Desalination and Water Treatment
www.deswater.com

doi: 10.5004/dwt.2021.27528

235 (2021) 39–65
September

Treatment of mine rejected-brine wastewater by indigenous halophilic bacterial species under aerobic and anaerobic conditions

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Received 18 April 2020; Accepted 18 June 2021

ABSTRACT

This study investigated the treatment of brine wastewater under aerobic and anaerobic conditions by indigenous halophilic bacterial strains. Series of experiments were conducted in batch reactors containing synthetic brine solutions as well as in batch reactors with natural brine wastewater supplemented with and without glucose as a source of carbon to ascertain the removal efficiency of halophilic bacteria under various environmental conditions such as pH (3–10), temperature (20°C–40°C), aeration (shaking speed of 100 rpm) and NaCl concentrations (0.8%–25% w/v). Culture-based methods and molecular techniques were used to identify the selected halophilic bacterial strains isolated from brine wastewater samples. The 16S rRNA gene sequences showed that these isolated strains were closely related to *Alishewanella tabrizica* (97%), *Bacillus anthracis* (97%), *Pseudomonas alcaliphila* (98%), *Serratia nematodiphila* (98%) and *Pseudomonas mendocina* (98%). These halophilic bacteria showed the ability to grow in a wide pH range from 3 to 10 (with an optimum value of pH 8) and a temperature range from 20°C to 40°C (with an optimum temperature of 28°C). Furthermore, *A. tabrizica* and *B. anthracis* were able to grow at NaCl concentrations of up to 10%, while *P. alcaliphila*, *S. nematodiphila* and *P. mendocina* could grow at NaCl concentrations of up to 20%. The results of the experimental studies for chemical content removal from brine wastewater showed that all the target halophilic bacteria were able to reduce the concentration of most chemical pollutants (B, Ba, Ca, Cu, Hg, K, Mg, Mn, Na, Ni, Pb, S, Si, Sr, U and Zn), which had initial concentrations ranging between 360 and 2,000 mg/L. No significant differences ($p > 0.05$) between bacterial isolates for the removal of pollutants in both anaerobic and aerobic conditions were noted in synthetic brine and natural brine with exception of natural brine media supplemented with glucose. This indicated that the type of media did not significantly affect the target isolates in their ability to remove pollutants. Moreover, the target isolates were able to remove high concentrations of chemical oxygen demand at a range of 69.58%–100% under aerobic conditions and at a range of 56.62%–98.68% under anaerobic conditions.

Keywords: Wastewater; Brine; Halophilic bacteria; Aerobic growth; Anaerobic growth

1. Introduction

Increasing freshwater scarcity has led to mounting competition for water among various sectors, such as industry, agriculture, and the domestic sector. In addition, water pollution has become a major global problem, especially in developing countries where in most cases, municipal and industrial wastewaters are discharged into the watercourse without any prior treatment [1]. In these countries, an average of 90%–95% of raw domestic sewage and 75%

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of raw industrial waste have been reported to be discharged into surface waters without any prior treatment [2]. The world is currently facing another tremendous challenge for the management of acid mine drainage, which is one of the typical pollutants of the country's freshwater environment. The United Nations even called it the second biggest problem facing the world after global warming. The United States Environmental Protection Agency stated that in the United States alone the cleaning up of just 156 mines can be estimated at approximately US$10 billion and this clean-up can result in the production of dangerous reject waste brine streams [3].

Many processes such as reverse osmosis, ion exchange and a batch mode saline wastewater treatment reactor have been employed for the removal of salt [4–6]. Nevertheless, several of these available options may be deemed not feasible for implementation due to the high cost. With the sharp population increase in many countries, particularly in many African countries, uncontrolled wastewater discharges and high demand for freshwater sources and the supply of potable water, innovative scientific and technological solutions for water recycling, wastewater reuse and reclamation as well as desalination are needed to increase the availability of freshwater sources.

In the process of biological treatment of brine, it has been reported that halophilic microorganisms might play a major role in the removal of dissolved minerals in brine [7–9]. These microorganisms are categorised as slight, moderate, or extreme halophiles, on the basis of their response to NaCl [10]. Even though halophiles are found mostly in the Archaea domain, bacterial halophiles and some Eukaryota such as the alga *Dunaliella salina* or fungus *Wallemia ichthyophaga* have been widely investigated by the study of Ying et al. [11]. Due to their unique halophilic properties, several studies have been carried out on different aspects such as their physiology, ecology, taxonomy and phylogenetic relationships (Ventosa [14]). Furthermore, halophilic microorganisms have been discovered to possess advantages for several desirable properties [12]. Evidence showed that moderately halophilic bacteria have strong potential for promising applications as a source of compatible solutes, fermented foods, enzymes, polymers and degradation of toxic compounds [13–15]. However, it should be mentioned that very few studies have been conducted on moderate halophiles when compared to extreme halophiles [16]. Though the biological treatment of brine is not popular, it appears to be the most efficient and affordable method for brine wastewater treatment [4].

Studies on the biological treatment of brine by using halophilic bacteria have been conducted both in biofilms and sequencing batch reactors to enhance the chemical oxygen demand (COD) removal at high salt content specifically in a rotating biological contactor [7,17]. However, to date, no comparative study has been conducted on the treatment of brine under aerobic and anaerobic conditions using moderately halophilic bacteria isolated in South African reject waste brine streams. Therefore, the aim of this study was to assess the effectiveness of moderately halophilic bacteria isolated from brine wastewater to treat both natural and synthetic brine wastewater under aerobic and anaerobic conditions.

2. Experimental procedures

2.1. Culture-based methods for selection of halophilic bacteria

In our previous study about a metagenomic approach applied using next-generation sequencing analysis for the profiling of bacterial communities of brine samples in 14 different stages of the eMalahleni Water Reclamation Plant resulted in the identification of 65 bacterial species, which were moderately halophilic bacteria [18]. Prior to ascertain their performance in treating reject waste brine, the cultured moderately halophilic bacteria were firstly isolated by filtering the collected brine samples through 0.22 µm membrane filters (Whatman®, GE Healthcare UK) and placing the filter disk on a moderately halophilic (MH) agar plate. This agar medium was prepared according to the study of Caton et al. [19]; the composition of the medium, expressed in g/L, was as follows: NaCl, 98 g; KCl, 2 g; $MgSO_4·7H_2O$, 1 g; $CaCl_2·2H_2O$, 0.36 g; $NaHCO_3$, 0.06 g; NaBr, 0.24 g; $FeCl_3·6H_2O$, 1 g; Bacto Tryptone (Difco), 10 g; glucose, 1 g; and agar, 20 g. The plates were then incubated at 30°C for 48 h and thereafter the colonies were streaked, purified and five different bacterial isolates were randomly selected based on morphological features, considering pigmentation and size and named as H1, H6, H9, H10 and H19. In order to identify these bacterial isolates, Gram's staining, cell morphology and motility tests were performed according to standard methods. The isolates were further identified using molecular techniques.

2.2. Polymerase chain reaction amplification and sequencing of halophilic bacterial isolates using 16S rRNA gene

The extraction of DNA from the halophilic bacterial isolates was carried out by using a fungal/bacterial DNA extraction kit (Zymo Research), according to the manufacturer's instructions. The polymerase chain reaction (PCR) random amplification of a 500 bp segment of the 16S rRNA gene sequence was done by using universal bacterial primers, and the forward primer was selected according to the study of Kamika and Momba [20] with nucleotide sequence 27F: (5′-GAGTTTGATCCTGGCTCAG-3′), while the reverse primer was selected according to the study of Lee et al. [21] with nucleotide sequence 518R: (5′-ATTACCGCGGCTGCTGG-3′). The PCR Master Mix was used and amplification was carried out under conditions as reported by the study of Sekar et al. [18].

2.3. Phylogenetic analysis of bacterial isolates

The 16S rRNA gene sequence results were provided on Sequence Scanner Software version 1.0 and the BLASTn sequence alignment program was used to compare the identity of the strains with the reference sequences listed in the GenBank database at the National Center for Biotechnology Information (NCBI). The phylogenetic analysis was conducted by using the MEGA version 6 Program after the alignment of sequences [22]. The confidence level of the phylogenetic tree topology was evaluated with a generation of 100 bootstrap sets. The generated 16S rRNA gene sequences of the isolates have also been deposited in the DNA Data Bank of Japan (DDBJ), a nucleotide sequence database, and are available from the above database (http://www.ddbj.nig.ac.jp) under accession numbers LC107905, LC107906, LC107907, LC107908 and LC107909 for H1, H6, H9, H10 and H19, respectively.

2.4. Preparation of synthetic brine solutions

Based on the characteristics of the natural brine, which was collected from the reclamation plant, synthetic brine solutions were prepared for two optimal concentrations of NaCl 7.5 and 12.5% (75,000 and 125,000 mg/L). Analytical grade sodium sulphate salt, potassium and calcium were weighed and dissolved in distilled water to obtain the various required concentrations of Na, SO_4, Ca, K, in solution (Tables S9 and S10). Characterisation of these synthetic solutions was carried out using ICP-MS and IC. Synthetic brine media were autoclaved at 121°C for 15 min.

2.5. Determination of growth/biomass of moderately halophilic bacterial isolates

The growth of moderately halophilic bacteria was monitored and measured after 8 h during 3 d in a shaking incubator (Scientific, Lasec South Africa) at a shaking speed of 100 r/min and a spectrophotometer (Spectroquant® Pharo 300, Merck Millipore, South Africa) at 600 nm, respectively. The growth rate of the bacteria was calculated using the formula reported by the study of Farrier-Pagès and Rassoulzadegan [23]:

$$\text{Growth rate per day: } y = \frac{\ln C_1 - \ln C_0}{t_1 - t_0} \quad (1)$$

where C_1: final concentration of the isolated cells; C_0: initial concentration of the isolated cells; $t_1 - t_0$: the time-lapse in a day between the intervals.

The biomass was calculated as follows:

$$Y = mx + c \quad (2)$$

where Y = absorbance of the isolate concentration; x = approximate number of isolated cells; m = constant value of the gradient of the curve (0.0003); c = the y-intercept of the curve (0.0002).

2.6. Determination of optimum environmental conditions for the growth of halophilic bacterial isolates

After identification, halophilic bacterial isolates were characterised for their tolerance to salt and for their preferred carbon source at different pH values and temperatures. Isolates were exposed to varying pH levels and temperature ranges in order to determine their optimum conditions for growth. An additional characterisation on the tolerance to salt was ascertained by exposing the isolates to different concentrations of NaCl ranging from 0.8%–25% (8,000–250,000 mg/L) for a period of 24 h on solid MH medium (nutrient agar) and MH nutrient broth at pH 7, 30°C and 100 r/min. The media were prepared according to the study of Caton et al. [19] by increasing the concentration of NaCl.

In order to determine the optimum temperature for the growth of each of the isolates, halophilic isolates at a constant concentration of 1.00×10^5 CFU/mL were separately inoculated in Erlenmeyer flasks containing 100 mL nutrient broth adjusted with specific optimum salt content and incubated at different temperatures ranging between 20°C–40°C (with a gradual scale of 2.5°C).

An aliquot of 5 mL was taken every 2 h for 32 h for bacterial growth analysis. For pH optimisation, halophilic bacterial isolates (1.00×10^5 CFU/mL) were separately inoculated in Erlenmeyer flasks containing 100 mL MH nutrient broth adjusted with optimum salt concentration, with a gradual increase of pH from 3–10 (with 1 unit interval) by using 1 N HCl for lowering the pH and 1 N NaOH for raising the pH. The inoculated flasks were incubated at the specific optimum temperature (28°C) for each isolate for 2 d.

In order to determine the optimum carbon source for each of the isolates, four carbon sources (glucose, sucrose, acetate and lactose) were selected based on their occurrence in the environment and their ease of degradation. The halophilic bacterial isolates were separately inoculated in sterile synthetic brine media containing different carbon sources, at different concentrations (10,000–50,000 mg/L) in order to study their effect on bacterial growth. The synthetic brine media were prepared according to the study of Caton et al. [19]. This experiment was done by using optimum conditions (pH, temperature and salt) of each bacterial isolates, as reported above, for a period of 48 h. All cited practical analysis was performed in triplicate.

2.7. Aerobic treatment of brine

In order to mimic the natural environment and to assess the suitability of selected isolates as potential candidates for the treatment of industrial brine samples, the efficiency of halophilic bacterial strains for natural brine treatment was tested in laboratory experiments under aerobic conditions. Prior to use, natural brine samples, considered as culture media, were filtered and adjusted with optimum conditions as described above. The culture media were autoclaved and cooled down at room temperature before use. A stock culture of the bacterial isolate was prepared 1 d before each experiment and then an Erlenmeyer flask containing 100 mL of the test brine sample was inoculated with an appropriate volume. All the inoculated flasks as well as the controls were initially incubated in a shaking incubator at a shaking speed of 100 r/min and exposed to a temperature of 30°C ± 2°C. During the 5 d of incubation, 5 mL sample aliquots were taken at 2 d intervals (Day 1 – Day 3 – Day 5) to determine the biomass and the concentrations of chemical contents (including B, Ba, Ca, Cu, Hg, K, Mg, Mn, Na, Ni, Pb, S, Si, Sr, U and Zn) removed from the brine wastewater samples [24]. The sterility of the culture media was checked by plating 100 µL of the media onto the MH medium agar plate. Only flasks containing the sterile medium were considered for further experimental study. The experiments were performed in triplicate.

2.8. Anaerobic treatment of brine

In order to perform the anaerobic treatment of the brine wastewater, the collected brine samples were filtered using a membrane filter (0.22 μm pore size) (Whatman®, Ø 150 mm, GE Healthcare UK Limited) and the filtrate was used to prepare culture media as stated above. Afterwards, the nitrogen gas was introduced via a pipe to remove the oxygen present in each flask fitted with a rubber stopper in order to promote anaerobic conditions. Using a sterile syringe, each serum bottle (50 mL) was inoculated with a 1 mL aliquot of the halophilic bacterial media and incubated for further measurements as reported in the previous section. Each experiment was done in triplicate.

2.9. Statistical analysis

The multivariate regression model was used to compare all halophilic bacterial strains adjusting for pH (8), carbon source (glucose), temperature (28°C) and NaCl concentrations. The comparison among data was carried out using the one-way analysis of variance (ANOVA) in SPSS Statistics procedure; the results of the two treatments in terms of NaCl concentration in the synthetic brine, natural brine supplemented with 5% glucose (50,000 mg/L) and natural brine without glucose under both aerobic and anaerobic conditions were expressed as *p*-values (p) and correlation coefficient for three replicates with a significant difference ($p < 0.05$). The interpretation was performed at the 95% confidence interval.

