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Degradation of the highly complex polycyclic aromatic hydrocarbon coronene by the halophilic bacterial strain *Halomonas caseinilytica*, 10SCRN4D

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Abstract: Polycyclic aromatic hydrocarbons (PAHs) are significant pollutants found in petroleum products. There is ample literature on the biodegradation of PAHs containing less than five rings, but little has been done on those with more than five rings. Coronene (CRN), a seven-ring-containing PAH, has only been shown to be degraded by one bacterial strain. In this study, a bacterial strain 10SCRN4D was isolated through enrichment in the presence of CRN and 10% NaCl (w/v). Analysis of the 16S rRNA gene identified the strain as *Halomonas caseinilytica*. The strain was able to degrade CRN in media containing 16.5–165 µM CRN with a doubling time of 9–16 hours and grew in a wide range of salinity (0.5–10%, w/v) and temperature (30–50°C) with optimum conditions of pH 7, salinity 0.5%–10% (w/v), and temperature 37°C. Over 20 days, almost 35% of 16.5 µM CRN was degraded, reaching 76% degradation after 80 days as measured by gas chromatography. The strain was also able to degrade smaller molecular weight PAHs such as benzo[a]pyrene, pyrene, and phenanthrene. This is the first report of *Halomonas caseinilytica* degrading CRN as the sole carbon source in high salinity, and thus highlights the potential of this strain in bioremediation.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important group of pollutants found in the environment. They are derived from petroleum products, oil exploration, transport, and exploitation (Patel et al. 2020). They can also come from pyrogenic sources, such as the incomplete combustion of organic matter, including wood (Lima et al. 2005, Pohl and Kostecki 2020). Several side effects have been associated with these compounds, including reduced immune response and reproduction rates in animals, and an increased incidence of cancer (Lee and v Vu 2010, Baali and Yahyaoui 2019, Patel et al. 2020).

Therefore, removing them from the environment remains a priority. Physical, chemical, and biological methods are used to remove these pollutants. However, the biological approach, which includes biodegradation or bioremediation, is a better alternative because it is more environmentally friendly and relatively cheaper than physical and chemical processes. This biodegradation relies on microorganisms that use these pollutants as a carbon source, leading to their removal.

PAHs are categorized into two groups based on their number of rings. The first group, known as low molecular weight PAHs (LMW-PAHs), consists of two- or three-ring-containing PAHs and includes mainly naphthalene, phenanthrene, and anthracene. The second group, known as high molecular weight PAHs (HMW-PAHs), includes PAHs with more than three rings, such as pyrene (four rings), benzopyrene (five rings), and coronene (CRN) (seven rings) (Patel et al. 2020). The higher the number of rings, the more resistant the compound is to degradation (Nzila and Musa, 2020). As a result, HMW-PAHs, particularly CRN, tend to accumulate

in the environment and have toxic effects on humans, animals, and ecosystems (Włodarczyk-Makuła, 2012).

The literature contains numerous studies on the microbial degradation of PAHs, particularly LMW-PAHs (Bamforth and Singleton 2005, Abbasian et al. 2015, Lawal 2017, Dhar et al. 2020). The degradation of HMW-PAHs, particularly pyrene and benzopyrene, has also been documented (Ghosal et al. 2016, Nzila 2018, Nzila and Musa 2020). However, to date, only three bacterial strains belonging to one of the species, *Burkholderia cepacia*, have been shown to degrade CRN (Juhasz et al. 1996, 1997, 2000). This is not surprising, as CRN is one of the most recalcitrant HMW-PAHs and is less amenable to degradation.

In the aforementioned studies on CRN biodegradation, the *B. cepacia* strains were initially isolated in the presence of pyrene and were later shown to degrade CRN, but this degradation was only pronounced in the presence of high bacterial cell density or the presence of another substrate (growth-substrate), a process known as co-metabolism (Juhasz et al. 1996, 1997). Additionally, one of the strains, which was later reclassified as *Stenotrophomonas maltophilia*, showed that the degradation of CRN was associated with low toxicity (mutagenicity) against *Salmonella typhimurium* strains (Juhasz et al. 2000).

To the best of our knowledge, only one bacterial species, *S. maltophilia* (formerly known as *Burkholderia cepacia*), has been reported to degrade CRN. This degradation was carried out under low salinity conditions [0.5% NaCl (w/v)]. In this study, we report the isolation and characterization of a halophilic bacterial strain capable of degrading CRN as the sole carbon source in the presence of high salinity (10% NaCl, w/v).

Materials and Methods

Materials

The following chemicals, CRN, benzo[a]pyrene, pyrene, anthracene, magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2$), monopotassium phosphate (KH_2PO_4), dipotassium phosphate (K_2HPO_4), ammonium nitrate (NH_4NO_3), ferric chloride ($FeCl_3$), and the agar used for the preparation of culture media were all procured from Sigma-Aldrich (St. Louis, MO, USA). CRN and the other PAHs had a purity of ≥ 96%. Chemicals for Luria–Bertani (LB) medium including tryptone, yeast extract, agar, and common salt (NaCl) were purchased from Difco (Detroit, MI, USA). The solvents chloroform and ethyl-acetate were of analytical grade and were also purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample collection

Contaminated soil samples were collected from the filling station at King Fahd University of Petroleum and Minerals (KFUPM), Saudi Arabia. The filling station (location, 26.309144410341673, 50.13994252761753) serves as a significant fuel depot for school buses within the KFUPM campus and it is known for its oil-contaminated soils, which is an ideal environment to isolate bacteria capable of degrading PAHs.

