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Research Article

Environmental stressor assessment of hydrocarbonoclastic bacteria biofilms from a marine oil spill

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ABSTRACT

The environmental and economic impact of an oil spill can be significant. Biotechnologies applied during a marine oil spill involve bioaugmentation with immobilised or encapsulated indigenous hydrocarbonoclastic species selected under laboratory conditions to improve degradation rates. The environmental factors that act as stressors and impact the effectiveness of hydrocarbon removal are one of the challenges associated with these applications. Understanding how native microbes react to environmental stresses is necessary for effective bioaugmentation. Herein, *Micrococcus luteus and M. yunnanensis* isolated from a marine oil spill mooring system showed hydrocarbonoclastic activity on Maya crude oil in a short time by means of total petroleum hydrocarbons (TPH) at 144 h: *M. luteus* up to 98.79 % and *M. yunnanensis* 97.77 % removal. The assessment of *Micrococcus* biofilms at different temperature (30 °C and 50 °C), pH (5, 6, 7, 8, 9), salinity (30, 50, 60, 70, 80 g/L), and crude oil concentration (1, 5, 15, 25, 35 %) showed different response to the stressors depending on the strain. According to response surface analysis, the main effect was temperature > salinity > hydrocarbon concentration. The hydrocarbonoclastic biofilm architecture was characterised using scanning electron microscopy (SEM) and atomic force microscopy (AFM). Subtle but significant differences were observed: pili in *M. luteus* by SEM and the topographical differences measured by AFM Power Spectral Density (PSD) analysis, roughness was higher in *M. luteus* than in *M. yunnanensis*. In all three domains of life, the Universal Stress Protein (Usp) is crucial for stress adaptation. Herein, the *usp*A gene expression was analysed in *Micrococcus* biofilm under environmental stressors. The *usp*A expression increased up to 2.5-fold in *M. luteus* biofilms at 30 °C, and 1.3-fold at 50 °C. The highest *usp*A expression was recorded in M. *yunnanensis* biofilms at 50 °C with 2.5 and 3-fold with salinities of 50, 60, and 80 g/L at hydrocarbon concentrations of 15, 25, and 35 %. *M. yunnanensis* biofilms showed greater resilience than *M. luteus* biofilms when exposed to harsh environmental stressors. *M. yunnanensis* biofilms were thicker than *M. luteus* biofilms. Both biofilm responses to environmental stressors through *usp*A gene expression were consistent with the behaviours observed in the response surface analyses. The *uspA* gene is a suitable biomarker for assessing environmental stressors of potential microorganisms for bioremediation of marine oil spills and for biosensing the ecophysiological status of native microbiota in a marine petroleum environment.

1. Introduction

Oil spills can have extensive environmental and economic impact. In 2018, the ultimate cost to BP for the Deepwater Horizon (DWH) oil spill in 2010 for 87 days into the Gulf of Mexico, was of $144.89 billion in the United States [1]. The intervention of microorganisms in the biodegradation processes is so important that for the DWH oil spill the half-lives of n-alkanes that leaked into the deep sea were shorter than expected, it meant 1–6 days [2]. Biotechnologies applied during a marine oil spill concern biostimulation, bioaugmentation, and biosurfactant dispersion.

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These processes can be carried out with indigenous or allochthonous microbiota [3]. Bioaugmentation with selected hydrocarbonoclastic species under laboratory conditions is used to improve hydrocarbon degradation rates [3]. Indigenous isolates obtained from environmental samples are applied to overcome the lack of adaptability of allochthonous microorganisms and are preferred for their catalytic abilities to accelerate the degradation of contaminants [3,4]. Also, the indigenous strain isolation is mainly carried out in environments where there is low number of autochthonous microbes with the ability to degrade the contaminants [4]. One of the biotechnological alternatives is the release of autochthonous microorganisms with active compounds into the marine environment attached to or inside particles made of polymers such as polyurethane–polyurea co-polymers, alginate, and chitosan of low or no toxicity that serve as a vehicle [3]. Due to the immobilization action, the bacterial cells were protected from being washed off into the sea [5]. In seawater, antibiotic resistance genes and stress response seem to be important components of the biofilm functional core of over 7300 marine biofilm-forming species, stress response aids bacteria in surviving in their natural environments and can start the formation of biofilms [6]. In this respect, biofilm-mediated bioremediation is a suitable strategy for the removal of toxic compounds in the environments [7]. Hydrocarbonoclastic bacteria use hydrocarbons as their sole nutrient source, their biofilms are potentially efficient due to their ability to utilise a wide range of crude oil compounds as their preferred energy and nutrient source, the biosurfactants they produce increase the availability of hydrocarbons to the microbial cells [8]. The low bioavailability of the hydrophobic compounds of hydrocarbons to microorganisms is one of the main factors limiting the effectiveness of the bioremediation process [7]. In this respect, biotechnological alternatives are available to immobilise cells and biofilms of microorganisms with catabolic capacities for hydrocarbon biodegradation [9–11]. Environmental conditions are one of the problems faced by these immobilization applications, as they exert stressors that influence the removal efficiency and biodegradation of hydrocarbons [11]. Subsequently, it is crucial to investigate and characterise the in vitro physiology and metabolic capacity of different hydrocarbon-degrading bacteria in specific conditions. Microbes are subject to environmental stressors all the time, their influence determines the regulation of genes, whether they are expressed or not during biofilm formation [12]. The universal stress proteins (Usps) are expressed in eukaryotes and prokaryotes. Usp, which is a serine and threonine autophosphorylating protein, a primary regulator of bacterial survival, has a role in adaptation to several pressures, such as oxidative stress, temperature changes, low pH, or hypoxia, and induces adaption to the hostile environments by promoting adhesion and motility [13]. On the other side, oil concentration, pH, and salinity have influence on the crude oil removal efficacy in hydrocarbonoclastic immobilised bacteria [11]. Moreover, understanding the mechanisms and enzymes/genes involved in biofilm formation will help to develop new strategies for the bioremediation of contaminated environments and hazardous pollutants [7]. *Micrococcus* can be found in contaminated soils, oil spills, and sludge, it degrades hydrocarbons and olefinic compounds [14]. It uses toxic organic molecules as carbon sources through dioxygenase and phenyl genes which makes it a suitable candidate for bioremediation processes [15]. *Micrococcus* is recognised as having the potential for enhanced bioremediation in marine environments [16]. Biosurfactants favour the hydrocarbon biodegradation by miscibility effect on hydrophobic compounds which makes them available to hydrocarbonoclastic microorganisms [17]. The *Micrococcus* hydrocarbon degrading activity has been widely demonstrated and applied in different biotechnological contexts, not only as cells in suspension, in biofilm or embedded in enrichment media with hydrocarbons [15, 18–20] but also with its extracellular organic matter for enhance *ex situ* biodegradation of soils contaminated with oils [21]. Immobilised *Micrococcus* has been evaluated for the biodegradation of the pyrethroid pesticide cypermethrin [22]. Encapsulated Micrococcus in electrospun fibers and alginate beads have been assessed to absorb large amounts of copper [23], as have models of porous composite nonwovens with hydrogel microparticles with encapsulated Micrococcus that influence in the copper bioremediation process [24]. Although it has been demonstrated its potential for biodegradation on and in different material and vehicle configurations as described above, and found to be a strong biofilm producer, which makes it a good candidate for crude oil biodegradation [20], in these situations, it is unclear or unstudied how environmental stressors and biofilm formation are related. The response of the indigenous microbiota in marine oil spill is modulated by local environmental factors, hence it is crucial to assess the microorganisms under these conditions [3,25]. According to the latter, in the case of marine hydrocarbonoclastic *Micrococcus* biofilms, the process of tolerance needs to be understood for their potential application in oil spills. In this regard, to the best of our knowledge, there are no specific assessment protocols established with this species as a field guide to evaluate certain environmental stressors affecting hydrocarbonoclastic biofilms that could adhere to the materials that support or transport them to be applied in a marine oil spill bioremediation process, which is paramount to preparing a ready-to-use bioremediation product. In this work, a basic strategy is proposed by optimising the high-throughput microtiter plate method as a rapid technique for the study and evaluation of responses to environmental stressors in biofilms of a marine hydrocarbonoclastic actinobacterium such as *Micrococcus* through the quantification of *usp*A gene expression (as a biomarker) under stressful conditions including pH, salinity, temperature, and hydrocarbon concentration, and by characterising the biofilm architecture by scanning electron microscopy (SEM) and atomic force microscopy AFM-PSD analysis.

2. Materials and methods

2.1. Sampling site

During August 2012, an oil spill from a mooring system was registered in Salina Cruz, Oaxaca, México (Lat 16.166389, −95.218858). At least eight beaches in the municipalities of Tehuantepec and Salina Cruz were impacted by the spill. The event had an influence on sea turtle arrivals as well. These beaches had oil layers covering the supratidal zone. Following the spill, early response decisions were made based on a situational analysis and to establish monitoring and sampling activities in accordance with guidelines issued by USEPA 2006 [26] and NOAA 2006 [27]. Identifying and validating the source, defining the spill zones, and selecting and implementing appropriate response measures were all part of the early stages of the leak. This followed the selection of water and sand samples from the affected zones. The most effective approach was a combination of strategies, utilising basic random and judgmental sampling and adhering to the characteristics and procedures of sampling as outlined in USEPA 1994 [28], USEPA 2001 [29], and USEPA 2002 [30]. Four sand samples (100–300 g) were taken along the line of the oil spill from a section of approximately 2 km of Salinas del Marqués, one of these nine beaches. Four water samples (1 L) were collected 10 m offshore from the intertidal zone of the same section beach. Samples were collected in sterilised amber glass bottles. They were immediately transported to the laboratory at 4 °C for analysis.

2.2. Isolation and screening of the hydrocarbonoclastic bacteria

Serial dilutions were carried out with 5 mL of seawater or 5 g of crude oil-impregnated sand in 100 ml of phosphate buffer (pH 7.4). For selective enrichment, one millilitre of dilution was transferred to 250 ml Erlenmeyer flasks containing 100 ml of Bushnell Haas mineral salts medium (BHM) for the isolation of crude oil-degrading bacteria. BHM medium contains (g/L)) KH_2PO_4, 1; K_2HPO4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaCl_2$, 0.02; NH_4NO_3, 1 [31,32] amended with 1 % (wt/vol) of sterilised Maya crude oil (22° API) as a carbon source for the hydrocarbonoclastic bacteria growth. The flasks were incubated for 8–10 days at 27 °C (the

same as the temperature of the sampling site) on a rotary shaker at 180 rpm. Five millilitre aliquots were then transferred to fresh BHM medium. After two further subcultures, the inoculum was removed from the flask, and colonies with distinct phenotypes were purified on BHM agar. To eliminate autotrophic and agar-using bacteria, phenotypically distinct colonies taken from the plates were plated on fresh BHM medium with and without crude oil. After repeating the process, isolates showing growth only on crude oil were retained for further analysis. The growth rates of the isolates were assessed on a routine basis by measuring the optical density at 620 nm in a UV–visible spectrophotometer.

2.3. Molecular identification

Several hydrocarbonoclastic bacteria were isolated from the oil spill; by comparisons of the significance (by one-way ANOVA) of differences between cultures ($p < 0.001$), *Micrococcus* showed the best hydrocarbonoclastic activity. Molecular identification of *Micrococcus* strains 2A1 and 2Sc was conducted by the 16S rRNA gene phylogenetic approach. DNA was extracted with an Ultraclean Microbial-DNA extraction kit (Qiagen). PCR amplifications of the 16S rRNA gene were performed with universal bacterial primers described by Relman [33]: NVZ-1. GCGGATCCGCGGCCGCTGCAGAGTTTGATCCTGGCTCAG and NVZ-2. GGCTCGAGCGGCCGCCCGGGTTACCTTGTTACGACTT-3. The PCR products were sequenced with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). The 16S rRNA gene sequence was compared to the GenBank nucleotide databases (NCBI) using BlastN and BlastX algorithms [34]. The nucleotide sequence was aligned in ClustalX [35]. The phylogenetic analyses were conducted using MEGA X [36]. Genetic distances were calculated by the Kimura two-parameter procedure [37]. The phylogenetic tree was constructed by the neighbour-joining method [38] and assessed with 1000 bootstrap replications [39]. The minimum value of the nucleotide similarity percentage to define the species at the taxonomic level was 97.5 % [40].

2.4. Crude oil biodegradation assays

The biodegradation assays were performed with the hydrocarbonoclastic bacteria *Micrococcus* strain 2A1 and 2Sc in BHM with artificial seawater prepared following the standard ASTM D1141 [41] (30 g/L salinity adjusted with a YSI-Multiparameter) with 1 %, 5 %, and 15 % (wt/vol) of Istmo crude oil (32° API), under sterile conditions. The microbial growth was measured by spectrophotometry at 620 nm every 12 h up to 150 h for both strains. The respective sample collection was conducted for the Total Petroleum Hydrocarbons (TPH) determination. No microorganisms were added to the control assays with 1 %, 5 %, and 15 % (wt/vol) of sterilised crude oil. Data were expressed as mean ± standard deviation (SD) of the triplicate experimental data. A lighter crude oil was chosen to ease handling, to be able to perform biofilm microtiter plate tests (which is experimentally difficult with heavy, viscous crude oils). Thus, there are the same experimental conditions in the biodegradation assessment.