3. Results

3.1. Isolation and identification of the halophilic bacterial strains

Results of morphological and biochemical tests revealed that strains H1 and H6 were able to grow at NaCl concentrations ranging between 5% (50,000 mg/L) and 10% (100,000 mg/L), while strains H9, H10 and H19 were able to grow at NaCl concentrations of up to 20% (200,000 mg/L). In addition, all the test strains were able to grow in the temperature range 20°C–40°C and pH range 3–10 (Table 1).

3.2. Nucleotide sequences and phylogenetic tree of halophilic bacterial strains

Based on the sequence analyses of bacterial 16S rRNA, the BLAST search on the GenBank databases at the NCBI indicated that the strains H1, H6, H9, H10, and H19 were most closely related with *Alishewanella tabrizica* (100% identity), *Bacillus anthracis* (100% identity), *Pseudomonas alcaliphila* (100% identity), *Serratia nematodiphila* (100% identity) and *Pseudomonas mendocina* (100% identity), respectively. However, when establishing a comparison between them, the phylogenetic analysis revealed that the two *Pseudomonas* sp. (*P. alcaliphila* and *P. mendocina*) were 74% identical, while the phylogenetic tree based on the sequence results of the isolated halophilic bacterial strains showed that the most closely related strains were the couples *A. tabrizica* and *Rheinheimera* with 100% identity; *A. tabrizica* and *Alishewanella* sp. with 100% identity; *Rheinheimera* and *Alishewanella* sp. with 100% identity, followed by *B. anthracis* and *Bacillus cereus* with 100% identity and finally *B. anthracis* and *Bacterium* MAS 16 with 100% identity (Fig. 1).

3.3. Optimisation conditions

Prior to treating synthetic and natural brine wastewater by using indigenous halophilic bacterial strains, it was important to determine their optimum growth conditions. Thus, different pH and temperature ranges and four organic carbon sources (glucose, lactose, sucrose and acetate) were tested.

3.3.1. Temperature optimisation

The identified halophilic bacterial strains H1, H6, H9, H10 and H19 were tested at various temperatures ranging between 20°C–40°C in 2.5°C increments. The results showed that all the test strains were able to grow within a temperature range of 20°C–40°C. The recovery counts of all strains were almost similar (approximately 10^5 CFU/mL) (Fig. 2). However, all strains were able to grow gradually during the study period and the highest growth occurred during the last day (Table S1). All bacterial strains showed higher growth at 30°C (H1: 9.805×10^5 CFU/mL, H6: 9.631×10^5 CFU/mL, H9: 10.25×10^5 CFU/mL, H10: 9.905×10^5 CFU/mL and H19: 10.106×10^5 CFU/mL).

Statistically, the adjusted model indicated that the growth of all the halophilic bacterial strains differed significantly ($p < 0.05$). Since the highest growth for bacterial isolates

Table 1
Main characteristics of selected halophilic bacterial strains

Feature	H1	H6	H9	H10	H19
Morphology	Rod	Rod	Rod	Rod	Rod
Size	0.5–1.0 × 2.0 μm	1–1.5 × 3–10 μm	0.3–0.5 × 1.5–3.0 μm	0.8–1.3 × 0.6–0.7 μm	0.7–0.8 × 1.4–2.8 μm
Pigmentation	Cream	Cream	Cream	Cream	Green
Motility	Non	Non	Motile	Motile	Motile
Facultative	+	+	+	+	+
Oxidase	+	+	+	+	+
NaCl (%)	5–10	5–10	5–20	5–20	5–20
Temperature range (°C)	20–40	20–40	20–40	20–40	20–40
pH range	3–10	3–10	3–10	3–10	3–10

H1: *Alishewanella tabrizica*; H6: *Bacillus anthracis*; H9: *Pseudomonas alcaliphila*; H10: *Serratia nematodiphila*; H19: *Pseudomonas mendocina*

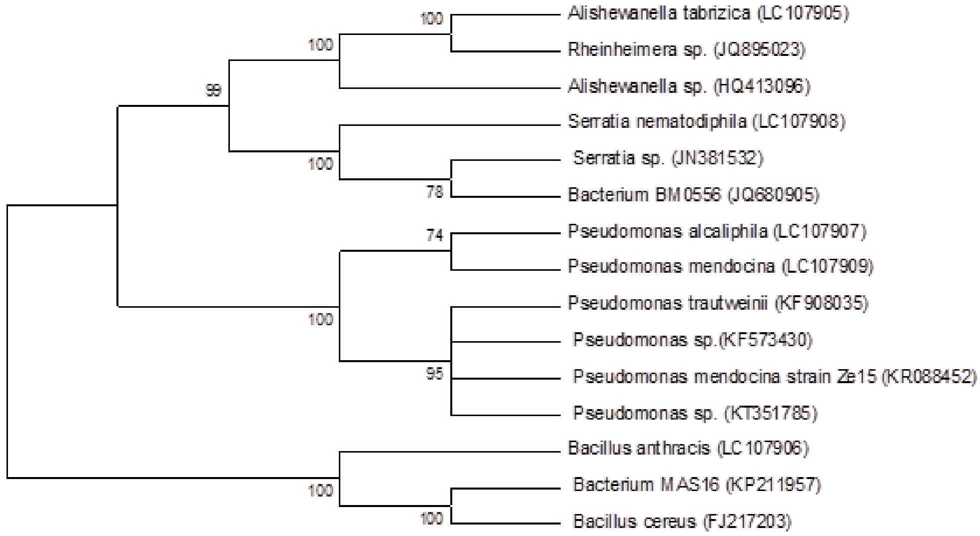

Fig. 1. Phylogenetic tree of the halophilic bacterial strains based on the 16S rRNA gene sequence analysis results.

was noted at 30°C, and a decline in growth was observed at temperatures ranging between 32.5°C to 40°C, for the sake of comparison, 28°C was hypothetically considered as the standard optimum temperature for all the isolates.

3.3.2. pH optimisation

Fig. 3 illustrates the growth of isolates in nutrient broth at 28°C at various pH levels ranging from 3 to 10 (with a gradual increase at a scale of 1). The recovery of all the test strains increased as the pH was increased from 3 to 10. The highest counts of all bacterial strains were observed at pH 8 (Table S2).

3.3.3. Carbon source optimisation

In order to determine the preferred carbon source of the bacterial isolates, four organic carbon sources including glucose, lactose, acetate and sucrose were tested. The experimental series were separately run at optimum temperature of 28°C and pH 8. In general, all isolated halophilic bacterial strains were able to utilise all the carbon sources tested. However, the halophilic strain H6 showed the lowest growth, compared with other strains, in all tested carbon sources with preferences in the following order: glucose > lactose > acetate > sucrose. In contrast, the growth of certain isolates such as strain H1, and H10 appeared to be enhanced in the presence of lactose and acetate for the former, and glucose for the latter (Fig. 4). Furthermore, bacterial isolates showed high growth in the presence of glucose highlighting that glucose might be the preferred carbon source of all isolates.

In general, the growth of halophilic bacteria was observed over 5 d under the following physicochemical parameters, namely pH (8), temperature (28°C) and carbon source (glucose). At pH 8, after 5 d of exposure, the growth rate of halophilic bacteria was in the following ascending order: H9 (*P. alcaliphila*: 0.0693 d^{-1}) > H19 (*P. mendocina*: 0.0681 d^{-1}) > H10 (*S. nematodiphila*: 0.0676 d^{-1}) > H1 (*A. tabrizica*: 0.0675 d^{-1}) > H6 (*B. anthracis*: 0.0674 d^{-1}) (Appendices Table S2). At 50,000 mg/L glucose, after 5 d exposure the growth rate of halophilic bacteria was in the following ascending order: H6 (*B. anthracis*: 0.1098 d^{-1}) > H1 (*A. tabrizica*: 0.0736 d^{-1}) > H19 (*P. mendocina*: 0.0722 d^{-1}) > H9 (*P. alcaliphila*: 0.0693 d^{-1}) > H10 (*S. nematodiphila*: 0.0684 d^{-1}) (Table S3).

3.3.4. Treatment of synthetic brine solutions under aerobic and anaerobic conditions

After optimisation of growth conditions of the isolated halophilic bacteria, their ability to treat brine wastewater was assessed using synthetic brine solutions under the set optimum conditions. Two salinity values of 7.5% (75,000 mg/L) and 12.5% (125,000 mg/L) NaCl were selected based on previous results, given in Tables 2 and 3, which showed that both H1 and H6 strains were able to grow in NaCl concentrations ranging between 5% (50,000 mg/L) and 10% (100,000 mg/L); while isolates H9, H10 and H19 strains were able to grow in the NaCl concentration range of 5–20% (50,000 mg/L–200,000 mg/L), thus, the average NaCl concentrations of 7.5% (75,000 mg/L) and 12.5% (125,000 mg/L) were selected to test these strains.

3.3.4.1. Treatment of synthetic brine solutions under aerobic conditions

In general, all halophilic bacterial strains exposed to aerobic conditions in synthetic brine solutions containing NaCl concentrations of 7.5% (75,000 mg/L) or 12.5% (125,000 mg/L) were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Tables 2 and S5). An increase in metal removal progressively occurred with time. After day 5 of exposure, results showed that all the halophilic bacteria were able to completely (at 100%) remove B, Cu, Ni, Pb, and Zn with

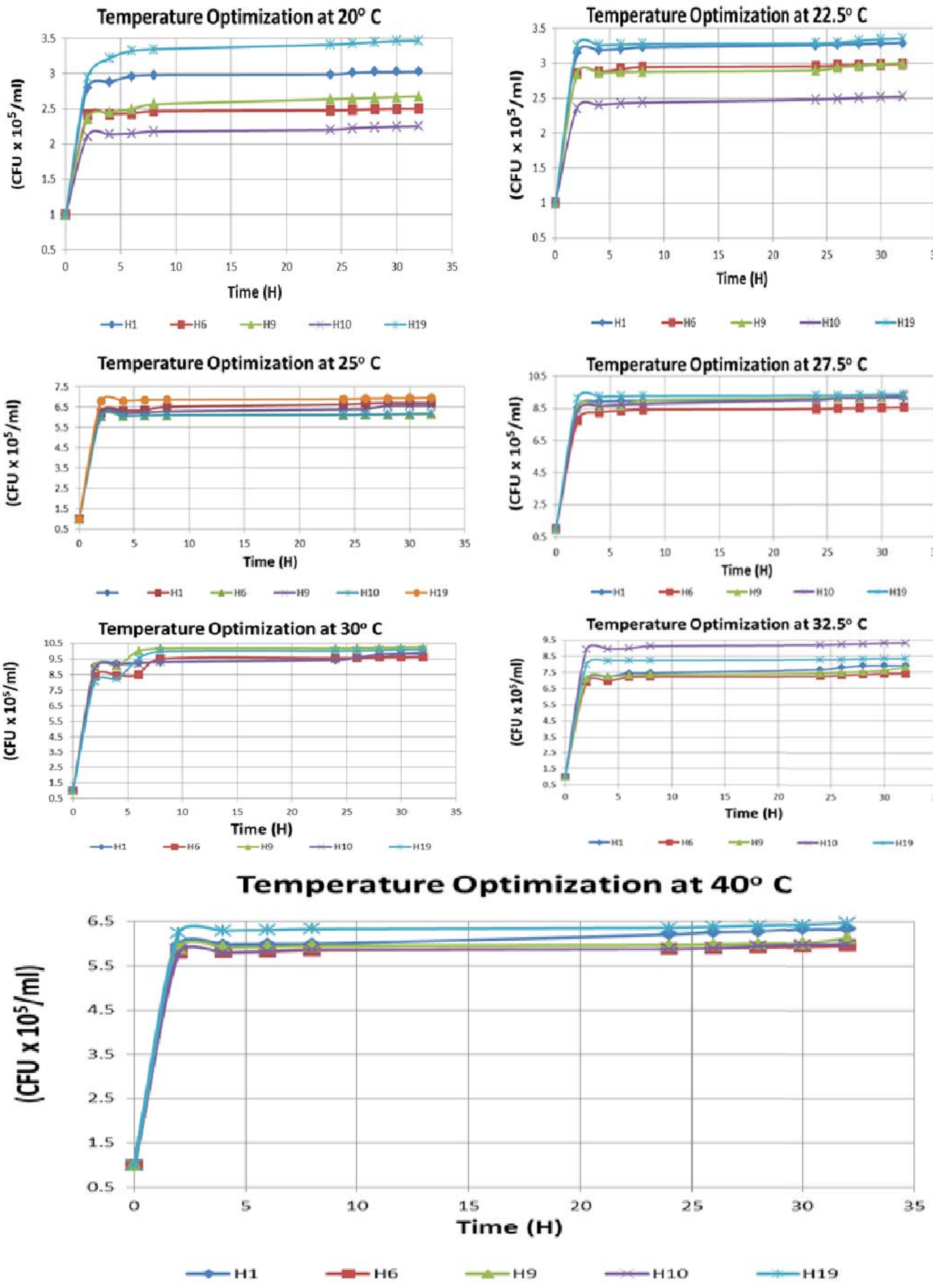

Fig. 2. Temperature optimisation from 20°C–40°C for halophilic bacterial strains.

