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# Halophilic bacterial community and their ability to remove zinc oxide and titanium dioxide nanoparticles from wastewater

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**ABSTRACT**

The microbial abundance of the brine samples in all the stages of plants was assessed using 16S ribosomal ribonucleic acid gene sequence analysis. A total of 35 genera (47% of the total population) except uncultured genera populated collected samples with *Halomonas* (8.48%) as the most dominant genus followed *Nesterenkonia* (6.29%), *Pseudomonas* (4.75%), *Serratia* (4.15%), *Shewanella* (3.64%), *Burkholderia* (2.47%), *Thauera* (2.14%), *Delftia* (2.18%), *Arthrospira* (2.18%) and others. Five moderately halophilic bacterial species namely *Serratia* sp. INBio 4041, *Bacillus cereus* strain CASA51-1, *Morganella morganii* strain AP28, *Citrobacter freundii* strain C09 and *Lysinibacillus* sp. NOSK was later isolated and assessed for the removal of nano-zinc oxide (nZnO) and nano-titanium dioxide ($nTiO_2$) in highly polluted wastewater samples. Prior removal experiment isolates showed the ability to thrive under an extensive range of operating conditions with 30°C, pH 7, 100 rpm (agitation speed), 4% NaCl and 2% sucrose as optimum growth conditions. Moderately halophilic bacterial isolates were gradually able to uptake nZnO and $nTiO_2$ at concentrations ranging from 1 to 200 mg/L over time. *Bacillus* sp. (100%–60%) appeared to have the highest nZnO-removal range, followed by *Serratia* sp. (100%–58%) and *Morganella* sp. (100%–50%), while *Citrobacter freundii* (97%–49%) and *Lysinibacillus* sp. (99%–43%) were observed to have the lowest removal efficiency. Individual isolates further demonstrated the following $nTiO_2$ removal trend: *Bacillus* sp. (98%–54%), *Serratia* sp., (99%–52%) and the lowest removal efficiency appeared to be associated with *Morganella* sp. (99%–43%) and *Citrobacter freundii* (99%–40%). The consortium of halophilic bacteria showed higher removal efficiency of nZnO (100%–63%) and $nTiO_2$ (100%–65%) with 100% over a wide range of metal oxide nanoparticles (NPs) concentration when compared to individual bacterial isolates. Bacterial isolates were also able to remove additional chemical pollutants with 100%–56% for Ag, Co, Cu, Ba, Ni, Li, Sr, and Ba, and 97%–1% for Na, S, Fe, P, Ca, Mg, As, and Sr upon exposure to various concentrations of both nZnO and $nTiO_2$ over exposure time. An increase of pH value was noted in the presence of $nTiO_2$ (pH 7 to pH 4) than in the presence of nZnO (pH 7 to pH 6). This study demonstrates the potential use of moderately halophilic bacteria for the bioremediation of highly polluted wastewater containing metal oxide nanoparticles and other metals.

*Keywords:* Moderate halophilic bacteria; Wastewater; Nanoparticles; Nano-zinc oxide; Nano-titanium oxide; Bioremediation

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## 1. Introduction

The need for new water resources worldwide has dramatically increased and this is due to the rapid global population growth, high water demand and high disposal of pollutants into the environment [1]. Considering the growing demand for potable water and the shortage of water supplies, water reuse will soon be an imperative requirement in densely populated areas. On the African continent, as of 2006, sub-Saharan Africa had the largest number of water-stressed countries compared to any other continent on the planet [2]. It has been estimated that 300 million out of 800 million people in Africa live in a water-stressed environment, and approximately 66% of the continent is arid or semi-arid.

In addition to the water scarcity, water pollution due to the disposal of microbiologically/chemically-contaminated effluents has been widely reported [3–5]. The discharge of these polluted effluents poses the biggest threat to the environment, public health, food safety, and access to safe drinking water for both human consumption and other aquatic species [6]. Like South Africa, many countries have shown significant industrial activities such as mining and metallurgy becoming the main drivers of their economy. Although these activities have resulted in the creation of wealth, they have also contributed to the tremendous challenges in terms of the pollution of the available water sources [7]. Therefore, there is a crucial and constant need to treat the wastewater into an effluent that can be either returned to the normal water cycle with minimal environmental effects to the end-users or can be reused for the benefit of the end-users.

To address these challenges, several techniques including chemical precipitation, chemical oxidation, ion exchange, and reverse osmosis (RO) have been developed for the removal of pollutants such as heavy metals, phosphates, nitrate, as well as organic matter. However, due to the emergence of new pollutants called "emerging pollutants" such as nanoparticles and pharmaceuticals in the environment, these techniques were found to be inadequate to an extent that the wastewater treatment plants are becoming an indirect source of pollution [8]. Even though nanoparticles are of great scientific interest due to their application in a wide variety of sectors such as electronics, cosmetics, pharmaceutical, information technology, agriculture, food, medical, water treatment, and environmental protection, there are growing concerns about their health and environmental impacts due to their overproduction. Metal oxide nanoparticles, such as titanium dioxide ($TiO_2$), aluminum oxide ($Al_2O_3$), silicon dioxide ($SiO_2$) and zinc oxide (ZnO), have received increased attention due to their widespread in industrial, medical and military applications [9]. They could have detrimental effects on ecosystems through their interactions with existing environmental contaminants [10]. Toxicology studies have shown the possibility of adverse effects on the immune system, oxidative stress-related disorders, and diseases such as cancer (tumor formation). However, the doses needed to produce these effects are generally high and it remains to be seen whether such exposure is possible via the environment (food, water or air) or at the workplace [11,12]. To avoid health hazards or any type of risk that these metal oxides nanoparticles could pose, it is essential to remove them from wastewater before disposal into water bodies.

To solve these new environmental challenges, several researches are being conducted to optimize the efficiency of remediation techniques [8]. Of these techniques, bioremediation techniques using microorganisms were also reported and are now the preferred process due to their eco-friendliness, efficiency, cost-effectiveness [3]. Studies on microorganisms such as halophiles are increasingly being done to gain a better understanding of their characteristics and with the ultimate aim of utilizing them in various applications [1,13]. Nevertheless, moderate halophiles have received little attention and that despite the number of questions related to their adaptability to a wide range of salinities, degradation, and transformation of pollutants. In the process of biological treatment of highly polluted wastewater, it has been demonstrated that the moderately halophilic bacteria have the potential to degrade large organic compounds [13]. The aim of this study was firstly to assess the bacterial community of the saline water collected from the eMalahleni Water Reclamation Plant in South Africa and secondly to establish the optimum growth conditions for the moderately halophilic bacteria isolated from brine samples of, with a view to being further explored for the bioremediation of nanoparticles in wastewater treatment. Lastly, this study determined the feasibility of employing moderately halophilic bacterial isolates for the bioremediation of wastewater contaminated with ZnO and titanium dioxide nanoparticles.