2.5. Determination of hydrocarbons

The hydrocarbon extraction and concentration were conducted following the EPA 3510 method [42]. The analytical method to determine the extent of TPH was followed according to ISO 16,703:2004 [43] by gas chromatography/flame ionization detection (GC/FID). To determine the concentration of TPH in the samples, the chromatograms were integrated considering the area under the curve of the resolved peaks and compared against a sample of the original crude oil. The following conditions were considered: equipment detection limit 0.02 mg / L, limit of quantification of the method: 0.05 mg / L, recovery rate: [91 %, 95 %] ± 1.5 %, matrix effect: [−9 %, −3 %] ±0.5 %, R2: [0.992, 0.998]. The equipment was an Agilent 7890 Series Gas Chromatograph with FID configurated as follows: 1) Split/splitless inlet; 2) Agilent Focus Liner, tapered, deactivated with glass wool; 3) Agilent 7890 Guard Chip; 4) Single detector 7890 flow path; 5) Agilent DB-5ht column, 5 m × 0.32 mm, 0.1 μm film; 6) Flame ionization detector; 7) Agilent 7693 automated Liquid sampler (ALS) with 10 μL syringe. The instrument conditions were as follows: Inlet: Splitless at 350 °C; Injection volume: 0.5 μL; Constant column flow 10 mL/min helium; Column temperature program: 40 °C for 0.5 min, 250 °C/min to 350 °C and 350 °C for 1.3 min; Intuvo flow path: Guard chip, track oven mode; Bus: default (350 °C); Detector Flame Ionization at 350 °C. An internal standard as a mixture of several hydrocarbons was used for the determinations of percentage of recovery rate and matrix effect. For calibration curve was used an alkane analytical standard, C_{10} - C_{40} (all even), 50 mg/l each. As an internal standard for recovery rate and matrix effects determinations was used a mix of deuterated Heptane (d-16), Octane (d-18), Dodecane (d-26), Pentadecane(d-32) and Eicosane (d-42) at 10 ng/ml from Sigma-Aldrich. The determination of hydrocarbons (even numbered) was conducted according to ISO 16,703:2004 [43]. The standard mixture of alkanes was used to optimise the chromatographic method, to identify alkanic components in petroleum, and to quantify alkanes during biodegradation assays. The percentage of biodegradation was calculated as follows:

$$\%BD = \frac{TPA\ i - TPAx}{TPA\ i}\ x\ 100$$

$$\%BD = \frac{(TPA\ i + TPAis) - (TPAx + TPAis)}{TPA\ i}\ x\ 100$$

TPAi = Initial Total Peak Area.
TPAx = Total Peak Area at time *x*.
TPAis = Total Peak Area of the internal standard mixture.

It means, a fixed concentration of the mixture of deuterated internal standards, which generates a constant total integration area (is), is added to the initial oil sample (*i*) and to the final sample at a given biodegradation time (*x*) to establish whether there are any effects that could interfere with and alter the results of the integration of the total area of the peaks obtained in the chromatograms.

2.6. Assessment of biofilm formation

The evaluation of the biofilms was made by microtiter plate assay [44] in 96-well polystyrene plates. After confirmation of hydrocarbonoclastic activity, *Micrococcus* strains were first subcultured on Luria Broth (LB) and incubated at 30 °C overnight whilst being shaken at 150 rpm to reach an exponential growth phase inoculum of 1.5×10^6 UFC/mL. In sterile conditions inside a biosafety hood, BHM aliquots of 200 μL were dispensed into the 96 wells of sterile polystyrene microplates, followed by the addition of the bacterial culture. Microplates were sealed with a sterile film and incubated at 30° and 50 °C for 24 h. The pH values tested were 5, 6, 7, 8, and 9 adjusted during BHM preparation. The concentrations of crude oil employed were 1 %, 5 %, 15 %, 25 %, and 35 % (wt/vol). The BHM salinity was set with artificial sea water, prepared following the ASTM D1141 standard [41], adjusting the salinity with a YSI-Multiparameter, with the following concentrations: 30, 50, 60, 70 and 80 g/L. Control wells had bacteria with BHM medium (pH 7) with the corresponding salinity, lacking hydrocarbons. The negative controls were wells with BHM and no bacteria. From preliminary tests on microtiter plates with the *Micrococcus* strains and with each of the stressors, it was observed that what most influenced the response in the biofilm was temperature > salinity > hydrocarbon concentration. It was therefore decided to set the conditions in the controls with the aim of obtaining a homogeneous response to biofilm growth that could be measured and compared with the rest of the microtiter experiments, and that would serve as a control to observe what

happened to the biofilm under different concentrations of hydrocarbons and the rest of the stressors. All experiments were carried out in triplicate. After incubation, the microtiter plates were washed twice with PBS (phosphate buffer saline) then stained with crystal violet (CV) 1 % with 150 µL of dye per well and incubated at room temperature for 10 min. CV was removed with 200 µL of alcohol-acetone (4:1) for 5 min. The microplates were read at 590 nm with a Synergy2-BioTek lector.

2.7. Statistical analyses

To assess for significant differences during the microbial growth between isolated hydrocarbonoclastic strains, including *Micrococcus,* under different hydrocarbon concentrations, data were analysed by means of a one-way ANOVA, and, if significant differences were found ($p < 0.05$), a Tukey test for multiple comparisons was applied. The effect of the temperature, salinity, hydrocarbon concentration, pH, and strain on the recorded absorbance of the biofilms was analysed by means of a five-way ANOVA, if significant differences were found ($p < 0.05$), a Tukey test for multiple comparisons was applied considering the log (optical density) as the response variable (biofilm growth). Statistical analyses were performed with the R-program v. 4.0.4 [45]. A significant level of $p < 0.05$ was used for all statistical tests.

2.8. Characterization of biofilm architecture by microscopy

2.8.1. Scanning electron microscopy (SEM)

Micrococcus biofilms developed on round polystyrene sheets after 24 h incubation in serologic bottles with BHM and Istmo crude oil (1 %) in artificial sea water (30 g/L) at 30 °C were analysed using a Scanning Electron Microscopy (SEM) Dual Beam Nova-2000 Nanolab Model 200 (Thermofisher) equipped with a field emission gun. The images were acquired in the secondary electron (SE) mode with the microscope operating at 15 kV and a working distance (WD) of ~ 5 mm. After the incubation time, in sterile conditions inside a biosafety hood, the polystyrene surfaces with the biofilms were picked up with tweezers and mounted on a microscope specimen holder. The samples were dried at room temperature during approximately 30 min prior to microscopic examination to remove moisture from the sample and to avoid contamination of the microscope column. Due to the characteristics of the microscope, no pre-treatment or pre-coating of the biological samples was necessary for observation. It was decided to use the biofilms developed under the described environmental conditions because they were suitable for obtaining biofilms of just the right thickness for microscopic observation, neither too thick nor too thin.

2.8.2. Atomic force microscopy (AFM)

To determine the topographical characteristics and the evolution of biofilm roughness, *Micrococcus* 2A1 and 2Sc hydrocarbonoclasts were cultured under sterile conditions on round polystyrene plates in BHM and Istmo crude oil (1 %) in artificial seawater (30 g/L) at 30 °C. These environmental conditions were sufficient to obtain biofilms of the correct thickness for manipulation and microscopic observation. Biofilm growth was observed after 8, 16, 24, 32, 40, 48, 60 and 72 h of exposure. All morphological measurements were performed in air at room temperature (20 °C) without any drying process using a MultiMode 4 AFM with a NanoScope V controller (Bruker), with RTESP silicon nitride tips with a nominal spring constant of 40 Nm^{-1} and drive amplitude of 0.2, in tapping mode over a maximum scanning area of 12.5 µm x 12.5 µm. A small area was defined to measure the nanoroughness factors [46]. Image processing was carried out in Nanoscope Analysis software (Bruker) to ensure that there were topographical differences in the two *Micrococcus* biofilms. Before conducting the roughness analyses, the images were flattened using the PlaneFit tool in the XY axes up to the second order [47]. Roughness was obtained by measuring five horizontal lines, each 12.5 µm in length with a spacing of approximately 2.2 µm. This data processing method was previously shown to provide a more accurate description of surface properties and characteristics when studied by AFM [48]. Mean roughness (Ra), root mean square roughness (Rq/RMS) and mean height roughness (Rz) were chosen as the three roughness parameters. Ra is the mean deviation of the height values from the mean line/plane. Similarly, Rq is the root mean square deviation from the mean/plane, i.e. the standard deviation from the mean. The mean roughness (Ra) is the arithmetic mean of the absolute values of the heights in each zone or profile of the surface $Z(x)$. It is mathematically described as follows:

$$R_a = \frac{1}{L}\int_0^L |Z(x)|dx \quad (1)$$

where $Z(x)$ is the function that details the area or profile of the surface being studied in terms of height (Z) and position (x) of the sample over the evaluation length, L [49].

$$R_q = \sqrt{\frac{1}{L}\int_0^L |Z^2(x)|dx} \quad (2)$$

The roughness of the mean height (Rz) was measured based on the methodology of Hamza et al. [50]. In addition, Power Spectral Density (PSD) has been used to study complex morphometric features such as biofilms. PSD provides topographical information on the vertical and lateral distribution as a function of surface spatial frequencies and can estimate the Rq parameter more accurately from the calculation of the integral of the PSD curve [48] without being limited by the scan size and resolution of the AFM. Mathematically, PSD is represented as:

$$PSD_{2D}(f_x, f_y) = L^{-2}\left[\sum_{m=1}^{N}\sum_{n=1}^{N} Z_{mm} e^{-2\pi\Delta L(f_x m + f_y n)}(\Delta L)^2\right]^2 \quad (3)$$

Statistical analysis was conducted by a paired student *t*-test to evaluate differences in biofilm growth between strains 2Sc and 2A1. The differences in the strain biofilm thickness (Rz) were evaluated by one-variable ANOVA [51,52].

2.9. Real time-PCR procedures

2.9.1. Primer designing

UspA protein from *Micrococcus* is related to its stress state [53]. It is a primary regulator of bacterial survival processes in *M. luteus. Usp* genes are widely conserved across diverse bacterial species [54]. The *usp*A gene sequence of Universal Stress Protein A was obtained from GeneBank at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) from the *M. luteus* strain SB1254 chromosome complete genome (GenBank CP026366.1) and was analysed by the BLAST program [34]. The highly conserved regions in the gene were considered for the design of PCR primers and for non-redundant proteins. The primers were designed with the Primer3 tool [55]; uspA-Forward: GAGCAGGCGACCCATGAAG; uspA-Reverse: CGTCACCGCCTACTCGATC (Tm: 60 °C). After the PCR's assays, the amplicon (259 bp) was sequenced and compared against the public database by the BLAST program, to ensure that the correct target was being amplified. Additionally, primers were probed with cDNA from the strain collected and the reference *M. luteus* ATCC 4698.

2.9.2. RNA extraction and RT-PCR

To remove any residual dye and previous step products from the biofilm, in sterile conditions inside a biosafety hood 200 µL of sterile buffer solution (3 g Na_2HPO_4; 2 g KH_2PO_4; 5 g NaCl; 0.25 g $MgSO_4$ $7H_2O$ per litre diluted in free RNAse water; pH 6.5) were added to each well. The biomass was then placed in an Eppendorf tube and washed once more with buffer solution. Subsequently, the pellet was washed and preserved in RNAprotect bacteria reagent (Qiagen). Total RNA was

isolated using TRIzol reagent (Invitrogen) and stabilised and purified through RNeasy Protect Kit (Qiagen). The extracted RNA was treated with RNase-free DNase (Qiagen). The mRNA was isolated and enriched using the MicrobExpress system (Ambion) to remove large rRNA. The reverse transcription reactions were conducted with OneStep RT-PCR kit (Qiagen) for gene-specific primers. The RT-PCR analyses were performed in triplicate with Power SYBR Green PCR Master Mix (Applied Biosystems) in a 7500 Fast Real-Time PCR System (Applied Biosystems). The detection limits of the assays were determined from a dilution series of RT-PCR products purified with the QIAquick PCR purification kit (Qiagen). Melting curve probes were developed to check the specificity of the amplicon. The RT-PCR runs were conducted with the following procedure: 2 min at 50 °C; a denaturation step of 10 min at 95 °C; 40 cycles of denaturation for 30 s at 95 °C; an annealing step for 1 min at 60 °C; an elongation step for 30 s at 72 °C and a final elongation for 5 min at 70 °C. The housekeeping gene 16S rRNA was used for normalization (primers 16S-F: CGAGCGTTGTCCGGAATTA; 16S-R: TCCACCGCTACACCAGGAAT) under the same experimental conditions. Each run included a negative control and a cDNA reaction without reverse transcriptase to rule out DNA contamination. The relative gene expression of the *Micrococcus* biofilm under the assessed stressors (temperature, salinity, and hydrocarbon concentration) was conducted in triplicate and compared to the respective controls, including the internal biological controls performed with *M. luteus* ATCC 4698. Data were analysed by student *t*-test ($p < 0.05$).

3. Results and discussion

3.1. Molecular identification of strains

The GenBank accession numbers of the *Micrococcus* strains are KM232741 and KM232742. The results of phylogenetic analyses and estimates of evolutionary divergence between the sequences (similarity matrix) identified the bacteria to species level (Fig. 1).

3.2. Crude oil biodegradation assays

Hydrocarbonoclastic bacteria were isolated from a mooring system oil spill in Salina Cruz, Oaxaca., Mexico. *Campylobacter, Alcaligenes, Chromobacterium, Enterobacteria, Pseudomonas, Vibrio, Veillonella, Flavobacterium,* and *Micrococcus* were found. *Micrococcus* showed the best hydrocarbonoclastic activity on Maya heavy crude oil (22° API). To confirm the hydrocarbonoclastic activity, the crude oil was tested as a sole carbon and energy source at 1 %, 5 %, and 15 % concentrations (Fig. 2). Both strains showed high removal activity by means of TPH; for *M. luteus* 2A1 isolated from seawater, at 24 h the removed TPH level was 96.76 %, 95.98 %, 90.43 %, respectively, at 48 h 98.79 %, 98.21 % and 92.81 %, respectively, and at 144 h 99.90 %, 99.11 %, and 96.30 % respectively. The strain *M. yunnanensis* 2Sc isolated from oily sand, resulted in a removed TPH level at 24 h of 96.72 %, 95.70 % and 90.70 %, respectively, and at 48 h these were 97.77 %, 96.58 % and 92.22 % respectively for the 1 %, 5 % and 15 % hydrocarbon concentrations. By 144 h the oil degradation was recorded to be 99.84 %, 99.80 % and 96.12 %, respectively. For both strains, 15 % concentration crude oil displayed 99 % biodegradation by 230 h. Significant difference ($p < 0.05$) was detected in hydrocarbon removal assays with 1 % and 5 % concentration for both strains, though *M. luteus* biodegraded the hydrocarbons faster than M. *yunnanensis*. During the growth period in the 15 % concentration crude oil, both strains behaved in a similar manner; there was no significant difference. *Micrococcus* belongs to Actinobacteria phylum, which is widely dispersed throughout marine environments, appearing in marine sediments, water column, marine snow, ocean-bottom sediments, and even marine vertebrates and invertebrates. These free-living, Gram-positive bacteria have a wide repertoire of biotechnological applications. They produce more than half of the known bioactive compounds derived from microbial sources [56], have a potential role in bioremediation of toxic organic pollutants and a tolerance to metals [18–24]. Despite its small genome, this actinobacterium can adapt to strict ecological niches and is widely used in the pharmaceutical industry to test and screen compounds for antibacterial activity because of the reduced set of penicillin-binding proteins and the absence of a *wbl*C gene. Furthermore, it can synthetize long-chain (C_{21}–C_{34}) aliphatic hydrocarbons and has been applied in ore dressing and bioremediation applications, since it is able to concentrate heavy metals from low-grade ores [57]. The marine *Micrococcus* strains in this work displayed a faster hydrocarbon biodegradation process, as well as much higher TPH removal rates with a heavy crude oil (96–99 %), occurring in just 6 days compared to the reported 15 days with crude oil removal values of 90–93.7 % with the same species [58].

