the case of other microorganisms exclusively identified in pristine soil samples, including *Streptococcus sp*, *Salmonella sp, Staphylococcus aureus,* and *Escherichia coli*. These microorganisms exhibit a notably higher susceptibility to kerosene toxicity, with survival rates within the range of 26.82% to 36.8% during the same exposure and incubation period. Their resilience against the toxic effects of kerosene is underpinned by a suite of resistance mechanisms, encompassing genetic adaptation, enzyme-mediated resistance, efflux pumps, biofilm metabolic activity, and outer membrane structural attributes (Nseabasi and Antai, 2012). Chemotaxis plays a crucial role in the behavior of bacteria, enabling them to navigate toward areas rich in chemicals that serve as valuable nutrients and energy sources. This process also empowers them to exploit resources that would, otherwise, be inaccessible, such as petroleum hydrocarbons. Bacteria are guided in their movement by specific chemical cues known as chemoattractants, while substances that repel them are referred to as chemorepellents (Murphy et al., 2020; Zhao and Ford, 2022). Interestingly, our research uncovered a positive chemotactic response to both hydrocarbons in the entirety of tested strains, suggesting their capability to utilize them as substrates or carbon sources. Intriguingly, bacterial migration towards crude oil surpassed that towards kerosene. Consistent findings demonstrated that *Pseudomonas* strains exhibited a strong attraction to refined petroleum oil. Our study further elucidated the role of bacterial adhesion and biofilm formation in confirming the directed mobility of bacteria towards refined oil (Dutta and Singh, 2016). Indeed, chemotactic microorganisms possess an interesting ability to detect changes in chemical gradients within their environments, leading to specific behavioral responses under the influence of this environmental stress. Based on the results obtained from the BATH analysis, it is evident that the various strains displayed a range of hydrophobic characteristics, fluctuating between being highly hydrophobic to distinctly hydrophilic. To provide a clearer understanding, strains were categorized based on a hydrophobicity index. Those with an index exceeding 80% were considered highly hydrophobic, while those with an index lower than 30% were regarded as strongly hydrophilic, as described by Wang et al. 2019. As per these criteria, our findings revealed that approximately 77.78% of the strains displayed a marked preference for hydrophobic interactions with crude oil. However, 22.22% of the strains exhibited a pronounced hydrophilic tendency when exposed to crude oil. Shifting the focus to kerosene, around 66.67% of the strains showcased different hydrophobic characteristics while 33.33% of strains manifested a hydrophilic phenotype in the presence of kerosene. Comparing the hydrophobicity of these strains across the two tested hydrocarbons, it was evident that our recovered strains exhibited a particularly intriguing preference for hydrophobic interactions with crude oil. These findings align partially with those described by Priji and colleagues, who observed significant hydrophobicity indices in *Pseudomonas sp*. strains toward crude oil in comparison to kerosene (Pijit et *al*., 2017). Notably, our results highlighted the exceptional hydrophilicity of the S30 strain, which is consistent with the findings of another study that characterized an environmental strain of *A. hydrophila* as having hydrophilic properties when exposed to hexadecane as a carbon source. In this case, the hydrophobicity indices reported were notably low (Kaczorek et al., 2010). Understanding the hydrophobicity of microbial surfaces is essential, as it plays a crucial role in adhesion to both abiotic and biotic surfaces, as well as in penetrating host tissues. Importantly, the hydrophobic properties of bacteria are integral to various beneficial processes, such as hydrocarbon degradation. Some environmental contaminants, such as toluene, are recognized for their high hydrophobicity and cell membrane-disrupting toxicity. Hydrophobic bacteria possess the ability to accumulate on such compounds and facilitate their degradation (Krasowska and Sigler, 2014). Remarkably, our assessment of the emulsification index unveils a compelling trait shared by a significant proportion of *Pseudomonas sp*. strains, as they exhibit noteworthy emulsifying capabilities when faced with crude oil and kerosene. Notably, the pinnacle of emulsification prowess was observed in *Pseudomonas* strains, with exceptional indices recorded in their interactions with diesel, kerosene, and motor oil, as elucidated by Viramontes-Ramos et al. 2010. Furthermore, our results reveal that *A. hydrophila* boasts substantial emulsification potential, particularly when confronted with crude oil. The emulsification activity of *A. hydrophila*, reaching an impressive 57.63%, surpassed that of *Acinitobacillus sp*. and *Vibrio parahaemolyticus* in the context of kerosene, as documented by Adetitun et al. in 2016. The analysis of statistical data has demonstrated a fascinating relationship, intricately tied to emulsification activity and chemotaxis towards both hydrocarbons. This is closely followed by the bacterial cell surface's hydrophobic characteristics. Notably, a robust correlation was observed between chemotaxis and hydrophobicity traits. However, The emulsification potential remained largely unaltered by other variables. The combined attributes of chemotaxis, cell surface hydrophobicity, and emulsification potential within these marine strains play a pivotal role in their resilience, capacity to adapt to hydrocarbon-induced stress, and ability to utilize these compounds as sources of energy and carbon. These findings hint at the potential of these strains to ameliorate water quality in sites contaminated by hydrocarbon contamination.

5. Conclusion

The behaviorome profiling of indigenous marine bacteria has yielded intriguing results in relation to the studied hydrocarbons. A pivotal discovery lies in their remarkable ability to form biofilms, enabling them to effectively combat hydrocarbon-induced stress. These bacterial strains have also exhibited substantial halotolerance, a crucial trait for survival in their marine habitat. Additionally, chemotaxis, a social behavior among these bacteria, has been emphasized. Notably, these marine strains exhibit an intriguing hydrophobicity and possess significant emulsifying potential, indicative of their significant capability in addressing hydrocarbon-related challenges. This emulsifying activity is intricately linked to the production of biomolecules, which serve as highly effective bioemulsifiers for hydrocarbons.

This study presents several noteworthy limitations, with one of the most significant concerns revolving around potential interactions between hydrophobicity and various surface properties. These interactions encompass factors like surface tension, the application of techniques such as PCR for molecular strain identification and HPLC for bioemulsifier detection, along with considerations related to their environmental relevance. From a broader perspective, the study explores the potential of these strains to produce valuable biomolecules for various biotechnological applications. The purification and molecular characterization of these metabolites will be facilitated through the use of analytical tools, including gel filtration chromatography and HPLC. Furthermore, the identification of robust producer strains will be accomplished by delving into their S16 rRNA profiles. In sum, these findings offer substantial support for the viability of employing these strains to promote the principles of sustainable development, particularly in scenarios marked by water contamination with hydrocarbons.

6. Conflicts of interest

The authors declare no conflict of interest

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