## 2. Material and methods

### 2.1. Site description and sample collection

Brine samples were collected from the 14 different sampling points of eMalahleni Water Reclamation Plant at eMalahleni (Fig. 1), which is located in Mpumalanga Province, South Africa (S 25°56′41.4, E 29°11′67.0). All samples were collected in sterile bottles and were kept in a cooler box and transported to the laboratory for analysis within 2 h after collection. It is important to mention that the experimental study was carried out in two phases, which included: (i) molecular profiling of 16S ribosomal ribonucleic acid (rRNA) gene sequences of halophilic bacterial communities in samples from all 14 sampling points and (ii) the phenotypic characterization of isolates and bioremediation assessment.

### 2.2. Physicochemical profile analysis of the brine samples

Except for the pH, temperature, and salinity that were analyzed in all 14 sampling points, the brine samples were further analyzed in terms of other chemical parameters (Fig. 1). The collected brine samples were allowed to settle for 2 h and thereafter filtered using Whatman Filter Paper No. 1 at ambient temperature and under strict aseptic conditions. The physicochemical parameters of the samples such as pH, dissolved oxygen (DO) and salinity were analyzed on-site using a pH probe (Model: PHC101, HACH, Germany) and DO probe (Model: LDO, HACH, Germany), respectively. Other parameters such as chemical constituents (metals, semi-metals, and non-metals) were also assessed from the filtered samples in the laboratory. The metal concentrations of the brine samples were determined using the inductively

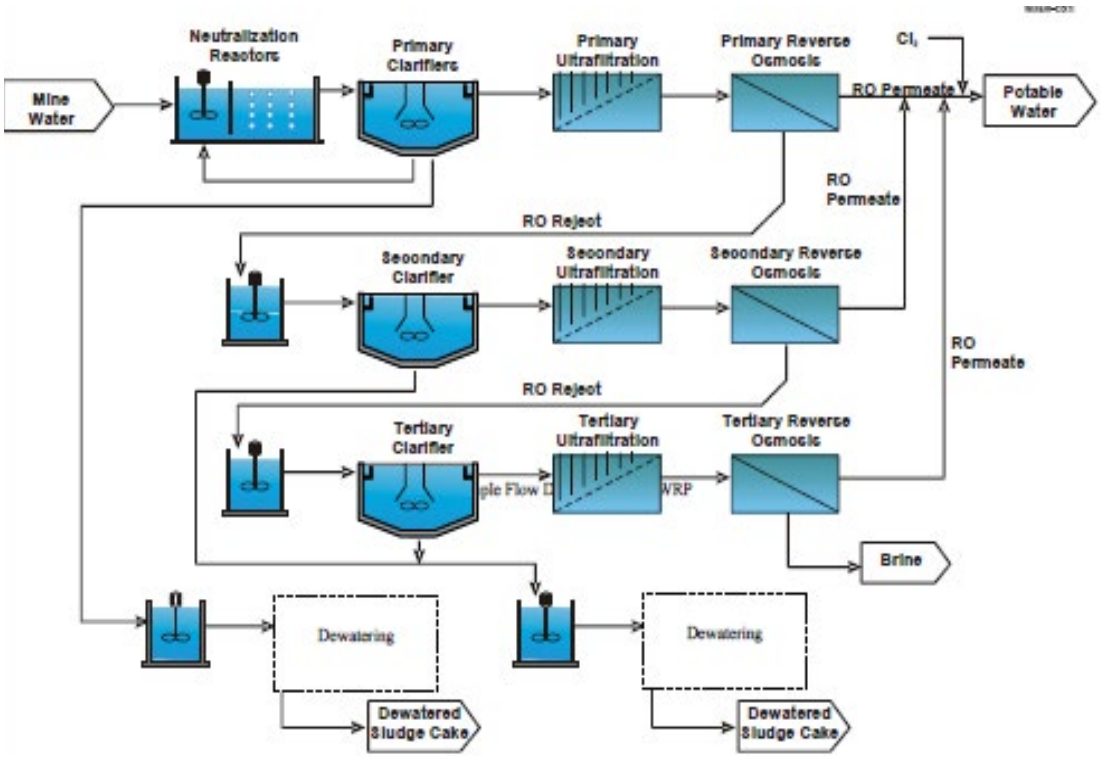


Fig. 1. Schematic representation of sampling points (Reactor A, Reactor B, Clarifier A, Stage II reactor, Stage III reactor, Stage II clarifier, Stage III Clarifier, Brine Dam, Reverse Osmosis (RO) Stage I, Reverse Osmosis Stage II, Reverse Osmosis Stage III) at eMalahleni Water Reclamation Plant, Mpumalanga

coupled plasma optical emission spectrometer (ICP-OES) (AMETEK-Spectro Analytical Instruments GmbH & Co. KG, Germany).

### 2.3. Bacterial profiling in brine samples

#### 2.3.1. Deoxyribonucleic acid extraction

The brine samples (10 mL) were filtered using filter paper with a pore size of 0.45 μm (Whatman Filter Paper No. 1). The harvested pellets containing microorganisms were mixed with 1X PBS. The metagenomic deoxyribonucleic acid (DNA) was extracted independently for all the samples with the ZR Fungal/Bacterial DNA Kit™ (Zymo Research, Pretoria, South Africa) as per the manufacturer's guidelines and finally eluted in 30 μL of ultrapure water. The integrity of the metagenomic DNA was assessed on 0.8% agarose gel. Thereafter, the DNA quantity, quality, and purity were measured using a NanoDrop ND-1000 Spectrophotometer (PeqLab, Germany).