Enrichment cultures

About 1.0 g of the soil sample was enriched with a 100 mL Bushnell-Hass (BH) culture medium, which consisted of 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4, 1.0 g NH_4NO_3, 0.05 g $FeCl_3$, at 10% and 15% w/v NaCl per liter, the final adjusted to pH 7.0. This suspension was then supplemented with 25 mg l^{-1} (82.5 µM) of CRN, as the sole carbon source, before culturing it at 37°C, at 120 rpm. After 3–4 weeks, the culture was transferred to a fresh culture medium (1/10, v/v) for another 2–3 weeks, and this transfer was repeated 3-4 times until the growth of bacteria was observed. Bacterial colonies were separated using solid agar culture, prepared in BH- medium (1%, w/v), and then incubated at 37°C for 15–21 days. The purity of the isolated individual colonies was confirmed by another solid agar culture (in the same conditions), and thereafter, these colonies were cryopreserved in 15% (v/v) glycerol.

Bacterial enumeration

Bacteria were enumerated or counted following serial dilutions of cultures by 10, 100, and 1000-fold, in phosphate buffer (PBS, pH7) containing NaCl (10%, w/v). Around 0.1 mL of the diluted culture was then spread onto solid agar plates prepared in rich medium LB containing NaCl (10%, w/v). Thereafter, the cultures were kept for 24 h at 37°C, the colony numbers were estimated, and the results were presented as colony-forming units per mL ($CFU \cdot mL^{-1}$).

Scanning electron microscopy (SEM)

About 1.0 mL of an aliquot of bacterial cells was cultured in 10 mL of LB medium in a shaker incubator at 37°C and 120 rpm for 12 hours. The bacterial cells were then centrifuged at 3500 × g for 5 min at 4°C to harvest the cells. The resulting cells were then suspended in 1 mL of phosphate buffer (PBS, pH7), and then 20 µL of 25% glutaraldehyde solution (v/v), the fixative agent, was added to the suspension and was incubated for 12 h at 25°C. Thereafter, around 100 µL of the suspension was fixed by spreading it on microscopic glass slides and incubated at 37°C for another 12 h. To dehydrate the bacterial cells, the slides were embedded in a serial dilution of ethanol, 10%, 25%, 50%, 75%, 96%, and 100% (v/v). The slides were then coated with gold for 5 minutes before being observed, using a JSM-T300 scanning electron microscope (JEOL, Japan).

Bacteria species identification by 16S rRNA gene sequencing and phylogeny

For bacterial species identification, the cryopreserved bacterial cells were pre-cultured in a 100 mL LB medium and centrifuged at 6000 × *g* for 5 minutes at 4°C to harvest the bacterial cells. The cells were lysed followed by DNA extraction and purification using a Qiagen Powerfecal Kit (Qiagen, Hilden, Germany). Then, the 16S rRNA gene was amplified and sequenced, as described elsewhere (Nzila et al. 2021). The analysis of the sequence was carried out using "the BLAST" software tools available in the National Center for Biotechnology Information, NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The program compares nucleotide sequences to those present in this database and calculates the statistical significance. In addition, the program will list the phylogenetically related sequences based on their statistical significance.

Assessment of bacterial degradation of CRN and the effects of temperature, pH, and salinity

These experiments were initiated with the preculturing of the bacterial strain in LB medium, and around 5 × 10^5 colony-

-forming units (CFUs) of bacteria were then cultured in 50 mL BH medium to test the effect of temperature, pH, and salinity on the degradation of CRN. More specifically, bacterial growth was tested in the presence of 16.5, 33, and 165 µM of CRN (initially dissolved in dimethyl sulfoxide, DMSO), at the temperatures of 30, 35, 40 and 45°C; the salinity of 0, 5, 10, 15 and 20% of NaCl (w/v), and pHs of 5, 6, 7 and 8. The ability of bacteria to grow in these various conditions was assessed by counting colonies (as described previously) and quantifying the remaining CRN in the culture. The ability of this strain to degrade separately the PAH benzo[a]pyrene, pyrene and anthracene was also evaluated. Cultures were initiated with 5×10^5 CFUs of bacterial cells in BH-medium, in the presence of 25 uM of each of the three tested PAHs (initially dissolved in DMSO), and were then separately cultured in 100 mL BH medium at 37°C, salinity 10% and pH 7 for 30 days. The remaining PAHs were extracted and quantified using the GC described below.

Quantification of CRN and other PAHs by GC

CRN was quantified following a 100 mL culture of the bacteria in the presence of CRN at various conditions. After each culture, 100 mL samples were collected, and the remaining CRN was first sonicated for 30 min, and then extracted twice, using 50 mL of ethyl acetate. The resulting organic layer was dehydrated using sodium sulfate before drying under a vacuum. The remaining residue (pellet) was then dissolved in 500 µL of chloroform before analysis in GC, as described below. The same protocol for pellet isolation was also employed for the other PAHs (Benzo[a]pyrene, pyrene and anthracene). However, the quantification procedure for CRN is different from that used for the other PAHs, as described below.