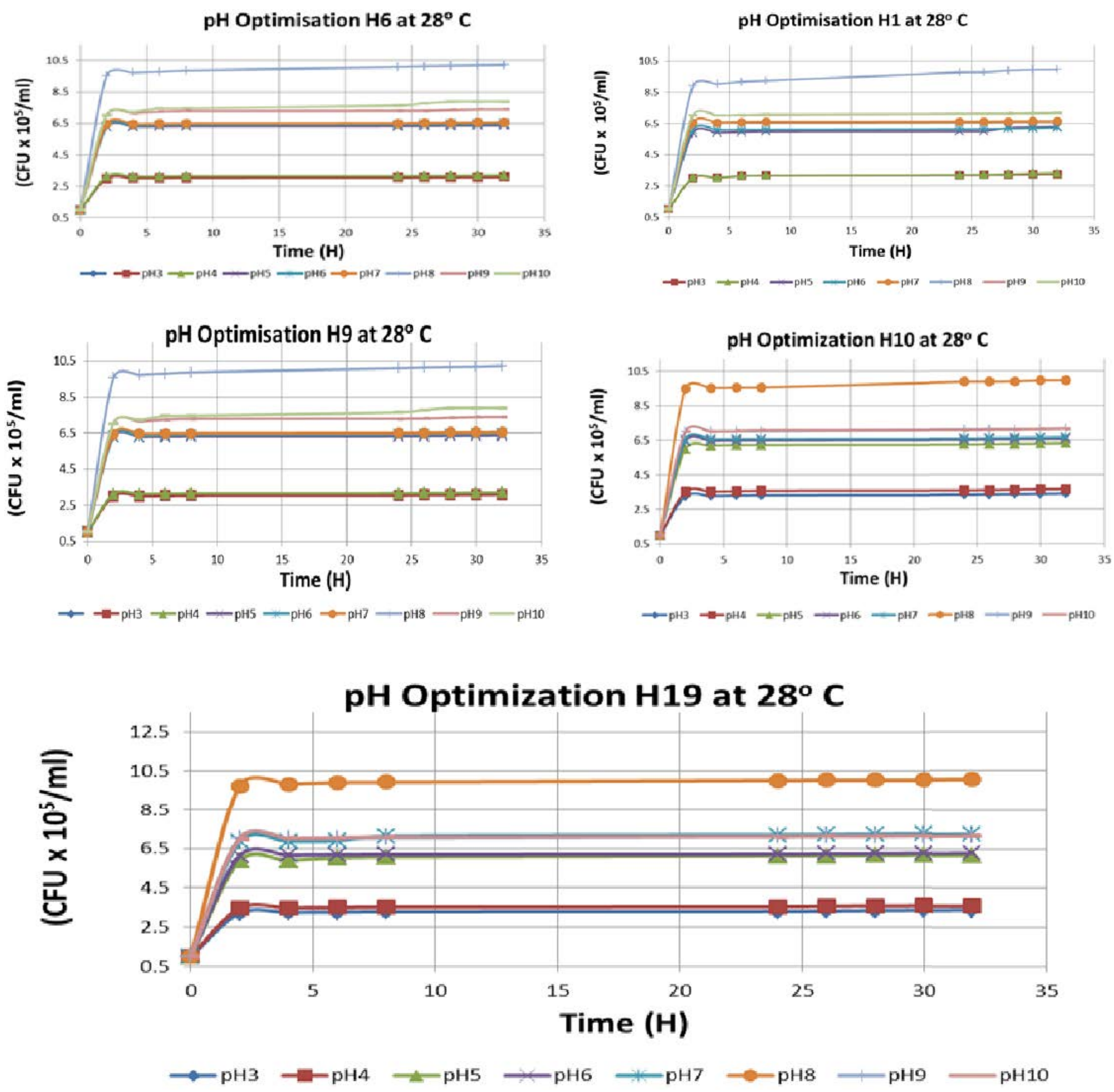

Fig. 3. pH optimisation ranged from 3–10 for the tested halophilic bacterial strains.

the exception of H6 and H19 that could completely remove Si and Na, respectively, from synthetic brine solutions under aerobic conditions.

Results also revealed that with the exception of K, which showed the lowest percentage removal of 82.84%, H1 had a removal efficiency ranging between 95.18% and 100% for all the metals after 5 d of exposure. This halophilic strain was able to completely remove a total of six metals (B, Cu, Ni, Pb, Zn and Zn) on the 5th day of the exposure to a synthetic brine solution containing 7.5% (75,000 mg/L) NaCl. Results also showed that the halophilic bacterial strain H6 had a removal efficiency ranging between 86.08% and 100% after 5 d of exposure, with K showing the lowest removal among all the metals. Strain H6 was able to completely remove a total of seven metals (B, Cu, Ni, Pb, Mn, Zn and Si) during the same period. Although K (2,500 mg/L) and Mg (1,000 mg/L) were removed at the highest concentrations compared to the other metals, the H1 strain was able to remove these metals up to 82.84 and 96.54 mg/L, and strain H6 removed up to 86.08 and 96.89 mg/L, respectively.

3.3.4.2. Treatment of synthetic brine solutions under anaerobic conditions

In general, all halophilic bacterial strains exposed to anaerobic conditions in synthetic brine solutions containing NaCl concentrations of 7.5% (75,000 mg/L) or 12.5% (125,000 mg/L) (Fig. S1) were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Table 3). An increase in metal removal progressively occurred with time. After day 5 of exposure,

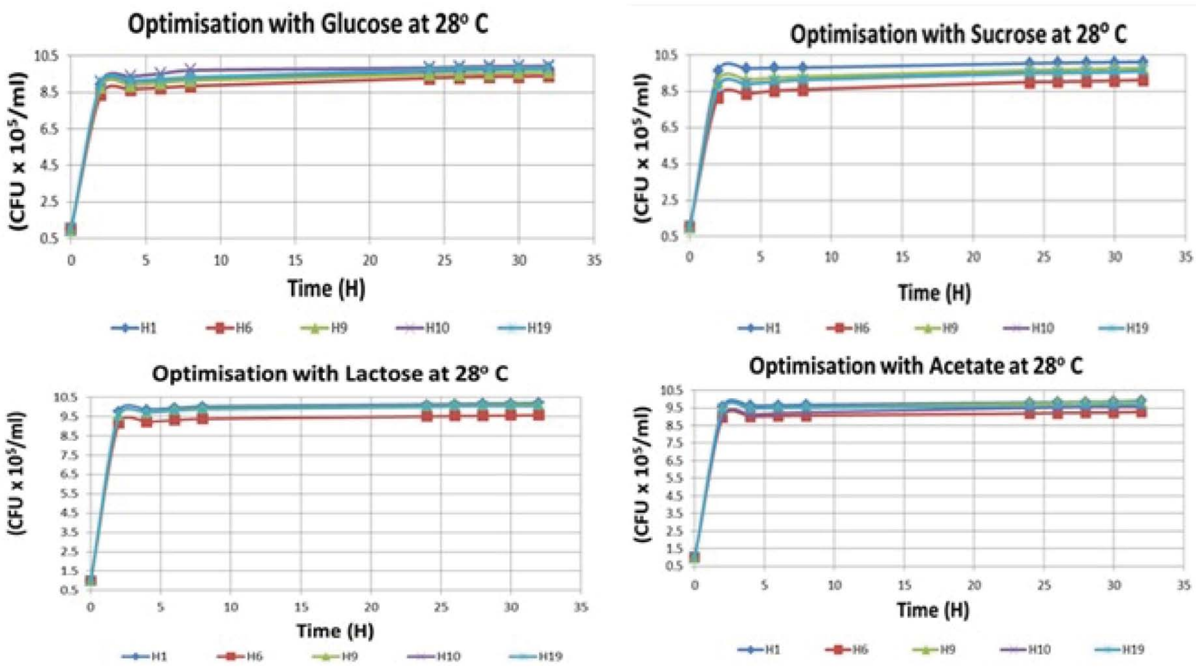

Fig. 4. Assimilation of glucose, lactose, sucrose and acetate as carbon sources.

results showed that all the halophilic bacteria were able to completely (at 100%) remove Ba, Cu, Hg, Ni, Pb and Zn but only H10 could completely remove K from synthetic brine solutions under anaerobic conditions (Tables 3 and S5).

Results also revealed that with the exception of Ca, which showed the lowest percentage removal of 44.3%, H1 had a removal efficiency ranging between 45.2% and 100% for all the metals after 5 d of exposure. This halophilic strain was able to completely remove a total of seven metals (B, Ba, Cu, Hg, Ni, Pb and Zn) on the 5th day of the exposure to a synthetic brine solution containing NaCl at a concentration of 7.5% (75,000 mg/L). Results also showed that the halophilic bacterial strain H6 had a removal efficiency ranging between 65.55% and 100% after 5 d of exposure, with Ca showing the lowest removal among all the metals. Strain H6 was able to completely remove a total of six metals (Ba, Cu, Hg, Ni, Pb and Zn) during the same period. Although K (2,500 mg/L) and Mg (1,000 mg/L) were removed at the highest concentrations compared to the other metals, the H1 strain was able to remove these metals up to 76.77 and 93.51 mg/L, and strain H6 removed up to 87.05 and 96.05 mg/L, respectively.

Exposure to a synthetic brine solution containing NaCl at a concentration of 12.5% (125,000 mg/L) showed that halophilic bacterial strains H9 (range 72.34%–100%), H10 (85.63%–100%) and H19 (85.52%–100%) were able to remove significant amounts of metals after 5 d. Strain H9 was able to completely remove eight metals (B, Ba, Cu, Hg, Ni, Pb, U and Zn), while H10 was capable of removing a total of nine metals (B, Ba, Cu, Hg, K, Ni, Pb, U and Zn) and H19 removed a total of eight metals (B, Ba, Cu, Hg, Ni, Pb, U and Zn) after 5 d of exposure. Potassium and Mg were also removed at the highest concentrations ranging between 72.34% and 96.1% by H9, between 100% and 95.59% by H10 and between 91.43% and 95.7% by H19 during the same period.

3.4. Treatment of natural brine under aerobic and anaerobic conditions

The ability of the halophilic isolates to treat natural brine under aerobic and anaerobic conditions was also investigated by conducting two sets of experiments. In the first experimental study, the natural brine wastewater was supplemented with glucose (5%) as source of carbon and the second experimental study was conducted without any carbon supplement (Tables S8 and S9).

3.4.1. Treatment of natural brine with glucose (50,000 mg/L) under aerobic conditions

In general, all halophilic bacterial strains exposed to aerobic conditions in natural brine containing 50,000 mg/L glucose were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Table 4). An increase in metal removal progressively occurred with time. After 5 d of exposure, results showed that all the halophilic bacteria were able to completely (at 100%) remove B, Cu, and Zn; however, strains H1 and H19 were capable of completely removing Ni (at 100%) from natural brine containing 50,000 mg/L glucose under aerobic conditions.

Results also revealed that with the exception of S, which showed the lowest percentage removal of 64.25%, strain H1 had a removal efficiency ranging between 66.90% and 100% for all the metals after 5 d of exposure (Table S7). This halophilic strain was able to completely remove a total of four

Table 2
Effectiveness of selected halophilic bacteria in removing metal contents in synthetic brine solutions containing 7.5% (75,000 mg/L) and 12.5% (125,000 mg/L) NaCl under aerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)		5,000	5,000	3,600	5,000	5,000	25,000	10,000	5,000	75,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000
Optimum NaCl: 7.5% (75,000 mg/L)																	
H1	Day 1	77.24	92.46	89.16	93.01	93.43	70.2	95.57	99.6	91.17	85.12	94.15	98.74	81.61	80.21	87.24	89.24
	Day 3	84.15	98.91	89.75	96.14	98.97	70.46	96.29	99.7	93.94	89.65	97.45	98.99	93.94	85.25	97.85	94.16
	Day 5	100	98.99	96.13	100	98.98	82.84	96.54	99.98	96.77	100	100	99.13	95.18	100	98.31	100
H6	Day 1	84.15	87.26	88.02	89.14	88.13	72.88	90.5	99.5	92.18	89.75	98.91	98.98	69.1	99.16	78.6	89.25
	Day 3	94.18	98.96	90.08	95.13	98.47	75.02	96.39	99.63	95.08	95.16	99.12	99	91.95	99.95	97.9	96.34
	Day 5	100	99.09	98.68	100	98.75	86.08	96.89	100	99.3	100	100	99.64	100	98.44	98.44	100
Optimum NaCl: 12.5% (125,000 mg/L)																	
H9	Day 1	78.52	88.59	87.73	85.75	89.65	65.54	91.48	99.48	96.25	85.45	99.09	98.6	72.7	99.22	80.16	78.45
	Day 3	85.63	91.56	89.51	95.16	98.2	73.64	96.82	99.82	98.93	94.13	99.91	98.92	94.5	99.98	98.13	89.46
	Day 5	100	99.17	98.83	100	99.09	87.34	97.93	100	99.86	100	100	99.62	94.82	100	98.71	100
H10	Day 1	86.66	86.72	87.15	98.22	87.23	67.08	89.72	99.43	97.98	98.85	98.77	99.14	68.5	99.15	77.65	89.45
	Day 3	89.67	88.55	97.75	98.33	89.36	82.78	91.1	99.98	98.64	99	99.06	99.34	72.5	99.24	80.77	94.14
	Day 5	100	99.67	98.85	100	99.42	87.44	96.73	100	99.87	100	100	99.76	97.06	100	98.93	100
H19	Day 1	85.26	86.84	89.54	98.17	87.68	71.8	89.9	95.13	98.14	98.85	98.83	98.91	67.9	99.03	77.17	78.25
	Day 3	94.56	88.36	98.09	98.34	89.28	87.58	91.17	98.25	98.22	99.09	99.03	99.35	73.2	99.31	80.69	89.36
	Day 5	100	99.2	98.86	100	99.01	96.64	97	100	100	100	100	99.6	95.68	100	98.36	100

Note: the removal of metal contents is expressed in percentage.

Table 3
Effectiveness of selected halophilic bacteria in removing metal components in synthetic brine solution containing 7.5% (75,000 mg/L) and 12.5% (125,000 mg/L) NaCl under anaerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)	Day 0	5,000	5,000	3,600	5,000	5,000	25,000	10,000	5,000	75,000	5,000	5,000	5,000	5,000	5,000	5,000	5,000
Optimum NaCl: 7.5% (75,000 mg/L)																	
H1	Day 1	66.167	89.78	13.26	65.12	59.32	40.47	84.74	8.8	50.58	75.62	84.15	82.17	93.59	40.08	87.49	82.12
	Day 3	71	92.34	22.53	79.68	75.12	47.34	86.36	31	56.21	85.41	92.13	86.4	93.65	48.94	92.15	85.36
	Day 5	88.45	100	44.3	100	100	76.77	93.51	45.2	80.28	100	100	95.31	96.31	75.31	99.87	100
H6	Day 1	78.93	85.46	34.81	68.52	78.24	62.79	81.52	90.82	68.09	58.78	65.23	75.04	94.86	61.75	87.48	85.26
	Day 3	78.98	92.34	44.84	89.18	86.53	63.84	89.97	96.11	69.47	89.56	89.75	88.6	95.27	84.21	92.56	94.25
	Day 5	94.53	100	65.55	100	100	87.05	96.05	97.47	88.71	100	100	95.24	96.65	93.15	99.88	100
Optimum NaCl: 12.5% (125,000 mg/L)																	
H9	Day 1	85.56	85.47	22.6	67.52	78.45	8.37	88.21	30.87	47.11	68.78	75.48	71.3	93.27	35.89	89.56	78.59
	Day 3	98.56	92.34	64.36	79.68	89.23	28.49	95.17	52.17	69.43	89.65	85.64	88.44	96.37	51.23	92.58	90.12
	Day 5	100	100	82.74	100	100	72.34	96.1	84.73	76.45	100	100	90.94	96.54	75.57	100	100
H10	Day1	71.57	75.37	46.2	59.23	65.29	90.61	89.11	68.64	71.5	58.12	68.95	78.49	94.13	70.34	78.15	89.28
	Day3	85.67	84.14	72.15	78.94	79.63	92.07	90.14	82.14	73.51	86.34	81.27	87.85	96.74	77.94	89.27	92.35
	Day5	100	100	85.63	100	100	100	95.59	88.77	92.62	100	100	91.57	97.48	89.9	100	100
H19	Day1	75.37	77.08	47.46	57.89	61.28	54.2	87.84	30.97	71.19	69.85	75.46	69.9	93.85	60.61	87.45	79.23
	Day3	98.82	85.12	66.51	82.38	78.94	55.01	90.25	84.34	74.3	79.89	94.52	88.74	96.48	77.79	93.65	85.41
	Day5	100	100	85.52	100	100	91.43	95.7	94.6	93.17	100	100	95.71	97.351	84.59	100	100

Note: the removal of metal contents is expressed in percentage.