#### 2.3.2. Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplification was performed as described by previous investigators [5,14]. Briefly, the PCR reaction was done on the extracted DNA samples using universal primers 27F [5'AGAGTTTGATCMTGG CTCAG3'] and 518R [5'GTATTACCGCGGCTGCTGG3'] amplifying approximately 500 bp and targeting the variable region V1-V3 of the 16S ribosomal DNA. The PCR reaction mixture consisted of a total volume of 50 μL, which contained 19 μL of nuclease-free water, 25 μL of DreamTaq™ PCR Master Mix (2x) (10 × DreamTaq™ buffer, 2 μM deoxyribonucleotide triphosphate mix and 1.25 U DreamTaq™ polymerase), 2 μL of each PCR primer (10 μM) (synthesised by Inqaba Biotechnical Industries, Pretoria) and 2 μL of genomic DNA (50 ng/μL prepared in a 200 μL PCR tube). The integrity of metagenomic DNA was visualized on 0.8% agarose gel and DNA samples with high purity were then sent to Inqaba Biotech (Pretoria, RSA) for sequence analysis using an Illumina MiSeq platform.

#### 2.3.3. Analysis of sequences

The raw sequence data-set was initially analyzed to remove artificial replicate reads and low-quality reads using ngsShoRT (next-generation sequencing Short Reads Trimmer) as described by [15] for improved downstream analysis. Following this initial process, all sequence reads were processed using the Mothur pipeline [16]. Reads shorter than 50 aligned nucleotides and reads with more than 2% ambiguities, or 7% homopolymers, respectively, were excluded from further processing. Sequences of mitochondrial origin were also excluded from the analysis and chimeric sequences were removed using UCHIME according to the de novo method. Non-chimeric rRNA reads were then aligned against the SILVA 16S rRNA gene database v-128 and a pairwise distance matrix was created from the curated aligned database to group sequences into operational taxonomic units at a

confidence threshold of 97%. Further classification of reads was done at the genus level using the Naïve Bayesian classifier algorithm against the SILVA 16S rRNA gene database with a confidence threshold of 80% to assign taxonomic identity.

### 2.4. Microbial isolation and characterization

#### 2.4.1. Isolation and purification of halophilic bacteria

Isolation and enrichment of halophilic bacteria were performed using halophiles moderate (HM) medium with and without agar. The HM with agar was supplemented with 98 g NaCl, 2 g KCl, 1 g $MgSO_4 \cdot 7H_2O$, 0.36 g $CaCl_2 \cdot 2H_2O$, 0.06 g $NaHCO_3$, 0.24 g NaBr, 1 g $FeCl_3 \cdot 6H_2O$, 10 g bacto tryptone (Difco), 1 g G-glucose and 20 g agar per 100 mL [17]. The media were autoclaved (121°C for 15 min) and thereafter incubated overnight at 37°C. Only media that showed no growth were used. To isolate halophilic bacteria, 1 mL of the brine sample was added to an Erlenmeyer Flasks containing 50 mL of HM liquid medium. After incubation in an orbital shaking incubator (shaking speed of 100 rpm) at 37°C for 30 min, 1 mL of supernatant was added to 9 mL of sterile saline water (0.85%) for serial dilutions (100, $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, … and $10^{-10}$). A 100 μL aliquot of each diluted sample was spread onto HM agar. After incubation at 37°C for 24–48 h, colonies were selected for isolation based on their morphological and physiological features, pigmentation, size, margin, or rate of growth. Colonies were transferred to fresh HM agar plates using the streak plate method. Well-isolated colonies (34) were re-picked and re-streaked on the HM agar plates at least five times to ensure purity of the colonies and then incubated at 37°C for 24–48 h. The isolates were maintained on HM agar slants supplemented with 50% glycerol and stored at –80°C until further use [17].

#### 2.4.2. Characterization and identification of halophilic bacterial isolates

Characterization of each isolate was based on morphological, physiological and biochemical tests. Colonies on respective HM agar plates were examined based on their color, form, catalase and oxidase tests. Gram-staining tests and electron microscopy (Hitachi, Japan) were conducted according to [18], while physiological test in terms of the growth of halophilic bacteria was determined on halophilic medium (with 10% salt concentration (w/v)) at 37°C and pH 7 [17]. Biochemical profiles for isolates were also generated using analytical profile index (API) 20E/20NE strips (BioMérieux Inc., Durham, NC). All API 20E/20NE tests were performed as per the manufacturer's instructions and profile numbers were determined after 24 h of incubation: Tests were performed in duplicates throughout the study.

#### 2.4.3. Phylogenetic analysis of the selected isolates based on the 16S rRNA gene sequences

After morphological, physiological and biochemical tests, five distinct isolates were determined and coded as H5, H8, H12, H15, and H28. Test isolates were further identified using molecular characterization. Bacterial genetic material (DNA) was extracted and amplified using 16S rRNA gene universal primers (27F/518R) and PCR amplification of target genes was done as stated above. The concentrated DNA samples were then stored at −20°C and dispatched to Inqaba Biotechnical Industries (Pretoria, South Africa) for sequencing. To carry out the phylogenetic analysis, the partial 16S rRNA gene sequences of the target isolates were blasted and their closely related strain was determined (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

### 2.5. Assessment of bacterial optimum growth conditions in HM broth culture media

The study was conducted in laboratory batch reactors, which consisted of 250 mL Erlenmeyer Flasks containing 100 mL of sterile HM broth culture media. Each laboratory batch reactor was inoculated with low initial bacterial cell densities (100 cfu/mL) for an individual organism. To obtain their optimum growth conditions, halophilic bacteria were separately exposed to various salt concentrations [(0.14, 0.26, 0.51, 0.68, 0.86, 1.28, 1.71, 2.56, 3.4, and 4.27 M) corresponding to (NaCl:0.8%, 1.5%, 4%, 5%, 7.5%, 10%, 15%, 20%, and 25% (w/v)], different pH levels (5–10, at a gradual scale of 1), varying temperature (20°C–40°C), various shaking speeds for aeration (40–120 rpm) and assorted carbon sources [10–50 g/L of d-glucose, fructose, sucrose, acetic acid corresponding to 1%–5% (w/v)]. The pH of the HM broth was adjusted using 1.0 M HCl and 1.0 M potassium hydroxide (Merck, SA). For each experimental study, all analyses were performed in triplicate.