A multi-reaction monitoring method (MRM) was developed to detect and quantify CRN using GC coupled with a tandem mass spectrometer (Shimadzu GC-MS/MS TQ8030, Japan). A Rxi 5 Sil MS capillary column (30m x id 0.25 mm x ft 0.25µm) (Restek, USA, Sr.No: 1652241) was used to separate CRN. High-purity helium (99.999%) gas was used as the carrier gas with a flow rate of 2 mL/min and the total run time was 17.5 min. The extract (1.0 µL) was injected using splitless mode. The injector temperature was maintained at 320°C. The column oven temperature was ramped from 200°C after a holding time of 1 min to 300°C at a rate of 15°C/min with a holding time of 10 min. The temperature of the detector was set at 280°C. The precursor's mass of CRN was 300.1; its detection mass ion and the collision energy for this transition were 150.10 m/z and 15 eV, respectively. A 5-point standard curve of CRN (0, 0.15, 0.3, 1.5 and 3 µmol L^{-1}) was used to assess the concentration. The percentage (%) of remaining CRN was assessed according to the formula: % degradation CRN=[(C–T)/C]*100, where C is the coronene concentration in the control sample (without bacteria), and T is coronene concentration in the tested sample (with bacteria).

As previously reported, the quantification of benzo[a] pyrene, pyrene, and anthracene was carried out using GC with a flame ionization detector (Nzila et al. 2017, 2021) This method involved using an HP-5 column (30 m × id 0.32 mm. The initial oven temperature was 120°C for 2 min, which increased to 250°C at 11°C. min^{-1}, and then held for 50 min. The injector and detector temperatures were 310°C and 320°C, respectively. The helium flow rate, the injected volume, and the split ratio were 15 mL. min^{-1}, 1.0 L and 10:1, respectively. The same formula, CRN=[(C–T)/C]* 100, was used to quantify the remaining PAHs. All these experiments were performed in duplicate. Multi points calibration curves were established in the range starting from 0.010 to 1.000 mg/L with a squared correlation coefficient >0.9.

Statistical analysis

The data were analyzed using one-way ANOVA, Student's t-test, and linear regression fitting model of the R software packages (Anonymous, 2023). The linearity of the data was determined using Pearson's correlation coefficient, and the significance level in all tests was set at a threshold of $p<0.05$.

Results and discussion

Enrichment, strain isolation and species identification

The enrichment experiments were carried out at 10% and 15% NaCl, in the presence of CRN as the sole carbon source. Growth, as determined by turbidity, was observed at 10% but not 15% NaCl. After spreading the culture on an agar plate, two bacterial colonies were identified by direct visualization. However, using a light microscope (1000X), both colonies were observed to be rod-shaped and punctiform, to have convex elevation, spherical, light yellow in color, and an entire margin. Thus, the colonies were identical and named 10SCRN4D.

Further studies have shown that this bacterial strain, 10SCRN4D, is Gram-positive, and scanning electron microscopy (SEM) confirmed that 10SCRN4D is rod-shaped with an average size of 2.1×0.5 µm (length x width) as shown in Fig. S1 of supporting information. The 16S rRNA gene sequencing, followed by the BLAST homology analysis of available 16S rRNA gene sequences in the National Center of Biotechnology Information (NCBI) database, showed that this strain belongs to the Halomonas caseinilytica species, based on the 99.93% homology with *Halomonas caseinilytica* strain NY-7 16S ribosomal RNA gene (NCBI, gene bank ref: OP815347.1).

Halomonas genus consists of halophilic (salt-tolerant) bacterial species, that grow in a salinity range of 5% to 20% NaCl. This genus is becoming increasingly attractive as a host for microbial cell factory engineering, due to its fast growth in high salt and pH conditions, leading to the absence of contamination during fermentation processes, without the need for sterilization (Xiao-Ran et al. 2018, Ye and Chen, 2021).

Concerning pollutant biodegradation, several species of *Halomonas* have been shown to degrade PAHs. For instance, degradation of naphthalene, pyrene, or benzo[a]pyrene has been reported in *Halomonas* sp. and *Halomonas shengliensis Halomonas smyrnensis* (Budiyanto et al. 2018, Govarthanan et al. 2020). The biodegradation of naphthalene has also been shown in *Halomonas pacifica* (Cheffi et al. 2020). The monoaromatic compounds phenol, catechol and para-aminoacetanilide have been demonstrated to be degraded in *Halomonas campisalis* and *Halomonas* sp. TBZ3 (Alva and Peyton 2003, Hajizadeh et al. 2015).

This is the first report on the degradation of the highly complex CRN by a *Halomonas* bacterial strain. Similarly, this report is also the first to describe the degradation of CRN in halophilic conditions.