Table 4
Effectiveness of selected halophilic bacteria in removing metal contents in natural brine wastewater containing 5% (50,000 mg/L) glucose under aerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)		650	321	1,689	500	350	2,564	1,785	254	9,859	145	251	754	895	785	796	780
	Day 1	58.12	72.14	34.1	78.21	73.44	36.36	54.9	95.15	82.45	79.56	75.81	29.11	86.66	98.66	79.22	89.63
H1	Day 3	85.95	73.19	43.39	86.18	81.85	45.1	60.74	95.76	83.66	97.25	96.84	40.2	98.8	98.8	80.03	94.16
	Day 5	100	82.21	66.90	100	82.30	67.8	75.54	98.68	91.83	100	97.81	64.25	99.57	99.57	97.94	100
	Day 1	82.53	72.74	39.52	76.3	72.67	41.49	57.47	94.52	82.75	91.25	91.94	36	46.64	92.54	81.2	59.56
H6	Day 3	95.48	74.95	40.85	85.21	77.12	43.11	57.56	94.78	83.26	95.06	95.9	37.73	51.17	95.36	82.75	85.17
	Day 5	100	78.17	41.27	100	78.61	43.91	59.44	95.35	92.92	99.72	96.82	40.1	53.68	98.94	83.27	100
	Day 1	68.79	73.22	39.93	52.13	73.9	43.01	58.12	95.41	82.55	99.71	93.84	38.06	52.51	98.56	79.73	85.26
H9	Day 3	82.19	74.7	41.05	78.49	77.04	43.38	59.24	95.7	83.66	99.73	96.31	39.79	53.11	98.71	82.26	95.26
	Day 5	100	74.73	45.14	100	78.44	46.72	60.39	95.92	90.3	99.79	96.81	41.71	54.41	98.8	83.08	100
	Day 1	78.69	56.2	34.81	58.12	77.24	36.58	54.58	49.4	81.33	99.28	90.82	28.44	51.73	80.38	79.17	74.58
H10	Day 3	83.64	69.1	39.87	79.95	77.45	40.15	57.08	94.86	83.46	99.36	92.47	33.11	51.78	82.15	80.38	91.26
	Day 5	100	72.46	93.57	100	81.57	41.96	57.5	95.7	90.04	99.68	97.29	34.68	52.78	98.83	82.15	100
	Day 1	59.68	71.86	37.03	79.56	73.74	41.61	58.62	92.28	83.26	99.72	84.16	35.67	52.55	74.25	80.87	78.41
H19	Day 3	79.32	73.09	40.05	85.2	82.71	41.9	58.87	93.97	86.61	99.81	96.59	37.75	56.2	86.5	82.57	89.6
	Day 5	100	77.25	41.38	100	82.75	43.37	60.03	95.55	91.86	100	97.5	38.46	58.71	98.7	83.41	100

Note: the removal of metal contents is expressed in percentage.

metals (B, Cu, Ni, and Zn) on the 5th day of the exposure to natural brine supplemented with 5% glucose (50,000 mg/L).

Results also showed that the halophilic bacterial strain H19 had a removal efficiency ranging between 38.46 and 100% after 5 d of exposure, with S showing the lowest removal among all the metals. Strain H19 was able to completely remove a total of four metals (B, Cu, Ni, and Zn) during the same period. All halophilic bacterial strains were able to remove Na (9,859 mg/L) which was at the highest concentration compared to the other metals that were removed up to 90%; the same results were revealed for Mn (254 mg/L), Pb (251 mg/L), and Sr (785 mg/L) during the same period.

3.4.2. Treatment of natural brine without glucose under aerobic conditions

In general, all halophilic bacterial strains exposed to aerobic conditions in natural brine without glucose were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Table 5). An increase in metal removal progressively occurred with time. After 5 d of exposure, results showed that all the halophilic bacteria were able to completely (at 100%) remove B, Cu, and Zn; however, H6, H9 and H10 were the ones capable to completely (at 100%) remove Mn while H19 was the only one for complete removal of Pb from natural brine water without glucose under aerobic condition (Table S6). Results also revealed that with the exception of Ca, which showed the lowest percentage removal of 39.9%, strain H1 had a removal efficiency ranging between 46.49% and 100% for all the metals after 5 d of exposure. This halophilic strain was able to completely remove a total of three metals (B, Cu, and Zn) on the 5th day of the exposure to natural brine water without glucose. All halophilic bacterial strains were able to remove more than 90% of Ni (145 mg/L), Pb (251 mg/L), and Sr (785 mg/L) during the same period (Table S4).

3.4.3. Treatment of natural brine supplemented with glucose (50,000 mg/L) under anaerobic conditions

Overall, all halophilic bacterial strains exposed to anaerobic conditions in natural brine containing 5% glucose (50,000 mg/L) were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Table 6). An increase in metal removal progressively occurred with time. After 5 d of exposure, results showed that all the halophilic bacteria were able to completely (at 100%) remove Ba, Cu, Hg, K, Ni, Pb, U, and Zn; however, strains H1, H6, and H9 were capable of completely removing (at 100%) B from natural brine containing 5% (50,000 mg/L) glucose under anaerobic conditions (Table S7). Results also revealed that with the exception of S, which showed the lowest percentage removal of 46.61%, strain H19 had a removal efficiency ranging between 83.29% and 100% for all the metals after 5 d of exposure. This halophilic strain was able to completely remove a total of eight metals (Ba, Cu, Hg, K, Ni, Pb, U, and Zn) on the 5th day of the exposure to natural brine supplemented with 5% glucose (50,000 mg/L).

All halophilic bacterial strains were able to remove more than 93.50% of Na (9,859 mg/L) which was at the highest concentration compared to the removal of other metals (above 90%); the same results were revealed for the removal of Mn (254 mg/L), Pb (251 mg/L), and Sr (785 mg/L) during the same period.

3.4.4. Treatment of natural brine without glucose under anaerobic conditions

In general, all the halophilic bacterial strains exposed to anaerobic conditions in natural brine without glucose were able to remove significant amounts of the metal components from day 1, regardless of their concentrations (Table 7). An increase in metal removal progressively occurred with time. After 5 d of exposure, results showed that all the halophilic bacteria were able to completely (at 100%) remove Cu, Hg, K, Ni, Pb, and Zn; however, strains H1, H6, H9, and H10 were capable of completely removing Ba (at 100%) from natural brine without glucose under anaerobic conditions.

Results also revealed that with the exception of Sr, which showed the lowest percentage removal of 70.27%, strain H19 had a removal efficiency ranging between 98.21% and 100% for all the metals after 5 d of exposure (Table S6). This halophilic strain was able to completely remove a total of five metals (Cu, Hg, Ni, Pb, and Zn) on the 5th day of the exposure to natural brine without glucose. Results also showed that the halophilic bacterial strain H1 had a removal efficiency ranging between 86.98% and 100% after 5 d of exposure, with Sr showing the lowest removal among all the metals. Strain H1 was able to completely remove a total of nine metals (B, Ba, Cu, Hg, K, Ni, Pb, U, and Zn) during the same period. All halophilic bacterial strains were able to remove Na (9,859 mg/L) which was at the highest concentration compared to the removal of the other metals (above 90%); the same results were revealed for Ca (1,782 mg/L), K (2,856 mg/L), Mn (254 mg/L), and S (856 mg/L) during the same period.

3.4.5. Statistical analysis for efficiency of halophilic bacteria in removing metal pollutants from synthetic and natural brine solutions

In the synthetic media, there were no significant differences ($p > 0.05$) between bacterial isolates for the removal of pollutants in both anaerobic and aerobic conditions with exception between H6 and H9 (aerobic conditions), and between H10 and H19 (anaerobic conditions) that showed significant differences ($p < 0.05$). Similar observations were noted in both natural brine wastewaters with and without glucose, where significant differences ($p < 0.05$) were only noted between H6 and H9, H6 and H19, H9 and H19 in aerobic condition, and H1 and H6, H1 and H19, and H6 and H9 under anaerobic condition. No statistic significant differences ($p > 0.05$) were noted when isolates were categorised in groups at their different media for their ability to remove pollutants from synthetic brine and natural brine media without glucose. This indicates that microbial isolates were not significantly affected by the type of media in their removal of pollutants. However,

Table 5
Effectiveness of selected halophilic bacteria in removing metal contents in natural brine wastewater without glucose under aerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)		650	321	1,689	50	350	2,564	1,785	254	9,859	145	251	754	895	785	796	78
H1	Day 1	85.26	72.24	33.89	76.2	74.62	56.51	62.43	93.32	41.06	84.23	97.67	52.78	45.86	56.56	78.45	86.23
	Day 3	96	74.71	33.96	89.6	75.18	58.07	62.8	98.12	41.27	95.21	97.94	53.71	47.65	85	80.4	92.13
	Day 5	100	77.3	39.9	100	75.92	58.22	63.72	99.54	46.49	99.59	98.54	54.77	50.39	98.49	82.72	100
H6	Day 1	84.28	75.01	34.28	75.26	75.02	57.02	64.4	90.27	40.86	95.42	98.33	54.17	34.3	55.98	81.59	78
	Day 3	98.26	78.34	40.37	89.26	77.1	59.88	65.35	95.62	45.37	96.32	98.68	56.23	39.05	98.66	83.54	89.23
	Day 5	100	79.9	53.58	100	79.28	68.48	68.48	100	57.55	99.86	99.41	64.52	45.08	98.69	88.12	100
H9	Day 1	81.25	74.08	37.77	72.89	75.42	60.15	64.39	90.26	43.85	95.41	97.76	55.17	42.34	58.53	80.96	91.32
	Day 3	88.56	75.56	37.83	84.56	77.2	60.25	65.35	95.62	46.49	96.32	98.46	56.83	49.16	90.25	84.73	94.26
	Day 5	100	80.34	44.74	100	79.38	66.1	69.41	100	53.24	99.86	99.48	62.93	55.58	98.72	85.39	100
H10	Day 1	78.29	71.52	13.85	56.32	70.45	46.56	54.56	98.42	27.83	87.46	97.39	42.44	30.89	54.39	77.13	77.77
	Day 3	85.85	74.28	25.1	78.45	80.15	56.51	61.06	99.18	38.98	94.16	98.52	52.51	44.02	97.56	82.09	81.25
	Day 5	100	77.52	39.63	100	82.44	62.55	66.05	100	48.82	99.56	99.26	57.16	46.81	98.44	83.66	100
H19	Day 1	82.65	72.44	35.76	55.56	78.14	57.48	62.96	98.97	42.94	90.21	98.83	7.55	40.44	58.4	64.44	73.24
	Day 3	93.24	77.88	38.83	78.46	79.41	59.49	64.59	99.42	44.92	95.24	99.06	56.69	46.53	98.43	82.91	89.25
	Day 5	100	81.05	81.05	100	82.34	61.07	66.19	99.57	45.02	99.66	100	57.55	49.32	98.78	83.6	100

Note: the removal of metal contents is expressed in percentage.

Table 6
Effectiveness of selected halophilic bacteria in removing metal components in natural brine wastewater containing 5% (50,000 mg/L) glucose under anaerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)		700	356	1,782	59	456	2,856	1,845	2,563	9,785	178	451	856	986	852	856	95
H1	Day 1	89.08	78.26	84.93	74.12	78.45	29.95	94.07	85.28	51.24	58.12	68.78	17.37	96.75	64.83	84.12	87.15
	Day 3	92.96	85.24	92.5	89.56	95.45	78.11	94.82	92.17	84.27	72.14	74.19	86.47	97.45	83.17	92.15	92.14
	Day 5	100	100	97.33	100	100	100	96.18	93.37	97.25	100	100	95.38	98.06	86.98	100	100
H6	Day 1	81.78	78.25	78.94	69.85	67.48	57.83	75.77	81.64	69.89	45.26	87.41	79.41	80.15	71.06	86.24	82.16
	Day 3	82.3	85.21	95.26	89.95	82.34	74.93	93.55	84.67	81.65	78.49	95.41	87.26	87.54	78.62	91.25	93.25
	Day 5	100	100	97.07	100	100	100	97.76	95.89	97.11	100	100	94.33	98.46	81.3	100	100
H9	Day 1	78.66	75.26	85.63	74.18	86.45	51.44	81.23	88.21	65.55	65.21	74.12	85.67	89.56	62.38	85.28	87.13
	Day 3	85.26	85.47	91.24	84.27	92.34	79.28	92.45	90.47	74.42	78.29	85.64	90.15	92.15	82.85	95.64	91.75
	Day 5	100	100	96.67	100	100	100	97.36	92.4	97.83	100	100	93.53	98.4	91.54	100	100
H10	Day 1	82.12	95.46	91.73	68.95	75.85	86.51	85.47	42.73	89.06	78.21	76.23	54.46	94.28	45.35	89.75	94.52
	Day 3	92.13	97.12	95.31	79.86	89.75	92.88	91.27	69.86	90.8	89.45	84.19	72.66	96.78	52.97	94.12	97.12
	Day 5	98.96	100	95.98	100	100	100	98.78	79.11	93.59	100	100	76.35	98.83	82.59	100	100
H19	Day 1	84.08	89.27	80.38	87.12	89.23	46.16	93.87	37.2	74.69	84.52	75.21	20.7	96.88	76.88	85.47	75.57
	Day 3	86.26	91.24	82.38	94.15	95.21	79.6	94.78	71.53	84.87	89.65	87.41	29.06	97.88	78.44	91.23	84.17
	Day 5	91.23	100	88.07	100	100	100	96.51	94.5	97.69	100	100	46.61	98.25	83.29	100	100

Note: the removal of metal contents is expressed in percentage.