3.3. Biofilm architecture by SEM and AFM

The *Micrococcus* biofilms developed on polystyrene surfaces in marine medium with crude oil were observed by SEM (Fig. 3). The cells of both strains were unfixed and displayed the typical morphology of *Micrococcus* consistent with previous studies [59]; the images were obtained without

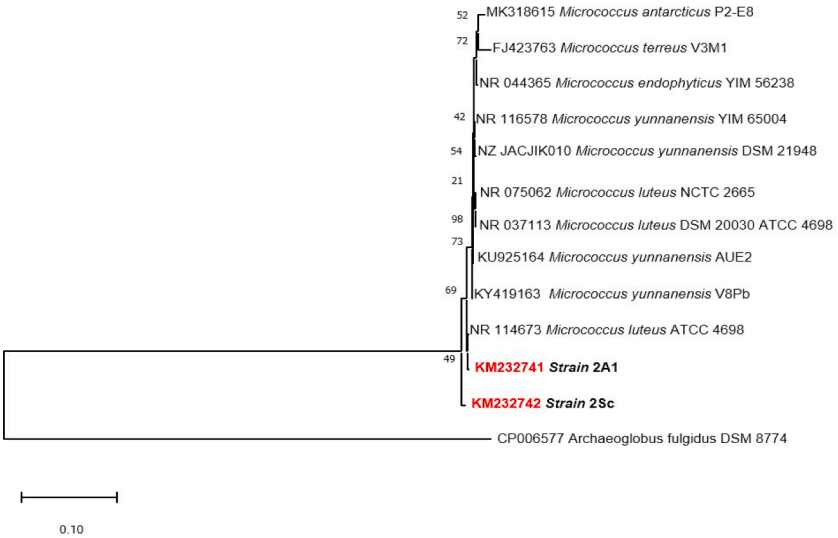

Fig. 1. Phylogenetic tree showing the evolutionary relationships of *Micrococcus* taxa. In bold letter the *Micrococcus* strains 2A1 and 2Sc with their respective GenBank numbers.

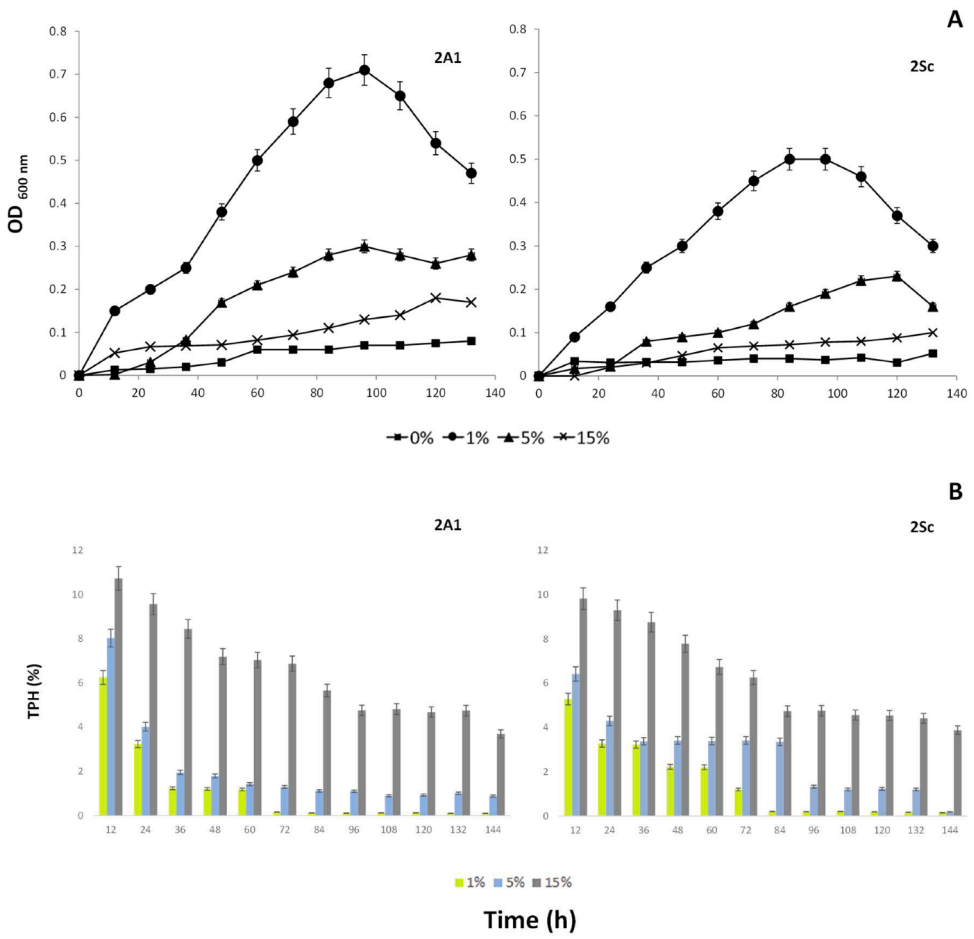

Fig. 2. Growth of hydrocarbonoclastic *Micrococcus* strains under different concentration of crude oil for 144 h and the total petroleum hydrocarbons during kinetics (mean ± standard deviation of three repeats). A) Absorbance at 600 nm in BHM at 30 °C; B) TPH (%) are shown for each strain over the time. *M. luteus* strain 2A1; *M. yunnanensis* strain 2Sc.

using conventional sample preparation methods. Despite being the same genus and sharing some morphological features, the different cell surface characteristics of each species became apparent. In the case of the *M. luteus* 2A1 strain, thin pili can be seen around the surface of the cell; pili are involved in adherence to surfaces, virulence, communication with other cells, as well as being implicated in the uptake of external DNA into the cell. Pili biogenesis has been documented in *Micrococcus* under certain nutritional conditions, such as starvation, as a general response to stress that leads to a competence state during growth [60]. The AFM results in tapping mode are shown in Fig. 4. Although the RTESP probe is stiff, it does not appear to cause any damage to the biofilm. This is due to the combination of the tapping mode and the low drive amplitude, which significantly reduces the shear force of the tip on the biofilms [61,62]. These probes have been widely used to study biofilms of different species without causing any apparent damage [63,64]. At 8 h the 2A1 biofilm showed higher surface colonization with respect to strain 2Sc. In both strains, the architecture of a mature biofilm was observed after 24 h. The presence of EPS is evident for both strains over time. However, in strain 2A1, the EPS formation starts as early as 8 h, while in strain 2Sc the EPS are not yet visible [65]. In general, roughness is quantified using linear roughness parameters (Rq, Ra, Rz, etc.) and area/surface roughness parameters (Sa, Sq, Sz, etc.). However, these treatments oversimplify the roughness value by reducing a large number of surface features to a single value [48,66]. The roughness parameters of the Micrococcus strains were obtained by averaging five sections homogeneously distributed over the entire micrograph (see Fig. 5). This method provides a more accurate roughness value, as shown in a previous study [48]. In both cases, the surface roughness of the biofilm is clearly modified (Fig. 5). As for the surface roughness determinations, at 0 h the topographical features were homogeneous, both in Ra and Rq the difference was less than 6 nm, bacterial growth was homogeneous and abundant, and it was in initial phase. The accumulation of cells was observed in the central zone of the biofilms and is corroborated by the lateral and vertical topographic evolution, which contributes to the increase in surface roughness. In some areas, small protuberances corresponding to typical *Micrococcus* individual cells were observed. At 32 h, the roughness as measured by Ra and Rq showed a decrease. For both strains, 2A1 and 2Sc, three characteristic successive phases of biofilm formation on the surfaces were detected by AFM: initial, growth period, and maturation [67]. The EPS is somewhat important, allowing the adherence of the cells in pores or grains on the surface, as already determined with other biofilm-forming bacterial cells [68,69]. AFM corroborates the topographical evolution of the biofilms laterally and vertically, which contributes to an increase in surface roughness [46,68]. The small protrusions observed corresponding to single cells and morphological characteristics of *Micrococcus* were confirmed. For the specific case of the surface roughness of strain 2A1 at 32 h, Rq showed a drastic decrease, being less than strain 2Sc due to the complexity of the biofilm, especially the appearance of the EPS and contribution of the porosity and topography of the substrate. This phenomenon may be due to the adsorption of proteins and the response or interaction of the cells on the surface [69–71]. The increase in roughness, with regards to Ra and Rq, contrasts to other experiments with hydrocarbonoclastic bacteria where the thickness of the biofilm during the first 7 days of growth measured between 50 and 200 nm [40]. Roughness in height (Rz) showed the highest values for both strains.

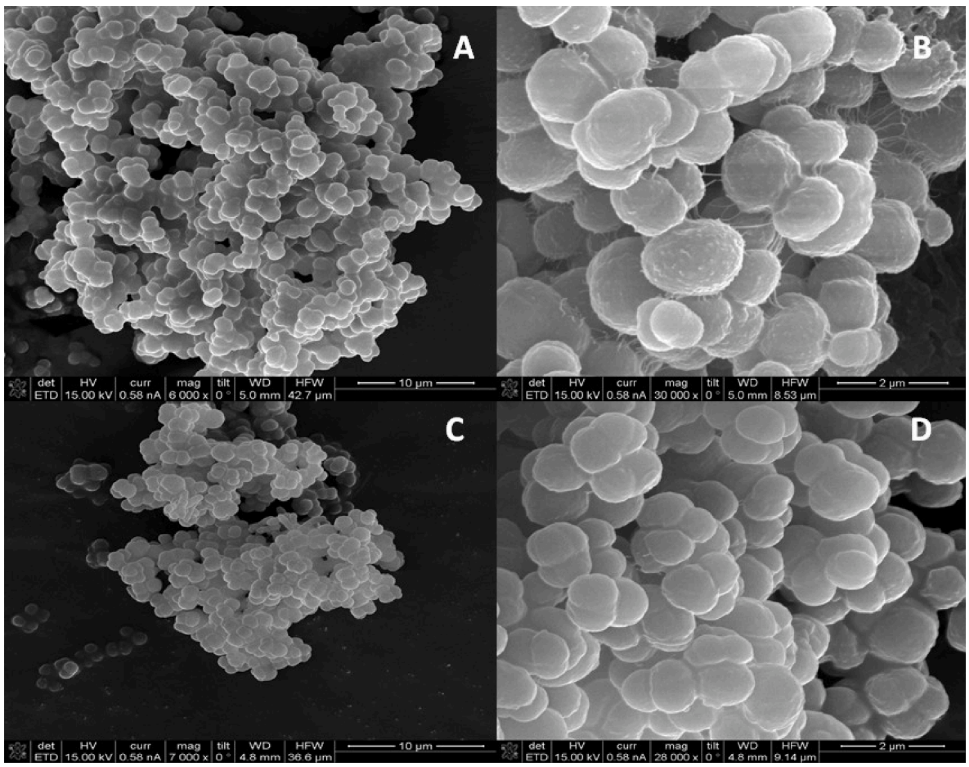

Fig. 3. SEM micrographs of the hydrocarbonoclastic biofilms (at 24 h/30 °C). A) and B) *Micrococcus luteus* 2A1 (GenBank: KM232741); C) and D) *Micrococcus yunnanensis* 2Sc (GenBank: KM232742).

Rz value is more sensitive to the presence of high peaks and deep valleys compared to Ra; however, Rz is underutilised in the AFM study of biofilm roughness [72]. In strain 2A1, Rz has a similar trend in behaviour to Rq, corroborating the drastic decrease in roughness at 32 h, because it easily estimates more pronounced irregularities, as described in other works [72, 73]. For strain 2Sc, a characteristic behaviour is displayed with respect to the other two parameters, showing a conditioning stage in the first 24 h, followed by maturation of the biofilm [67]. Biofilm formation on surfaces is a dynamic and complex process. As in other fields, the topographic evolution of biofilms is described by traditional roughness parameters [67, 68,74], which can qualitatively and quantitatively mask the real roughness distribution [75], which is related to the maturation phases of biofilms. PSD allows to describe this process considering the lateral and vertical characteristics of the surface, complementing the traditional roughness parameters. In this study, PSD curves of both strains showed flat regions at low frequencies and a slope associated with medium frequencies (Fig. 6) [76], and characteristic of surfaces with complex roughness [77] such as biofilms. Strain 2A1 shows a rapid increase in PSD after the first 8 h of biofilm growth, which is associated with increased roughness [78] due to the presence of EPS (48). At 32 h, the PSD confirms the decrease in roughness, corresponding to the decrease in power density values at the low and medium frequency limits [78], and then a steady increase up to 72 h. In contrast to Ra and Rq, the PSD shows small changes in roughness with minimal variations in power density at all frequencies [78]. In 2Sc, at 8 h, the PSD curve showed an elongated flat region at low frequencies, which is associated with longer lengths or at micrometre scales [79], as it is possible to observe individual cocci with sizes close to 1 µm and without the presence of EPS [60]. The roughness of 2Sc tends to decrease in the first few hours and then increases significantly. As seen in Ra and Rq of 2Sc, the roughness increases dramatically at 32 h, mimicking homogeneous growth over the entire surface; however, this increase is due to the appearance of a large protrusion in a region of the biofilm and not over the entire surface. The PSD contrasts these features by increasing power values in the low frequency range associated with micrometre lengths, i.e., the protrusion and minimal variation at intermediate frequencies associated with nanometre features [79]. Prior literature's surface analyses are limited to the organisation or colonisation of biofilms by hydrocarbonoclastic bacteria [80,81], only in some cases go further by measuring mechanical parameters [82]. AFM analysis highlighted significant species-specific visual differences and mechanisms of *Micrococcus* biofilm formation over the time. However, the ANOVA analysis ($p < 0.05$) to determine the biofilm topographical distinctions in *M. yunnanensis* and *M. luteus* showed that there are no significant differences between both strains, regarding roughness parameters, with $p =$ values 0.85, 0.77 and 0.75 for Ra, Rq, and Rz, respectively. This highlights the ability of both strains to form mature biofilms under marine conditions with 1 % concentration crude oil at 30 °C. Notwithstanding the foregoing, AFM analyses highlighted differences in the surface roughness in both *Micrococcus* biofilms over the time; these cell aggregates are related to the ability to immobilise compounds by biosorption and to interact with surfaces and other microbial metabolic products [7]. AFM techniques enables one to relate the morphology and roughness values to environmental responses of the biofilm to the surrounding medium. This allows a more complete insight into what happens to a biofilm during its development under certain conditions. Further AFM studies are required that consider the combination of the rest of the variables to establish the morphological difference at the surface level in each *Micrococcus* species. There is a methodological restriction of obtaining biofilms in the microtiter system and the ability to adapt it to the features of the AFM equipment. Experimental trials to overcome this problem are ongoing. Nonetheless, the application of AFM is a powerful tool for the characterization of biofilms, as it allows a fine and detailed description of the physical and mechanical characteristics at the cellular level, the results of which can be associated with the state of maturation of hydrocarbon degrading biofilms from oil spills.