To evaluate the behavior of each isolate based on each of the operating conditions, aliquot samples were taken every day for five consecutive days. The growth of halophilic bacteria and their growth rates were monitored and calculated as stated below. The isolates were incubated in a shaking incubator (Lasec, Pretoria, SA) at a specific agitation speed and temperature and separately supplemented with a specific carbon source according to [17]. The optical density ($OD_{600}$) values were converted to cfu/mL using a factor previously determined from a calibration curve relating to the biomass of the isolates as described by [19]:

$$Y = mx + C \tag{1}$$

where $Y$ = is the absorbance at 600 nm; $m$ = constant value of the gradient of the curve (0.3797); $x$ = the actual biomass of the bacterial isolates; $C$ = the $y$-intercept of the curve (0.000).

The growth rate (μ) of the test halophilic bacteria was calculated by using the following mathematical expression:

$$\mu = \left(\ln X - \ln X_0\right) \times \left(t - t_0\right)^{-1} \tag{2}$$

where the values $X$ and $X_0$ are the concentrations of cultured microorganisms at times $t$ and $t_0$, respectively.

### 2.6. Bioremediation of ZnO and $TiO_2$ nanoparticle

The experimental study series to determine bioremediation potential were conducted in separate batch reactors,

which consisted of 250 mL Erlenmeyer Flasks containing 100 mL of the culture media. To prepare the culture media, only the screened domestic wastewater samples containing very low Zn and Ti concentrations (<1 mg/L) were considered for the experimental studies. The following optimum conditions, which allowed the growth of the moderately halophilic bacteria, were considered: 4% NaCl, 2% sucrose, $MgSO_4 \cdot 7H_2O$ (0.5 g/L) and $KNO_3$ (0.18 g/L). They were added to serve as a carbon source and nutrient supplement for the culture media. The culture media were also supplemented with nano-zinc oxide (nZnO) or nano-titanium dioxide ($nTiO_2$) at various concentrations (of 1, 5, 10, 20, 50, 100, 150, and 200 mg/L), and the pH was adjusted to pH 7, using 1.0 M HCl and 1.0 M NaOH (Merck, SA). Prior to the experiment, overnight fresh cultures of halophilic bacterial isolates were prepared, and each flask was aseptically inoculated with an initial concentration of approximately $10^2$ cfu/mL. Two supplementary culture media were set up as negative (culture media with nanoparticles only) and positive controls (culture media free of nanoparticles but inoculated with specific moderately halophilic bacterial isolates). All the batch reactors for the bioremediation study as well as the controls were incubated in an incubator with a shaking speed set at 100 rpm and temperature at 30°C. Aliquot samples were taken every day for 5 d to evaluate the ability of each of the isolates to remove test nanoparticles. The aliquot samples were filtered using 0.45 μm syringe filters and the concentrations of nZnO and $nTiO_2$ were determined using the ICP-OES. Other physicochemical parameters such as pH, temperature, chemical oxygen demand, DO, nitrate and phosphate were also determined as stated above to identify whether they influence the bioremediation efficiency of halophilic bacterial isolates. All analyses were performed in triplicate. The growth performance of each bacterial isolate was evaluated by measuring changes in optical density at 600 nm with a spectrophotometer (HACH, Loveland, CO, USA). The bacterial counts were calculated using Eq. (1). It should be mentioned that nZnO or $nTiO_2$ purchased from Sigma-Aldrich (Johannesburg, South Africa) were characterized before usage in terms of size distribution, morphology, particle size and chemical composition using high resolutions transmission electron microscope images analysis that were performed on a JEOL-JEM 2100 operating at accelerating voltage of 100 KV (JEOL, Japan). The transmission electron microscopy (TEM) images revealed that both test NPs had different shapes (rods, hexagon, rectangle, spherical and irregular). The micrographs analysis obtained showed a lattice structure of crystallinity with average size distribution ranging between 10 and 100 nm as indicated by the vendor.

### 2.7. Statistical analysis

The data were statistically analyzed using STATA V13 statistical software. Regression analysis was performed to compare the average bacterial growth of each bacterial isolate under various growth conditions (NaCl concentrations, pH, temperature, and shaking speed) and the growth rate during each experiment.

## 3. Results

### 3.1. Physicochemical profile of the brine samples

#### 3.1.1. Brine samples collected from brine sampling points

Table 1 illustrates the physicochemical profile of the brine samples collected at the brine sampling points of the eMalahleni Water Reclamation Plant during the study period. Brine samples were highly polluted with various concentrations of metals, metalloids, and non-metals. Calcium (928.42 mg/L) was the metal with the highest concentration, while Si (71.3 mg/L) and S (14,400.00 mg/L) were the metalloid and non-metal with the highest concentrations, respectively. The samples were found to be highly alkaline with pH values ranging between pH 7.44 and 10.69 from the various treatment stages, while the temperature values ranged between 24.78°C and 26.82°C. The samples from different stages also had a salinity ranging from 1.47 to 3.48 g/L as shown in Table 2. The pH values, temperature, and salinity increased as the brine was subjected to various treatments.

#### 3.1.2. Relative abundance of halophilic bacteria in brine samples based on 16S rRNA gene sequences

Prior to sequencing, the integrity of the set of genomic fragments was assessed and purified. For metagenomic studies, the 16S rRNA gene sequences were used to assess the microbial abundance of the brine samples in all the stages of the water reclamation plant. Out of 50,253 sequences

Table 1
Physicochemical profile of the brine samples collected from the brine sampling points of eMalahleni Water Reclamation Plant, South Africa

| Element | B (mg/L) | Ba (mg/L) | Fe (mg/L) | K (mg/L) | Mg (mg/L) |
|---|---|---|---|---|---|
| Mean ± Standard deviation | 2.53 ± 0.08 | 0.07 ± 0.003 | 0.11 ± 0.009 | 175.00 ± 0.02 | 18,600 ± 0.02 |
| Element | Mn (mg/L) | Ni (mg/L) | P (mg/L) | S (mg/L) | Sr (mg/L) |
| Mean ± Standard deviation | 0.11 ± 0.003 | 0.07 ± 0.001 | 0.53 ± 0.006 | 14,400.00 ± 0.04 | 5.71 ± 0.05 |
| Element | Si (mg/L) | Zn (mg/L) | Cu (mg/L) | Co (mg/L) | Na (mg/L) |
| Mean ± Standard deviation | 71.30 ± 0.02 | 0.21 ± 0.003 | 0.02 ± 0.02 | 0.02 ± 0.001 | 104.00 ± 0.06 |
| Element | Ca (mg/L) | pH | DO (mg/L) | Conductivity (mS/cm) | Salinity (g/L) |
| Mean ± Standard deviation | 928.42 ± 0.01 | 9.37 ± 0.03 | 7.93 ± 0.01 | 175.00 ± 0.02 | 186.00 ± 0.02 |

DO: dissolved oxygen.