Table 7
Effectiveness of selected halophilic bacteria in removing metal components in natural brine wastewater without glucose under anaerobic conditions

		B	Ba	Ca	Cu	Hg	K	Mg	Mn	Na	Ni	Pb	S	Si	Sr	U	Zn
Initial conc. (mg/L)		700	356	1,782	59	456	2,856	1,845	2,563	9,785	178	451	856	986	852	856	95
	Day 1	89.08	85.14	84.93	85.21	45.12	29.95	94.07	55.96	51.24	45.25	54.19	17.37	89.45	64.83	81.23	87.56
H1	Day 3	92.32	91.3	92.5	94.21	78.65	78.11	94.82	92.17	84.27	87.16	78.82	86.47	91.25	83.17	96.15	94.12
	Day 5	100	100	97.33	100	100	100	96.18	93.37	97.25	100	100	95.38	98.06	86.98	100	100
	Day 1	91.05	98.99	89.43	89.78	75.26	80.32	92.87	36.59	74.26	76.35	89.25	92.19	98.12	70.24	95.08	85.67
H6	Day 3	97.8	99.96	96.33	95.26	84.57	80.99	98.46	65.68	87.64	89.12	92.35	96.48	98.36	74.09	99.91	89.75
	Day 5	98.21	100	99.9	100	100	96.55	99.99	92.4	99.75	100	100	99.2	99.79	88.69	100	100
	Day 1	81.56	78.56	79.63	68.45	78.1	69.75	94.06	55.74	77.56	85.16	75.49	37.71	97.79	83.79	95.38	79.85
H9	Day 3	91.1	89.65	89.6	85.14	84.16	81.4	96.82	66.53	86.23	94.78	89.45	38.82	99.54	86.47	99.87	89.56
	Day 5	97.64	100	99.97	100	100	98.47	100	93.43	99.85	100	100	99.43	99.84	86.47	100	100
	Day 1	86.09	79.85	84.55	78.21	85.47	75.98	94.88	61.82	81.81	78.95	85.46	11.03	98.29	78.43	56.28	78.21
H10	Day 3	91.49	89.78	94.55	92.35	91.24	92.9	98.24	72.36	94.33	86.18	92.37	79.99	99.1	83.03	78.95	85.2
	Day 5	97.83	100	99.98	100	100	98.46	100	93.22	99.85	100	100	99.25	99.86	93.4	100	100
	Day 1	76.75	89.65	81.91	52.12	56.38	65.1	92.65	56.14	74.54	87.24	85.67	36.18	97.27	23.22	94.93	78.59
H19	Day 3	97.88	93.54	91.24	68.21	74.26	84.26	95.34	94.21	91.24	93.26	96.34	98.67	98	64.22	95.52	85.32
	Day 5	99.61	99.98	99.86	100	100	98.66	99.97	98.21	99.72	100	100	99.02	99.73	70.27	99.93	100

Note: the removal of metal contents is expressed in percentage.

natural brine media supplemented with glucose was the exception when comparing those under aerobic and anaerobic conditions ($p < 0.05$).

In order to evaluate the impact of growth parameters on the removal of pollutants, further statistical analysis was carried using a Spearman's rank-order correlation to determine the relationship between pH, temperature and glucose for each of the 5 parameters. A perfect positive correlation between temperature and glucose, temperature and pH and pH and glucose was noted and this was statistically significant ($r_s(10) = 0.1$, $p < 0.05$). This shows that change on one of parameters could affect the removal ability of bacterial isolates.

3.5. Chemical oxygen demand removal by selected halophilic bacterial strains

3.5.1. COD removal under aerobic conditions

As it can be seen from Fig. 5, results revealed that halophilic bacterial isolates were able to remove significant COD from the natural brine with and without glucose as well as from the synthetic brine solution under aerobic conditions at 28°C and pH 8. In all scenarios, the COD removal efficiency was even observed from the first day after the exposure (Table S10). Thereafter a progressive and significant increase in COD removal occurred with an increase in exposure time.

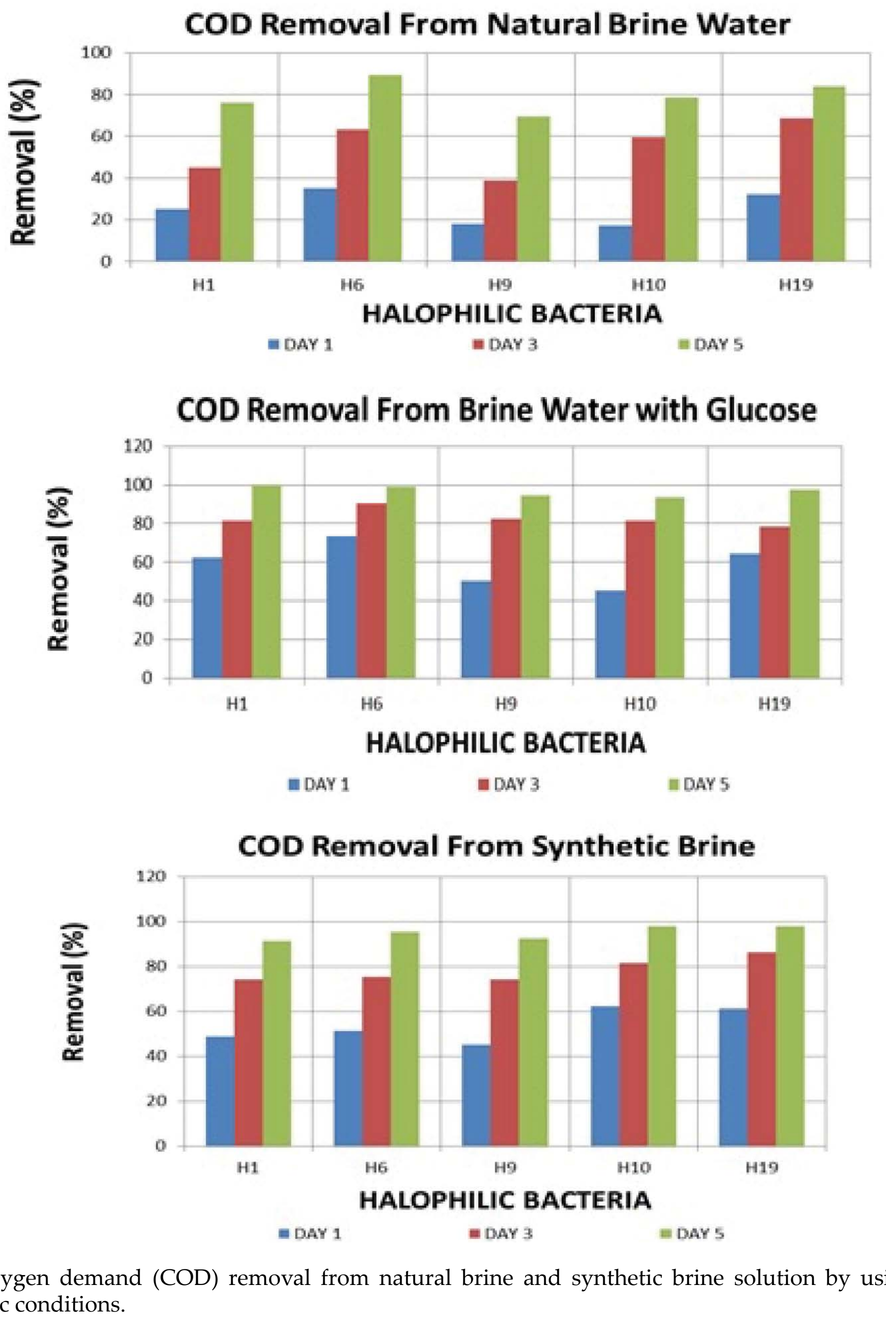

Fig. 5. Chemical oxygen demand (COD) removal from natural brine and synthetic brine solution by using halophilic bacterial strains under aerobic conditions.

After 5 d of exposure to natural brine wastewater without glucose, the removal of COD was observed in the following descending order: 89.23% for H6 (*B. anthracis*); 84.15% for H19 (*P. mendocina*); 78.45% for H10 (*S. nematodiphila*); 76.23% for H1 (*A. tabrizica*); and 69.58% for H9 (*P. alcaliphila*); with a standard deviation of 3.01. Higher COD removal rates (93.65%–100%) were recorded when the isolates were exposed to brine supplemented with glucose as a source of carbon. The highest COD removal of 100% was observed with H1 (*A. tabrizica*), followed by 99.25% for H6 (*B. anthracis*), 94.28% for H9 (*P. alcaliphila*), 93.65% for H10 (*S. nematodiphila*), and 97.28% for H19 (*P. mendocina*), with a standard deviation of 2.56 after 5 d of treatment.

When exposed to synthetic brine, the COD removal rate by all the target halophilic bacteria was above 90% in the following descending order: 97.98% (H19 *P. mendocina*); >97.85% (H10 *S. nematodiphila*); >95.46% (H6 *B. anthracis*); >92.34% (H9 *P. alcaliphila*); and >91.24% (*A. tabrizica*); with a standard deviation of 2.77 after 5 d of treatment. Regardless of the high rate of COD removal, this study showed no significant difference between the test halophilic bacterial isolates ($p > 0.05$) in synthetic brine, natural brine with or without glucose, separately. However, when comparing the removal by type of media, the statistical analysis showed significant difference ($p < 0.05$) with synthetic brine having the highest mean value.

3.5.2. COD removal under anaerobic conditions

All halophilic bacterial isolates exposed to natural brine without glucose under anaerobic conditions were able to remove COD at a rate above 60% with the exception of H9 (*P. alcaliphila*) showing a lower percentage (Fig. 6). The removal of COD was observed in the following descending order: 69.29% (H6 *B. anthracis*); >67.43% (H1 (*A. tabrizica*); >66.41% (H19 *P. mendocina*); >65.89% (H10 *S. nematodiphila*); and >56.62% (H9 *P. alcaliphila*), with a standard deviation of 4.4 after 5 d of treatment.

Exposure of halophilic bacterial isolates to natural brine supplemented with glucose resulted in COD removal at a rate above 85% (Fig. 6, Table S11). The overall COD removal was recorded in the following descending order: 98.68% (H6 *B. anthracis*); >98.12% (H1 *A. tabrizica*); >95.62% H19 (*P. mendocina*); >89.65% (H9 *P. alcaliphila*); and >87.26% (H10 *S. nematodiphila*), with a standard deviation of 4.6 after 5 d of treatment.

The target halophilic bacteria exposed to synthetic brine solutions under anaerobic conditions after 5 d of treatment were able to remove COD at a rate above 85%, which was different from one strain to another in the following descending order: 97.23% (H19 *P. mendocina*); >95.23% (H10 *S. nematodiphila*); >92.36% (H6 *B. anthracis*); >87.26% (H9 *P. alcaliphila*); and >85.26% H1 (*A. tabrizica*), with a standard deviation of 4.57 after 5 d of treatment (Fig. 6). Similarly to aerobic treatment, in anaerobic condition the statistical analysis also showed no significant difference between halophilic bacterial isolates in natural brine. Furthermore, synthetic brine also revealed no significant difference ($p > 0.05$) among the mean values of test bacterial isolates. However, when comparing natural to the synthetic brines, a statistical significant difference ($p < 0.05$) was noted.

3.5.3. Statistical analysis for efficiency of halophilic bacteria in removing COD from natural brine and synthetic brine solutions

The data were statistically analysed using the SPSS computer software. One-way ANOVA in SPSS package was used for the comparison among the COD data for the treatments in the synthetic brine under both aerobic and anaerobic conditions. The values were expressed as *p*-values (p) for three replicates with a significant difference ($p < 0.05$).

4. Discussion

Hypersaline wastewaters (brine wastewaters) generated from mining and mineral processing industries, pickling processes, meatpacking plants, dyestuffs, pesticides, herbicides, polyhydric compounds, organic peroxides, and pharmaceutical industries have become an environmental and public health concern as they contain large amounts of chemical pollutants [6]. This situation has drastically increased due to fast industrialisation, and increased population growth, worldwide. Of the methods used to treat brine wastewaters, the biological treatment of saline wastewater has been seen as by far the most popular treatment method [25]. The present study investigated the ability of indigenous bacterial species isolated from brine wastewater for the bioremediation of this water source under aerobic and anaerobic conditions.

Culture-based methods in general and molecular techniques such as 16S rRNA gene sequence analysis in particular have resulted in the identification of *A. tabrizica* for H1 (97% identity), *B. anthracis* for H6 (97% identity), *P. alcaliphila* for H9 and H19 (98% identity), and *S. nematodiphila* for H10 (98% identity). *B. anthracis* presented some phenotypic and phylogenetic similarities with non-pathogen *B. cereus* and *Bacillus megaterium* [26,27]. Most of these halophilic bacterial strains have also been isolated from halophilic environmental samples in several other studies [28–32]. *A. tabrizica* sp. nov. was for the first time isolated from Qurugöl Lake (in Azerbaijan) by the study of Tarhriz et al. [33]. Nevertheless, *A. tabrizica* sp. was formerly not known in the classified taxa of Gram-negative bacteria from South Africa. These halophilic bacteria could be considered as obligate moderate due to their ability to grow at minimum NaCl concentrations of 5% (50,000 mg/L) (w/v) and mainly at optimal NaCl concentrations of 7.5%–12.5% (75,000–125,000 mg/L) (w/v) (Fig. S1). It has been pointed out that halophiles that are capable of growing in media containing more than 20% (200,000 mg/L) are restricted to saline environments [34]. In a study conducted by the study of Osman et al. [30], it was reported that *Bacillus* spp. were isolated from hypersaline environments and were able to grow in NaCl concentrations of up to 17.55% (175,500 mg/L) (w/v). *Bacillus* spp. and *Pseudomonas* spp. are considered the most common bacteria isolated from brine or salted water [31,35,36].