3.4. Assessment of biofilms formation under stressors

The growth of *M. luteus* biofilms was observed in hydrocarbon

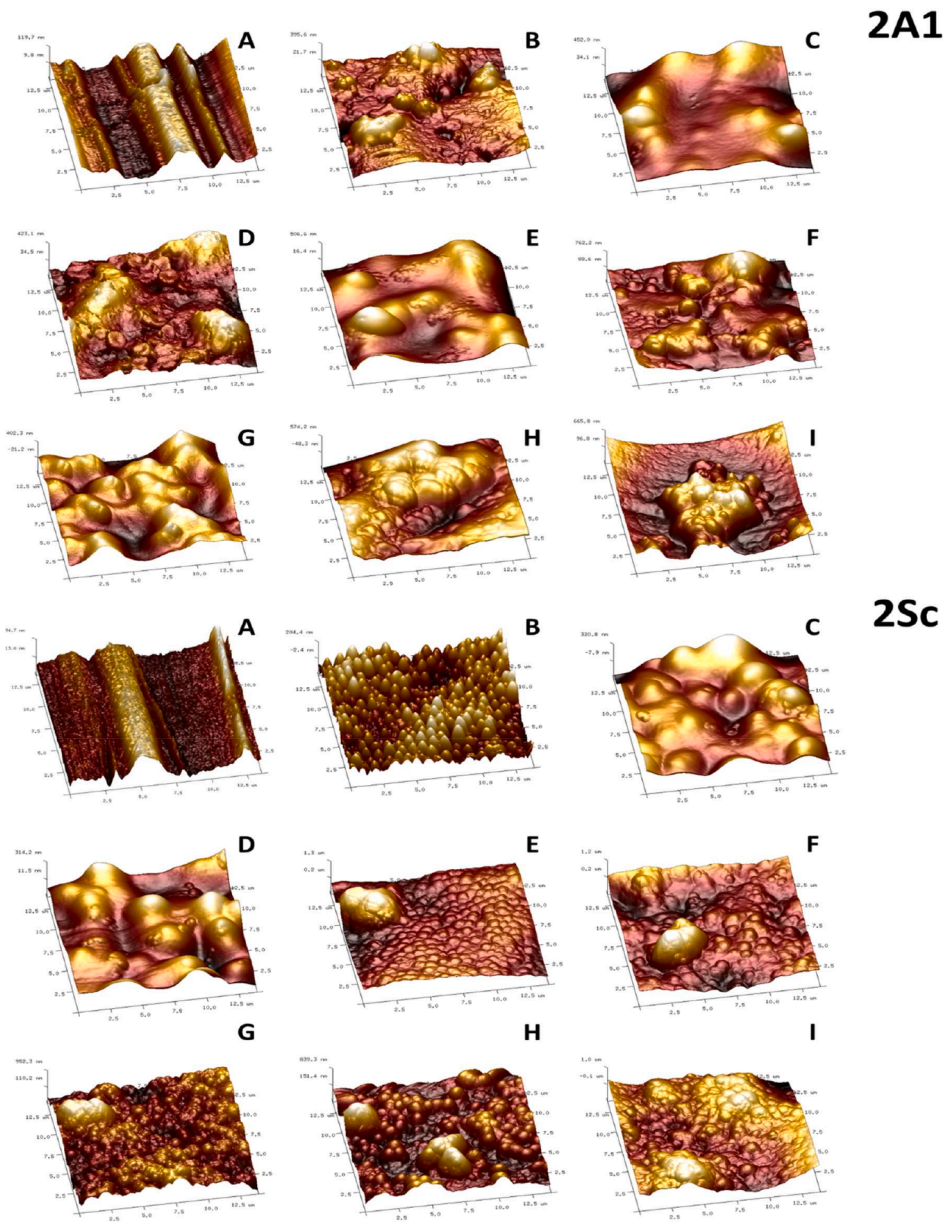

Fig. 4. AFM images. *M. luteus* 2A1 and *M. yunnanensis* 2Sc biofilms incubated under marine conditions at 30 °C, pH 7. Strain 2A1: A) polystyrene surface B) biofilm at 8 h; C) at 16 h; D) at 24 h; E) at 32; F) at 40 h; G) at 48 h; H) at 60 h e I) at 72 h. Strain 2Sc: A) polystyrene surface; B) biofilm at 8 h; C) at 16 h; D) at 24 h; E) at 32; F) at 40 h; G) at 48 h; H) at 60 h e I) at 72 h.

concentrations from 1 % to 35 % at 30 °C. Said growth, however, was lower at 50 °C in all hydrocarbon concentrations for salinity ranging from 30 to 80 g/L resulting in weak biofilms (Fig. 7), contrary to the findings with *M. yunnanensis* (Fig. 8). *M. yunnanensis* biofilm growth was not affected by hydrocarbon concentrations from 1 % to 35 % at 30 °C. The biofilm growth doubled under 35 % HC, salinity from 30 to 80 g/L, and 30 °C, when compared to *M. luteus* under the same conditions. *M. yunnanensis* biofilms at the higher temperature of 50 °C displayed less growth in 1 % concentration hydrocarbons, but not in the concentrations from 5 % to 35 % at 50 °C. At this temperature, the difference in biofilm growth compared to *M. luteus* is noticeable; *M. yunnanensis* 2Sc biofilms were more robust than *M. luteus* 2A1. Regarding the statistical analysis of biofilm formation, a model including the strain as a factor, where the pH variable had no significant effect on the response variable under the conditions assessed was analysed. The variables did have a substantial effect on both *Micrococcus* strains. Significant effects of the variables: temperature, salinity, and hydrocarbon concentration were found, as well as the interaction between them and the interaction with each hydrocarbonoclastic strain. The analyses showed that the effect of the three variables depends on the strain. What most influences the response is the strain and then, in descending order, temperature > salinity > hydrocarbon concentration. Based on this finding, a statistical analysis was then performed for each strain separately. Important point of emphasis is that the microtiter plate is a highly adaptable and easy-to-operate biofilm reactor that provides good repeatability and reproducibility for high-throughput screening evaluation methods, when used properly in conjunction with statistical analysis and experimental design [83]. Studies with microtiter plates and *Micrococcus* species have been conducted with antibiotics and other substances on their biofilms [84–86] and to evaluate them with other species [87], but not in a hydrocarbon-containing environment as has been done with other bacteria [11,88–90]. Forming biofilms on hydrophobic surfaces is essential for oil spill bioremediation process in marine environment since this is how the microorganisms adhere to oil droplets in natural

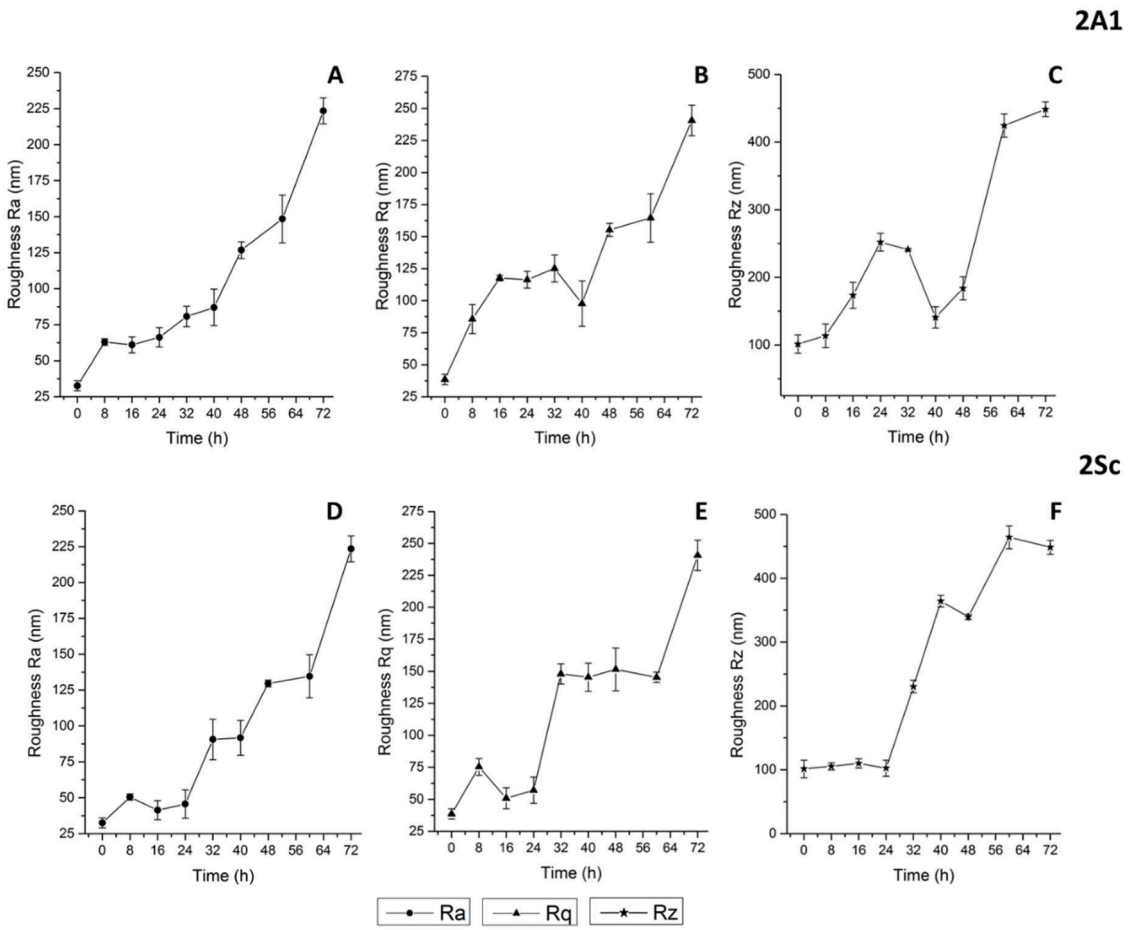

Fig. 5. Roughness parameters: Roughness average (Ra), Roughness mean square (Rq) and Roughness in height (Rz). Obtained by AFM image processing of *M. luteus* 2A1 and *M. yunnanensis* 2Sc biofilms incubated in marine conditions at 30 °C, pH 7. Strain 2A1: A) Ra; B) Rq; C) Rz. Strain 2Sc: D) Ra; E) Rq and F) Rz.

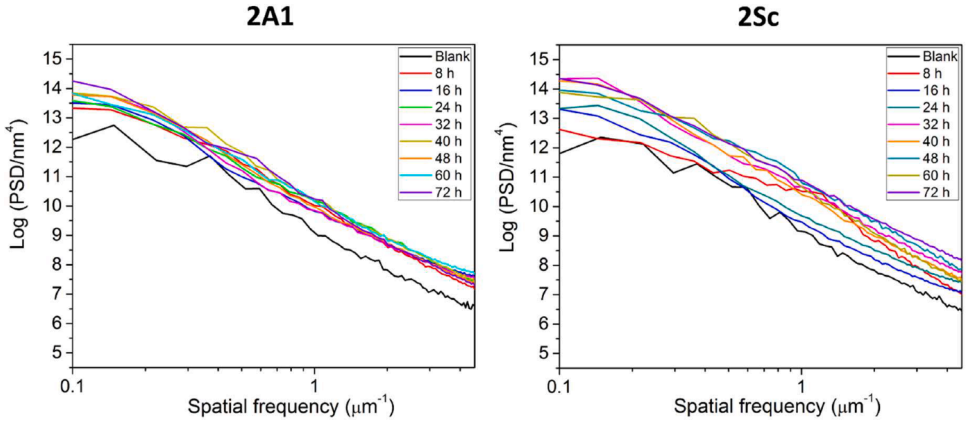

Fig. 6. PSD_{2D} curves of the biofilms. Obtained by AFM image processing of *M. luteus* 2A1 and *M. yunnanensis* 2Sc.

settings [91]. The marine oil-degrading bacteria for oil spill bioremediation can stabilise oil droplets in oil-in-water emulsions due to interaction with surfactant decorated oil water interface [92]. It is essential to comprehend how *Micrococcus* interacts with stressors and the surfaces on which it forms a biofilm in order to develop a potential model for use in bioremediation applications in the future.