Table 2
Distribution of the halophilic bacterial isolates based on pH, temperature, and salinity of sampling sites

| Sampling sites | pH | Temperature (°C) | Salinity (g/L) | Halophilic bacterial isolate |
|---|---|---|---|---|
| S I Reactor | 9.44 | 24.78 | 2.11 | H7 |
| S I Reactor | 9.56 | 25.13 | 2.78 | H29 |
| S I Reactor | 9.64 | 25.22 | 2.65 | H30 |
| S I Reactor | 9.62 | 25.11 | 2.55 | H32 |
| S II Reactor | 10.69 | 26.33 | 2.44 | H17 |
| S II Reactor | 10.65 | 25.24 | 2.24 | H24 |
| S III Reactor | 9.88 | 25.22 | 2.21 | H20 |
| Reactor A | 9.56 | 24.89 | 2.04 | H6 |
| Reactor A | 9.53 | 25.51 | 2.09 | H8 |
| Reactor A | 9.59 | 25.61 | 2.56 | H31 |
| Reactor B | 10.38 | 25.22 | 2.70 | H11 |
| Reactor B | 10.44 | 25.36 | 2.67 | H14 |
| Reactor B | 10.34 | 25.04 | 2.61 | H23 |
| S I Clarifier | 9.45 | 25.09 | 2.12 | H25 |
| S I Clarifier | 10.60 | 25.21 | 2.11 | H28 |
| S II Clarifier | 10.58 | 25.53 | 2.35 | H16 |
| S II Clarifier | 10.67 | 25.33 | 2.22 | H27 |
| S III Clarifier | 10.46 | 25.7 | 2.34 | H1 |
| S III Clarifier | 10.44 | 26.20 | 2.17 | H4 |
| Clarifier A | 10.15 | 25.41 | 2.49 | H19 |
| Clarifier A | 10.11 | 25.30 | 2.34 | H34 |
| Clarifier B | 9.79 | 25.01 | 2.12 | H3 |
| Clarifier B | 9.69 | 26.01 | 2.45 | H12 |
| Clarifier B | 9.77 | 26.14 | 2.23 | H26 |
| S I R O | 9.1 | 26.31 | 1.82 | H2 |
| S I R O | 9.3 | 25.41 | 1.78 | H9 |
| S I R O | 9.3 | 25.00 | 1.93 | H15 |
| S II R O | 8.79 | 25.34 | 1.68 | H13 |
| S III R O | 8.82 | 25.42 | 1.47 | H21 |
| S III R O | 8.84 | 26.82 | 1.53 | H5 |
| S III RO | 8.88 | 25.14 | 1.62 | H10 |
| Brine Dam I | 7.58 | 24.88 | 3.38 | H18 |
| Brine Dam I | 7.44 | 24.89 | 3.28 | H22 |
| Brine Dam II | 7.52 | 25.54 | 3.48 | H33 |

obtained from the collected samples, 27,267 valid sequences were obtained after the removal of low reads (shorter than 50 aligned nucleotides) and only sequences with more than 97% similarity were considered. After the assignment of the sequences to a taxonomic identity using the Naïve Bayesian classifier, the relative abundance of each taxon from the samples was determined by plotting the number of specific taxonomic sequences against the total number of valid sequences used. The brine samples appeared to have a diverse microbial population with a total of 35 genera (47% of the total population) excluding the uncultured bacteria.

The putative classification of the collected brine samples was consistent with microorganisms belonging to *Halomonas* (8.48%), *Nesterenkonia* (6.29%), *Pseudomonas* (4.75%), *Serratia* (4.15%), *Shewanella* (3.64%), *Burkholderia* (2.47%), *Thauera* (2.14%), *Delftia* (2.18%), *Arthrospira* (2.18%), *Propionibacterium* (0.83%), *Staphylococcus* (0.8%), *Variovorax* (0.7%), Flavobacterium (0.64%), *Aeribacillus* (0.64%), *Stenotrophomonas* (0.55%), *Actinoplanes* (0.5%), *Klebsiella* (0.5%), *Morganella* (0.5%), *Alicycliphilus* (0.41%), *Geobacillus* (0.35%), *Clavibacter* (0.35%), *Streptomyces* (0.35%), *Thioflavicoccus* (0.35%), *Raoultella* (0.32%), *Lysinibacillus* (0.32%), *Thioalkalivibrio* (0.32%), *Cupriavidus* (0.28%), *Ralstonia* (0.28%), *Laribacter* (0.28%), *Bacillus* (0.23%), *Rubrivivax* (0.23%), *Halothiobacillus* (0.23%), *Paenibacillus* (0.23%), *Arthrobacter* (0.23%), *Micrococcus* (0.23%), *Aeromonas* (0.07%) and uncultured bacteria (53%) (Fig. 2).

#### *3.1.3. Distribution of the halophilic bacterial isolates based on pH, temperature, and salinity of sampling sites*

Using culture-based methods, a total of 34 halophilic bacteria were isolated from brine samples of the different sampling sites of the eMalahleni Water Reclamation Plant. A summary of the distribution of halophilic bacterial isolates is given in Table 2. A large number of different isolates (4) was observed in Stage I Reactor, and a total of three halophilic bacterial strains were isolated from each of the following stages: Stage I and III Reverse Osmosis, Reactors A and B, and Clarifier B. At least one or two halophilic bacterial isolates were identified in the remaining stages. All the bacteria isolated in Stage I Reactor were present under the following conditions: pH ranging between 9.44 and 9.62, temperature ranging between 24.78°C and 25.22°C, salinity ranging between 2.13 and 2.78 g/L, followed by Stage I and III Reverse Osmosis with a pH ranging from 9.1–9.3 and 8.82–8.88, respectively; temperature ranges of 25.00°C–26.31°C and 25.14°C–26.82°C, respectively, and salinity ranges of 1.78–1.93 g/L and 1.47–1.62 g/L, respectively. In Reactors A and B, the following conditions were observed: pH 9.53–9.59 and 10.34–10.44, respectively, temperature ranges of 24.89°C–25.61°C and 25.04°C–25.36°C, respectively, and salinity ranges of 1.78–1.93 and 1.47–1.62 g/L, respectively. In Clarifier B, a pH ranging between 9.69 and 9.79 was observed, with a temperature range of 25.01°C–26.14°C, and salinity ranging between 2.12–2.45 g/L. The highest number of bacterial isolates were observed in Reactors A and B and Clarifier B. Of the 34 bacterial isolates, a total of 14 strains were successfully isolated based on their macroscopic characteristics and identified by streaking a nutrient agar plate and repeating the number of streaks to obtain pure colonies. To gain an understanding of the behavior of halophilic bacteria in extreme saline conditions, 5 isolates were randomly selected at various stages for further characterization. These random isolates were coded as H5, H8, H12, H15, and H28 based on the high diversity of bacteria present in the brine presumed to be halophiles (H). It should be noted that the samples were collected from different sites in the same sampling areas.

