


# Biodegradation of diesel oil by cold-adapted bacterial strains of *Arthrobacter* spp. from Antarctica

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**Abstract:** Bioremediation has been proposed as a means of dealing with oil spills on the continent. However, the introduction of non-native organisms, including microbes, even for this purpose would appear to breach the terms of the Environmental Protocol to the Antarctic Treaty. This study therefore aimed to optimize the growth conditions and diesel degradation activity of the Antarctic native bacteria *Arthrobacter* spp. strains AQ5-05 and AQ5-06 through the application of a one-factor-at-a-time (OFAT) approach. Both strains were psychrotolerant, with the optimum temperature supporting diesel degradation being 10–15°C. Both strains were also screened for biosurfactant production and biofilm formation. Their diesel degradation potential was assessed using Bushnell–Haas medium supplemented with 0.5% (v/v) diesel as the sole carbon source and determined using both gravimetric and gas chromatography and mass spectrophotometry analysis. Strain AQ5-06 achieved 37.5% diesel degradation, while strain AQ5-05 achieved 34.5% diesel degradation. Both strains produced biosurfactants and showed high biofilm adherence. Strains AQ5-05 and AQ5-06 showed high cellular hydrophobicity rates of 73.0% and 81.5%, respectively, in hexadecane, with somewhat lower values of 60.5% and 70.5%, respectively, in tetrahexadecane. Optimized conditions identified via OFAT increased diesel degradation to 41.0% and 47.5% for strains AQ5-05 and AQ5-06, respectively. Both strains also demonstrated the ability to degrade diesel in the presence of heavy metal co-pollutants. This study therefore confirms the potential use of these cold-tolerant bacterial strains in the biodegradation of diesel-polluted Antarctic soils at low environmental temperatures.

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**Key words:** Antarctica, *Arthrobacter*, bioremediation, cold-tolerant, diesel, one-factor-at-a-time

## Introduction

Antarctica remains a largely untouched region of the planet. However, even here, areas have been contaminated by petroleum hydrocarbons, notably diesel oil, associated with anthropogenic activities through national governmental research stations as well as the rise in tourism in recent decades, both primarily supported by ships (Tin *et al.* 2009, Hughes 2014). Currently, over 100 facilities are established on the continent, primarily supporting research activities (Hughes 2014). Petroleum hydrocarbons represent the major source of energy used to run facilities in Antarctica, and they are the primary source of hydrocarbon pollution in this region (Ruberto *et al.* 2005).

Diesel oil, the most commonly used hydrocarbon fuel in the Antarctic region, consists of a complex mixture of aliphatic and aromatic hydrocarbons and is made by the fractional distillation of petroleum (Gallego *et al.* 2001). It also contains toxic compounds such as phenolics that tend to persist in contaminated environments, along with heavy metals including cadmium (Cd), chromium (Cr) and lead (Pb) (Lee *et al.* 1992). Heavy metals have been reported to alter the activities of Antarctic microbial communities and to affect the enzymes involved in various metabolic processes, including the impairment of microbial diesel degradation. Oil spillage as a result of accidental discharge during transportation and leakage from storage tanks and pipelines has become a serious and persistent threat in

Antarctica (Aislabie *et al.* 2004, Hughes 2014), particularly affecting the seasonally melted active layer of the soil profile overlying permafrost. Hydrocarbon contamination of Antarctic soils can alter the microbial community's structure and function and affect the abundance of small invertebrate organisms. Spilt oil tends to persist for a long time in the Antarctic environment owing to the chronically low temperatures, limited nutrient availability and dry conditions of the environment, which slow the rate of microbial processes and abiotic degradation (Aislabie *et al.* 2006).

The use of microorganisms in order to remediate contaminated soil has increased significantly in recent years, surpassing conventional methods as they have proved to be ecologically friendly, simple to apply, cheap and effective (Aislabie *et al.* 2006, Ahmad *et al.* 2018). However, in Antarctica, the use of indigenous Antarctic microorganisms for such activities is required, as the importation and release of non-native biota are not permitted under the Environmental Protocol to the Antarctic Treaty (Aislabie *et al.* 2000). Various studies have confirmed the ability of native microbes to break down petroleum hydrocarbon contaminants in the harsh environmental conditions of Antarctica (Hughes *et al.* 2007). The most frequently isolated hydrocarbon-degrading bacteria from contaminated Antarctic soils belong to the genera *Acinetobacter*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* genera (Ruberto *et al.* 2005, Aislabie *et al.* 2006) and are known to be capable of degrading alkanes (Shukor *et al.* 2009) and aromatics (Aislabie *et al.* 2000).

One of the most significant characteristics of petroleum hydrocarbon-degrading bacteria is their ability to emulsify hydrocarbons in an aqueous solution by producing surface active molecules that cause a reduction in surface and interfacial tension in aqueous solutions and hydrocarbon mixtures (Ibrahim *et al.* 2013). Biosurfactants are used extensively in bioremediation as they can improve microbial growth on hydrophobic surfaces and also increase the nutrient intake of hydrophobic substrates, thereby making the hydrocarbon pollutant accessible to microorganisms (Ibrahim *et al.* 2013). The use of microbes capable of degrading hydrocarbons as well as of producing biosurfactants can speed up the bioremediation of hydrocarbon-polluted sites. The utilization of hydrocarbons by bacteria has been reported to depend strongly on environmental variables, including temperature, pH, salinity and nitrogen sources (Habib *et al.* 2018). Thus, these environmental variables are important factors affecting the efficiency of diesel biodegradation. Optimization of the conditions for diesel degradation by microorganisms is essential in order to ensure successful treatment of diesel. Optimization means establishing the foremost solution, representing the best compromise amidst several varying demands subject to predefined

requirements called restrictions (Karamba *et al.* 2016). Optimization is very significant in biotechnological processes. Optimum conditions for the rapid growth of a microorganism must be attained in order to achieve desirable responses. Optimizing the variables required for biological processes is crucial to overcoming any difficulties that are identified. The application of optimization in a biotechnological process tends to make such processes efficient and achievable (Ibrahim *et al.* 2015, Karamba *et al.* 2016). Traditionally, optimization has been carried by examining the effects of one factor at a time (OFAT) on an experimental response. However, one major drawback of this approach is that it does not study the interaction effects between the examined variables. Previous studies have successfully used the traditional OFAT approach in order to determine the optimal conditions for substrate degradation (Shukor *et al.* 2009, Habib *et al.* 2018, Lee *et al.* 2018). Therefore, in order to achieve the maximum rate of diesel degradation, it is important to optimize bacterial growth and degradation. This study was conducted in order to determine the optimum conditions for diesel degradation by the known Antarctic soil bacterial strains *Arthrobacter* spp. strains AQ5-05 and AQ5-06 using the traditional OFAT method.

## Materials and methods

### *Samples and media*

The isolates of *Arthrobacter* spp. strains AQ5-05 and AQ5-06 used for this study were originally isolated from Antarctic soil obtained on King George Island, South Shetland Islands (Lee *et al.* 2018). *Arthrobacter* spp. strains AQ5-05 (ref. KX946130-KX946131) and AQ5-06 (ref. KX946127) nucleotide sequences are deposited in the National Center for Biotechnology Information (NCBI) database. The diesel fuel used for the experiment was obtained from a Petronas filling station in Selangor, Malaysia, and was used as the sole source of carbon in all of the experiments. Bushnell–Haas (BH) broth was used for screening and growth optimisation of diesel-degrading bacteria (Bushnell & Haas 1941). The medium used was composed of MgSO<sub>4</sub> (0.2 g l<sup>-1</sup>), CaCl<sub>2</sub> (0.02 g l<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (1.0 g l<sup>-1</sup>), NH<sub>4</sub>NO<sub>3</sub> (1.0 g l<sup>-1</sup>) and FeCl<sub>3</sub> (0.05 g l<sup>-1</sup>). The pH of the medium was adjusted to 7.0 ± 0.2 at 25°C prior to sterilization. In order to ascertain the ability of the strains to use diesel oil as the only source of carbon, a non-inoculated BH broth was used as a control. All of the experiments were performed in triplicate.