Furthermore, the present study revealed that the target isolates were able to grow in brine solutions with pH values

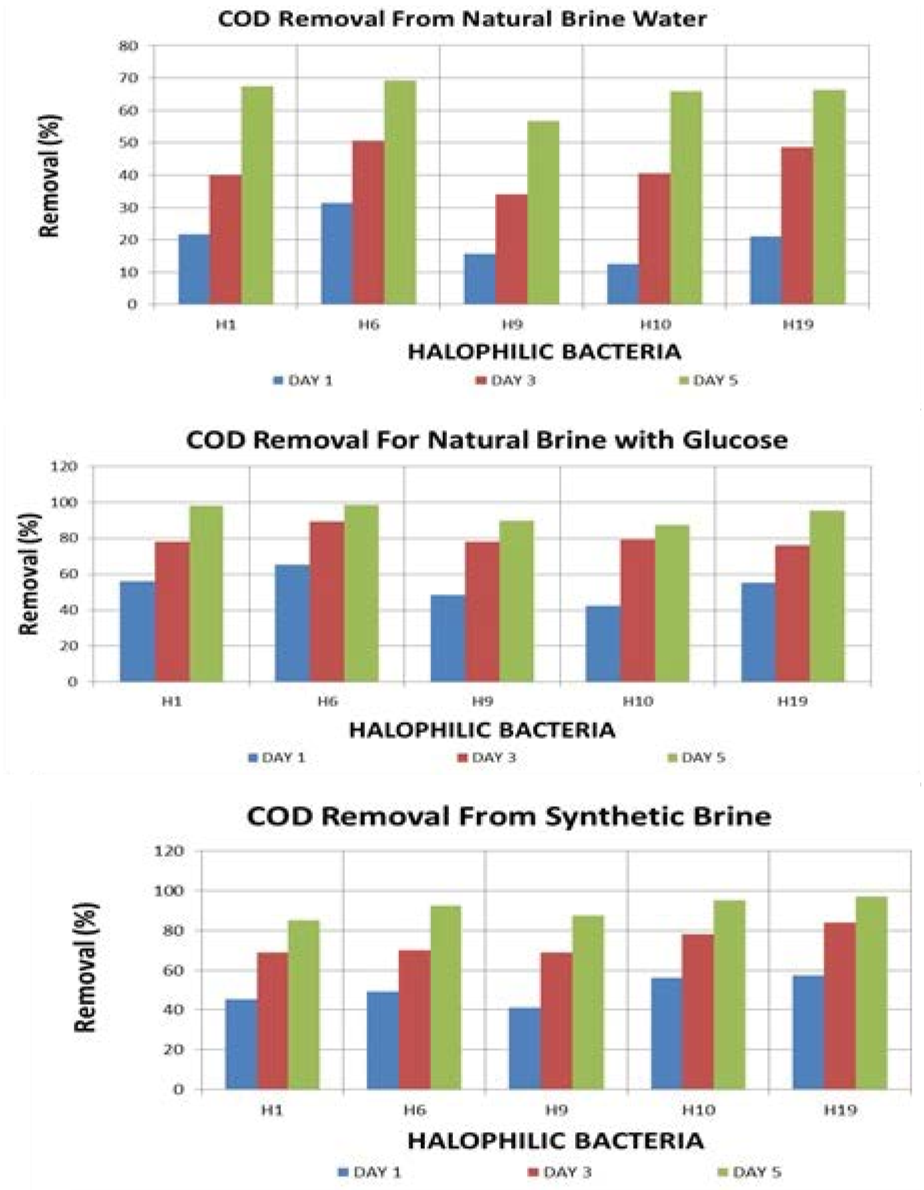

Fig. 6. Chemical oxygen demand (COD) removal from natural brine and synthetic brine solution by using halophilic bacterial strains under anaerobic conditions.

ranging from pH 3 to 10 and at temperatures ranging from 20°C to 40°C. These results corroborated those of Kroll [37] who found that these microorganisms can grow over the pH range of less than pH 1.0 to approximately pH 13.0. Kargi and Dinçer [4] also found that the pH of brine wastewater ranged between 4 and 5.80 with high NaCl contents (116 mg/L) highlighting that these microorganisms can grow under acidic conditions.

During the study period, isolated halophilic bacterial species were used to remove chemical pollutants including salt content from synthetic brine solutions and natural brine wastewater collected from the eMalahleni Water Reclamation Plant. The findings showed a low percentage of metal removal in the natural brine (39.63%–100% under aerobic conditions and 70.27%–100% under anaerobic conditions) compared with the synthetic brine solution (82.84%–100% under aerobic conditions and 44.3%–100% under anaerobic conditions) and this could be due to the absence of sufficient carbon source and other nutrient supplements. Furthermore, the percentage metal removal was found to be significantly enhanced in natural brine supplemented with glucose (Table 3). By comparing the natural brine with glucose and natural brine without glucose under aerobic conditions, it was revealed that four chemical components (B, Cu, Ni, and Zn) out of 16 were completely removed in natural brine with glucose, three chemical components (B, Cu, Zn) out of 16 were equally removed in both types of brine, while only four (K, Mn, Pb, and S) out of 16 were removed from natural brine without glucose (Table S1). In the meantime, under anaerobic conditions, nine chemical components (B, Ba, Cu, Hg, K, Ni, Pb, U, and Zn) out of 16 were completely removed in natural brine with glucose; six chemical components (Ni, Cu, Hg, Pb, U, and Zn) out of 16 were equally removed in both brines while only seven (Ca, Mg, Mn, Na, S, Si, and Sr) were removed from natural brine without glucose (Table S2). The same process was repeated with synthetic brine solutions under both aerobic and anaerobic conditions. Results revealed that nine chemical components (B, Cu, Mn, Na, Ni, Pb, Si, Sr and Zn) out of 16 were completely removed in aerobic conditions, four chemical components (Ni, Cu, Pb, and Zn) out of 16 were equally removed both in aerobic and anaerobic conditions, while four (Ba, Hg, K, and U) out of 16 were only removed in anaerobic conditions (Table S3). The assessment of these results proved that the oxidation under aerobic conditions fostered the halophilic bacteria in natural brine with glucose to significantly enhance the percentage removal of the chemical components compared to the percentage removal under anaerobic conditions.

With natural brine containing glucose under aerobic conditions, H1 (*A. tabrizica*) strain was the halophile capable of removing in high percentage six chemical components out of nine (82.21% of Ba, 75.54% of Mg, 100% of Ni, 99.57% of Si, 99.57% of Sr and 97.94% of U); followed by H19 (*P. mendocina*) that was able to remove two chemical components out of nine (82.75% of Hg and 100% of Ni); and finally, H6 (*B. anthracis*) and H10 (*S. nematodiphila*) were able to remove 92.92% of Na and 93.57% of Ca out of nine chemical components, respectively. Under similar conditions in natural brine, H6 (*B. anthracis*) was the halophile capable of removing in high percentage three chemical components out of four (68.48% of K, 100% of Mn and 64.52% of S); followed by H9 (*P. alcaliphila*), H10 (*S. nematodiphila*) and H19 (*P. mendocina*) that achieved complete removal (100%) of Mn and Pb. Finally, under aerobic conditions either with natural brine without or with glucose, all halophilic bacteria were able to entirely remove B (100%), Cu (100%) and Zn (100%).

With natural brine containing glucose under anaerobic condition, all the strains were able to remove 100% of Ba and K, while only H1 (*A. tabrizica*), H6 (*B. anthracis*) and H9 (*P. alcaliphila*) were able to remove 100% of B. Under the same conditions, Ni, Cu, Hg, Pb, U and Zn were completely removed (100%) in both brines by all the strains, while in the natural brine without glucose the halophile H10 (*S. nematodiphila*) was capable of removing 99.98% of Ca, 100% of Mg, 99.85% of Na, 100% of Si, 100% of Sr, followed by H9 (*P. alcaliphila*) that was capable of removing 100% of Mg, 99.85% of Hg and 99.43% of S, and finally, H19 was only able to remove 98.21% of Mn.

When exposed to synthetic brine solutions under anaerobic conditions, all the strains were able to completely remove Ba and Hg, while H10 (*S. nematodiphila*) was only able to remove 100% of K, and H9 (*P. alcaliphila*), H10 (*S. nematodiphila*) and H19 (*P. mendocina*) were able to completely remove U (100%). Under aerobic conditions, all the strains were able to remove 100% of B while 98.86% of Ca was only removed by H19 (*P. mendocina*); 97.93% of Mg was only removed by H9 (*P. alcaliphila*); 100% of Mn was completely removed by H6 (*B. anthracis*), H9 (*P. alcaliphila*), H10 (*S. nematodiphila*) and H19 (*P. mendocina*); 100% of Sr was removed by H1 (*A. tabrizica*); H9 (*P. alcaliphila*); H10 (*S. nematodiphila*) and H19 (*P. mendocina*); and finally 100% of Na, 99.76% of S and 100% of Si were removed by H19 (*P. mendocina*), H10 (*S. nematodiphila*) and H6 (*B. anthracis*), respectively. After a 5 d treatment, all the strains were able to completely remove Ni, Cu, Pb and Zn (100% removal) from synthetic brine solutions under both aerobic and anaerobic conditions.

The performance of the biological process in treating saline wastewater in terms of COD removal has been observed to be poor, and this has been reported to be due to the negative impact of the salt content on microbial communities [8]. It has been reported that high salt concentrations (>1%) lead to an intense osmotic pressure on the cell wall leading to cell dehydration and to the loss of microbial activity [8]. In the present experimental study, five halophilic bacterial strains were investigated for their ability to removal COD from natural brine and synthetic brine solutions under aerobic and anaerobic conditions. The results of this study revealed that all the halophilic bacterial strains have the ability to remove COD at a range of 69.58%–100% under aerobic conditions and at a range of 56.62%–98.68% under anaerobic conditions from all tested brine wastewaters after a 5 d treatment. However, the highest COD removal was shown in brine wastewater containing glucose (95% COD removal) and this could be due to the addition of one organic compound, such as glucose as glucose-salts medium that can support the growth of many microbes [38]. Results highlighted the fact that *A. tabrizica* was more active under aerobic conditions for the removal of COD as it could remove up to 100% of the COD content under aerobic conditions in natural brine containing glucose and 98.12% of the COD content under aerobic conditions when exposed to the same brine wastewater (Figs. 5 and 6). A similar observation was noted in the presence of yeast and bacteria during the experimental study conducted by the study of Dan et al. [39]. In two laboratory-scale studies consisting of yeast and bacteria membrane bioreactor systems that aimed to treat high salinity wastewater containing high organic load (5,000 mg/L COD) and salt content (32 g/L NaCl), this author observed the COD removal of above 90% at a hydraulic retention time of 5 h under the same operating conditions. In another study, a salt-tolerant microorganism strain *Staphylococcus xylosus* was used in the biological aerobic treatment of saline wastewater in a membrane bioreactor system. It was found that the treatment performance in terms of COD removal rates improved and reached up to 93.4% [6]. Kargi and Dinçer [4] reported that *Halobacterium* spp. in activated sludge culture could continue removing COD even at 50 g salt/L. Findings of the present study therefore demonstrated the efficiency of halophilic bacterial strains such as *A. tabrizica*, *B. anthracis*, *P. alcaliphila*, *S. nematodiphila*, and *P. mendocina* to remove COD from saline wastewater. Although these halophilic bacteria were isolated from South African brine wastewater, the results of this study corroborate the findings of previous studies in other countries showing the capability of other halophilic bacterial strains in removing COD from saline wastewater.

5. Conclusion

Halophilic bacterial strains *A. tabrizica* (H1), *B. anthracis* (H6), *P. alcaliphila* (H9), *P. mendocina* (H19) and *S. nematodiphila* (H10) are found in the waste brine stream of the eMalahleni Water Reclamation Plant. The most important contribution of this study is the isolation of *A. tabrizica* from waste brine streams in South Africa, a species that has not yet been reported in any previous study on brine wastewaters in South Africa. It is clear that the indigenous halophilic bacterial strains isolated from South African waste brine streams are able to survive in an aquatic environment which contains NaCl concentrations of up to 20% (200,000 mg/L) w/v. Moreover, this study revealed the ability of all target indigenous halophilic bacteria to reduce (K, Na, Ni, Si, Mg, Mn, and Sr) or to completely remove various metal components (B, Ba, Cu, Hg, Pb, U, and Zn) as well as to significantly reduce COD concentrations in brine wastewater (up to 95%) under aerobic and 80% under anaerobic conditions. This study therefore recommends the biological treatment of waste brine streams using indigenous halophilic bacteria, which showed high effectiveness in metal and/or COD removal. A combination of these halophilic bacteria with physical and/or chemical treatment processes could enhance the treatment of waste brine streams and produce higher quality effluents that can be reused for multiple purposes.

Declarations

Availability of data and materials

The data generated in this review, supplementary tables and figures are found in the supplementary file.

Authors' contributions

AAEAZ conducted research and wrote the draft manuscript. IK and MNBM conceived the research, reviewed the manuscript and made considerable input for final submission. All authors read and approved the final manuscript.

Conflicts of interest statement

The authors declare no conflict of interest.

Ethics approval

This article does not contain any studies concerned with experiment on human or animals.

Funding

This research article received funding from the Department of Science and Technology/ the National Research Foundation/ South African Research Chairs Initiative in Water Quality and Wastewater management. Opinions expressed and conclusions arrived at are those of the authors and are not necessarily to be attributed to the funders.

References

[1] M. Dungeni, M.N.B. Momba, The abundance of *Cryptosporidium* and *Giardia* spp. in treated effluents produced by four wastewater treatment plants in the Gauteng Province of South Africa, Water SA, 36 (2010), doi: 10.4314/wsa.v36i4.58413.

[2] W. Carty, Towards an urban world, Earthwatch, 43 (1991) 2–4.

[3] G. Naidu, S.C. Ryu, R. Thiruvenkatachari, Y.K. Choi, S.Y. Jeong, S. Vigneswaran, A critical review on remediation, reuse, and resource recovery from acid mine drainage, Environ. Pollut., 247 (2019) 1110–1124.

[4] F. Kargi, A.R. Dinçer, Use of halophilic bacteria in biological treatment of saline wastewater by fed-batch operation, Water Environ. Res., 72 (2000) 170–174.

[5] M. El-Kady, F. El-Shibini, Desalination in Egypt and the future application in supplementary irrigation. Desalination, 136 (2001) 63–72.

[6] S.I. Abou-Elela, M.M. Kamel, M.E. Fawzy, Biological treatment of saline wastewater using a salt-tolerant microorganism, Desalination, 250 (2010) 1–5.

[7] C.R. Woolard, R.I. Irvine, Biological treatment of hypersaline wastewater by a biofilm of halophilic bacteria, Water Environ. Res., 66 (1994) 230–235.

[8] F. Kargi, A. Dincer, Enhancement of biological treatment performance of saline wastewater by halophilic bacteria, Bioprocess Eng., 15 (1996) 51–58.