#### *3.1.4. Molecular identification of selected culturable halophilic bacterial isolates*

Selected isolates were subjected to molecular identification and phylogenetic analysis. Sequences were compared to those deposited in the public database using the

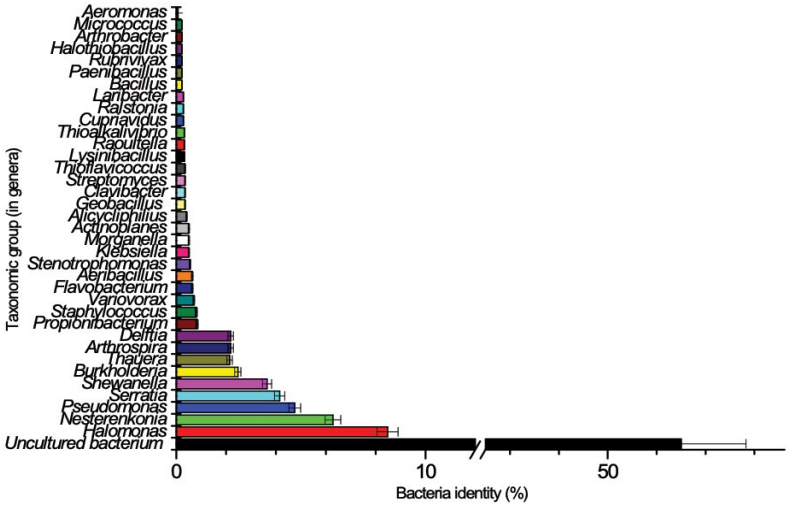


Fig. 2. Microbial abundance (at genus level) of brine samples.

Basic Local Alignment Search Tool program. Phylogenetic analysis using the neighbor-joining method with 100 bootstrap replicates indicated that the H5, H8, H12, H15, and H28 isolates belonged to the genera *Serratia*, *Bacillus*, *Morganella*, *Citrobacter*, and *Lysinibacillus*, respectively (Table 3). The isolated bacterial species were most closely related to *Serratia* spp. INBio 4041 [KM242484.1], *Bacillus cereus* strain CASA51-1 [HQ179148.1], *Morganella morganii* strain AP28 [DQ358125.1], *Citrobacter freundii* strain C09 [KM222617.1], and *Lysinibacillus* spp. NOSK [KM241862.1] at a 16S rRNA gene sequence similarity of approximately 98%, 80%, 99%, 99%, and 100%, respectively (Table 3). The isolated bacterium H8 was found to have the lowest similarity (80%) when compared to the other isolates.

### *3.2. Determination of optimum growth conditions of the halophilic bacteria*

#### *3.2.1. Effect of NaCl concentration on growth rate*

As halophilic bacteria required sodium ions for their growth, the present study assessed the salt tolerance of the isolated microbial species (Fig. 3). In general, the results revealed that bacterial isolates could grow in the presence of NaCl concentrations ranging between 0.8% and 25%, with higher growth observed when exposed to a range of 0.8% and 10% regardless of the organism. The best growth for each of the halophilic bacteria was noted when exposed to 4% NaCl on the 4th day of exposure irrespective of the organism. Although H28 and H15 showed a higher growth starting from day 3 compared to the other target isolates, no significant difference was observed ($p > 0.005$). Nevertheless, the growth of the bacterial isolates appeared to be inversely proportional to NaCl concentrations. At an NaCl concentration of 4%, the growth of halophilic bacteria was observed to be above $1 \times 10^9$ cfu/mL after 4 d of exposure, in the following order, from highest to lowest: H5 ($1.99 \times 10^{10}$ cfu/mL) > H8 ($1.98 \times 10^{10}$ cfu/mL) > H12 ($1.95 \times 10^{10}$ cfu/mL) > H28 ($1.94 \times 10^{10}$ cfu/mL) = H15 ($1.94 \times 10^{10}$ cfu/mL).

It should be mentioned that the decline in the growth rate of halophilic bacterial isolates was noted from 15% NaCl for all isolates. The isolates H5 and H8 demonstrated the highest growth rate (4.751 and 4.750 $d^{-1}$, respectively), whereas H28 had the lowest growth rate (4.745 $d^{-1}$) compared to the rest of the selected halophiles. The results of regression analyses comparing the bacterial growth at 4% NaCl showed no significant difference ($p > 0.05$) between all test isolates. However, all the isolates showed a significant difference over time ($p < 0.05$).

#### *3.2.2. Effect of pH on growth rate*

Since the pH of the culture media is among the vital factors that govern cell growth, the present study investigated the

Table 3
BLAST results from raw data (non-aligned)

| Name of isolates | Primers | Predicted organism | GenBank accession number | Similarity |
|---|---|---|---|---|
| H5 | 27F/518R | *Serratia* sp. | KM242484.1 | 98% |
| H8 | 27F/518R | *Bacillus* sp. | HQ179148.1 | 80% |
| H12 | 27F/518R | *Morganella* sp. | DQ358125.1 | 99% |
| H15 | 27F/518R | *Citrobacter* sp. | KM222617.1 | 99% |
| H28 | 27F/518R | *Lysinibacillus* sp. | KM241862.1 | 100% |