### *Screening for diesel-degrading ability*

Strains were pre-cultured in nutrient broth medium for 24 h in a shaking incubator at 150 rpm and 10°C. Following incubation, cultures were centrifuged at

5000 rpm for 10 min, and the resulting pellets were washed twice using 1× phosphate-buffered saline (137 mM NaCl and 2.7 mM KCl in 10 mM phosphate buffer, pH 7.4) and then re-suspended to an OD<sub>600</sub> of 1 in BH broth containing 0.5% (v/v) diesel as the sole source of carbon and energy. The diesel medium was incubated at 10°C for 168 h in a shaking incubator at 150 rpm. The 10°C incubation temperature was chosen in order to mimic typical summer soil surface temperatures at King George Island (Lee *et al.* 2016). The diesel-degrading activity of each bacterial strain was observed at intervals of 24 h, with bacterial growth determined by measuring the optical density of the medium (OD<sub>600</sub>). The residual hydrocarbon in diesel oil from each culture was extracted every 12 h using a solvent extraction method by destructive sampling from a triplicate flask containing culture (50 ml of BH media in a 250 ml conical flask). *n*-Hexane (1:1 media to *n*-hexane) was used in order to separate the cellular material. After evaporation, the remaining residual hydrocarbon in diesel was quantified gravimetrically (Patowary *et al.* 2017). The diesel degradation percentage was calculated as in Eq. (1):

$$\text{Hydrocarbon degradation (\%)} = \frac{X - Y}{X} \times 100 \quad (1)$$

where *X* is the original mass of diesel and *Y* is the mass of residual diesel oil in the test sample.

After 7 days of culture, the amount of degraded hydrocarbon was analysed in the extracted diesel oil samples.

Furthermore, in order to confirm the degradation ability of both strains, the *n*-hexane-separated samples of treated diesel oil and the abiotic control were further analysed using a gas chromatograph mass spectrometer (GC/MS-QP2010 Ultra, Shimadzu, Japan) equipped with an auto-injector (GC2010). The GC programme was optimized and all of the analyses were performed with a 10:1 split ratio. Helium was used as the carrier gas with a flow rate of 0.8 ml min<sup>-1</sup>, maintaining an injection temperature of 250°C. The column oven temperature was set at 50°C with a hold time of 10 min and was subsequently increased to 300°C with a ramp of 8°C min<sup>-1</sup>, with a final hold time of 37 min. The mass spectrometric data were acquired in electron ionization mode (70 eV). The ion source temperature and interface temperature for MS were set at 200°C and 250°C, respectively. The mass range (*m/z*) was selected as 40–700 for the entire analysis. The chromatograms were analysed with GC/MS solution software, the compound identification was carried out using the NIST 11 library database and alkane standard solution (C<sub>8</sub>–C<sub>20</sub>) was used as an internal standard.

### *Diesel hydrocarbon degradation kinetics*

The degradation of diesel was evaluated using the first-order reaction kinetics expressed in Eq. (2):

$$\ln_{cr} = \ln_{co} - kt \quad (2)$$

where  $\ln_{cr}$  is the concentration of the residual diesel oil in the medium at any given time (mg l<sup>-1</sup>),  $\ln_{co}$  is the initial concentration of diesel oil, *k* signifies the rate constant representing the rate of degradation and *t* represents time (days). The half-life of diesel oil (*t*<sub>1/2</sub>) was evaluated using Eq. (3):

$$t_{1/2} = \ln 2/k \quad (3)$$

where *k* denotes the degradation rate constant (days<sup>-1</sup>).

### *OFAT optimization of diesel degradation and growth conditions*

The growth of bacteria and diesel degradation activity were optimized using the OFAT method based on the following selected parameters: temperature, nitrogen source, initial pH, salinity and initial substrate concentration. The influence of the selected parameters was assessed in 50 ml of BH media contained in a 250 ml conical flask shaken at 150 rpm and 10°C. Each tested parameter was optimized while keeping all other parameters at a constant level for the traditional scaling-up approach. Optimization was performed sequentially (pH, temperature, nitrogen source, nitrogen concentration, salinity and substrate concentration (diesel)). Thus, each subsequent parameter was examined after considering the previously optimized parameters. The influence of temperature was examined at 5°C, 10°C, 15°C, 20°C and 30°C, while the effects of pH were optimized using acetate buffer (pH 5.0–6.0), phosphate buffer (pH 6.0–7.5) and Tris-HCl buffer (pH 7.0–8.0). Salt tolerance was studied by adding various concentrations of sodium chloride (w/v) to the medium (0%, 1%, 2%, 3%, 4% and 5%). The influence of initial diesel concentration was evaluated at 0.5%, 1.0%, 1.5%, 2.0%, 3.0%, 4.0%, 5.0%, 6.0% and 7.0% (v/v). Nitrogen sources evaluated included ammonium nitrate, glutamate, ammonium sulphate, ammonium carbonate, sodium nitrate, ammonium chloride, leucine and aspartic acid. After selection of the most effective source, it was tested at concentrations from 0 to 1.4 g l<sup>-1</sup>. All experiments were conducted in triplicate, including the negative control. Prior to these experiments, bacterial cell suspensions were prepared by growing the isolates in 100 ml of nutrient broth for 24 h at 10°C and 150 rpm. Bacterial cells were then centrifuged at 500 *g* for 10 min and washed twice by re-suspending in 1× phosphate buffer solution. Finally, the cell suspension was adjusted to an absorbance of 1 (OD<sub>600</sub>), equivalent to 2.75 × 10<sup>7</sup> colony-forming

units ml<sup>-1</sup>. Bacterial growth and diesel degradation were evaluated after 7 days of incubation.

#### *Microbial adhesion to hydrocarbon assay*

The cellular hydrophobicity of both isolates was measured as previously described by Rosenberg (1984). Briefly, 24 h grown bacterial culture was collected, washed and rinsed twice with saline. The bacteria were then re-suspended to an absorbance of 1 (OD<sub>600</sub>) measured on a spectrophotometer (UV mini 1240, Shimadzu, Japan). A total of 300 ml each of hexadecane and tetrahexadecane were added to 5 ml of bacterial cell suspension in a clean borosilicate round-bottom glass tube. The mixture in the tube was vortexed for 2 min, and then was set to stand for 15 min to allow hexadecane and tetrahexadecane to separate from the aqueous phase. The sample was carefully removed from the aqueous phase and absorbance was measured at 600 nm. Bacterial adhesion to the hydrocarbon was calculated using Eq. (4):

$$1 - A_{600} \text{ of final absorbance} / A_{600} \text{ of initial absorbance} \quad (4)$$

#### *Screening assays for biosurfactant production*

Bacterial strains were grown in nutrient broth medium at 10°C and 150 rpm for 24 h. Following incubation, cultures were centrifuged (5000 rpm, 10 min), rinsed twice and then adjusted to an absorbance of 1 (OD<sub>600</sub>) in BH medium containing Na<sub>2</sub>HPO<sub>4</sub> (2.2 g l<sup>-1</sup>), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.01 g l<sup>-1</sup>), NaCl (0.05 g l<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (1.4 g l<sup>-1</sup>), CaCl<sub>2</sub> (0.02 g l<sup>-1</sup>) and MgSO<sub>4</sub>·7H<sub>2</sub>O (0.6 g l<sup>-1</sup>). The BH medium was supplemented with 0.5 ml of filtered-sterilized trace element solution containing: H<sub>3</sub>BO<sub>3</sub> (0.56 g l<sup>-1</sup>), ZnSO<sub>4</sub> (0.29 g l<sup>-1</sup>), CoCl<sub>2</sub>·6H<sub>2</sub>O (0.42 g l<sup>-1</sup>), CuSO<sub>4</sub>·5H<sub>2</sub>O (1.78 g l<sup>-1</sup>) and MnSO<sub>4</sub> (0.39 g l<sup>-1</sup>) and 3% (v/v) of sterilized diesel (polytetrafluoroethylene 0.4 µm) as the sole carbon source (Sriram *et al.* 2011). The medium pH was adjusted to 7.2, and it was sterilized at 121°C for 15 min. Bacterial suspensions were inoculated into 100 ml medium in 250 ml flasks and incubated at 10°C for 7 days at 150 rpm.

Following incubation, bacterial cultures were centrifuged at 3000 rpm for 5 min. Pellets were then re-suspended in phosphate buffer. The resulting pellets and supernatants were used for biosurfactant screening, oil displacement testing, drop collapse testing and emulsification testing (Sriram *et al.* 2011). All of the experiments were conducted in triplicate, and sterile distilled water served as a control.

#### *Drop collapse test*

A drop collapse test was conducted using mineral oil as the hydrocarbon substrate, as described by Youssef *et al.* (2004).

Approximately 10 µl of mineral oil was set on a grease-free glass slide, followed by drops of each of supernatant or pellet being placed onto the centre of the oil film. Drop collapse activity was assessed by examining the oil drop shape after 1 min. A positive outcome was recorded if the shape of the oil drop collapsed or flattened, while negative outcome was recorded if the shape remained rounded.

#### *Oil displacement test*

Oil displacement assessment was performed in triplicate. A total of 100 µl of used engine oil was added to Petri dishes containing 50 ml of distilled water. Then, 10 µl each of cell-free culture supernatant, pellets or water (control) was carefully added to the oil surface. Following 30 min of incubation, the diameter of the clear zone was measured by comparing it to the negative control (Rodrigues *et al.* 2006).