Effect of concentration of CRN

PAHs are known to be toxic to living organisms in general and bacteria in particular. To evaluate the extent of this toxicity, the 10SCRN4D strain was grown in the presence of increasing concentrations of CRN (16.6, 33 and 166.5 µM), pH of 7, temperature of 37°C, and salinity of 10% NaCl. Before commencing the study, the effect of DMSO (0.1% v/v) was assessed by monitoring the growth of a culture of around 10^5 CFU mL^{-1} 10SCRN4D for 30 days. No bacterial growth was observed; thus these bacteria could not utilize DMSO as their carbon source. As a result, in all subsequent studies, CRN was dissolved in DMSO, as a neutral solvent.

Fig. 1 (A) shows bacterial growth in the presence of 16.6, 33.3, and 166.5 µM of CRN as a function of time (30 days). Starting from an aliquot of around 5×10^5 CFU.mL^{-1}, the optimum growth was reached within 9 days for the three tested concentrations. However, the maximum counts differed. At the lowest concentration (16.6 µM CRN), the maximum count was around 7×10^{11} CFU.mL^{-1} while this value decreased to 10^{11} CFU mL^{-1} at 33.3 µM CRN. The lower maximum count was observed with the highest concentration (10^{10} CFU mL^{-1}). All these observations were supported by the computation of the doubling times (dt), with values of 8.78, 11.13, and 15.71 hours respectively at 16.6, 33.3, and 166.5 µM of CRN (Fig. 1 (B)). The difference of dt values pertaining to these 3 concentrations was statistically significant (ANOVA test, $p<0.05$), and the trend analysis indicated a linear relationship equation of $dt = 0.001 \times C + 0.37$ ($R2 = 0.93$, p-value <0.05, where C is the CRN concentration).

The effect of CRN concentration was also assessed by quantifying the remaining CRN using the GC technique. Before the analysis, a 5-point CRN concentration (0, 0.15, 0.3, 1.5 and 3 µM) was quantified. The data was plotted on a linear x and y graph, and the results showed a squared correlation coefficient of R^2=0.9996, which was used as the standard curve for determining the unknown CRN concentrations.