3.4.1. Effects of variables on M. luteus 2A1 biofilm and M. yunnanensis 2Sc biofilm

In the case of *M. luteus* 2A1, the significant effects ($p < 0.01$) had the following order: salinity > temperature > hydrocarbon concentration; as well as the interaction of salinity with temperature. When a multiple linear regression model was built, including the independent variables as continuous (hydrocarbon concentration, temperature, and salinity), the significant effects ($p < 0.01$) had the following order: temperature > salinity > hydrocarbon concentration. The effect of temperature was negative on the biofilm development (Fig. 9). The behaviour of *M. yunnanensis* biofilm showed significant effects as follows: temperature > salinity > hydrocarbon concentration; the main interaction was salinity with temperature. The temperature and salinity had a negative effect on

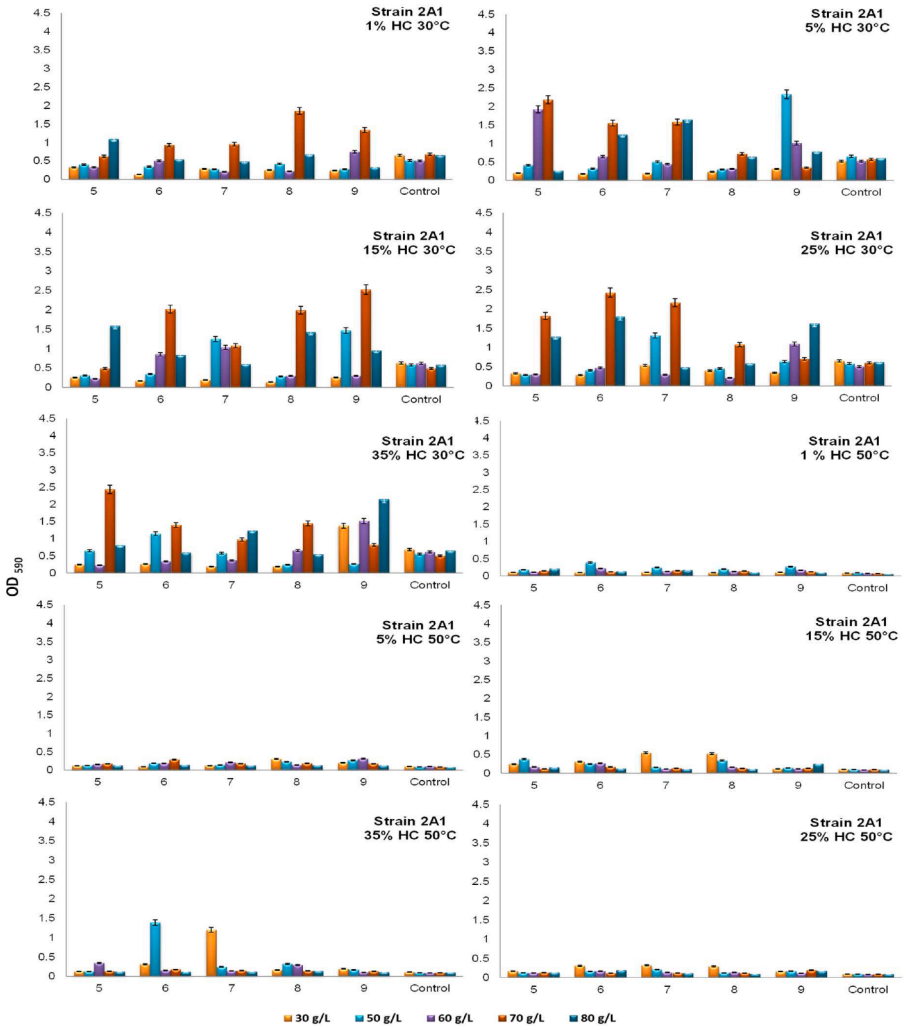

Fig. 7. Microtiter plate assays. Effect on *M. luteus* strain biofilm formation in BHM under different environmental conditions. Crude oil concentration: 1 %, 5 %, 15 %, 25 %, 35 % (wt/vol); temperature: 30 °C and 50 °C; pH 5, 6, 7, 8, and 9; salinity (g/L): 30, 50, 60, 70, 80 (mean ± standard deviation of three repeats).

the response variable. When a multiple linear regression model was built, the significant effects ($p < 0.01$) were followed the order temperature > hydrocarbon concentration > salinity. The interaction between the temperature and salinity had a negative effect on the *M. yunnanensis* biofilm growth (Fig. 9). The strain *M. luteus* 2A1 biofilms grew more than the controls and were thicker at 30 °C, high salinity, and high hydrocarbon concentrations, according to the results. Temperature was a sensitive factor for strain 2A1, as evidenced by the first biofilm it formed at 50 °C. Treatments with hydrocarbon concentrations of 15 %, 25 %, and 35 % showed higher biomass than the corresponding controls. Under nearly all salinity and hydrocarbon concentration treatments at 30 °C, biofilms for strain 2Sc remained thicker. At 30 °C, the maximum biofilm growths occurred at 15 %, 25 %, and 35 % hydrocarbons and at salinities of 30, 50, and 60 g/L, just like in the controls. Whereas at 50 °C, the 2SC biofilms were much more robust at 15 %, 25 %, and 35 % hydrocarbons and higher salinities of 70 and 80 g/L. The degree of biofilm formation exhibited by controls of *M. luteus* strain 2A1 is congruent with that documented by Malic et al. [87] for a *M. luteus* species assessed using a microtiter plate in tryptone soy broth (TSB) and brain heart infusion broth (BHI) media at 37 °C for 24 h, with optical density (OD) values ranging from 0.15 to 0.20 at 595 nm. As well as for *Micrococcus* from rhizosphere soil, which was grown in a microtiter plate with nutrient broth for 4 days at 37 °C, resulting in a biofilm with an OD of 2.05 [93]. However, the results of microtiter tests using the halophiles *Halomonas* and *Kushneria*, supplemented with 60 g/L NaCl, at 37 °C for 24 h revealed 2.5 and 0.5 OD, respectively [94]; these recorded behaviours are similar to those observed in 2A1 and 2Sc. Additionally, marine *Micrococcus* biofilm in microtiter plate had 0.511 (OD) incubated at 37 °C for 24 h, it was isolated from hydrocarbon contaminated site in the northwest coast of Borneo, Brunei, Southeast Asia [20], this behaviour also observed in *Micrococcus* 2A1 and 2Sc strains. As well as the hydrocarbonoclastic bacteria *Exiguobacterium*, isolated from sea sediment microcosms and immobilised for the removal of crude oil from marine environments, which produced a biofilm with 2.0 (OD) in microtiter plate at 30 °C for 3–7 days [11]. The aforementioned findings also give insight into the biofilm development in control strain 2Sc under the environmental conditions assayed; behaviour changes substantially for both *Micrococcus* 2A1 and 2Sc in the treatments when temperature, salinity, and crude oil concentration are increased. Microtiter plate tests were carried out with hydrocarbonoclastic biofilm-forming bacteria such as *Pseudomonas, Bacillus, and Rhodococcus*; the incubation time of 14 days in certain alkanes and diesel gave optical densities at 590 nm of 0.2–0.8 at alkane concentrations of 1–2 %. The OD of the biofilms was 0.5–0.2 at the same incubation time for alkane and diesel concentrations of 3–4 % [88]. These results contrast with the findings of *Micrococcus* 2A1 and 2Sc to form biofilms at very high crude oil concentrations, up to 35 % in a shorter time. On the other hand, microtiter plate assays have been used to enumerate, screen, and characterise hydrocarbonoclastic bacteria from hydrocarbon-contaminated soils, sands, and culture enrichments and to evaluate their potential use in the bioremediation of oil-contaminated sites [89,90,95,96], but not for

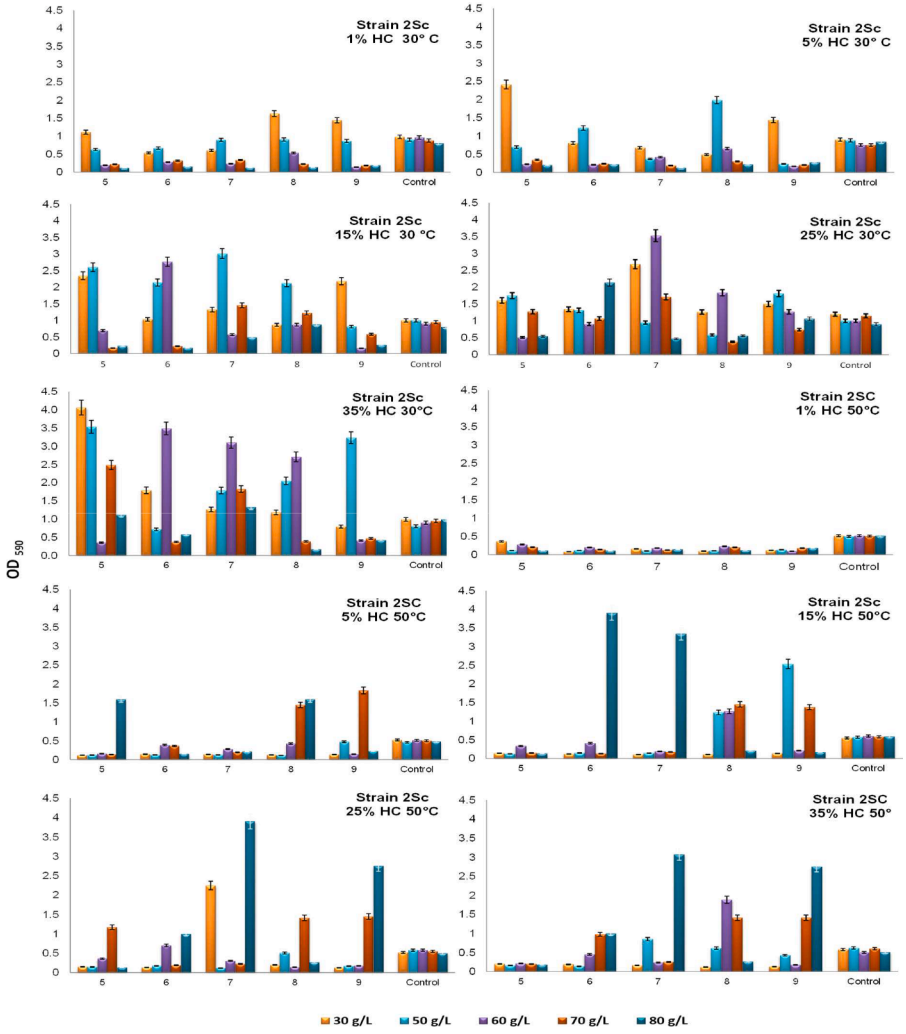

Fig. 8. Microtiter plate assays. Effect on *M. yunnanensis* 2Sc biofilm formation in BHM under different environmental conditions. Crude oil concentration: 1 %, 5 %, 15 %, 25 %, 35 % (wt/vol); temperature: 30 °C and 50 °C; pH 5, 6, 7, 8, and 9; salinity (g/L): 30, 50, 60, 70, 80 (mean ± standard deviation of three repeats).

the case of marine biofilms of *Micrococcus* with prospective use in marine oil spills. The results were compared with previous studies of *Micrococcus* biofilms isolated from other locations and for other biotechnological purposes, as no studies were found evaluating marine biofilms of hydrocarbonoclastic *Micrococcus* under the environmental conditions tested here. According to the findings, hydrocarbon concentration was not a limiting factor for 2Sc biofilm growth as it was for 2A1. The greatest influence was temperature and salinity for both strains, although the effect of temperature was more pronounced for *M. luteus*. Despite being the same genus, *M. yunnanensis* has a wider range of optimum growth temperatures, from 4 to 45 °C [97]. Regarding the temperature, it changes drastically the biofilm morphology, thickness, and cell density. This reflects the temperature-dependent regulation of EPS production, and consequently, its formation seems to be a three-dimensional mesh prepared for unstable environmental conditions as is the case observed with *Micrococcus* 2A1 [12]. Temperature affects the synthesis of extracellular EPS, which is known to enhance bacterial cell adhesion; molecular changes in EPS as a function of temperature have been recorded in lipids, lipid-like molecules, organic acids, nucleosides, nucleotides, organo-heterocyclic compounds, benzenoids, phenylpropanoids and polyketides, polysaccharides, phenols, binding proteins, and carboxylic acids [98]. Additionally, temperature is also known to stress mesophilic bacteria that are found in cold water; depending on the physiology of each bacterial species, a decrease in biofilm biomass has also been observed with increasing ambient temperature [99]. Overall, the hydrocarbon concentration was not limiting due to the high metabolic capacity of *Micrococcus* to degrade complex organic compounds such as petroleum hydrocarbons [56–58]. In addition, biofilm formation with crude oil has shown a large number of microorganisms near the oil-water interface and a higher biomass compared to the biofilm produced with a different carbon source, such as glucose [100], this is observed with the biofilms produced in the crude oil-free controls. Furthermore, it has been observed that biofilms undergo temperature-induced adaptations that are independent of the nutrient source and that facilitate the differential adherence of biofilms to different surfaces [99]. Overall, the growth of 2A1 and 2Sc biofilms was significantly higher than that of non-hydrocarbon controls at high salinity and crude oil concentrations; biofilms formed with crude oil as the sole carbon source are thicker and have more biomass than biofilms formed with glucose alone [100]. Additionally, at higher salinities, *Micrococcus* 2Sc was found to form slightly more robust biofilms than 2A1; biofilm formation was variable for the strains, depending on the type of surface and salt concentration [94], also, high concentrations of salt induce to form more compact biofilms by reducing the charge of EPS through double layer compression [101]. A difference was observed between the 2A1 and 2Sc biofilms and the control biofilms; the former were more robust than those of the controls (without hydrocarbon), despite containing the same salinities and being incubated at the same time and temperatures, but not the carbon source; as the amount of salt stress increased, the complex medium promoted greater biomass formation and, consequently, higher production of EPS [94]. *Micrococcus* has an

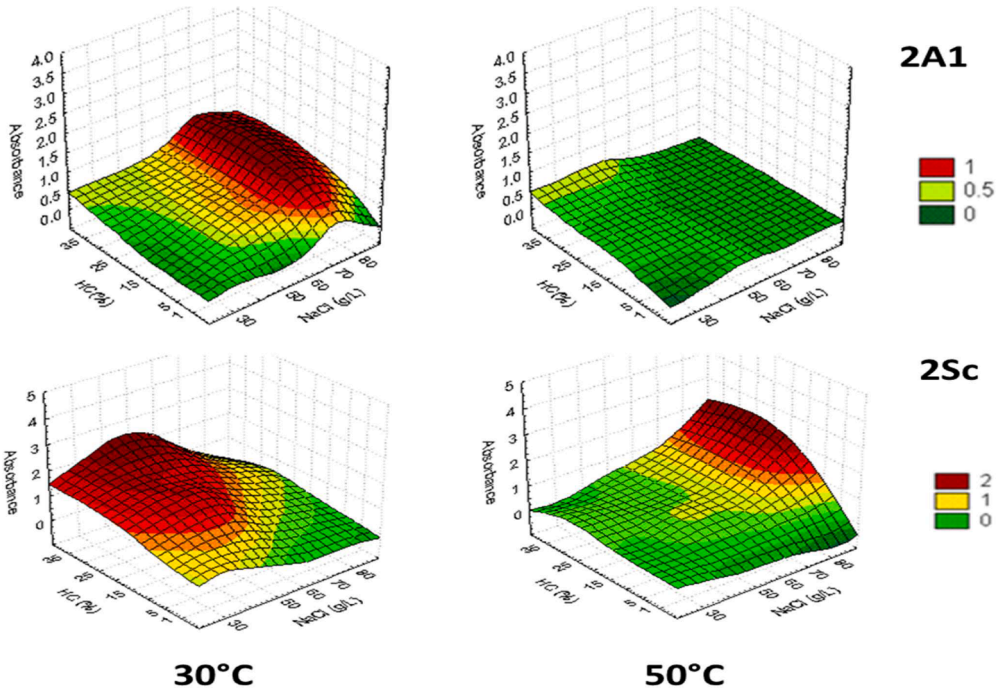

Fig. 9. Response surface for evaluating the assessed factors, salinity and crude oil concentration, and their interactions under two temperatures in biofilms development (response variable). The plots are shown for each strain: *M. luteus* strain 2A1 and *M. yunnanensis* strain 2Sc.

extraordinary ability to produce EPS in a short period of time that can cause biofouling problems in a variety of environments [102] and the marine bacterial EPS influence the fate and ultimate degradation of hydrocarbon pollutants in the ocean, particularly during oil spills [103]. With regard to pH in this study, the lowest pH evaluated was 5 and the highest was 9 for Micrococcus biofilms; nevertheless, no significant effect was found for either strain. *Micrococcus,* as an actinobacterium, is a promising candidate for bioaugmentation treatments of marine oil spills because of its ability to metabolise environmental contaminants for growth, its tolerance to high concentrations of contaminants and survival in various environmental circumstances, making it an economical and ecotoxicologically safe biotechnological method for the removal or transformation of environmental contaminants [4,16]. In aquatic environments, natural oil-degrading species, such as *Micrococcus,* that are capable of biofilm formation degrade oil much faster and more efficiently than planktonic cells on their own. There is a notable increase in degradation for each naturally occurring oil-degrading strain, with varied degrees of biofilm-forming capacity. Following an oil spill, naturally occurring oil-degrading microorganisms can congregate near the oil-water interface, which significantly lessens the *in situ* bioremediation's bioavailability restriction [100].