#### *Emulsification test*

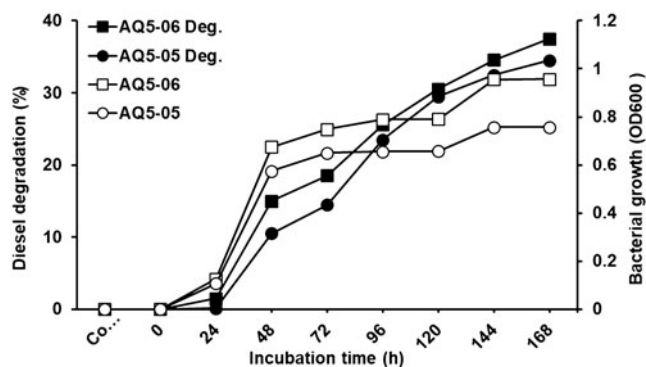
The emulsification assay was performed as previously described by Cooper & Goldenberg (1987). A total of 4 ml of each supernatant and pellet suspension was added to an equal amount of hexadecane and diesel. The resulting mixture was then vortexed for 5 min. The height of the emulsion layer was then measured after 24 h. The emulsification activity index was calculated as the ratio of the height of the emulsion layer to the total height of the liquid as in Eq. (5):

$$E_{24} = (h \text{ emulsion} / h \text{ total}) \times 100 \quad (5)$$

where E<sub>24</sub> is the emulsification activity index after 24 h, *h* emulsion is the emulsion layer height and *h* total is the height of the total liquid.

#### *Biofilm formation test*

The biofilm formation test relies on the ability of bacterial strains to form biofilms on plastics, typically polypropylene (PPE) and polystyrene (PLS) (Tribelli *et al.* 2012). Bacterial strains were cultivated in Luria–Bertani (LB) broth and incubated at 10°C for 24 h at 150 rpm. Following incubation, the overnight-grown bacterial culture was readjusted to an optical density of 0.3 (OD<sub>600</sub>) in the same LB broth. Several 96 well plates of PPE and PLS containing 3 µl of bacterial culture, 15 µl of sterilized hydrocarbon (3% v/v; diesel and hexadecane) as the sole source of carbon and 285 µl of minimal medium were prepared in triplicate. As the negative control, 3 µl of sterile LB medium was used. The plates were then sealed with parafilm, wrapped in aluminium foil and then incubated for 7 days at 10°C in static



**Fig. 1.** Growth of strains AQ5-05 (empty circles) and AQ5-06 (empty squares) in Bushnell–Haas media containing 0.5% (v/v) diesel as the sole carbon source at 10°C and diesel degradation by strains AQ5-05 (filled circles) and AQ5-06 (filled squares) based on gravimetric analysis ( $n = 3$ ). Error bars represent the mean  $\pm$  standard deviation for the triplicates. Non-inoculated samples serve as controls.

conditions as described by Tribelli *et al.* (2012), with modification of the incubation temperature in order to mimic the maximum Antarctic summer temperature. After incubation, bacterial growth was estimated by measuring the absorbance at 600 nm (absorbance of bacterial growth; ABG). Then, the PLS and PPE plates were aspirated with 100  $\mu$ l of 10 mM MgSO<sub>4</sub> for 20 min. The wells were then washed and stained with crystal violet solution for another 20 min. The dye was then washed by rinsing with water and extracted with 96% ethanol. The absorbance of crystal violet (ACV) was then measured at 550 nm. The adherence indices were estimated using Eq. (6):

$$\text{ACV/ABG} \quad (6)$$

### Statistical analysis

All of the experiments were performed in triplicate. The data obtained are presented as the mean  $\pm$  standard deviation. One-way analysis of variance (ANOVA) was used to compare data within treatment groups, and Turkey's test was carried out in order to conduct *post hoc* pairwise analyses if a significant difference was observed.

## Results

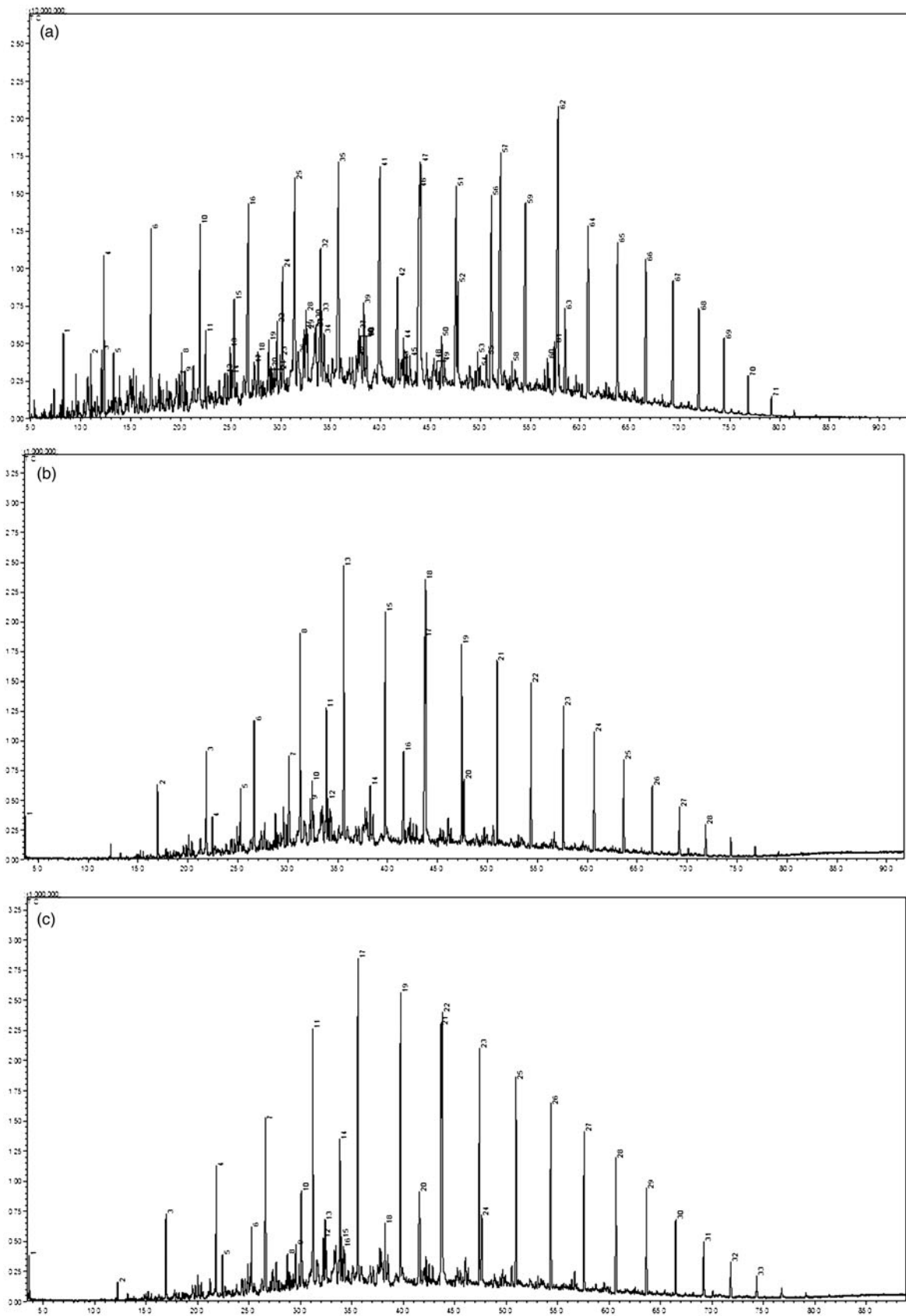
### Evaluation of diesel degradation ability

The ability of *Arthrobacter* spp. strains AQ5-05 and AQ5-06 to metabolize diesel oil was evaluated by incubating both strains in BH medium supplemented with 0.5% (v/v) diesel as the sole carbon source at 10°C and 150 rpm. Strain AQ5-06 showed greater growth ( $\text{OD}_{600} = 0.956$ ) over the 168 h incubation period than did AQ5-05 ( $\text{OD}_{600} = 0.758$ ) (Fig. 1). Gravimetric analysis showed that strain AQ5-06 degraded 37.5% of