[9] A. Shahalam, Treatment of nitrogen and phosphorus in brine of RO process refining effluent of biological processes treating municipal wastewater, Eur. J. Sci. Res., 28 (2009) 514–521.

[10] D.S. Shiladitya, A. Priya, Encyclopedia of Life Sciences, Nature Publishing Group, University of Massachusetts, Amherst, Massachusetts, 2001. Available at: www.els.net

[11] J.-Y. Ying, Z.-P. Liu, B.-J. Wang, X. Dai, S.-S. Yang, S.-J. Liu, *Salegentibacter catena* sp. nov., isolated from sediment of the South China Sea, and emended description of the genus *Salegentibacter*, Int. J. Syst. Evol. Microbiol., 57 (2007) 219–222.

[12] Z. Chan, Z. Wang, Z. Yi, R. Zeng, Haloalkaliphilic protease production by a newly isolated moderately halophilic bacterium *Pontibacillus* sp. SY-8, Oceanography: Open Access J., 2 (2014) 1–7.

[13] D. Cánovas, C. Vargas, M.I. Calderón, A. Ventosa, J.J. Nieto, Characterization of the genes for the biosynthesis of the compatible solute ectoine in the moderately halophilic bacterium *Halomonas elongata* DSM 3043, Syst. Appl. Microbiol., 21 (1998) 487–497.

[14] A. Ventosa, Taxonomy of moderately halophilic heterotrophic eubacteria, Halophilic Bacteria, 1 (1988) 71–84.

[15] J.A. Mata, J. Martínez-Cánovas, E. Quesada, V. Béjar, A detailed phenotypic characterisation of the type strains of *Halomonas* species, Syst. Appl. Microbiol., 25 (2002) 360–375.

[16] S. Pal, A. Sar, B. Dam, Moderate halophilic bacteria, but not extreme halophilic Archaea can alleviate the toxicity of short-alkyl side chain imidazolium-based ionic liquids, Ecotoxicol. Environ. Saf., 184 (2019) 109634, doi: 10.1016/j.ecoenv.2019.109634.

[17] C.R. Woolard, R.L. Irvine, Treatment of hypersaline wastewater in the sequencing batch reactor, Water Res., 29 (1995) 1159–1168.

[18] S. Sekar, A.A.E.A. Zintchem, J. Keshri, I. Kamika, M.N.B. Momba, Bacterial profiling in brine samples of the eMalahleni Water Reclamation Plant, South Africa, using 454-pyrosequencing method, FEMS Microbiol. Lett., 359 (2014) 55–63.

[19] T.M. Caton, L.R. Witte, H.D. Ngyuen, J.A. Buchheim, M.A. Buchheim, M.A. Schneegurt, Halotolerant aerobic heterotrophic bacteria from the Great Salt Plains of Oklahoma, Microbiol. Ecol., 48 (2004) 449–462.

[20] I. Kamika, M.N.B. Momba, Microbial diversity of Emalahleni mine water in South Africa and tolerance ability of the predominant organism to vanadium and nickel, PloS One, 9 (2014) e86189.

[21] J.-C. Lee, W.-J. Li, L.-H. Xu, C.-L. Jiang, C.-J. Kim, *Lentibacillus salis* sp. nov., a moderately halophilic bacterium isolated from a Salt Lake, Int. J. Syst. Evol. Microbiol., 58 (2008) 1838–1843.

[22] K. Tamura, G. Stecher, D. Peterson, A. Filipski, S. Kumar, MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol., 30 (2013) 2725–2729.

[23] C. Farrier-Pagès, F.N. Rassoulzadegan, Mineralization in planktonic protozoa, Limnol. Oceanogr., 39 (1994) 411–419.

[24] E.W. Rice, R.B. Baird, A.D. Eaton, L.S. Clesceri, APHA, Standard Methods for the Examination of Water and Wastewater, 22nd ed., 2012.

[25] O. Lefèbvre, R. Moletta, Treatment of organic pollution in industrial saline wastewater: a literature review. Water Res., 40 (2006) 3671–3682.
[26] E.G. Dib, S.A. Dib, D.A. Korkmaz, N.K. Mobarakai, J.B. Glaser, Nonhemolytic, nonmotile Gram-positive rods indicative of *Bacillus anthracis*, Emerging Infect. Dis., 9 (2003) 1013–1015.
[27] K. Shimizu, H. Nakamura, M. Ashiuchi, Salt-inducible bionylon polymer from *Bacillus megaterium*, Appl. Environ. Microbiol., 73 (2007) 2378–2379.
[28] H. Larsen, Halophilic and halotolerant microorganisms—an overview and historical perspective, FEMS Microbiol. Lett., 39 (1986) 3–7.
[29] P. Shivanand, G. Jayaraman, Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast, Process Biochem., 44 (2009) 1088–1094.
[30] O. Osman, H. Tanguichi, K. Ikeda, P. Park, S. Tanabe-Hosoi, S. Nagata, Copper-resistant halophilic bacterium isolated from the polluted Maruit Lake, Egypt, J. Appl. Microbiol., 108 (2010) 1459–1470.
[31] K. Al'abri, Use of Molecular Approaches to Study the Occurrence of Extremophiles and Extremodures in Non-extreme Environments, Doctoral Thesis, University of Sheffield, 2011.
[32] M. Rameshpathy, G. Jayaraman, V. Devi Rajeswari, A.S. Vickram, T.B. Sridharan, Emergence of a multidrug resistant *Halomonas hydrothermalis* strain VITP09 producing a Class A β-Lactamase, isolated from Kumta Coast, Int. J. Pharm. Pharm. Sci., 4 (2012) 639–644.
[33] V. Tarhriz, G. Nematzadeh, S.Z. Vahed, M.A. Hejazi, M.S. Hejazi, *Alishewanella tabrizica* sp. nov., isolated from Qurugöl Lake, Int. J. Syst. Evol. Microbiol., 62 (2012) 1986–1991.
[34] C. Sáiz-Jiménez, L. Laiz, Occurrence of halotolerant/halophilic bacterial communities in deteriorated monuments, Int. Biodeterior. Biodegrad., 46 (2000) 319–326.
[35] C. Ash, J.A.E. Farrow, S. Wallbanks, M.D. Collins, Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences, Lett. Appl. Microbiol., 13 (1991) 202–206.
[36] O.A. Osman, S. Bertilsson, Isolation and Characterization of Surface-Active Compounds Produced by Halophilic Bacteria, Book of Proceedings: 5th International Symposium on Biosorption and Bioremediation, ICT Press, Prague, 2012, pp. 164–168.
[37] R. Kroll, Alkalophiles, Microbiol. Extreme Environ., McGraw-Hill, New York, NY, 1990, pp. 55–92.
[38] D. Mara, N.J. Horan, Handbook of Water and Wastewater Microbiology, Academic Press, 2003.
[39] N.P. Dan, C. Visvanathan, C. Polprasert, R. Ben Aim, High salinity wastewater treatment using yeast and bacterial membrane bioreactors, Water Sci. Technol., 46 (2002) 201–209.

Supplementary information

Wastewater brine treatment with halophilic bacteria

Table S1
Growth rate (d^{-1}) of halophilic bacteria during temperature optimisation studies

Halophilic bacteria	20°C	22.5°C	25°C	27.5°C	30°C	32.5°C	35°C	37.5°C	40°C
	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate
Alishewanella	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
tabrizica	0.0082 (D3)	0.0135 (D3)	0.0218 (D3)	0.0412 (D3)	0.0562 (D3)	0.0452 (D3)	0.0326 (D3)	0.0315 (D3)	0.0306 (D3)
	0.0346 (D5)	0.0372 (D5)	0.0594 (D5)	0.0692 (D5)	0.0713 (D5)	0.0646 (D5)	0.0607 (D5)	0.0626 (D5)	0.0576 (D5)
Bacillus anthracis	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
	0.0061 (D3)	0.0128 (D3)	0.0224 (D3)	0.0425 (D3)	0.0574 (D3)	0.0451 (D3)	0.0324 (D3)	0.0312 (D3)	0.0307 (D3)
	0.0286 (D5)	0.0342 (D5)	0.0567 (D5)	0.0675 (D5)	0.0707 (D5)	0.0625 (D5)	0.0593 (D5)	0.0617 (D5)	0.0557 (D5)
Pseudomonas	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
alcaliphila	0.0056 (D3)	0.0113 (D3)	0.0212 (D3)	0.0428 (D3)	0.0582 (D3)	0.0458 (D3)	0.0312 (D3)	0.0332 (D3)	0.0329 (D3)
	0.0307 (D5)	0.0343 (D5)	0.0589 (D5)	0.0697 (D5)	0.0727 (D5)	0.0641 (D5)	0.0607 (D5)	0.0631 (D5)	0.0566 (D5)
Serratia	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
nematodiphila	0.0054 (D3)	0.0185 (D3)	0.0261 (D3)	0.0432 (D3)	0.0578 (D3)	0.0457 (D3)	0.0345 (D3)	0.0338 (D3)	0.0332 (D3)
	0.0253 (D5)	0.0288 (D5)	0.0569 (D5)	0.0699 (D5)	0.0716 (D5)	0.0696 (D5)	0.0632 (D5)	0.0638 (D5)	0.0559 (D5)
Pseudomonas	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
mendocina	0.0059 (D3)	0.0197 (D3)	0.0284 (D3)	0.0435 (D3)	0.0598 (D3)	0.0475 (D3)	0.0349 (D3)	0.0341 (D3)	0.0333 (D3)
	0.0388 (D5)	0.0377 (D5)	0.0606 (D5)	0.0699 (D5)	0.0723 (D5)	0.0722 (D5)	0.0625 (D5)	0.0631 (D5)	0.0583 (D5)

D1: Day 1, D3: Day 3, D5: Day 5

Table S2
Growth rate (d^{-1}) of halophilic bacteria during pH optimisation studies at 28°C

Halophilic bacteria	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9	pH 10
	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate	Growth rate
	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
Alishewanella tabrizica	0.0601 (D3)	0.0580 (D3)	0.0638 (D3)	0.0547 (D3)	0.0590 (D3)	0.0635 (D3)	0.0639 (D3)	0.0570 (D3)
	0.0623 (D5)	0.0594 (D5)	0.0652 (D5)	0.0568 (D5)	0.0663 (D5)	0.0675 (D5)	0.0645 (D5)	0.0584 (D5)
	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
Bacillus anthracis	0.0622 (D3)	0.0217 (D3)	0.0574 (D3)	0.0589 (D3)	0.0671 (D3)	0.0684 (D3)	0.0317 (D3)	0.0306 (D3)
	0.0651 (D5)	0.0231 (D5)	0.0589 (D5)	0.0594 (D5)	0.0678 (D5)	0.0695 (D5)	0.0624 (D5)	0.0624 (D5)
	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
Pseudomonas alcaliphila	0.0439 (D3)	0.0448 (D3)	0.0377 (D3)	0.0326 (D3)	0.0334 (D3)	0.0414 (D3)	0.0425 (D3)	0.0461 (D3)
	0.0458 (D5)	0.0478 (D5)	0.0389 (D5)	0.0348 (D5)	0.0552 (D5)	0.0589 (D5)	0.0446 (D5)	0.0472 (D5)
	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
Serratia nematodiphila	0.0606 (D3)	0.0565 (D3)	0.0530 (D3)	0.0502 (D3)	0.0330 (D3)	0.0284 (D3)	0.0394 (D3)	0.0402 (D3)
	0.0621 (D5)	0.0578 (D5)	0.0546 (D5)	0.0516 (D5)	0.0645 (D5)	0.0676 (D5)	0.0398 (D5)	0.0414 (D5)
	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)	0.0000 (D1)
Pseudomonas mendocina	0.0221 (D3)	0.0255 (D3)	0.0265 (D3)	0.0209 (D3)	0.0223 (D3)	0.0217 (D3)	0.0226 (D3)	0.0277 (D3)
	0.0363 (D5)	0.0375 (D5)	0.0408 (D5)	0.0326 (D5)	0.0402 (D5)	0.0681 (D5)	0.0235 (D5)	0.0284 (D5)

D1: Day 1, D3: Day 3, D5: Day 5

Table S3
Growth rate (d^{-1}) of halophilic bacteria during carbon source optimisation studies at 28°C

Halophilic bacteria	Glucose	Lactose	Sucrose	Acetate
	Growth rate	Growth rate	Growth rate	Growth rate
Alishewanella tabrizica	0.0000 (D1) 0.0523 (D3) 0.0796 (D5)	0.0000 (D1) 0.0213 (D3) 0.0465 (D5)	0.0000 (D1) 0.0456 (D3) 0.0785 (D5)	0.0000 (D1) 0.0211 (D3) 0.0303 (D5)
Bacillus anthracis	0.0000 (D1) 0.0596 (D3) 0.1098 (D5)	0.0000 (D1) 0.0203 (D3) 0.0422 (D5)	0.0000 (D1) 0.0412 (D3) 0.0897 (D5)	0.0000 (D1) 0.0185 (D3) 0.0239 (D5)
Pseudomonas alcaliphila	0.0000 (D1) 0.0326 (D3) 0.0793 (D5)	0.0000 (D1) 0.0218 (D3) 0.0501 (D5)	0.0000 (D1) 0.0411 (D3) 0.0691 (D5)	0.0000 (D1) 0.0201 (D3) 0.0346 (D5)
Serratia nematodiphila	0.0000 (D1) 0.0328 (D3) 0.0884 (D5)	0.0000 (D1) 0.0289 (D3) 0.0739 (D5)	0.0000 (D1) 0.0402 (D3) 0.0691 (D5)	0.0000 (D1) 0.0222 (D3) 0.0451 (D5)
Pseudomonas mendocina	0.0000 (D1) 0.0356 (D3) 0.0722 (D5)	0.0000 (D1) 0.0245 (D3) 0.0418 (D5)	0.0000 (D1) 0.0456 (D3) 0.0699 (D5)	0.0000 (D1) 0.0182 (D3) 0.0230 (D5)

D1: Day 1, D3: Day 3, D5: Day 5

Table S4
Determination of metal components in natural brine wastewater and their removal (%) after five days of treatment by using halophilic bacterial strains under aerobic conditions