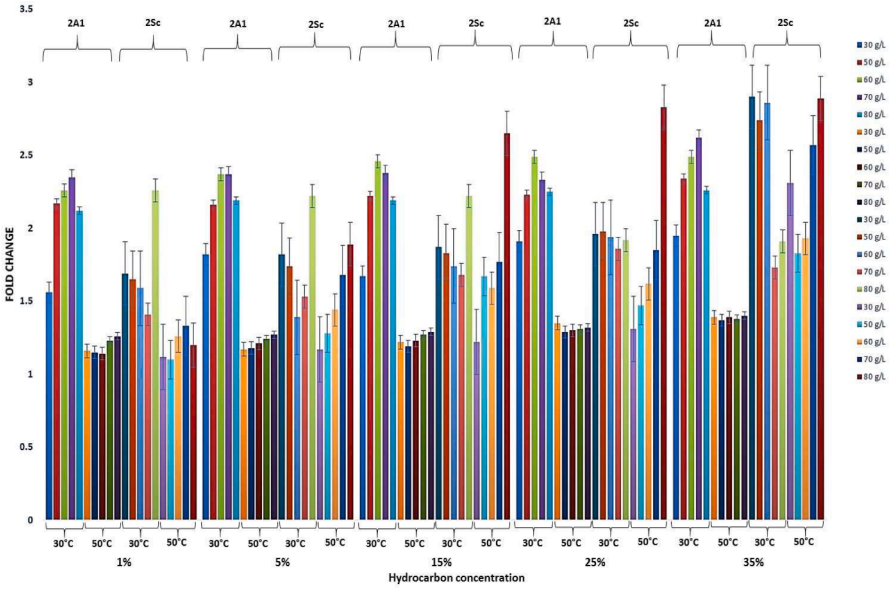

Fig. 10. Relative quantification of gene expression of *usp*A in *M. luteus* 2A1 and *M. yunnanensis* 2Sc biofilms under assessed stressors (temperature, salinity, and hydrocarbon concentrations). Gene expression was normalized to the 16S rRNA gene. The *usp*A gene relative quantification values were obtained after scaling to controls.

3.5. Transcriptional induction of uspA gene under stress conditions

The biofilm growth of both strain and their transcript quantification under described conditions suggest that stressors induce the expression of the *usp*A gene (Fig. 10). Overall, it was observed that *usp*A expression doubled at 30 °C in *M. luteus* 2A1, and in some cases a 2.5-fold increase was observed with 60 g/L and 70 g/L in 15 %, 25 %, and 35 % crude oil. This agrees with the findings described in the evaluation by response surface analyses, where there is a significant effect following the sequence: temperature > salinity > hydrocarbon concentration. At 50 °C, the *usp*A transcription had 1.15–1.46-fold increase; in this case, the biofilms detected were weaker than those found at 30 °C with the stressors evaluated, which is related to the behaviour in the response surface for this strain in the assays at 50 °C. In the case of *M. yunnanensis* 2Sc, the *usp*A expression performed significantly different at assessed temperatures. At 30 °C, with hydrocarbon concentrations of 1–15 %, a 1.6-to-1.8-fold increase was observed. For the 25 % and 35 % crude oil concentrations, a factor of 2–3 increase of gene expression was measured respectively. This pattern was reflected in the response surface analyses of the strain 2Sc (high temperature of 50 °C, salinity of 70 g/L and 80 g/L and high hydrocarbon concentrations) where the biofilms were thicker. In particular, the *usp*A overexpression was remarkably a factor of 3 higher at the high salinity and high hydrocarbon concentrations assessed. The large changes in *usp*A are related to the mature growth level of the *Micrococcus* 2Sc biofilm. These behaviours align with the previously discussed effects of environmental stressors on development of 2A1 and 2Sc biofilms. That is, where the biofilms were more robust, there was a higher expression of *usp*A in their fold change. The Usp in bacteria has a role in adaptation to several pressures, e.g., oxidative stress, temperature changes, low pH, and contributes to the adaption to the hostile environments through adhesion and motility [13]. Likewise, Usp proteins are required for resistance against DNA-damaging agents [13]; mutation in the *usp*A gene leads to reduced stress tolerance [104]. Due to the different physiological and biological needs of these actinobacteria, *M. luteus* 2A1 and *M. yunnanensis* 2Sc biofilm growth responses depending on environmental conditions. For instance, biofilm growth was weaker in strain 2A1 at 50 °C; the registered gene expression suggests that the stressor induces expression when the cells are in a certain physiological steady state. *usp*A transcription and protein production are related to the microbial growth phase. Here, growth-arrested cells, subject to growth disrupted by stress, are more focused on maintaining their current state, using the nutritional resources that are available under the specific environmental stressors [105]. This means that cellular processes like genome duplication and cell division stop. Furthermore, Usp is a primary regulator of bacterial survival through the non-replicative persistent (NRP) state in *M. luteus* [54]. Stress responses have a relevant importance in bacterial processes like quorum sensing and biofilm formation, as well as dormancy, which consist of persistence state in bacteria. In *Micrococcus* it has been reported that in this dormant state, the UspA protein is highly expressed and can induce and modulate a viable but nonculturable (VBNC) response in cells to hostile environmental conditions [53]. According to the above, strain 2A1 was possibly in a steady state without growth under temperature, salinity, and hydrocarbon stress while *usp*A expression was gradually increasing in number. These physiological and genetic characteristics must be taken into account when considering the use of a hydrocarbonoclastic microorganism for the bioremediation of marine crude oil spills. In live Cell array systems with the enterobacterium *E. coli* to identify novel modes of toxicities of the oil spill-affected sediments, the bacteria displayed *usp*A down-regulated expressions from 2 to 0.25-fold changes [106]. Similarly, another proteobacterium, *Sphingomonas*, inoculated in sand contaminated with dibenzo-p-dioxins and dibenzofurans, saw an *usp*A down-regulated expression by 1 h after inoculation [107]. Despite the existence of diverse responses in gene expression among bacteria belonging to different phyla, *Micrococcus* remains a preferred and suitable model for the assessment of environmental stress tolerance. Unlike *E. coli*, the actinobacterium *Micrococcus* exposed to stress rapidly shift metabolic processes to enable short-term survival, leading to a metabolically active lag state with reduced proliferation [54]. This means that the individual bacteria mature without doubling their biomass and enzymes. RNA and various molecules are synthesised. Specifically in *M. luteus*, protein level reorganization rapidly enhances short-term survival by switching metabolism from energy consumption to increased synthesis of survival proteins, such as UspA, and molecular species, leading to a potential lag-phase adaptation to stress, the so-called “short-term tolerance” [54]; These genetic and physiological characteristics make *Micrococcus* suitable as a model for tolerance mechanisms that could be used as an application model for marine oil spill bioremediation. Notwithstanding the above, further studies are needed to get the full picture of what happens to the *usp*A expression over time for the present *Micrococcus* case under the stressor evaluated. On the other hand, *usp* gene expression levels are determined by the carbon source as a substrate, in the case of some actinobacteria such as *Mycobacterium*, citrate (tricarboxylic acid) could regulate the *usp* expression and cause a factor of 10 increase [108]. For *Micrococcus*, to the knowledge of the present authors, there are no specific quantitative data of *usp*A gene expression using crude oil as a substrate under stress conditions. Moreover, Usp represent components of a reproducible protein signature for the microbial survival states and mediate general responses to different external stresses [53]. To understand the physiological and metabolic state of microorganisms with biotechnological potential that are subject to specific environmental conditions, Usp proteins become a suitable biomarker in the development of such biotechnological approaches. Further molecular studies need to be performed in order to describe the role of the *usp*A gene in the hydrocarbonoclastic *Micrococcus* cell cycle phases, monitoring these molecular regulators of *usp*A during the crude oil biodegradation process and under physiological stress by other factors. However, this is the first report that we are aware of regarding the expression of the *usp*A gene in marine *Micrococcus* biofilms and the assessment of the impact of environmental factors, like hydrocarbon concentrations, using a microtiter plate approach.

4. Conclusion

M. luteus and *M. yunnanensis*, showed the highest biodegradation capacity in heavy crude oil. Scanning electron microscopy and atomic force microscopy provided insights into the surface features, such as the roughness and development dynamics of the biofilms formed by both strains. The response to the environmental were different for each *Micrococcus* species. The interaction between the salinity and temperature remains significant for the two strains. *usp*A expression was greater in *Micrococcus* 2Sc than in 2A1, particularly at higher salinities and at 50 °C temperature, and hydrocarbon concentrations of 25 and 35 %. Overall, 2Sc biofilms were thicker than 2A1 biofilms under the conditions examined. The *usp*A expression up-regulated from 1.5 to 2.5-fold in 2A1 biofilms at 30 °C at all salinities with the hydrocarbon concentrations tested; at 50 °C *usp*A expression up-regulated to 1.3-fold for the same strain. The *usp*A gene is a suitable biomarker to assess the physiological state of microorganisms with biotechnological potential, especially in oil related environments. Future research should further investigate the relationship between the cell cycle phases and specific metabolism of *Micrococcus,* and the expression of the *usp*A gene under biodegradation of petroleum hydrocarbons. Furthermore, there should be an investigation introducing omics platforms to elucidate metatransciption levels and expressed protein profiles during the bioprocess. The survival and degradation capacity of microorganisms introduced to a site impacted by an oil spill is highly dependent on environmental conditions. Microorganisms grown in rich media under laboratory conditions become stressed when exposed to fields where temperature, salinity, and nutrient concentrations are substantially different. The best approach to bridging these barriers is the use of autochthonous

microbiota. Therefore, for a successful *in situ* bioremediation strategy, it is crucial to assess the environmental stressors of indigenous microorganisms.

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CRediT authorship contribution statement

I. Zapata-Peñasco: Conceptualization, Investigation, Validation, Resources, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation. **I.A. Avelino-Jiménez:** Investigation, Methodology, Formal analysis, Writing – original draft. **J. Mendoza-Pérez:** Investigation, Methodology, Formal analysis, Resources, Funding acquisition. **M. Vázquez Guevara:** Methodology, Formal analysis, Validation. **M. Gutiérrez-Ladrón de Guevara:** Conceptualization, Data curation, Methodology, Investigation, Formal analysis. **M. Valadez-Martínez:** Methodology, Formal analysis, Investigation, Conceptualization. **L. Hernández-Maya:** Methodology, Formal analysis, Investigation, Conceptualization. **V. Garibay-Febles:** Methodology, Resources, Formal analysis. **T. Fregoso-Aguilar:** Validation, Resources. **J. Fonseca-Campos:** Validation, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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References

[1] Y.G. Lee, X. Garza-Gomez, R.M. Lee, Ultimate costs of the disaster: seven years after the deepwater horizon oil spill, J. Corp. Finance 29 (2018) 69–79, https://doi.org/10.1002/jcaf.22306.

[2] T.C. Hazen, E.A. Dubinsky, T.Z. Desantis, G.L. Andersen, Y.M. Piceno, N. Singh, J. K. Jansson, et al., Deep-sea oil plume enriches indigenous oil-degrading bacteria, Science 330 (2010) 204–208, https://doi.org/10.1126/science.1195979.

[3] F. Mapelli, A. Scoma, G. Michoud, F. Aulenta, N. Boon, S. Borin, Biotechnologies for marine oil spill cleanup: indissoluble ties with microorganisms, Trends Biotechnol. 35 (2017) 860–870, https://doi.org/10.1016/j.tibtech.2017.04.003.

[4] C.-I. Mawang, A.-S. Azman, A-S.M. Fuad, M. Ahamad, Actinobacteria: an eco-friendly and promising technology for the bioaugmentation of contaminants, Biotechnol. Rep. 32 (2021) e00679, https://doi.org/10.1016/j.btre.2021.e00679.

[5] S.S. Radwan, R.H. Al-Hasan, Potential application of coastal biofilm-coated gravel particles for treating oily waste, Aquatic Microb. Ecol. 23 (2001) 113–117, https://doi.org/10.3354/ame023113.

[6] W. Zhang, W. Ding, Y.X. Li, et al., Marine biofilms constitute a bank of hidden microbial diversity and functional potential, Nat. Commun. 10 (2019) 517, https://doi.org/10.1038/s41467-019-08463-z.

[7] S. Mishra, Y. Huang, J. Li, X. Wu, Z. Zhou, Z.Q. Lei, P. Bhatt, S. Chen, Biofilm-mediated bioremediation is a powerful tool for the removal of environmental pollutants, Chemosphere 294 (2022) 133609, https://doi.org/10.1016/j.chemosphere.2022.133609.

[8] Y. Kasai, H. Kishira, T. Sasaki, K. Syutsubo, K. Watanabe, S. Harayama, Predominant growth of Alcanivorax strains in oil-contaminated and nutrient-supplemented sea water, Environ. Microbiol. 4 (3) (2002) 141–147, https://doi.org/10.1046/j.1462-2920.2002.00275.x.

[9] L. Shan, Y. Gao, Y. Zhang, W. Yu, Y. Yang, S. Shen, S. Zhang, et al., Fabrication and use of alginate-based cryogel delivery beads loaded with urea and phosphates as potential carriers for bioremediation, Ind. Eng. Chem. Res. 55 (2016) 7655–7660, https://doi.org/10.1021/acs.iecr.6b01256.