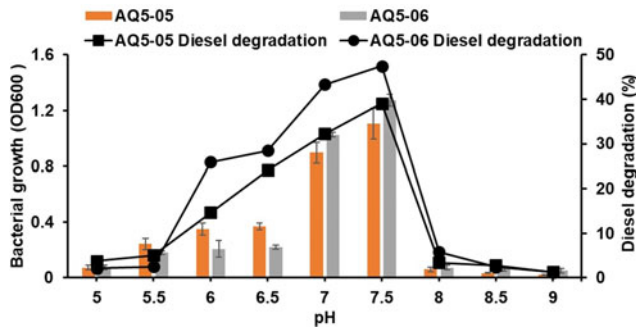
the diesel provided, while strain AQ5-05 degraded 34.5% of the diesel provided. The GC/MS analyses of the residual diesel oil extracted from strain AQ5-05 and AQ5-06 cultures were carried out after 7 days and compared with an abiotic control under the same conditions. Figure 2 displays the results of the obtained chromatograms. Based on the chromatograms, it was observed that the samples treated with *Arthrobacter* spp. strains AQ5-05 and AQ5-06 clearly reduced diesel oil as compared to the abiotic control sample. Short- and medium-chain alkanes were removed more efficiently than long-chain alkanes by both strains, and the rate of removal decreased as the carbon chain length increased. The highest degradation abilities of both isolates were observed for C<sub>8</sub> and C<sub>10</sub> (45–50%), lower degradation abilities were observed for C<sub>13</sub> and C<sub>22</sub> (15–30%) and the lowest degradation abilities were observed for > C<sub>25</sub>. Removal of aromatic compound was also observed. The length of the *n*-alkane chain influenced its degradation significantly, as after 7 days of incubation C<sub>8</sub> and C<sub>10</sub> were reduced to 50% by both strains. Similarly, C<sub>13</sub> was reduced by 15–30%, whereas C<sub>25</sub> was only reduced by 10%. In addition, reductions in aromatic compounds were also observed. These results therefore validate the gravimetric analysis results, as well as suggesting the significant effectiveness of both bacterial strains for degrading various components of hydrocarbons. The specific degradation rates of AQ5-05 and AQ5-06 were 0.023 and 0.029 days<sup>-1</sup>, respectively. The degradation rate constant was higher in AQ5-06 ( $k = 0.047$  days<sup>-1</sup>,  $t_{1/2} = 14.7$  days<sup>-1</sup>) than in AQ5-05 ( $k = 0.031$  days<sup>-1</sup>,  $t_{1/2} = 22.3$  days<sup>-1</sup>), which is again consistent with a faster degradation process.

### Growth optimization using OFAT

The effects of the various selected environmental parameters of pH, temperature, NaCl concentration, nitrogen source and substrate concentration were evaluated using the OFAT technique. Figure 3 shows the influence of initial pH on *Arthrobacter* spp. strains AQ5-05 and AQ5-06, indicating that pH 7.5 supported the optimum growth of both strains. Significant differences between pH treatment groups overall were detected by ANOVA for growth ( $F(8, 18) = 188.640$ ,  $P < 0.001$  and  $F(8, 18) = 796.937$ ,  $P < 0.001$ , respectively) and substrate degradation ( $F(8, 18) = 206.123$ ,  $P < 0.001$  and  $F(8, 18) = 156.608$ ,  $P < 0.001$ , respectively) in both strains, with the optimum being at pH 7.5. *Post hoc* tests comparing growth and degradation at pH 7.5 and the other tested pH values indicated that the mean growth and degradation values were significantly greater at the optimum pH for both strains. As the pH level increased towards alkaline pH 8–9, a rapid decrease in growth was apparent for both strains.



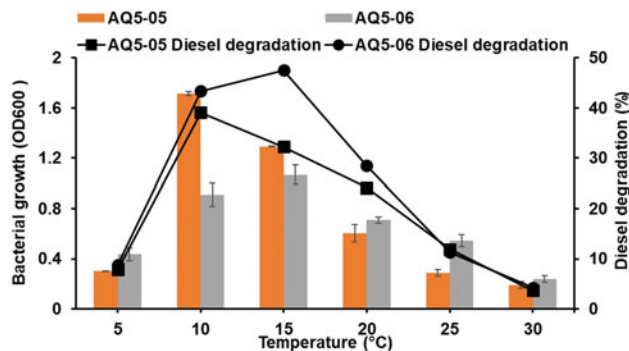
**Fig. 2.** Gas chromatography/mass spectrometry profile of diesel oil extracted from the aqueous phase of the medium after 7 days of incubation with and without strains AQ5-05 and AQ5-06. **a.** Chromatograph of abiotic control. **b.** Chromatograph of diesel oil treated with strain AQ5-05. **c.** Chromatograph of diesel oil treated with strain AQ5-06.



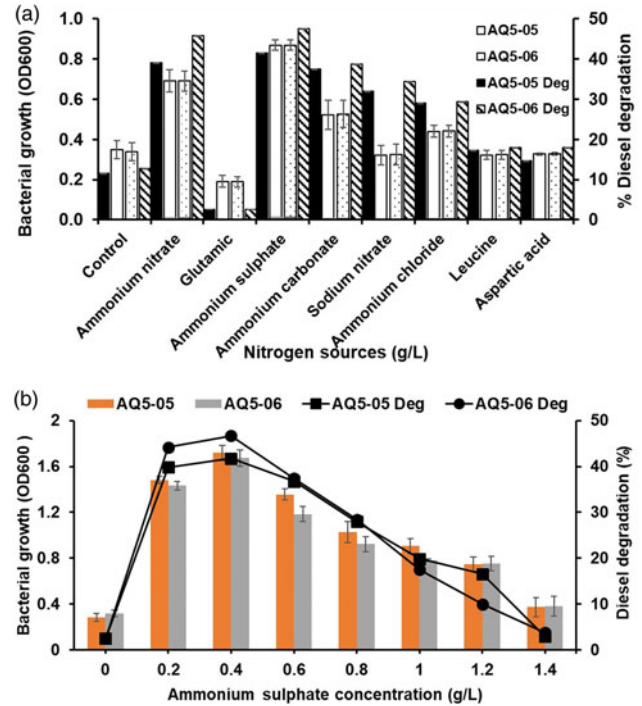
**Fig. 3.** Effects of initial pH on growth of strains AQ5-05 and AQ5-06 in diesel oil medium and diesel degradation by strains AQ5-05 (filled squares) and AQ5-06 (filled circles) at 10°C and with 1.0 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Error bars represent mean ± standard deviation for the triplicates.

The effects of temperature on growth and degradation achieved by strains AQ5-05 and AQ5-06 are illustrated in Fig. 4. Both strains showed optimum growth between 10°C and 15°C, with growth decreasing at higher temperatures. Strain AQ5-05 showed optimal growth and the highest degradation at 10°C, with significant differences between culture temperatures ( $F(5, 12) = 2092.797$ ,  $P < 0.001$  and  $F(5, 12) = 82.789$ ,  $P < 0.001$ , respectively), whereas AQ5-06 attained maximum growth and the highest diesel reduction at 15°C, again with significant differences overall ( $F(5, 12) = 165.485$ ,  $P < 0.001$  and  $F(5, 12) = 121.376$ ,  $P < 0.008$ , respectively). *Post hoc* comparisons showed that there were significant differences between the mean value at 10°C ( $M = 1.7140$ ,  $SD = 0.010$ ) and the other tested temperatures for AQ5-05 and at 15°C ( $M = 1.066$ ,  $SD = 0.055$ ) and the other tested temperatures for AQ5-06.

The influences of various nitrogen sources on the growth and degradation achieved by strains AQ5-05 and AQ5-06

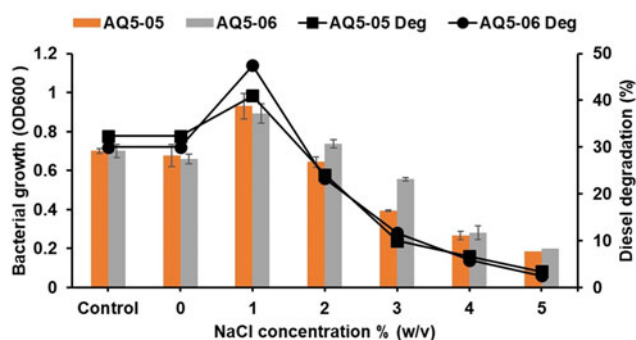


**Fig. 4.** Effects of temperature on the growth of strains AQ5-05 and AQ5-06 in diesel medium at 10°C and diesel degradation by strains AQ5-05 (filled squares) and AQ5-06 (filled circles) at 7.5 pH and with 1.0 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Error bars represent the mean ± standard deviation for the triplicates.



**Fig. 5. a.** Effects of various nitrogen sources on the growth of strains AQ5-05 (empty bars) and AQ5-06 (striped bars) and diesel degradation by strains AQ5-05 (dark bars) and AQ5-06 (dotted bars) at 7.5 pH, 10°C and 15°C and with 1.0 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Error bars represent mean ± standard deviation for the triplicates. **b.** Effects of various ammonium concentrations on the growth of strains AQ5-05 and AQ5-06 in diesel medium and diesel degradation by strains AQ5-05 (filled squares) and AQ5-06 (filled circles) at 7.5 pH and 10°C and 15°C. Error bars represent mean ± standard deviation for the triplicates.