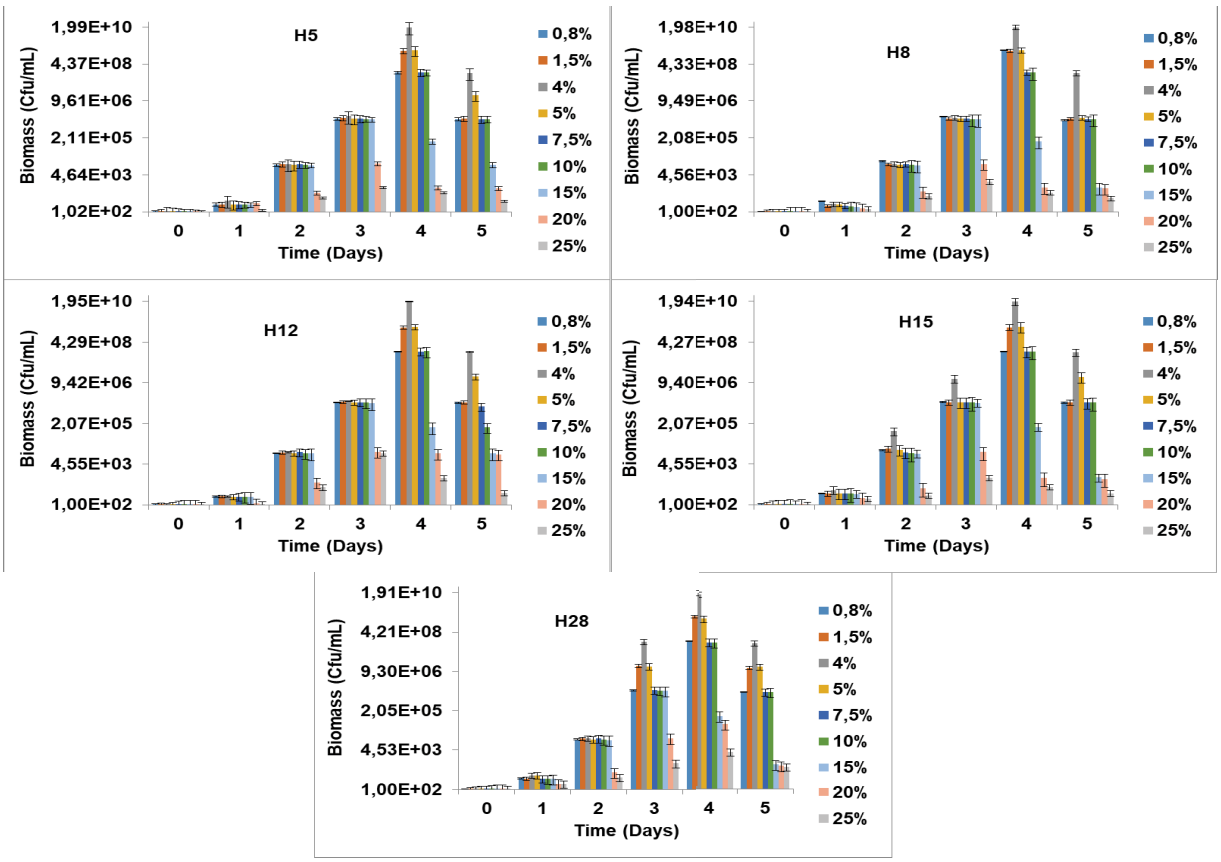


Fig. 3. Optimum growth conditions for the halophilic bacteria with NaCl at different concentrations (0.8%–25%).

optimum pH of each halophilic bacterial isolate by varying the pH value from 5 to 10 (Fig. 4).

In general, the bacterial isolates showed growth at all pH values from 5 to 10. The optimum pH for each isolate was observed to be at pH 7 with the highest growth rate observed at (4.736 $d^{-1}$) and (4.772 $d^{-1}$), respectively. Fig. 4 shows the highest bacterial growth at pH 7 on day 4, although statistically, the difference between pH values 6, 7 and 8 was not significant. At this exposure period and pH 7, the growth of halophilic bacteria was observed to be above $10^{10}$ cfu/mL, in the following order, from highest to lowest: H8 ($1.96 \times 10^{10}$ cfu/mL) > H28 ($1.94 \times 10^{10}$ cfu/mL) > H5 ($1.92 \times 10^{10}$ cfu/mL) > H15 ($1.92 \times 10^{10}$ cfu/mL) > H12 ($1.90 \times 10^{10}$ cfu/mL). Slow growth was noted at pH 9 and pH 10 for all isolates, while H28 was the only isolate with low growth compared to the rest of the isolates observed on day 2 at pH 9–10. Regardless of the slow growth noted at pH 9 and pH 10, no significant difference was observed between the days of exposure for all isolates. However, when considering isolates individually, significant differences ($p < 0.05$) were noted from the first day to the 5th day of the experiment.

### 3.2.3. Effect of temperature on growth rate

The investigation on the effect of temperature was carried out by inoculating isolates into media containing optimum conditions of NaCl and pH and then incubated at various temperatures ranging from 20°C to 40°C. Growth of the isolated moderate halophilic bacteria was observed throughout the experiment (Fig. 5). The bacterial isolates showed a significant growth rate at temperatures ranging from 27.5°C and 40°C from the second day of the experiment. The highest optimum growth rate was observed at 30°C on the 4th day (2.274 $d^{-1}$) and the lowest growth observed (2.864 $d^{-1}$) at 40°C. Under these conditions, the highest counts for all halophilic bacteria were observed to be above $10^9$ cfu/mL, in the following order, from highest to lowest: H28 ($1.93 \times 10^9$ cfu/mL) > H8 ($1.92 \times 10^9$ cfu/mL) > H12 ($1.88 \times 10^9$ cfu/mL) > H15 ($1.80 \times 10^9$ cfu/mL) > H5 ($1.53 \times 10^9$ cfu/mL). The experiment revealed bacterial isolates to be significantly different in terms of the effects of temperature ($p < 0.05$). The test moderately halophilic isolates showed the ability to progressively grow over a wide range of temperatures making them suitable for biotechnological use (Fig. 5).