[10] B.M. Dellagnezze, S.P. Vasconcellos, A.L. Angelim, V.M.M. Melo, S. Santisi, S. Cappello, V.M. Oliveira, Bioaugmentation strategy employing a microbial consortium immobilized in chitosan beads for oil degradation in mesocosm scale, Mar. Pollut. Bull. 107 (2016) 107–117, https://doi.org/10.1016/j.marpolbul.2016.04.011.

[11] Ch. Sakdapetsiri, N. Kaokhum, O. Pinyakong, Biodegradation of crude oil by immobilized *Exiguobacterium* sp. AO-11 and shelf life evaluation, Sci. Rep. 11 (2021) 12990, https://doi.org/10.1038/s41598-021-92122-1.

[12] M. Toyofuku, T. Inaba, T. Kiyakawa, N. Obana, Y. Yawata, N. Nomura, Environmental factors that shape biofilm formation, Biosci. Biotechnol. Biochem. 80 (1) (2016) 7–12, https://doi.org/10.1080/09168451.2015.1058701.

[13] K. Kvint, L. Nachin, A. Diez, T. Nystöm, The bacterial universal stress protein: function and regulation, Curr. Opin. Microbiol. 6 (2) (2003) 140–145, https://doi.org/10.1016/S1369-5274(03)00025-0.

[14] W.Q. Zhuang, J.H. Tay, A.M. Maszenan, L.R. Krumholz, S.T.L. Tay, Importance of gram-positive naphthalene-degrading bacteria in oil-contaminated tropical marine sediments, Lett. Appl. Microbiol. 36 (2003) 251–257, https://doi.org/10.1046/j.1472-765X.2003.01297.x.

[15] A. Ghosh, S.A. Chaudhary, S.R. Apurva, T. Tiwari, S. Gupta, A.K. Singh, K. H. Katudia, et al., Whole-genome sequencing of Micrococcus luteus strain Modasa, of Indian origin, Genome Announc. 1 (2013) 10–1128, https://doi.org/10.1128/genomea.00076-13.

[16] H.R. Dash, N. Mangwani, J. Chakraborty, S. Kumari, S. Das, Marine bacteria: potential candidates for enhanced bioremediation, Appl. Microbiol. Biotechnol. 97 (2013) 561–571, https://doi.org/10.1007/s00253-012-4584-0.

[17] J.S. Oliveira, W.J. Araújo, R.M. Figueiredo, R.C.B. Silva-Portela, A. de Brito Guerra, S. Carla da Silva Araújo, C. Minnicelli, Biogeographical distribution analysis of hydrocarbon degrading and biosurfactant producing genes suggests that near-equatorial biomes have higher abundance of genes with potential for bioremediation, BMC Microbiol. 17 (2017) 1–10, https://doi.org/10.1186/s12866-017-1077-4.

[18] Z. Bayat, M. Hassanshahian, M.A. Hesni, Enrichment and isolation of crude oil degrading bacteria from some mussels collected from the Persian Gulf, Mar. Pollut. Bull. 101 (2015) 85–91, https://doi.org/10.1016/j.marpolbul.2015.11.021.

[19] H.S. Titah, H. Pratikno, A. Moesriati, M.F. Imron, R.I. Putera, Isolation and screening of diesel degrading bacteria from ship dismantling facility at Tanjungjati, Madura, Indonesia, J. Eng. Technol. Sci. 50 (2018) 99–109.

[20] D. Khoo, p. Shivanand, h. Taha, Determination of crude oil degradation efficiency of biofilm producing bacteria isolated from oil contaminated site, Biodiversitas 23 (8) (2022), https://doi.org/10.13057/biodiv/d230835.

[21] A. Bodor, N. Bounedjoum, G. Feigl, Á. Duzs, K. Laczi, Á. Szilágyi, G. Rákhely, et al., Exploitation of extracellular organic matter from *Micrococcus luteus* to enhance ex situ bioremediation of soils polluted with used lubricants, J. Hazard. Mater. 417 (2021) 125996, https://doi.org/10.1016/j.jhazmat.2021.125996.

[22] P.N. Tallur, S.I. Mulla, V.B. Megadi, M.P. Talwar, H.Z. Ninnekar, Biodegradation of cypermethrin by immobilized cells of *Micrococcus* sp. strain CPN 1, Braz. J. Microbiol. 46 (2015) 667–672, https://doi.org/10.1590/S1517-838246320130557.

[23] I. Letnik, R. Avrahami, R. Port, A. Greiner, E. Zussman, J.S. Roken, C. Greenblatt, Biosorption of copper from aqueous environments by Micrococcus luteus in cell suspension and when encapsulated, Int. Biodeterior. Biodegrad. 116 (2017) 64–72, https://doi.org/10.1016/j.ibiod.2016.09.029.

[24] M. Mafi, A. Greiner, Bioremediation of copper-ions by polymer encapsulated and immobilized Micrococcus luteus, Macromol. Biosci. 21 (2021) 2100086, https://doi.org/10.1002/mabi.202100086.

[25] M. Nikolopoulou, N. Pasadakis, N. Kalogerakis, Evaluation of autochthonous bioaugmentation and biostimulation during microcosm-simulated oil spill, Mar. Pollut. Bull. 72 (2013) 165–173, https://doi.org/10.1016/j.marpolbul.2013.04.007.

[26] US EPA (2006). Guidance on systematic planning using the data quality objectives process; EPA/240/B-06/001.

[27] NOAA, Special Monitoring of Applied Response Technologies. US Coast Guard, National Oceanic and Atmospheric Administration, US Environmental Protection Agency, Centers for Disease Control and Prevention and Minerals Management Service, 2006. https://response.restoration.noaa.gov/oil-and-chemicalspills/oilspills/resources/smart.html.

[28] US EPA, General Field Sampling Guidelines, United States Environmental Protection Agency, 1994. Document ref. SOP# 2001, November 1994, https://nepis.epa.gov/Exe/ZyPURL.cgi?Dockey=P100C9V0.txt.

[29] US EPA, Methods for Collection, Storage and Manipulation of Sediments for Chemical and Toxicological Analyses: Technical Manual, Office of Water, 2001. EPA-823-B-01-002.

[30] US EPA (2002). Guidance on choosing a sampling design for environmental data collection.

[31] L.D. Bushnell, H.F. Hass, The utilization of certain hydrocarbons by microorganisms, J. Bacteriol. 41 (5) (1940) 653–673.

[32] M. Mahjoubi, A. Jaouani, A. Guesmi, S.B. Amor, A. Jouini, H. Cherif, A. Najjari, et al., Hydrocarbonoclastic bacteria isolated from petroleum contaminated sites in Tunisia: isolation, identification and characterization of the biotechnological

potential, New Biotechnol. 30 (2013) 723–733, https://doi.org/10.1016/j.nbt.2013.03.004.
[33] D.A. Relman, Universal bacterial 16S rRNA amplification and sequencing, in: Persing, Smith, Tenover, White (Eds.), Diagnostic Molecular Microbiology: Principles and Applications, ASM Press, Washington DC, 1993.
[34] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (3) (1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.
[35] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools, Nucleic Acids Res. 25 (24) (1997) 4876–4882, https://doi.org/10.1093/nar/25.24.4876.
[36] S. Kumar, G. Stecher, M. Li, C. Knyaz, K. Tamura, X. MEGA, Molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (6) (2018) 1547–1549, https://doi.org/10.1093/molbev/msy096.
[37] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (1980) 111–120, https://doi.org/10.1007/BF01731581.
[38] N. Saitou, M. Nei, The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. 4 (4) (1987) 406–425, https://doi.org/10.1093/oxfordjournals.molbev.a040454.
[39] J. Felsenstein, Confidence limits on phylogenies: an approach using the bootstrap, Evolution (N Y) 39 (4) (1985) 783–791, https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.
[40] R. Rosselló-Mora, R. Amann, The species concept for prokaryotes, FEMS 25 (1) (2001) 39–67, https://doi.org/10.1111/j.1574-6976.2001.tb00571.x.
[41] ASTM D1141-98.2013. Standard practice for the preparation of substitute ocean water active standard. 2013.
[42] U.S. EPA. SW-846 test method 3510C: separatory funnel liquid-liquid extraction for isolating water-insoluble and slightly soluble organic compounds from aqueous samples in preparation for a variety of chromatographic procedures. Washington, DC.1996.
[43] ISO 16703:2004. Soil quality — determination of content of hydrocarbon in the range C10 to C40 by gas chromatography. 2004.
[44] G.A. OToole, L.A. Pratt, P.I. Watnick, D.K. Newman, V.B. Weaver, R. Kolter. Genetic approaches to study of biofilms. Meth. Enzymol. 310 (1999) 91–109, 10.1016/S0076-6879(99)10008-9.
[45] R Core Team, R: A Language and Environment For Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2021. URL, https://www.R-project.org/.
[46] V.K. Truong, R. Lapovok, Y.S. Estrin, S. Rundell, J.Y. Wang, C.J. Fluke, R. J. Crawford, et al., The influence of nano-scale surface roughness on bacterial adhesion to ultrafine-grained titanium, Biomaterial 31 (13) (2010) 3674–3683, https://doi.org/10.1016/j.biomaterials.2010.01.071.
[47] H.K. Gumber, A.S. Louyakis, T. Sarma, K.I. Fabijanic, R. Paul, K. Mellenbruch, L. Kilpatrick-Liverman, Effect of a stannous fluoride dentifrice on biofilm composition, gene expression and biomechanical properties, Microorganisms 10 (2022) 1691, https://doi.org/10.3390/microorganisms10091691.
[48] M.A. Valadez-Martínez, L.G. Hernández-Maya, I.A. Avelino-Jiménez, J. Menchaca-Arredondo, C. Garibay-Febles, I. Zapata-Peñasco, Influence of roughness on the early corrosion of API steels by atomic force microscopy (AFM): a real-time assessment, Mater. Today Commun. 36 (2023) 106519, https://doi.org/10.1016/j.mtcomm.2023.106519.
[49] M. Raposo, Q. Ferreira, P.A Ribeiro, A guide for Atomic Force Microscopy Analysis of Soft-Condensed Matter, in: J.D.A. Méndez-Vilas (Ed.), Modern Research and Educational Topics in Microscopy Formatex., 2007, pp. 758–769.
[50] M. Hamza, B.A. Mei, Z. Zuo, Effect of surface roughness on the oil distribution and the heat transfer coefficient for piston cooling gallery, Case Stud. Therm. Eng. 33 (2022) 101960, https://doi.org/10.1016/j.csite.2022.101960.
[51] J. Dong, K.S.L. Signo, E.M. Vanderlinde, K.Y. Christopher, T.E.S. Dahmas, Atomic force microscopy of a ctpA mutant in Rhizobium leguminosarum reveals surface defects linking CtpA function to biofilm formation, Microbiology 157 (11) (2011) 3049–3058, https://doi.org/10.1099/mic.0.051045-0.
[52] Q. Huang, H. Wu, P. Cai, J.B. Fein, W. Chen, Atomic force microscopy measurements of bacterial adhesion and biofilm formation onto clay-sized particles, Sci. Rep. 5 (1) (2015) 16857, https://doi.org/10.1038/srep16857.
[53] S. Mali, M. Mitchell, S. Havis, A. Bodunrin, J. Rangel, G. Olson, W.R. Widger, et al., A proteomic signature of dormancy in the *Actinobacterium Micrococcus luteus*, J. Bacteriol. 199 (14) (2017) e00206–e00217, https://doi.org/10.1128/JB.00206-17.
[54] S. Havis, A. Bodunrin, J. Rangel, R. Zimmerer, J. Murphy, J.D. Storey, T. D. Duong, et al., A universal stress protein that controls bacterial stress survival in Micrococcus luteus, J. Bacteriol. 201 (24) (2019), https://doi.org/10.1128/JB.00497-19 e00497-19.
[55] A. Untergasser, I. Cutcutache, T. Koressaar, J. Ye, B.C. Fairchloth, M. Remm, S. G. Rozen, Primer3-new capabilities and interfaces, Nucleic Acids Res. 40 (15) (2012) e115, https://doi.org/10.1093/nar/gks596.
[56] G. Zhang, T. Cao, J. Ying, Y. Yang, L. Ma, Diversity and novelty of actinobacteria in Arctic marine sediments, Antonie Van Leeuwenhoek 105 (2014) 743–754, https://doi.org/10.1007/s10482-014-0130-7.
[57] M. Young, V. Artsatbanov, H.R. Beller, G. Chandra, K.F. Chater, L.G. Dover, E. B. Goh, Genome sequence of the fleming strain of *Micrococcus luteus*, a simple free-living actinobacterium, J. Bacteriol. 192 (3) (2010) 841–860, https://doi.org/10.1128/JB.01254-09.
[58] A.H. Kawo, H.Y. Bacha, Crude oil degradation by Bacillus and Micrococcus species isolated from soil compost in Kano, Nigeria, Bayero J. Libr. Inf. Sci. 9 (1) (2016) 108–117, https://doi.org/10.4314/bajopas.v9i1.17.
[59] M.C. de Goffau, D.S. Charnock-Jones, G.C.S. Smith, J. Parkhill, Batch effects account for the main findings of an in utero human intestinal bacterial colonization study, Microbiome 9 (1) (2021) 6, https://doi.org/10.1186/s40168-020-00949-z.
[60] A. Angelov, P. Bergen, F. Nadler, P. Hornburg, A. Lichev, M. Übelacker, F. Pachl, et al., Novel Flp pilus biogenesis-dependent natural transformation, Front. Microbiol. 6 (84) (2015) 1–11, https://doi.org/10.3389/fmicb.2015.00084.
[61] E. Thormann, T. Pettersson, J. Kettle, P.M. Claesson, Probing material properties of polymeric surface layers with tapping mode AFM: which cantilever spring constant, tapping amplitude and amplitude set point gives good image contrast and minimal surface damage? Ultramicroscopy 110 (4) (2010) 313–319, https://doi.org/10.1016/j.ultramic.2010.01.002.
[62] Y. He, Y. Yan, Y. Geng, Morphology measurements by AFM tapping without causing surface damage: a phase shift characterization, Ultramicroscopy 254 (2023) 113832, https://doi.org/10.1016/j.ultramic.2023.113832.
[63] Q. Huang, H. Wu, P. Cai, J.B. Fein, W. Chen, Atomic force microscopy measurements of bacterial adhesion and biofilm formation onto clay-sized particles, Sci. Rep. 5 (2015) 16857, https://doi.org/10.1038/srep16857.
[64] A. Zdarta, E. Kaczorek, Nanomechanical changes in probiotic bacteria under antibiotics exposure: implications on Lactobacillus biofilm formation, Biochim. Biophys. Acta, Mol. Cell Res. 1870 (7) (2023) 119533, https://doi.org/10.1016/j.bbamcr.2023.119533.
[65] Y. Chao, T. Zhang, Surface-enhanced Raman scattering (SERS) revealing chemical variation during biofilm formation: from initial attachment to mature biofilm, Anal. Bioanal. Chem. Res. 404 (2012) 1465–1475, https://doi.org/10.1007/s00216-012-6225-y.
[66] J.F. González-Martínez, I. Nieto-Carvajal, J. Abad, J. Colchero, Nanoscale measurement of the power spectral density of surface roughness: how to solve a difficult experimental challenge, Nanoscale Res. Lett. 7 (1) (2012) 174, https://doi.org/10.1186/1556-276X-7-174.
[67] C. Dussud, C. Hudec, M. George, P. Fabre, P. Higgs, S. Bruzaud, A.M. Delort, et al., Colonization of non-biodegradable and biodegradable plastics by marine microorganisms, Front. Microbiol. 9 (2018) 1–13, https://doi.org/10.3389/fmicb.2018.01571.
[68] A.V. Singh, V. Vyas, R. Patil, V. Sharma, P.E. Scopelliti, G. Bongiorno, A. Podestà, et al., Quantitative characterization of the influence of the nanoscale morphology of nanostructured surfaces on bacterial adhesion and biofilm formation, PLoS ONE 6 (9) (2011) e25029, https://doi.org/10.1371/journal.pone.0025029.
[69] P.E. Scopelliti, A. Borgonovo, M. Indrieri, L. Giorgetti, G. Bongiorno, R. Carbone, A. Podestà, et al., The effect of surface nanometre-scale morphology on protein adsorption, PLoS ONE 5 (7) (2010) e11862, https://doi.org/10.1371/journal.pone.0011862.
[70] R. Carbone, I. Marangi, A. Zanardi, L. Griorgetti, E. Chierici, G. Berlanda, A. Podestà, et al., Biocompatibility of cluster-assembled nanostructured TiO_2 with primary and cancer cells, Biomaterials 27 (17) (2006) 3221–3229, https://doi.org/10.1016/j.biomaterials.2006.01.056.
[71] M. Belicchi, S. Erratico, P. Razini, M. Meregalli, A. Cattaneo, E. Jacchetti, A. Farini, et al., Ex vivo expansion of human circulating myogenic progenitors on cluster-assembled nanostructured TiO_2, Biomaterials 31 (20) (2010) 5385–5396, https://doi.org/10.1016/j.biomaterials.2010.03.021.
[72] N.A. Hamzah, N.A.A. Razak, M.S.A. Karim, S.Z. Salleh, Validation of a roughness parameters for defining surface roughness of prosthetic polyethylene Pe-Lite liner, Sci. Rep. 12 (1) (2022) 1–9, https://doi.org/10.1038/s41598-022-05173-3.
[73] E. Csanády, E. Magoss, Surface roughness. Mechanics of Wood Machining, Springer, Berlin, Heidelberg, 2013.
[74] I. Drame, C. Lafforgue, C. Formosa-Dague, M.-P. Chapot-Chartier, J.-C. Piard, M. Catelain, E. Dague, Pili and other surface proteins influence the structure and the nanomechanical properties of *Lactococcus lactis* biofilms, Sci. Rep. 11 (1) (2021) 4846, https://doi.org/10.1038/s41598-021-84030-1.
[75] R.S. Matos, E.P. Pinto, G.Q. Ramos, M.D.F. de Albuquerque, H.D. da Fonseca Filho, Stereometric characterization of kefir microbial films associated with Maytenus rigida extract, Microsc. Res. Tech. 83 (11) (2020) 1401–1410, https://doi.org/10.1002/jemt.23532.
[76] E. Babich, S. Scherbak, E. Lubyankina, V. Zhurikhina, A. Lipovskii, Power spectral density analysis for optimizing SERS structures, Sensors 22 (2) (2022) 593, https://doi.org/10.3390/s22020593.
[77] S. Schröder, A. Duparré, L. Coriand, A. Tünnermann, D.H. Penalver, J.E. Harvey, Modeling of light scattering in different regimes of surface roughness, Opt. Express 19 (10) (2011) 9820–9835, https://doi.org/10.1364/OE.19.009820.
[78] J. Reitbauer, F. Harrer, R. Eckhart, W. Bauer, Focus variation technology as a tool for tissue surface characterization, Cellulose 28 (2021) 6813–6827, https://doi.org/10.1007/s10570-021-03953-0.
[79] C. Cutler, C.-B. Lee, J.W. Thackeray, C. Mack, J. Nelson, J. DeSisto, M. Li, E. Aqad, X. Hou, T. Marangoni, J. Kaitz, R. Rena, Utilizing roughness power spectral density variables to guide resist formulation and understand impact of frequency analysis through process, J. Photopolym. Sci. Technol. 31 (6) (2018) 679–687, https://doi.org/10.2494/photopolymer.31.679.
[80] M. Omarova, L.T. Swientoniewski, I.K.M. Tsengam, D.A. Blake, V. John, A. McCormick, G.D. Bothun, et al., Biofilm formation by hydrocarbon-degrading marine bacteria and its effects on oil dispersion, ACS Sustain. Chem. Eng. 7 (17) (2019) 14490–14499, https://doi.org/10.1021/acssuschemeng.9b01923.
[81] B. Klein, P. Bouriat, P. Goulas, R. Grimaud, Behavior of *Marinobacter hydrocarbonoclasticus* SP17 cells during initiation of biofilm formation at the