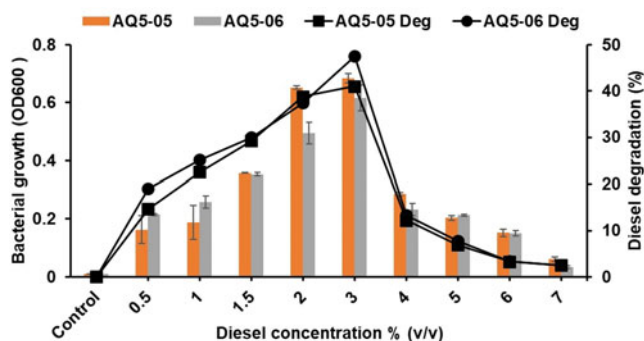
are illustrated in Fig. 5a. Ammonium nitrate and ammonium sulphate gave the greatest enhancement in the growth and degradation of both strains, demonstrating overall significant differences between the different nitrogen sources ( $F(8, 9) = 42.899$ ,  $P < 0.001$  and  $F(8, 9) = 47.556$ ,  $P < 0.001$ , respectively). *Post hoc* analysis showed no significant differences between the two best sources. Due to its availability and widespread use as a cheap nitrogen source for bioremediation, ammonium sulphate was therefore selected as the primary source of nitrogen for the next stage of this study. The impacts of various ammonium sulphate concentrations on the growth and degradation achieved by strains AQ5-05 and AQ5-06 are presented in Fig. 5b, with both bacterial strains showing optimum growth at 0.4 g l<sup>-1</sup> ammonium sulphate. Analyses of variance again identified significant differences in growth ( $F(7, 16) = 66.673$ ,  $P < 0.001$  and  $F(7, 16) = 71.648$ ,  $P < 0.001$ , respectively) and diesel degradation ( $F(7, 16) = 775.906$ ,  $P < 0.007$  and  $F(7, 16) = 78.712$ ,  $P < 0.001$ , respectively) between the various ammonium sulphate concentrations. *Post hoc* tests



**Fig. 6.** Effects of various salt concentrations on the growth of strains AQ5-05 and AQ5-06 in diesel medium at 10°C and diesel degradation by strains AQ5-05 (filled squares) and AQ5-06 (filled circles) at 7.5 pH, 10°C and 15°C and 0.4 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Error bars represent mean ± standard deviation for the triplicates.

showed that the mean values at 0.4 g l<sup>-1</sup> were significantly greater than those at the other tested concentrations in both strains.

The impacts of various salt concentrations on the growth and degradation achieved by strains AQ5-05 and AQ5-06 are illustrated in Fig. 6. Growth of both strains was best at zero- or low-salt concentrations (0% and 1% w/v). As salt concentration increased above this level, there was a corresponding decrease in bacterial cell growth. Analysis of variance identified significant overall differences in growth achieved by strains AQ5-05 and AQ5-06 at various salt concentrations ( $F(5, 12) = 120.263$ ,  $P < 0.001$  and  $F(5, 12) = 139.795$ ,  $P < 0.001$ , respectively), as well as in degradation ( $F(5, 12) = 701.790$ ,  $P < 0.002$  and  $F(5, 12) = 102.532$ ,  $P < 0.001$ , respectively). Pairwise analyses confirmed that there were also significant differences at 0% and 1% (w/v) salt concentrations in both growth and degradation for



**Fig. 7.** Effects of various diesel concentrations on the growth of strains AQ5-05 and AQ5-06 and diesel degradation by strains AQ5-05 (filled squares) and AQ5-06 (filled circles) at 7.5 pH, 10°C and 15°C and with 0.4 g l<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 1% (w/v) NaCl. Error bars represent mean ± standard deviation for the triplicates.

both strains. The mean values achieved at the optimum salt concentration (1% w/v) were significantly greater than those achieved with the other tested concentrations.

The effects of initial diesel concentration on the growth and degradation achieved by strains AQ5-05 and AQ5-06 are illustrated in Fig. 7. The optimum initial diesel concentration supporting the growth of both isolates was between 2% and 3% (v/v). The differences in growth achieved at various initial concentrations were significant in both strains ( $F(8, 18) = 207.284$ ,  $P < 0.001$  and  $F(8, 18) = 190.391$ ,  $P < 0.001$ , respectively), as well as for degradation ( $F(8, 18) = 323.757$ ,  $P < 0.001$  and  $F(8, 18) = 273.105$ ,  $P < 0.015$ , respectively). Pairwise tests confirmed that there were no significant differences in growth or degradation between 2% and 3% initial concentrations. However, significant differences were apparent between the optimum initial diesel concentration (2% and 3% v/v) and the other tested concentrations.

#### Microbial adhesion to hydrocarbon assay

The effects of hydrocarbons on bacterial growth are presented in Table I. Both strains showed good hydrophobicity characteristics. Following extraction with hexadecane and tetrahexadecane, strain AQ5-05 demonstrated cellular hydrophobicity levels of 73.0% and 60.5% to hexadecane and tetrahexadecane, respectively. Slightly higher values (81.5% and 70.5%, respectively) were obtained for strain AQ5-06. These data indicate that the bacterial cells tend to float easily in hydrocarbon oils, a vital property in petroleum hydrocarbon degradation.

#### Screening assay for biosurfactant production

The results of the biosurfactant production test are summarized in Table II. Both the pellet suspensions and the culture supernatants of strains AQ5-05 and AQ5-06 showed positive results in the drop collapse test. The mineral oil drop became flattened within 1 min, strongly suggesting the presence of biosurfactants. Similarly, in the oil displacement test, strain AQ5-06 culture supernatants showed a clear halo zone with a diameter > 1 cm, while strain AQ5-05 culture supernatants generated a halo of 0.9 cm. Both strains exhibited good emulsification activity. The two strains demonstrated the

**Table I.** Microbial adhesion to hydrocarbons in strains AQ5-05 and AQ5-06.

Strain	Hexadecane (%)	Tetrahexadecane (%)
AQ5-05	73.0	60.5
AQ5-06	81.5	70.5



**Table II.** Results of the biosurfactant test using supernatant (SPT) and pellet suspension (PLT).

Strain	Drop collapse		Oil displacement halo diameter (cm)		Emulsification test			
	SPT	PLT	SPT	PLT	(emulsification activity, %)			
					Hexadecane		Diesel	
	SPT	PLT	SPT	PLT	SPT	PLT	SPT	PLT
AQ5-05	++	+	0.9	0.5	60	40	SLY	SLY
AQ5-06	++	+	1.1	0.7	67	42	SLY	SLY

+ = positive results for drop fattening, ++ = strongly positive results for drop fattening, SLY = slight layer.

strongest emulsification in hexadecane (> 50%), whereas in diesel both strains demonstrated relatively low emulsification activities.

#### Biofilm formation test

Both strains demonstrated greater biofilm adherence in the presence of hexadecane and diesel than in the control conditions (Table III). Strain AQ5-06 displayed the greatest tendency to form a biofilm in the presence of hexadecane, 15–30 times greater than the control in PPE plates and 17–40 times greater than the control in PLS plates.

#### Effects of heavy metals on diesel metabolic activity of AQ5-05 and AQ5-06

The effects of various heavy metals on growth and diesel degradation activity were evaluated by growing strains AQ5-05 and AQ5-06 in BH medium containing diesel as the sole carbon source, with a 1 ppm concentration of various heavy metal ions. Mercury (Hg) and silver (Ag) greatly impeded the growth and diesel degradation activity of both strains (Fig. 8a & b). The growth ( $F(9, 10) = 72029.644$ ,  $P < 0.001$  and  $F(9, 20) = 3335.543$ ,  $P < 0.001$ , respectively) and degradation activity ( $F(9, 10) = 105.21$ ,  $P < 0.001$  and  $F(9, 20) = 246.021$ ,  $P < 0.001$ , respectively) of both strains were significantly influenced by the various heavy metals trialled. *Post hoc* tests identified no significant differences between the effects of Hg and Ag for both strains. In the absence of either Hg or Ag, strains AQ5-05 and AQ5-06 degraded 37.3% and 46.6% diesel, respectively, which reduced to

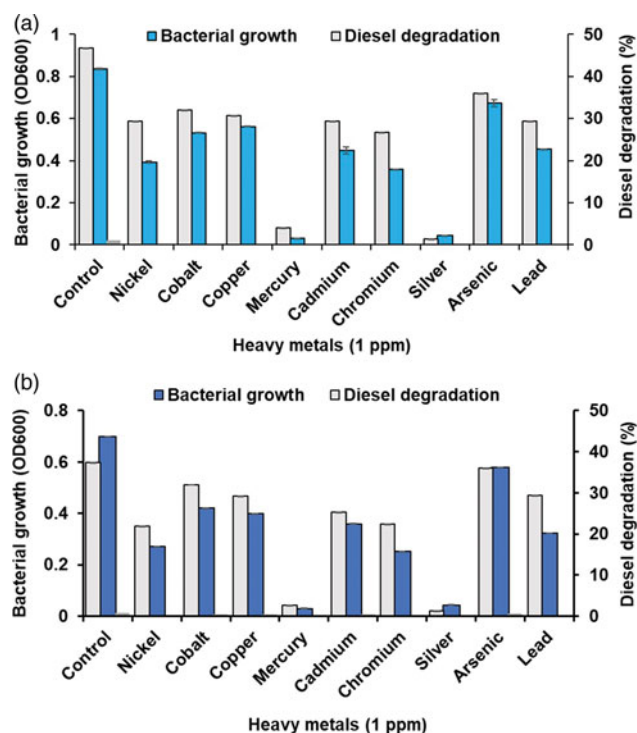
**Table III.** Biofilm formation indices of strains AQ5-05 and AQ5-06 on polystyrene (PLS) and polypropylene (PPE) plates in hexadecane and diesel (OD<sub>600</sub>/OD<sub>550</sub>).