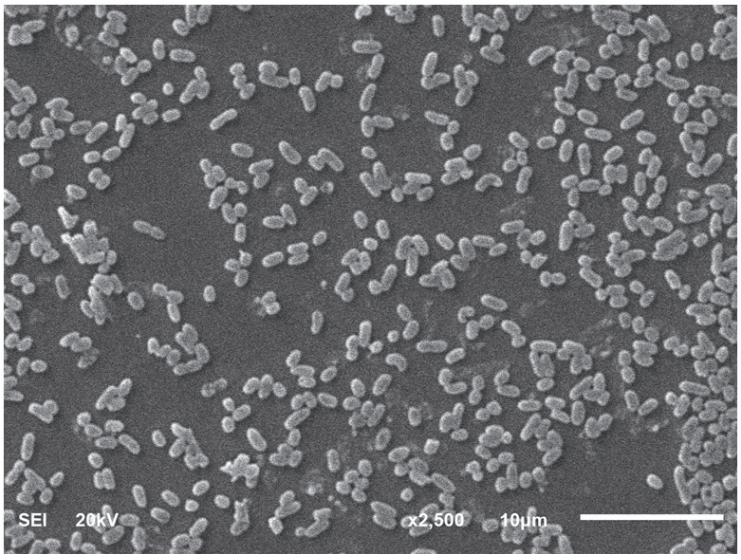

Fig. S1. Electron microscopy picture of the 10SCRN4D strain

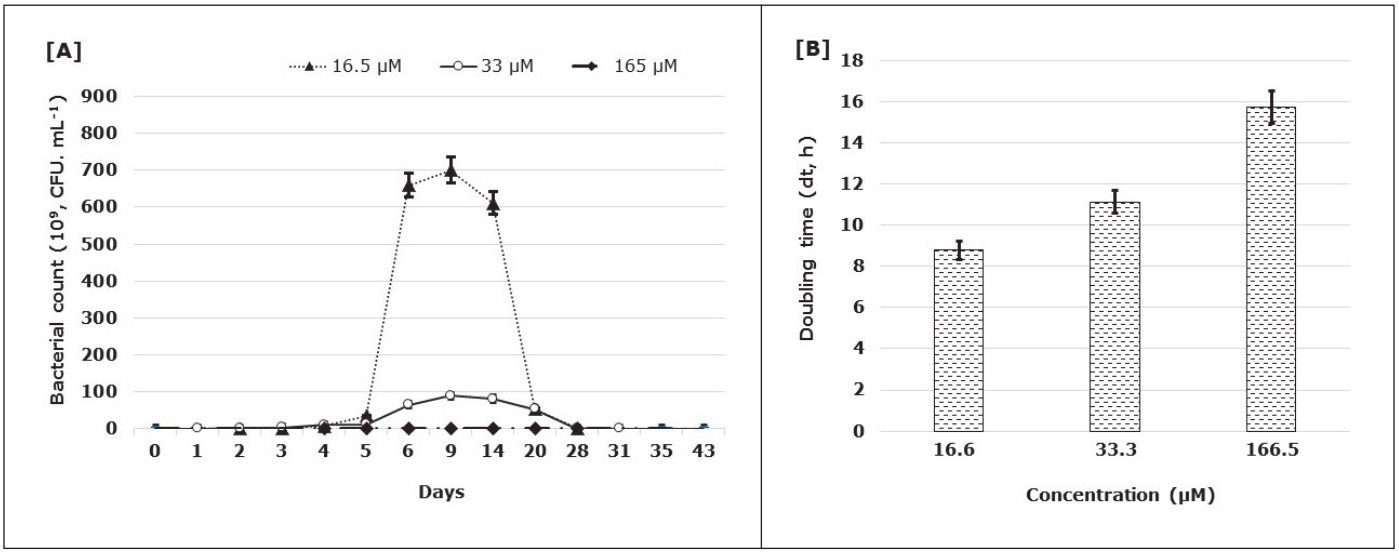

Fig. 1. The growth profile of Halomonas caseinilytica, 10SCRN4D strain, as a function of coronene concentration (A). Doubling time (h) of the growth of this stain as a function of coronene concentration. The dt differences between these 3 concentrations were statistically significant (B).

As shown in Fig.2, the degradation of CRN decreases as the concentration of CRN used in the medium increases. For instance, at 16.6 µM, after 20 days, the degradation rate was 48%, and this value decreased to 38% and 25% at 33.3 and 165 µM respectively, a clear illustration of the toxicity of the CRN. The ANOVA test showed that these values were statistically different ($p<0.05$), while the trend analysis indicates a quadratic model according to the equation: degradation (%)= 0.0013 x C^2 − 0.27 x C + 52 (Adj = 0.8618, p-value < 0.05).

These results, showing the decrease of bacterial growth as CRN concentrations increase, are consistent with previously reported studies using other PAHs. For instance, a decrease in growth was observed with the increasing anthracene concentration in *Bacilluslicheniformis*, *Ochrobactrum* sp. and a co-culture of *Ralstonia pickettii* and *Thermomonas haemolytica* (Arulazhagan and Vasudevan 2011, Swaathy et al. 2014, Nzila et al. 2017), phenanthrene in a co-culture of *Pseudomonas citronellolis* and S. *maltophilia* (Nzila et al. 2017); pyrene in *Ochrobactrum sp., Achromobacter xylosoxidans*, and in the halophilic strains of *Halomonas shengliensis* and *Halomonas smyrnensis* (Arulazhagan and Vasudevan 2011, Budiyanto et al. 2018, Nzila et al. 2018).

Temperature effect

In relation to the temperature effect, the growth of the 10SCRN4D strain was monitored at 30, 37 and 50°C, while keeping the CRN concentration, pH and salinity fixed at 16.6 µM, 7 and 10% (NaCl, w/v) respectively. The highest growth rate was observed at 30 and 37°C, with the culture reaching a maximum count of around 10^{12} CFU mL^{-1} on day 20 at 37°C, and on day 28 at 30°C (Fig. 3(A)). In comparison, at 50°C, bacterial counts remained below 10^9 CFU mL^{-1}, throughout the experimental period. The computation of doubling time (dt) showed values of 9.81+0.001 and

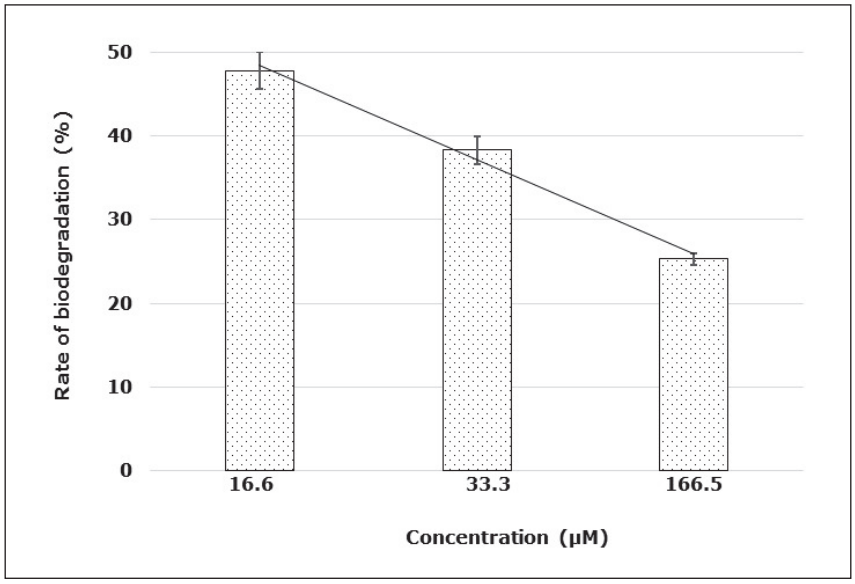

Fig. 2. Quantification of coronene degradation by Halomonas caseinilytica, 10SCRN4D strain using Gas chromatography