alkane–water interface, Biotechnol. Bioeng. 105 (3) (2010) 461–468, https://doi.org/10.1002/bit.22577.
[82] C.B. Volle, M.A. Ferguson, K.E. Aidala, E.M. Spain, M.E. Núñez, Spring constants and adhesive properties of native bacterial biofilm cells measured by atomic force microscopy, Colloids Surf. B 67 (1) (2008) 32–40, https://doi.org/10.1016/j.colsurfb.2008.07.021.
[83] J. Allkja, F. van Charante, J. Aizawa, I. Reigada, C. Guarch-Pérez, J.A. Vazquez-Rodriguez, P.C. Tom-Coenye, Interlaboratory study for the evaluation of three microtiter plate-based biofilm quantification methods, Sci. Rep. 11 (2021) 13779, https://doi.org/10.1038/s41598-021-93115-w.
[84] M. Ntakatsane, P. Chen, J. Liu, P. Mosebi, L. Xu, K. Senoko, Optimization of microbiological plastic film test plate conditions for rapid detection of antibiotics in milk, J. Food Meas. Charact. 14 (2020) 3473–3482, https://doi.org/10.1007/s11694-020-00576-0.
[85] V. Celiksoy, R.L. Moses, A.J. Sloan, R. Moseley, C.M. Heard, Synergistic in vitro antimicrobial activity of pomegranate rind extract and zinc (II) against *Micrococcus luteus* under planktonic and biofilm conditions, Pharmaceutics 13 (2021) 851, https://doi.org/10.3390/pharmaceutics13060851.
[86] S. Liao, G. Gong, X. Wang, L. Tian, Membrane damage mechanism of protocatechualdehyde against *Micrococcus luteus* and its effect on pork quality characteristics, Sci. Rep. 12 (2022) 18856, https://doi.org/10.1038/s41598-022-23309-3.
[87] S. Malic, K.E. Hill, R. Playle, D.W. Thomas, D.W. Williams, In vitro interaction of chronic wound bacteria in biofilms, J. Wound Care 20 (2011) 569–577, https://doi.org/10.12968/jowc.2011.20.12.569.
[88] H. Mehdi, E. Giti, Investigation of alkane biodegradation using the microtiter plate method and correlation between biofilm formation, biosurfactant production and crude oil biodegradation, Biodegradation 62 (2008) 170–178, https://doi.org/10.1016/j.ibiod.2008.01.004.
[89] K.K. Kadali, K.L. Simons, P.P. Skuza, R.B. Moore, A.S. Ball, A complementary approach to identifying and assessing the remediation potential of hydrocarbonoclastic bacteria, J. Microbiol. Methods 88 (2012) 348–355, https://doi.org/10.1016/j.mimet.2011.12.006.
[90] M. Ejaz, B. Zhao, X. Wang, S. Bashir, F.U. Haider, Z. Aslam, M. Khan, et al., Isolation and characterization of oil-degrading *Enterobacter* sp. from naturally hydrocarbon-contaminated soils and their potential use against the bioremediation of crude oil, Appl. Sci. 11 (2021) 3504, https://doi.org/10.3390/app11083504.
[91] M. Ganesan, R. Mani, S. Sai, G. Kasivelu, M.K. Awasthi, R. Rajagopal, N.I. W. Awasti, Bioremediation by oil degrading marine bacteria: an overview of supplements and pathways in key processes, Chemosphere 303 (2022) 134956, https://doi.org/10.1016/j.chemosphere.2022.134956.
[92] M. Bookstaver, A. Bose, A. Tripathi, Interaction of Alcanivorax borkumensis with a surfactant decorated oil–water interface micelle, Langmuir 31 (2015) 5875–5881, https://doi.org/10.1021/acs.langmuir.5b00688.
[93] A. Dubey, A. Kumar, M.L. Khan, D.K. Payasi, Plant growth-promoting and bio-control activity of *Micrococcus luteus* strain AKAD 3-5 isolated from the soybean (Glycine max (L.) Merr.) rhizosphere, Open Microbiol. J. 15 (2021) 188–197, https://doi.org/10.2174/1874285802115010188.
[94] A.W. Qurashi, A.N. Sabri, Biofilm formation in moderately halophilic bacteria is influenced by varying salinity levels, J. Basic Microbiol. 52 (2011) 566–572, https://doi.org/10.1002/jobm.201100253.
[95] J.R. Haines, B.A. Wrenn, E.L. Holder, K.L. Strohmeier, R.T. Herrington, A. D. Venosa, Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure, J. Ind. Microbiol. Biotechnol. 16 (1996) 34–41, https://doi.org/10.1007/BF01569919.
[96] S.J. Varjani, V.N. Upasani, Biodegradation of petroleum hydrocarbons by oleophilic strain of *Pseudomonas aeruginosa* NCIM, Bioresour. Technol. 5514 (222) (2016) 195–201, https://doi.org/10.1016/j.biortech.2016.10.006.
[97] G.-Z. Zhao, J. Li, S. Qin, Y.-Q. Zhang, W.-Y. Zhu, C.-L. Jiang, L-H. Xu, et al., *Micrococcus yunnanensis* sp. nov., a novel actinobacterium isolated from surface-sterilized *Polyspora axillaris* roots, Int. J. Syst. Evol. Microbiol. 59 (10) (2009) 2383–2387, https://doi.org/10.1099/ijs.0.010256-0.
[98] W. Li, M.S. Siddique, N. Graham, W. Yu, Influence of temperature on biofilm formation mechanisms using a gravity-driven membrane (GDM) system: Insights from microbial community structures and metabolomics. Environ. Sci. Technol. 56 (12) (2022) 8908–8919, https://doi.org/10.1021/acs.est.2c01243.
[99] K. Bisht, A.R. Luecke, C.A. Wakeman, Temperature-specific adaptations and genetic requirements in a biofilm formed by *Pseudomonas aeruginosa*, Front. Microbiol. 13 (2023) 1032520, https://doi.org/10.3389/fmicb.2022.1032520.
[100] D. Dasgupta, R. Ghosh, T.K. Sengupta, Biofilm-mediated enhanced crude oil degradation by newly isolated pseudomonas species, ISRN Biotechnol. 5 (2013) 250749, https://doi.org/10.5402/2013/250749.
[101] C. Sun, T. Leiknes, J. Weitzenböck, B. Thorstensen, Salinity effect on a biofilm-MBR process for shipboard wastewater treatment, Sep. Purif. Technol. 72 (2010) 380–387, https://doi.org/10.1016/J.SEPPUR.2010.03.010.
[102] L. Feng, X. Li, P. Song, G. Du, J. Chen, Physicochemical properties and membrane biofouling of extra-cellular polysaccharide produced by a *Micrococcus luteus* strain, World J. Microbiol. Biotechnol. 30 (2014) 2025–2031, https://doi.org/10.1007/s11274-014-1627-y.
[103] T. Gutierrez, D. Berry, T. Yang, S. Mishamandani, L. McKay, A. Teske, M. D. Aitken, Role of bacterial exopolysaccharides (EPS) in the fate of the oil released during the deepwater horizon oil spill, PLoS ONE 8 (6) (2013) e67717, https://doi.org/10.1371/journal.pone.0067717.
[104] W.-T. Liu, M.H. Karavolos, D.M. Bulmer, A. Allaoui, R.D.C.E. Hormaeche, J.J. Lee, C.M.A. Khan, Role of the universal stress protein UspA of *Salmonella* in growth arrest, stress and virulence, Microb. Pathog. 42 (1) (2007) 2–10, https://doi.org/10.1016/j.micpath.2006.09.002.
[105] Ö. Persson, A. Valadi, T. Nystöm, A. Farewell, Metabolic control of the Escherichia coli universal stress protein response through fructose-6-phosphate, Mol. Microbiol. 65 (4) (2007) 968–978, https://doi.org/10.1111/j.1365-2958.2007.05838.x.
[106] D. Jung, M. Guan, S. Lee, C. Kim, H. Shin, S. Hong, U.H. Yim, et al., Searching for novel modes of toxic actions of oil spill using *E. coli* live cell array reporter system-A Hebei Spirit oil spill study, Chemosphere 169 (2017) 669–677, https://doi.org/10.1016/j.chemosphere.2016.11.078.
[107] S.K. Moreno-Forero, J. Roelof van der Meer, Genome-wide analysis of *Sphingomonas wittichii* RW1 behaviour during inoculation and growth in contaminated sand, ISME 9 (1) (2015) 150–165, https://doi.org/10.1038/ismej.2014.101.
[108] S. Samanta, P. Biswas, A. Banerjee, S. Nambi, D.K. Saini, S.S. Visweswariah, A universal stress protein in Mycobacterium smegmatis sequesters the cAMP-regulated lysine acyltransferase and is essential for biofilm formation, J. Biol. Chem. 295 (6) (2020) 1500–1516, https://doi.org/10.1074/jbc.RA119.011373.