Strain	Hexadecane		Diesel	
	PPE	PLS	PPE	PLS
Control	2.5	1.4	2.4	2.1
AQ5-05	55.3	44.5	4.5	36.3
AQ5-06	60.5	52.5	5.6	41.2

~4% of the initial diesel amount in the presence of either heavy metal ion.

#### Discussion

Antarctica is a remote and dry continent. Its chronically low temperatures are a challenge for microbial metabolic activity. However, human activities in the southern polar region are associated with the risk of pollution events. King George Island is one of the largest islands in the South Shetlands archipelago, and it is subjected to some of the most intense levels of human activity in Antarctica, with multiple heavily populated research stations run by various national operators within the Antarctic Treaty System (ATS) and several of the most

**Fig. 8.** Effects of various heavy metals on the growth and degradation activity of a. AQ5-06 and b. AQ5-05. Error bars represent mean  $\pm$  standard deviation for the triplicates.

visited tourist sites in the region (Tin *et al.* 2009, Eduardo *et al.* 2015). Human activity on the island is inevitably associated with the use of fossil fuels and therefore the occurrence of accidental fuel spills (Hughes *et al.* 2007). Fuel spills may both provide nutrients supporting specific microbial growth and produce harmful effects on other microbial growth and activity.

Persistent hydrocarbon spills are now evident on King George Island, specifically in areas close to several research stations near the Fildes Peninsula. Leakage from fuel storage tanks is to date the primary source of petroleum hydrocarbon pollution here, a consequence of noncompliance with ATS regulations and poor waste management by the operators responsible (Braun *et al.* 2014). These hydrocarbons enter the local soils and sediments. Remediation of polluted sites is an urgent challenge facing the Antarctic Treaty Parties generally, and specifically those countries operating the affected stations (Hughes & Convey 2014). Bioremediation requires the use of appropriate microorganisms, and as the introduction of non-native microbes is prohibited under the Environmental Protocol to the Antarctic Treaty, the identification and development of protocols using local Antarctic microbes are important.

Various studies have documented the existence of Antarctic hydrocarbon-degrading bacteria and fungi (e.g. Aislabie *et al.* 2000, Hughes *et al.* 2007). Bacteria belonging to the genera *Pseudomonas*, *Acinetobacter*, *Sphingomonas* (Gram-negative) and *Rhodococcus* and *Arthrobacter* (Gram-positive) are the most commonly encountered taxa present in hydrocarbon-polluted sites (Whyte *et al.* 2002, Shukor *et al.* 2009, Habib *et al.* 2018). *Arthrobacter* spp. strains AQ5-05 and AQ5-06 are cold-tolerant strains isolated from chronically polluted Antarctic soil that can utilize diesel as their sole source of carbon and energy, and as shown here, they are capable of significant diesel degradation. After an initial acclimatization period of 24 h, both strains achieved maximum diesel oil degradation within 7 days. Aliphatic hydrocarbon degradation by microbes is initiated by oxidation of the terminal methyl group to form a primary alcohol, which is then oxidized to produce aldehyde and lastly fatty acid derivatives. Nonetheless, at times,  $\omega$ -hydroxy fatty acids are produced when both ends of the alkane molecules are included in the oxidation process. The  $\omega$ -hydroxy fatty acids produced are then transformed into dicarboxylic acids via  $\beta$ -oxidation. Secondary alcohols are formed by sub-terminal oxidation, and these are further converted to ketone and afterwards oxidized to an ester by Baeyer–Villiger monooxygenase. Hydroxylation of esters to produce alcohols and fatty acids is facilitated by an esterase enzyme. The broad ranges of alkanes (C<sub>8</sub>–C<sub>33</sub>) comprising the short-chain, medium-chain and long-chain alkanes, as well as aromatic compounds existing in the abiotic control sample, were reduced by

both bacterial strains. Both strains tended to degrade short- and medium-chain alkanes more efficiently. However, reduction of long-chain alkanes and aromatic compounds was evident. Generally, alkanes of lower molecular weight tended to degrade faster than those of greater molecular weight due to presence of shorter carbon chains. Habib *et al.* (2018) also reported high rates of degradation of short-chain alkanes with Antarctic bacteria. Alkanes ranging from C<sub>9</sub> to C<sub>14</sub> are the most commonly detected in Antarctic soil, and this aliphatic fraction represents the major sources of pollution near Antarctic stations. Thus, microbial populations with the competence to degrade C<sub>12</sub>–C<sub>18</sub> are needed for the biodegradation and bioremoval of oil spillages around Antarctic research station, as diesel oil typically consists of *n*-alkanes in this range. Whyte *et al.* (2002) reported the detection and characterization of alkane-degrading (*alkB* and *alkM*) genotypes in both hydrocarbon-contaminated and hydrocarbon-non-contaminated Antarctic soils. Whole-genome sequencing of the bacterial strain AQ5-05 revealed that this strain harbours *alkB* and *alkM* and naphthalene dioxygenase (*ndoB*) genes, which are responsible for the degradation of a wide range of alkanes (C<sub>8</sub>–C<sub>33</sub>) and aromatic compounds.

Previous studies have reported species of *Enterobacter*, *Rhodococcus*, *Bacillus*, *Pseudomonas* and *Arthrobacter* to be diesel degraders (Yousaf *et al.* 2010). Other studies (e.g. Dussán & Numpaque 2012) have reported non-diesel-degrading strains such as *Arthrobacter* sp. PQ II. The genera *Arthrobacter* and *Rhodococcus* have been identified among the major hydrocarbon-degrading bacteria (Whyte *et al.* 2002). Studies of non-polluted soils have also revealed psychrophilic or cold-tolerant members of the *Arthrobacter* genus to be important members of bacterial communities in Antarctic soils (Dsouza *et al.* 2015). Margesin *et al.* (2013) and Lee *et al.* (2018) also reported *Arthrobacter* spp. strains capable of degrading petroleum hydrocarbons (*n*-alkanes and phenol) at low temperatures.

Temperature is a significant parameter controlling microbial growth and enzymatic activity, and it greatly affects the rate of bacterial hydrocarbon degradation. The increased viscosity of spilled oil at low temperatures reduces the volatilization of short-chain alkanes, thereby increasing their toxicity and delaying the onset of microbial degradation (Margesin & Schinner 1999). Reaction rates generally follow an Arrhenius relationship, meaning that as temperature decreases, there is a corresponding decrease in biodegradation. Antarctic soil bacteria are predominantly psychrotolerant rather than psychrophilic, which is consistent with the temperature variation characteristic of terrestrial environments in this region (Convey *et al.* 2018). Therefore, bioremediation is normally planned and performed in the summer period

when soils are unfrozen soils and liquid water is available. The temperature optimization experiments carried out here confirmed that both strains were psychrotolerant, growing best at temperatures between 10°C and 15°C. This concurs with the findings of Shukor *et al.* (2009), Margesin *et al.* (2013) and Lee *et al.* (2018).

Both bacterial strains grew most effectively on diesel at initial pH values of 7.0–7.5, demonstrating a strong affinity towards phosphate buffer. Much less growth was observed at pH values of 6.0 and 8.0, demonstrating that both bacterial strains are pH sensitive. Several studies have reported optimal growth in diesel-degrading bacteria at near-neutral pH (Habib *et al.* 2018).

Salinity can also have a significant influence on bacterial growth (Karamba *et al.* 2016). High salt concentrations can inhibit bacterial metabolic activity due to the increase in osmotic pressure affecting the solubility and movement of essential ions. Both strains studied here were intolerant of high salt concentrations. However, salt-resistant strains are potentially advantageous in the decontamination of hydrocarbon pollution in marine and coastal polluted areas (Durán & Esposito 2000). The composition and content of salts vary considerably in Antarctic soils, most notably with proximity to the coast or to dense colonies of marine vertebrates, as well as with the age of the soil in some areas (Convey *et al.* 2014). A number of diesel-degrading bacteria have been reported to demonstrate peak diesel degradation activities at low salt concentrations, in accordance with the data obtained here (Habib *et al.* 2018).