	Initial conc. (mg/L)	Natural brine with 5% glucose					Natural brine without glucose				
		H1	H6	H9	H10	H19	H1	H6	H9	H10	H19
B	650	100	100	100	100	100	100	100	100	100	100
Ba	321	82.21	78.17	74.73	72.46	77.25	77.3	79.9	80.34	77.52	81.05
Ca	1,689	66.9	41.27	45.14	93.57	41.38	39.9	53.58	44.74	39.63	81.05
Cu	50	100	100	100	100	100	100	100	100	100	100
Hg	350	82.3	78.61	78.44	81.57	82.75	75.92	79.28	79.38	82.44	82.34
K	2,564	67.8	43.91	46.72	41.96	43.37	58.22	68.48	66.1	62.55	61.07
Mg	1,785	75.54	59.44	60.39	57.5	60.03	63.72	68.48	69.41	66.05	66.19
Mn	254	98.68	95.35	95.92	95.7	95.55	99.54	100	100	100	99.57
Na	9,859	91.83	92.92	90.3	90.04	91.86	46.49	57.55	53.24	48.82	45.02
Ni	145	100	99.72	99.79	99.68	100	99.59	99.86	99.86	99.56	99.66
Pb	251	97.81	96.82	96.81	97.29	97.5	98.54	99.41	99.48	99.26	100
S	754	64.25	40.1	41.71	34.68	38.46	54.77	64.52	62.93	57.16	57.55
Si	895	99.57	53.68	54.41	52.78	58.71	50.39	45.08	55.58	46.81	49.32
Sr	785	99.57	98.94	98.8	98.83	98.7	98.49	98.69	98.72	98.44	98.78
U	796	97.94	83.27	83.08	82.15	83.41	82.72	88.12	85.39	83.66	83.6
Zn	78	100	100	100	100	100	100	100	100	100	100

Table S5
Comparison of the percentage removal of chemical components in synthetic brine solution under both aerobic and anaerobic conditions after five days of treatment

Metals	Aerobic conditions (% per strain(s))	Symbol	Anaerobic conditions (% per strain(s))
B	100 (all strains)	>	100 (H9; H10; H19)
Ba	99.67 (H10)	<	100 (all strains)
Ca	98.86 (H19)	>	85.63 (H10)
Hg	99.42 (H10)	<	100 (all strains)
K	96.64 (H19)	<	100 (H10)
Mg	97.93 (H9)	>	96.1 (H9)
Mn	100 (H6; H9; H10; H19)	>	97.47 (H6)
Na	100 (H19)	>	93.17 (H19)
S	99.76 (H10)	>	95.71 (H19)
Si	100 (H6)	>	97.48 (H10)
Sr	100 (H1; H9; H10; H19)	>	93.15 (H6)
U	98.93 (H10)	<	100 (H9; H10; H19)
Ni	100 (all strains)	=	100 (all strains)
Cu	100 (all strains)	=	100 (all strains)
Pb	100 (all strains)	=	100 (all strains)
Zn	100 (all strains)	=	100 (all strains)

NBG: natural brine with glucose; NB: natural brine; =equal to; >higher than; <lower than

Table S6
Natural brine with glucose vs. natural brine without glucose under aerobic conditions after five days of treatment

Metals	NBG (% per strain(s))	Symbol	NB (% per strain(s))
B	100 (all strains)	=	100 (all strains)
Ba	82.21 (H1)	>	81.05 (H19)
Ca	93.57 (H10)	>	81.05 (H19)
Hg	82.75 (H19)	>	82.44 (H10)
K	67.8 (H1)	<	68.48 (H6)
Mg	75.54 (H1)	>	69.41 (H9)
Mn	98.68 (H1)	<	100 (H6; H9; H10)
Na	92.92 (H6)	>	57.55 (H6)
Ni	100 (H1; H19)	>	99.86 (H6; H9)
Pb	97.81 (H1)	<	100 (H19)
S	64.25 (H1)	<	64.52 (H6)
Si	99.57 (H1)	>	55.58 (H9)
Sr	99.57 (H1)	>	98.78 (H19)
U	97.94 (H1)	>	88.12 (H6)
Cu	100 (all strains)	=	100 (all strains)
Zn	100 (all strains)	=	100 (all strains)

NBG: natural brine with glucose; NB: natural brine; =equal to; >higher than; <lower than

Table S7
Natural brine with glucose vs. natural brine without glucose under anaerobic conditions after five days of treatment

Metals	NBG (% per strain(s))	Symbol	NB (% per strain(s))
B	100 (H1; H6; H9)	>	100 (H1)
Ba	100 (all strains)	>	100 (H1; H6; H9; H10)
Ca	97.33 (H1)	<	99.98 (H10)
K	100 (all strains)	>	100 (H1)
Mg	98.78 (H10)	<	100 (H9; H10)
Mn	95.86 (H6)	<	98.21 (H19)
Na	97.83 (H19)	<	99.85 (H9; H10)
S	95.38 (H1)	<	99.43 (H9)
Si	98.83 (H10)	<	99.86 (H10)
Sr	91.54 (H9)	<	93.4 (H10)
U	100 (all strains)	=	100 (all strains)
Ni	100 (all strains)	=	100 (all strains)
Cu	100 (all strains)	=	100 (all strains)
Hg	100 (all strains)	=	100 (all strains)
Pb	100 (all strains)	=	100 (all strains)
Zn	100 (all strains)	=	100 (all strains)

NBG: natural brine with glucose; NB: natural brine; =equal to; >higher than; <lower than

Table S8
Determination of metal contents in natural brine water and their removal (%) after 5 days treatment by using halophilic bacterial strains under aerobic conditions

	Initial Conc. (mg/L)	Natural brine with 5% glucose					Natural brine without glucose				
		H1	H6	H9	H10	H19	H1	H6	H9	H10	H19
B	650	100	100	100	100	100	100	100	100	100	100
Ba	321	82.21	78.17	74.73	72.46	77.25	77.3	79.9	80.34	77.52	81.05
Ca	1689	66.9	41.27	45.14	93.57	41.38	39.9	53.58	44.74	39.63	81.05
Cu	50	100	100	100	100	100	100	100	100	100	100
Hg	350	82.3	78.61	78.44	81.57	82.75	75.92	79.28	79.38	82.44	82.34
K	2564	67.8	43.91	46.72	41.96	43.37	58.22	68.48	66.1	62.55	61.07
Mg	1785	75.54	59.44	60.39	57.5	60.03	63.72	68.48	69.41	66.05	66.19
Mn	254	98.68	95.35	95.92	95.7	95.55	99.54	100	100	100	99.57
Na	9859	91.83	92.92	90.3	90.04	91.86	46.49	57.55	53.24	48.82	45.02
Ni	145	100	99.72	99.79	99.68	100	99.59	99.86	99.86	99.56	99.66
Pb	251	97.81	96.82	96.81	97.29	97.5	98.54	99.41	99.48	99.26	100
S	754	64.25	40.1	41.71	34.68	38.46	54.77	64.52	62.93	57.16	57.55
Si	895	99.57	53.68	54.41	52.78	58.71	50.39	45.08	55.58	46.81	49.32
Sr	785	99.57	98.94	98.8	98.83	98.7	98.49	98.69	98.72	98.44	98.78
U	796	97.94	83.27	83.08	82.15	83.41	82.72	88.12	85.39	83.66	83.6
Zn	78	100	100	100	100	100	100	100	100	100	100

Table S9
Determination of metal components in natural brine and their removal (%) after five days of treatment by using halophilic bacterial strains under anaerobic conditions

		Natural brine with 5% glucose					Natural brine without glucose				
	Initial conc. (mg/L) *A*/*B*	H1	H6	H9	H10	H19	H1	H6	H9	H10	H19
B	700/650	100	100	100	98.96	91.23	100	98.21	97.64	97.83	99.61
Ba	356/321	100	100	100	100	100	100	100	100	100	99.98
Ca	1,782/1,689	97.33	97.07	96.67	95.98	88.07	97.33	99.9	99.97	99.98	99.86
Cu	59/50	100	100	100	100	100	100	100	100	100	100
Hg	456/350	100	100	100	100	100	100	100	100	100	100
K	2,856/2,564	100	100	100	100	100	100	96.55	98.47	98.46	98.66
Mg	1,845/1,785	96.18	97.76	97.36	98.78	96.51	96.18	99.99	100	100	99.97
Mn	256/254	93.37	95.86	92.4	79.11	94.5	93.37	92.4	93.43	93.22	98.21
Na	9,785/9,859	97.25	97.11	97.83	93.59	97.69	97.25	99.75	99.85	99.85	99.72
Ni	178/145	100	100	100	100	100	100	100	100	100	100
Pb	451/251	100	100	100	100	100	100	100	100	100	100
S	856/754	95.38	94.33	93.53	76.35	46.61	95.38	99.2	99.43	99.25	99.02
Si	986/895	98.06	98.46	98.4	98.83	98.25	98.06	99.79	99.84	99.86	99.73
Sr	852/785	86.98	81.3	91.54	82.59	83.29	86.98	88.69	86.47	93.4	70.27
U	856/796	100	100	100	100	100	100	100	100	100	99.93
Zn	95/78	100	100	100	100	100	100	100	100	100	100

A (Initial concentration of metal components in natural brine with 5% glucose);
B (Initial concentration of metal components in natural brine without glucose).

Table S10
Chemical oxygen demand (COD) removal (%) from natural brine wastewater and synthetic brine solution by using halophilic bacterial strains under aerobic conditions

COD removal (%) from natural brine					
Initial COD (mg/L)	1,320	1,234	1,285	1,396	1,378
Strains	H1	H6	H9	H10	H19
Day 1	25	35.12	18.24	17.12	32.15
Day 3	45.12	63.28	38.75	59.68	68.79
Day 5	76.23	89.23	69.58	78.45	84.15
COD removal (%) from natural brine + glucose					
Initial COD (mg/L)	1,378	1,256	1,295	1,425	1,412
Strains	H1	H6	H9	H10	H19
Day 1	62.31	73.26	50.29	45.26	64.25
Day 3	81.24	90.24	82.32	81.24	78.19
Day 5	100	99.25	94.28	93.65	97.28
COD removal (%) from synthetic brine solution					
Initial COD (mg/L)	1,424	1,326	1,310	1,413	1,416
Strains	H1	H6	H9	H10	H19
Day 1	48.75	51.24	45.16	62.31	61.24
Day 3	74.16	75.46	74.26	81.59	86.24
Day 5	91.24	95.46	92.34	97.85	97.98

Table S11
Chemical oxygen demand (COD) removal (%) from natural brine wastewater and synthetic brine solution by using halophilic bacterial strains under anaerobic conditions

COD removal (%) from natural brine					
Initial COD (mg/L)	1,895	1,689	1,458	1,578	1,548
Strains	H1	H6	H9	H10	H19
Day 1	21.68	31.43	15.68	12.58	21.15
Day 3	40.12	50.65	33.94	40.52	48.55
Day 5	67.43	69.29	56.62	65.89	66.41
COD removal (%) from natural brine + glucose					
Initial COD (mg/L)	0.02	0.013	0.018	0.022	0.025
Strains	H1	H6	H9	H10	H19
Day 1	56.25	65.28	48.45	42.35	55.23
Day 3	78.25	89.56	78.24	79.25	76.38
Day 5	98.12	98.68	89.65	87.26	95.62
COD removal (%) from synthetic brine solution					
Initial COD (mg/L)	0.015	0.016	0.019	0.024	0.028
Strains	H1	H6	H9	H10	H19
Day 1	45.26	49.23	41.23	56.23	57.23
Day 3	68.91	70.23	68.94	78.26	84.12
Day 5	85.26	92.36	87.26	95.23	97.23

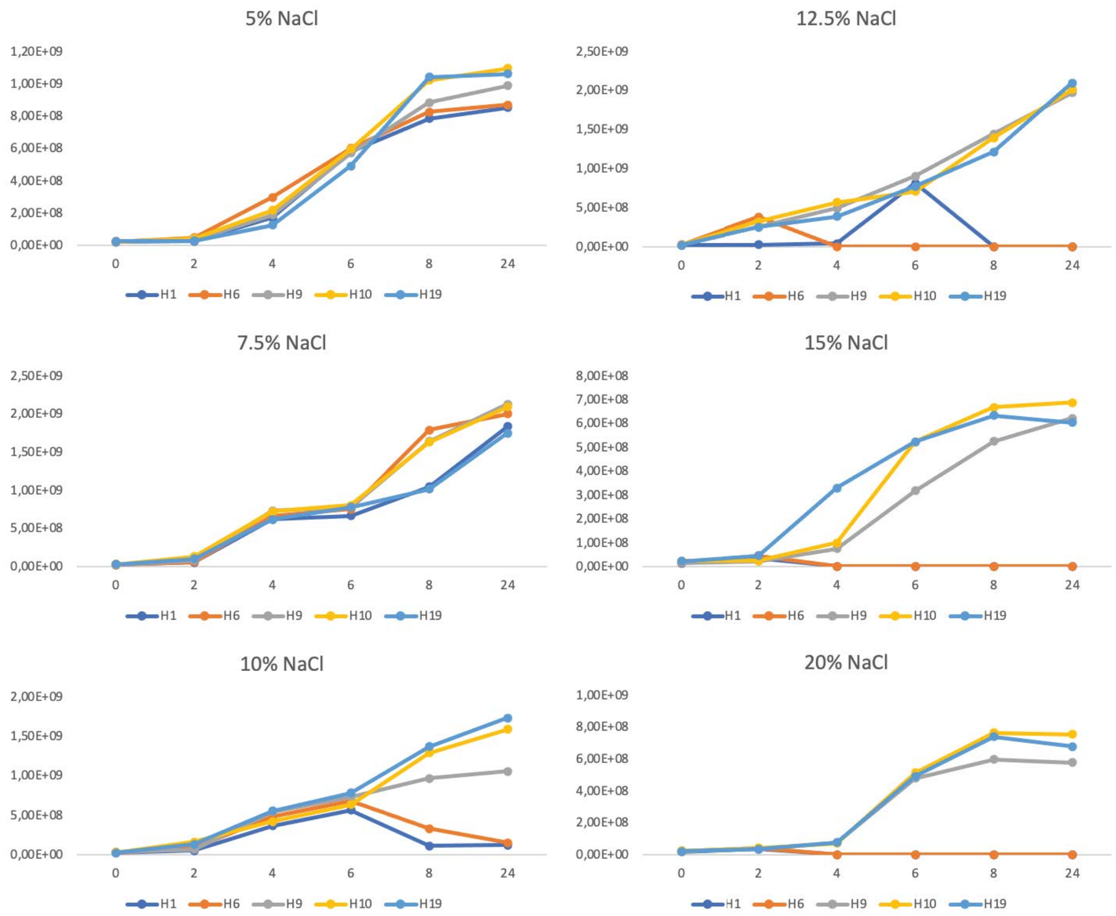

Fig. S1. Growth curves of halophilic bacteria during NaCl optimisation studies.