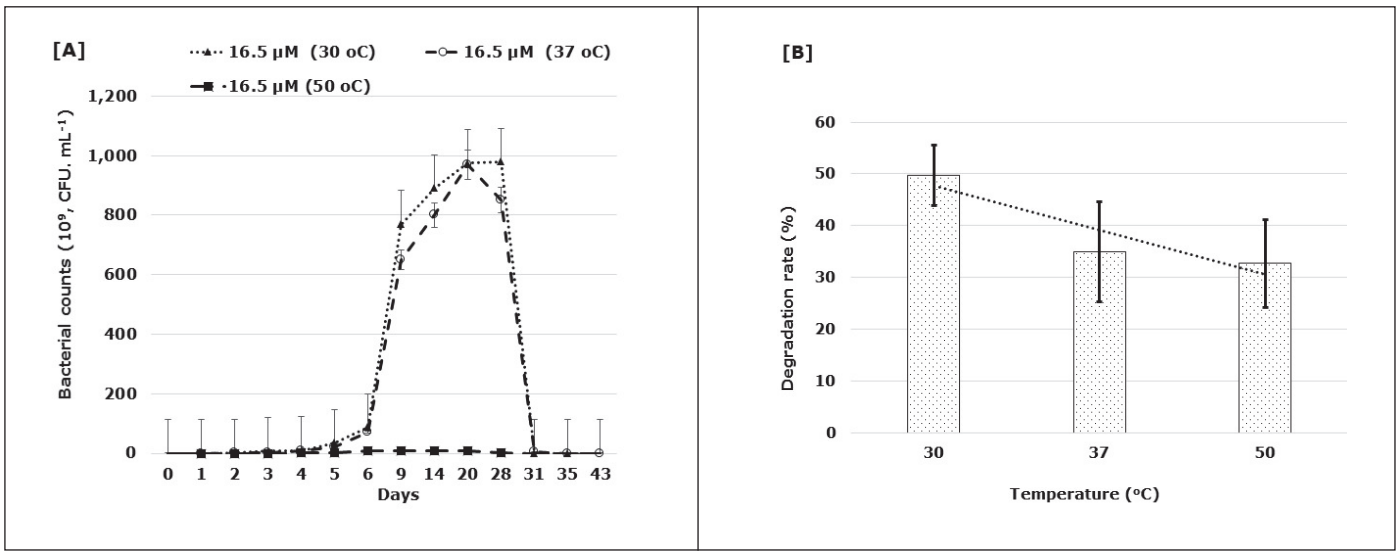

Fig. 3. Growth profile of Halomonas caseinilytica, 10SCRN4D strain as a function of temperature (A) and effect of temperature on its ability to degrade coronene (B)

10.51+0.01 h at 30 and 37°C respectively, while the value for 50°C was higher (14.73+0.02 h), further illustrating that this strain grows less efficiently at 50°C.

The quantification of remaining CRN following the *in-vitro* culture demonstrates that a higher rate of degradation occurred at 30° followed at 37°C, and finally at 50°C, with the percentage of degradation being approximately 50%, 34% and 33% respectively (Fig. 3 (B)). However, these differences were not statistically significant (p>0,05).

Although, as mentioned, these differences were not statistically significant, a temperature of 30°C appeared to be associated with a higher rate of CRN degradation (50% at 30°C compared to 35% at 37°C). Interestingly, even at 50°C (associated with low bacterial growth), the degradation reached 33%. Thus, this species of bacteria has a relatively wide temperate range for growth and CRN degradation. This is consistent with previous reports on the ability of different *Halomonas* species to grow at a wide range of temperatures, including strains belonging to species such as *Halomonas axialensis, Halomonas hydrothermalis, Halomonas neptunia* and *Halomonas sulfidaeris* (Kaye et al. 2004, Harrison et al. 2015, Yin et al. 2015).

pH effect

In addition to pH 7, used in the aforementioned studies, further bacterial growth was assessed at pHs 3 and 10. These studies were performed at 37°C, 16.6 µM, and 10% NaCl (w/v) respectively. However, no growth was observed at the acidic pH 3 or the alkaline pH 10. Generally, the optimum pH range for PAH degradation falls between pH 6 and 8, with the neutral pH being the most commonly reported (Leahy and Colwell 1990, Margesin and Schinner 2001). Nevertheless, PAH biodegradation has also been reported under extreme pH conditions. For instance, pyrene degradation has been reported at pH 9 by the alkaliphilic *Mycobacterium* sp. strain MHP-1 (Habe et al. 2004), and the degradation of naphthalene and phenanthrene has been reported in acidophilic conditions as low as pH 3 (Stapleton et al. 1998). Bacteria belonging to the genera *Clavibacter, Arthrobacter,* and *Acidocella* have also been reported to grow in the presence of naphthalene at a pH as low as 3.5 (Dore et al. 2003). Bacterial degradation of PAHs has also been reported in high alkaline or acidophile conditions by bacteria belonging to genera such as *Marinobacter*, *Pseudomonas,* and *Stappia* (Al-Awadhi et al. 2007).

In relation to the *Halomonas* genus bacteria, pyrene degradation at pH 7 and 9 has been reported in *H. shengliensis* and *H. smyrnensis* salinity, at 10% and 20% respectively (Budiyanto et al. 2018). The strain isolated in this study was unable to grow at either high or low pH (10 or 3). However it cannot be ruled out that this strain can grow at moderate-high or low pHs (7 to 9 or 7 to 4).