The availability of a suitable nitrogen source is an essential factor controlling bioremediation, although bacterial growth can be hindered by higher concentrations of nitrogen. Ammonium sulphate and ammonium nitrate best supported the growth of the two studied strains. Ammonium sulphate was selected for further study as it is readily and cheaply available and is the most used source of nitrogen by bacteria (Shukor *et al.* 2009). Various diesel-degrading bacteria have been reported to use ammonium sulphate as their sole nitrogen source (Shukor *et al.* 2009). In the natural environment in Antarctica, ammonium dispersed from vertebrate colonies has been shown to be an important driver of local terrestrial diversity (Bokhorst *et al.* 2019).

The influence of substrate concentration on bacterial growth has been documented in experiments using a range of substrate concentrations (Ruberto *et al.* 2005). Most studies conducted on diesel degradation have used relatively low diesel concentrations (between 0.5% and 1.5%) due to the toxicological effect of diesel on bacterial membranes (Shukor *et al.* 2009). The two bacterial strains studied here showed some resistance to diesel concentration, with best growth observed at 3% (v/v). Shukor *et al.* (2009) also reported a diesel-degrading *Pseudomonas* strain isolated from Antarctic soil that grew

optimally at 3.5% (v/v) diesel concentration. Kwapisz *et al.* (2008) even reported degradation at 6% (v/v) diesel, but this required the addition of glucose (0.2% w/v) and yeast extract (0.1% w/v). After optimization of all of the growth parameters via the OFAT method, strains AQ5-05 and AQ5-06 demonstrated enhanced diesel degradation efficiency, withstanding up to 3.0% diesel (v/v) and breaking down 41.0% and 47.5% diesel, respectively, as confirmed by gravimetric analysis and validated by GC/MS analysis.

The production of biosurfactants and biofilm formation are crucial for the successful and effective application of diesel-degrading bacterial strains in polluted soil. The formation of biofilms stabilizes the soil substrate, while biosurfactants produced by bacterial cells significantly increase pollutant bioavailability (Tribelli *et al.* 2012). Both bacterial strains studied here showed biosurfactant production. Ibrahim *et al.* (2013) reported 50–90% emulsification, a positive result for the drop collapse assay and a 20–51 mm oil displacement test for strains of bacteria isolated from crude oil-polluted soil. Patowary *et al.* (2017) reported a positive drop collapse assay, a surface tension reduction from 51.8 to 29.6 mN m<sup>-1</sup> and 81.8% hydrocarbon degradation by biosurfactant-producing *Pseudomonas aeruginosa* PG1. Similarly, Kumari *et al.* (2012) reported 60.6% and 49.5% petroleum hydrocarbon degradation by biosurfactant-producing *Pseudomonas* sp. BP10 and *Rhodococcus* sp. NJ2, respectively. These results are consistent with our findings. When biosurfactant-producing bacteria are exposed to polluted substrates, the improved biodegradation achieved is attained through mobilization, solubilization or emulsification of the oil (Ibrahim *et al.* 2013). Biosurfactants boost microbial hydrocarbon degradation, making the substrate accessible by increasing its water solubility through emulsification and enabling bacterial cell contact with hydrophobic substrates, thus reducing bacterial cell surface hydrophobicity (Kumari *et al.* 2012).

Emulsification activity is one of the most regularly and extensively employed techniques for testing for the presence of biosurfactants, although the results may differ considerably depending on the molecular weight of the hydrocarbons emulsified (Tribelli *et al.* 2012, Ibrahim *et al.* 2013). The use of hexadecane and diesel here showed that diesel cannot be easily emulsified as it is heavier than hexadecane and comprises a combination of complex compounds. Both isolates displayed poor formation of biofilms in diesel, in contrast with their performance with hexadecane. Bacterial strains capable of forming biofilms may become attached to diesel-containing substrates, giving them greater access compared to planktonic communities. In addition, cells in biofilms typically have improved metabolic competence and greater cell density than planktonic

cells (Ibrahim *et al.* 2013), which can therefore lead to enhanced and more effective degradation.

Strains AQ5-05 and AQ5-06 demonstrated significant maximum degradation competence, degrading 41.0% and 47.5% of diesel in the test system over 7 days, respectively. Zhang *et al.* (2014) reported 30–60% diesel degradation using various surfactant-producing bacterial isolates. Other studies have also reported effective diesel-degrading bacterial strains obtained from Antarctic soils (Aislabie *et al.* 2004, Habib *et al.* 2018). Strain AQ5-06 displayed the greatest degradation effectiveness and demonstrated a good biofilm adherence index, along with the production of biosurfactants.

In summary, the two psychrotolerant diesel-degrading Antarctic bacterial strains studied here proved to be competent at degrading diesel up to 3% (v/v) within 7 days at 10°C. Optimization of physical environmental factors including temperature, substrate concentration, pH, salt concentration and nitrogen source significantly enhanced the diesel degradation activity of these strains. Both AQ5-05 and AQ5-06 achieved maximum diesel degradation at between 10°C and 15°C, confirming that the Antarctic summer soil environment provides appropriate environmental conditions for diesel remediation by these strains. The presence of features such as biofilm formation, biosurfactant production and emulsification activity may also help in boosting the diesel degradation activity of these strains. Further optimization through statistical response surface methodology, combined with the purification and characterization of the biosurfactants produced and the identification of the pathway used for diesel degradation, will assist in the development and application of these two strains in field bioremediation approaches.

#### Author contributions

S.A. Ahmad and M.Y. Shukor carried out the research. M. Abdulrasheed and N.N. Zakaria conducted the research and drafted the manuscript. A.F. Ahmad Rosle and A. Zulkharnain carried out the statistical data analyses. S. Napis and P. Convey edited and reviewed the manuscript. G. Gonzalez-Rocha and S.A. Alias collected the soil samples used for the bacterial isolation.

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#### Details of data deposit

The isolates of *Arthrobacter* spp. strains AQ5-05 and AQ5-06 used for this study were originally isolated from Antarctic soil obtained on King George Island, South Shetland Islands (Lee *et al.* 2018). *Arthrobacter* spp. strains AQ5-05 (ref. KX946130-KX946131) and AQ5-06 (ref. KX946127) nucleotide sequences are deposited in the NCBI database.

#### References

- AHMAD, S.A., ASOKAN, G., YASID, N.A., NAWAWI, N.M., SUBRAMANIAM, K., ZAKARIA, N.N., *et al.* 2018. Effect of heavy metals on biodegradation of phenol by Antarctic bacterium: *Arthrobacter bambusae* strain AQ5-003. *Malaysian Journal of Biochemistry and Molecular Biology*, **21**, 47–51.
- AISLABIE, J., FOGHT, J. & SAUL, D. 2000. Aromatic hydrocarbon-degrading bacteria from soil near Scott Base, Antarctica. *Polar Biology*, **23**, 183–188.
- AISLABIE, J., SAUL, D.J. & FOGHT, J.M. 2006. Bioremediation of hydrocarbon-contaminated polar soils. *Extremophiles*, **10**, 171–179.
- AISLABIE, J.M., BALKS, M.R., FOGHT, J.M. & WATERHOUSE, E.J. 2004. Hydrocarbon spills on Antarctic soils: effects and management. *Environmental Science and Technology*, **38**, 1265–1274.
- BOKHORST, S., CONVEY, P. & AERTS, R. 2019. Nitrogen inputs by marine vertebrates drive abundance and richness in Antarctic terrestrial ecosystem. *Current Biology*, **29**, 10.1016/j.cub.2019.04.038.
- BRAUN, C., HERTEL, F., MUSTAFA, O., NORDT, A., PFEIFFER, S. & PETER, H. 2014. Environmental assessment and management challenges of the Fildes Peninsula Region. In TIN, T., LIGGETT, D., MAHER, P. & LAMERS, M., eds. *Antarctic futures*. Berlin: Springer, 169–191.
- BUSHNELL, L.D. & HAAS, H. 1941. The utilization of certain hydrocarbons by microorganisms. *Journal of Bacteriology*, **41**, 653–673.
- CONVEY, P., COULSON, S.J., WORLAND, M.R., & SJOBLOM, A. 2018. The importance of understanding annual and shorter term temperature patterns and variation in the upper layers of polar soils for terrestrial biota. *Polar Biology*, **41**, 1587–1605.
- CONVEY, P., CHOWN, S.L., CLARKE, A., BARNES, D.K.A., BOKHORST, S., CUMMINGS, V., *et al.* 2014. The spatial structure of Antarctic biodiversity. *Ecology of Monographs*, **84**, 203–244.
- COOPER, D.G. & GOLDENBERG, B.G. 1987. Surface-active agents from two *Bacillus* species. *Applied and Environmental Microbiology*, **53**, 224–229.
- DSOUZA, M., TAYLOR, M.W., TURNER, S.J. & AISLABIE, J. 2015. Genomic and phenotypic insights into the ecology of *Arthrobacter* from Antarctic soils. *BMC Genomics*, **16**, 10.1186/s12864-015-1220-2.
- DURÁN, N. & ESPOSITO, E. 2000. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. *Applied Catalysis*, **28**, 83–99.
- DUSSÁN, J. & NUMPAQUE, M. 2012. Degradation of diesel, a component of the explosive ANFO, by bacteria selected from an open cast coal mine in La Guajira, Colombia. *Journal of Bioprocessing and Biotechniques*, **2**, 1–5.