Salinity effect

The effect of salinity on bacterial growth was also assessed at 0.5%, 10% and 20% NaCl, while the concentration and temperature were fixed at 16.6 µM and 37°C respectively. As shown in Fig. 4(A), growth profiles at 0.5 and 10% NaCl were almost similar, with the maximum growth attained at day 20 (7.10^{11} CFU mL^{-1}), and the corresponding dt values were 9.86+0.004 and 10.42+0.001 h for 0.5% and 10% NaCl, respectively. The bacterial growth was substantially reduced at a salinity of 20%, with a maximum growth of around 10^{11}, within 9 days, and a dt of 11.78+0.01.

The higher ability of this strain to grow at 0.5% is confirmed by the quantification of the remaining CRN *in vitro*, which shows a higher rate of degradation of 57% at 0.5% NaCl, and this rate decreases as the salinity increases, at 5, 10, and 20% NaCl (Fig. 4(B)). Interestingly, at 20% NaCl, the highest salinity, the degradation rate was around 19%, indicating that bacteria were still active at this salinity level (Fig. 4(B)). However, the ANOVA test indicated that these differences were not statistically significant (p>0.05).

These data suggest that this particular strain is active within a wide range of salt concentrations, from 0.5% to 20%. Similar results were reported in the degradation of another PAH, pyrene, in *Halomonas* strains (Budiyanto et al. 2018). In that study, two strains of *H. shengliensis* and *H. smyrnensis* were found to degrade pyrene within a range of 5–15% and 5–20% NaCl respectively.

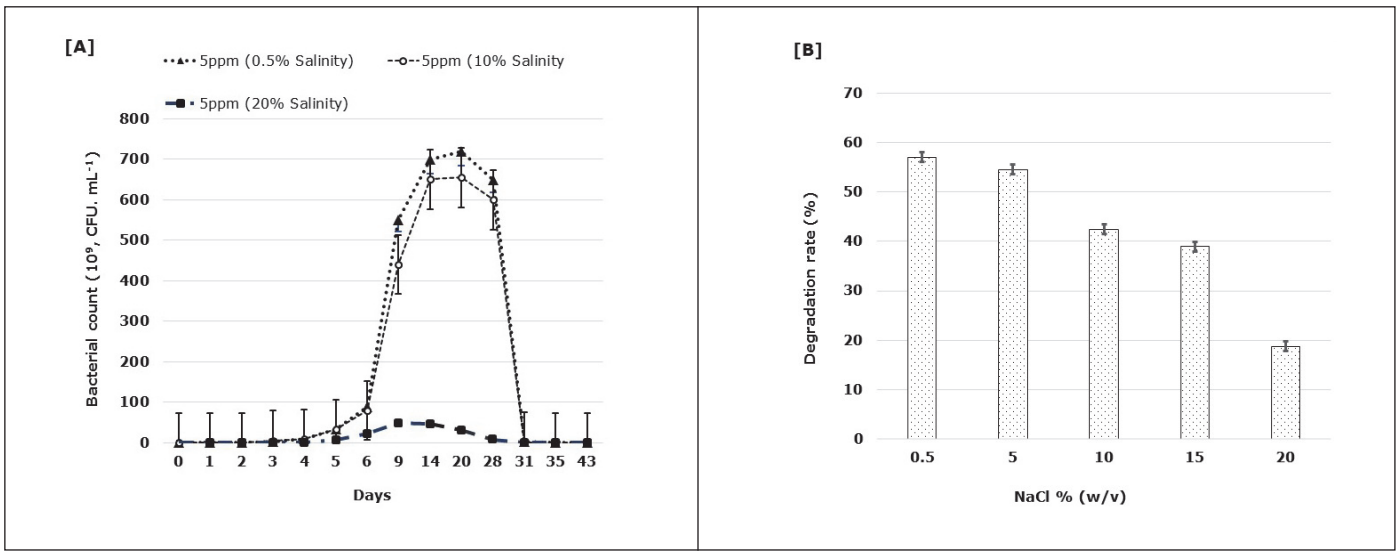

Fig. 4. Growth profile of Halomonas caseinilytica, 10SCRN4D strain as a function of salinity (NaCl concentrations) (A), and the effect of salinity on its ability to degrade coronene (B)

The first reported strain of *Halomonas caseinilytica* in the literature could grow at NaCl concentrations of 3–6% (Wu et al. 2008), a relatively narrow range, compared to the wide range of 0.5–20% NaCl reported in this current study. However, it is worth noting that a wide – range of salt tolerance has also been reported in other species within the *Halomonas* genus. For example, a strain of *Halomonas populi* has been reported to grow at NaCl concentrations of 0.5–18% (Xu et al. 2021), *Halomonas endophytica* at 0.5–6.0% (Chen et al. 2018), *Halomonas lactosivorans* at 1–12% (Ming et al. 2020), and *Halomonas azerica* at 1–17% (Wenting et al. 2021). Thus, it appears that the ability to grow in a wide range of salinities is a common characteristic among *Halomonas* bacteria.