- EDUARDO, A., PADEIRO, A., DE FERRO, A., MOTA, A., LEPPE, M., VERKULICH, S. *et al.* 2015. Assessing trace element contamination in Fildes Peninsula (King George Island) and Ardley Island, Antarctic. *Marine Pollution Bulletin*, **97**, 523–527.
- GALLEGO, J.L.R., LOREDO, J., LLAMAS, J.F., VÁZQUEZ, F. & SÁNCHEZ, J. 2001. Bioremediation of diesel-contaminated soils: evaluation of potential *in situ* techniques by study of bacterial degradation. *Biodegradation*, **12**, 325–335.
- HABIB, S., AHMAD, S.A., WAN JOHORI, L.W., SHUKOR, M.Y.A., ALIAS, S.A., KHALIL, K.A., *et al.* 2018. Evaluation of conventional and response surface level optimisation of *n*-dodecane (*n*-C<sub>12</sub>) mineralisation by psychrotolerant strains isolated from pristine soil at Southern Victoria Island, Antarctica. *Microbial Cell Factories*, **17**, 1–21.
- HUGHES, K.A. 2014. Threats to soil communities: human impacts. In COWAN, D., *ed.* *Antarctic terrestrial microbiology*. Berlin: Springer, 263–277.
- HUGHES, K.A., & CONVEY, P. 2014. Alien invasions in Antarctica - is anyone liable? *Polar Research*, **33**, 10.3402/polar.v33.22103.
- HUGHES, K.A., BRIDGE, P. & CLARK, M.S. 2007. Tolerance of Antarctic soil fungi to hydrocarbons. *Science of the Total Environment*, **372**, 539–548.
- IBRAHIM, M.L., IJAH, U.J.J., MANGA, S.B., BILBIS, L.S. & UMAR, S. 2013. Production and partial characterization of biosurfactant produced by crude oil degrading bacteria. *International Biodeterioration and Biodegradation*, **81**, 28–34.
- IBRAHIM, S., SHUKOR, M.Y., ABDUL KHALIL, K., HALMI, M.I.E., SYED, M.A. & AHMAD, S.A. 2015. Application of response surface methodology for optimising caffeine-degrading parameters by *Leifsonia* sp. strain SIU. *Journal of Environmental Biology*, **36**, 1215–1221.
- KARAMBA, K.I., AHMAD, S.A., ZULKHARNAIN, A., SYED, M.A., KHALIL, K.A., SHAMAAN, N.A., *et al.* 2016. Optimisation of biodegradation conditions for cyanide removal by *Serratia marcescens* strain AQ07 using one-factor-at-a-time technique and response surface methodology. *Rendiconti Lincei*, **27**, 533–545.
- KUMARI, B., SINGH, S.N. & SINGH, D.P. 2012. Characterization of two biosurfactant producing strains in crude oil degradation. *Process Biochemistry*, **47**, 2463–2471.
- KWAPISZ, E., WSZELAKA, J., MARCHUT, O. & BIELECKI, S. 2008. The effect of nitrate and ammonium ions on kinetics of diesel oil degradation by *Gordonia alkanivorans* S7. *International Biodeterioration and Biodegradation*, **61**, 214–222.
- LEE, G.L.Y., AHMAD, S.A., YASID, N.A., ZULKHARNAIN, A., CONVEY, P., JOHARI, W.L.W., *et al.* 2018. Biodegradation of phenol by cold-adapted bacteria from Antarctic soils. *Polar Biology*, **41**, 553–562.
- LEE, I.S., HAGWALL, M., DELFINO, J.J. & RAOTVG, P.S. 1992. Partitioning of polycyclic aromatic hydrocarbons from diesel fuel into water. *Environmental Science and Technology*, **26**, 2104–2110.
- LEE, J.-Y., LIM, H.S. & YOON, H.I. 2016. Thermal characteristics of soil and water during summer at King Sejong Station, King George Island, Antarctica. *Geosciences Journal*, **20**, 503–516.
- MARGESIN, R. & SCHINNER, F. 1999. Biological decontamination of oil spills in cold environments. *Journal of Chemical Technology and Biotechnology*, **74**, 381–389.
- MARGESIN, R., MOERTELMAIER, C. & MAIR, J. 2013. Low-temperature biodegradation of petroleum hydrocarbons (*n*-alkanes, phenol, anthracene, pyrene) by four actinobacterial strains. *International Biodeterioration and Biodegradation*, **84**, 185–191.
- PATOWARY, K., PATOWARY, R., KALITA, M.C. & DEKA, S. 2017. Characterization of biosurfactant produced during degradation of hydrocarbons using crude oil as sole source of carbon. *Frontiers in Microbiology*, **8**, 10.3389/fmicb.2017.00279.
- RODRIGUES, L., BANAT, I.M., TEIXEIRA, J. & OLIVEIRA, R. 2006. Biosurfactants: potential applications in medicine. *Journal of Antimicrobial Chemotherapy*, **57**, 609–618.
- ROSENBERG, M. 1984. Bacterial adherence to hydrocarbons: a useful technique for studying cell surface hydrophobicity. *FEMS Microbiology Letters*, **22**, 289–295.
- RUBERTO, L.A.M., VÁZQUEZ, S., LOBALBO, A. & MAC CORMACK, W.P. 2005. Psychrotolerant hydrocarbon-degrading *Rhodococcus* strains isolated from polluted Antarctic soils. *Antarctic Science*, **17**, 47–56.
- SHUKOR, M.Y., HASSAN, N.A.A., JUSOH, A.Z., PERUMAL, N., SHAMAAN, N.A., MACCORMACK, W.P., *et al.* 2009. Isolation and characterization of a *Pseudomonas* diesel-degrading strain from Antarctica. *Journal of Environmental Biology*, **30**, 1–6.
- SRIRAM, M., GAYATHIRI, S., GNANASELVI, U., JENIFER, P., MOHAN, R. & GURUNATHAN, S. 2011. Novel lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal tolerant bacterium *Escherichia fergusonii* KLU01 as a potential tool for bioremediation. *Bioresource Technology*, **102**, 9291–9295.
- TIN, T., FLEMING, Z.L., HUGHES, K.A., AINLEY, D.G., CONVEY, P., MORENO, C.A., *et al.* 2009. Impacts of local human activities on the Antarctic environment. *Antarctic Science*, **21**, 3–33.
- TRIBELLI, P.M., MARTINO, C.D., LOPEZ, N.I. & RAIGER I.L.J. 2012. Biofilm lifestyle enhances diesel bioremediation and biosurfactant production in the Antarctic polyhydroxyalkanoate producer *Pseudomonas extremaustralis*. *Biodegradation*, **23**, 645–651.
- WHYTE, L.G., SCHULTZ, A., VAN BEILEN, J.B., LUZ, A.P., PELLIZARI, V., LABBÉ, D., *et al.* 2002. Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. *FEMS Microbiology Ecology*, **41**, 141–150.
- YOUSAF, S., ANDRIA, V., REICHENAUER, T.G., SMALLA, K. & SESSITSCH, A. 2010. Phylogenetic and functional diversity of alkane degrading bacteria associated with Italian ryegrass (*Lolium multiflorum*) and birdsfoot trefoil (*Lotus corniculatus*) in a petroleum oil-contaminated environment. *Journal of Hazardous Materials*, **184**, 523–532.
- YOUSSEF, N.H., DUNCAN, K.E., NAGLE, D.P., SAWAGE, K.N., KNAPP, R.M. & MCINERNEY, M.J. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. *Journal of Microbiological Methods*, **56**, 339–347.
- ZHANG, Q., WANG, D., LI, M., XIANG, W.N. & ACHAL, V. 2014. Isolation and characterization of diesel degrading bacteria, *Sphingomonas* sp. and *Acinetobacter junii* from petroleum contaminated soil. *Frontiers of Earth Science*, **8**, 58–63.