Effect of time on degradation

The data discussed in all the aforementioned experiments were obtained within 30 days. Since the degradation increases as time increases, we sought to assess the extent of degradation over 80 days. This investigation was carried out at 37°C, with a salinity of 10% NaCl, pH 7, and a CRN concentration of 16.6 µM. As Fig. 5 shows, at day 20, the degradation rate was around 37%, and this rate steadily increased over time. At day 80, corresponding to the end of the experiment, almost 76% of CRN was degraded. These values of degradation rates were statistically different (ANOVA test, $p<0.00001$), and the trend analysis indicated a linear model of degradation (%) = 0.7×days + 21.4 (R2 = 0.96, p-value <0.000001), giving rise to a rate of degradation of 0.116 µM CRN day^{-1} (0.7% of 16.6 µM per day). This is the first report on the degradation rate of CRN in a bacterial cell, so these results cannot be compared with any reported ones. The closest comparison can be made with the five-ring PAH benzo[a] pyrene, for which the reported degradation rates in various bacterial strains fell within 0.04–0.3 µM day^{-1} (Nzila et at. 2021). The rate reported in the current work falls within this range. Thus, this *Halomonas caseinilytica,* 10SCRN4D strain can degrade the seven-ring PAH CRN as efficiently as other bacterial strains degrade benzo[a]pyrene.

Biodegradation of PAHs of lower molecular weights

The ability of 10SCRN4D to degrade small PAHs such as benzo[a]pyene, pyrene, and phenanthrene was investigated. The experiments were conducted at 37°C, pH 7, and 10% NaCl for 30 days in the presence of 20 µM of each of the tested PAH, and PAH degradation was quantified using GC. Under these conditions, the 10SCRN4D strain degraded 43.68% ± 19.36%, 51.75% ± 13.67%, and 41.83% ± 16.24% of benzo[a]pyene, pyrene, and phenanthrene, respectively.

Bacteria gradually break down PAHs by initial oxidation following by ring opening, resulting in PAHs with lower molecular weights, and eventually monoaromatic rings and aliphatic derivatives. Bacteria that can degrade high molecular weight PAHs can also degrade lower molecular weight PAHs. For example, bacterial strains belonging to the genus *Ochrobactrum*, *Cellulosimicrobium*, *Hydrogenophaga*, *Rhizobium tropici*, and *Staphylococcus*, selected for their ability to degrade benzo[a]pyrene, have also been shown to degrade other PAHs of lower molecular weight such as pyrene, phenanthrene, anthracene, and naphthalene (Wu et al. 2009, Arulazhagan and Vasudevan 2011, Yessica et al. 2013, Qin et al. 2018, Nzila et al. 2021). This CRN-degrading strain can also degrade lower molecular weight PAHs, making it a promising candidate for bioremediation.

Conclusion

As shown in this work, a halophilic bacterial strain, *Halomonas caseinilytica*, 10SCRN4D, capable of degrading CRN when used as a sole source of carbon, has been isolated and characterized for the first time. It can degrade CRN at a rate as high as those reported for lower molecular-weight PAHs. In addition, it can degrade CRN within a wide range of salinity (0.5–10% NaCl), making it a useful bacterial strain to be used in the context of bioremediation of an environment contaminated with CRN. However, further studies involving "Omics" such as metabolomics, transcriptomics, proteomics, and a whole genome analysis are needed to establish the biochemical

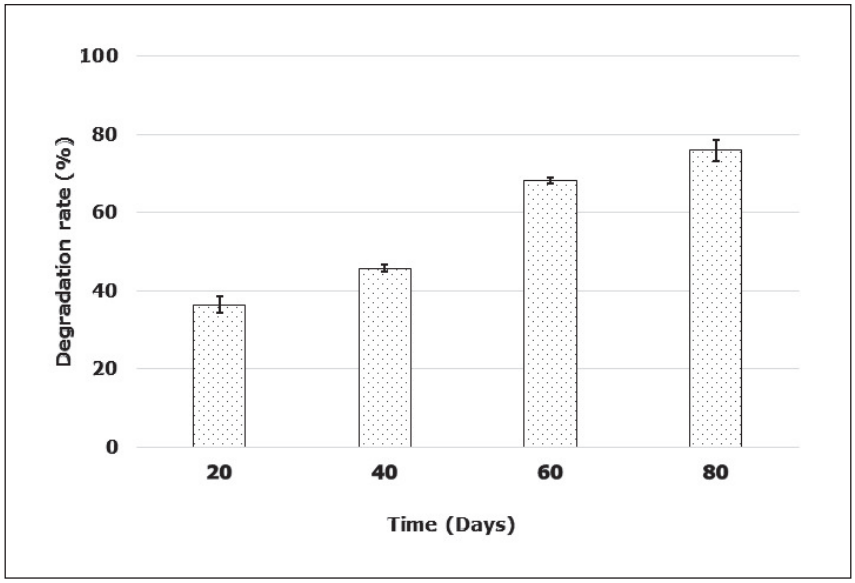

Fig. 5. Ability of Halomonas caseinilytica, 10SCRN4D to degrade coronene over 80 days

pathways of CRN degradation and the enzymes involved in this degradation. Such studies can lead to the identification of enzymes that can not only be used in bioremediation, but also in biocatalytic transformations.

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