### 3.2.4. Effect of carbon source on growth rate of halophilic isolates

During this study, 4 different carbon sources namely glucose, fructose, sucrose, and acetic acid were used (1%–5%) (1–5 g/100 mL). Overall, all the halophilic isolates grew in the presence of all carbon sources, but sucrose was found to be the best carbon source regardless of the concentrations (Figs. 6a–d). It was observed that H5, H8, and H28 had the highest growth when exposed to 1%–3% sucrose from day 2

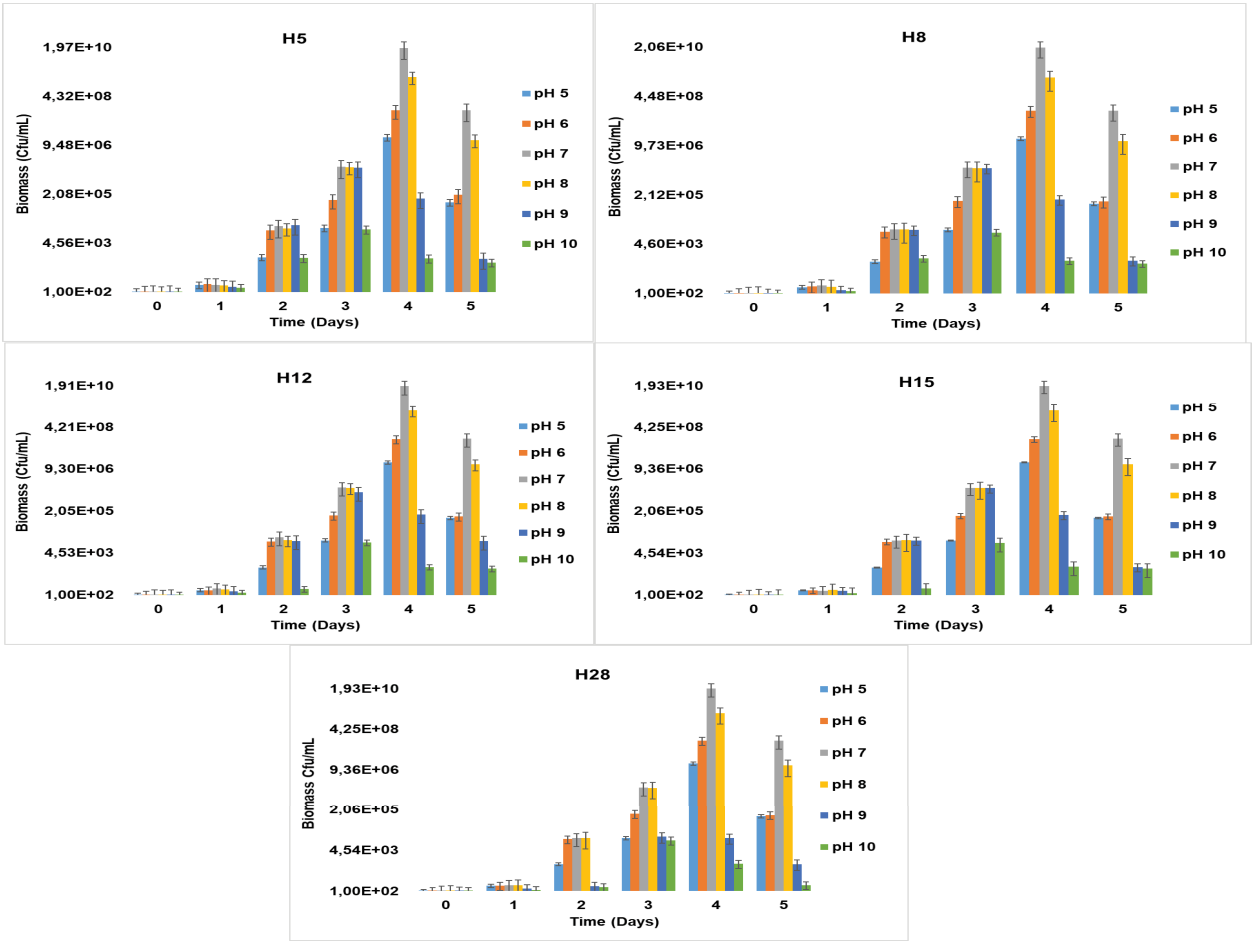


Fig. 4. Optimum growth conditions for halophilic bacteria with pH at different scales (5–10).

(1.939–4.678 $d^{-1}$), while for H12 and H15 the best growth was noted at 1%–4% sucrose from day 2 (1.923–4.617 $d^{-1}$). In spite of these differences, the highest growth of all the moderately halophilic bacteria occurred at 2% sucrose and their cell counts were found to be in the following order, from highest to lowest: H5 ($7.98 \times 10^{10}$ cfu/mL) > H12 ($3.93 \times 10^{10}$ cfu/mL) > H8 ($1.97 \times 10^{10}$ cfu/mL) > H15 ($1.96 \times 10^{10}$ cfu/mL) > H28 ($1.92 \times 10^{10}$ cfu/mL) (Fig. 6a).

Glucose appeared to be the second most suitable carbon source for the isolates with a gradual bacterial exponential growth observed up to day 4, regardless of its concentrations, and thereafter a decline in growth occurred on day 5. The highest bacterial growth rates were observed when isolates were exposed to the following glucose concentrations: 1%–3% glucose for H5 (0.957–2.422 $d^{-1}$); 1%–4% for H8, H12, and H28 (0.845–2.411 $d^{-1}$); and 1%–5% (1–5 g/100 mL) for H15 (0.957–1.870 $d^{-1}$). The bacterial counts were observed to be in the following order, from highest to lowest: H5 ($2.94 \times 10^{8}$ cfu/mL) > H8 ($1.94 \times 10^{8}$ cfu/mL) > H15 ($1.93 \times 10^{8}$ cfu/mL) > H28 ($1.92 \times 10^{8}$ cfu/mL) > H12 ($1.92 \times 10^{8}$ cfu/mL) (Fig. 6a).

Fructose was the third most suitable carbon source and all the isolated halophiles could grow in the presence of fructose, with an optimum ranging between 1%–3% throughout the experiment. A stationary phase was observed on day 4 and a lower response observed on day 5. The highest positive response varied from one percentage to another; with H5 and H8 being the isolates with the highest response observed at 1% and 4% fructose and the lowest at 1% and 2% (Fig. 6b). The lowest growth rates were observed with H28 at 1% fructose and with H15 at 4% and 5%. The best growth rate ranged from 0.968 to 2.380 $d^{-1}$, when H5, H8, H12, and H15 were exposed to fructose concentrations ranging from 1% and 3% and the lowest observed with H28 exposed to 1%–2% (0.959–2.366 $d^{-1}$) (Fig. 6). Their bacterial counts were observed in the following order, from highest to lowest: H5 ($1.55 \times 10^{5}$ cfu/mL) > H8 ($1.51 \times 10^{5}$ cfu/mL) > H12 ($1.50 \times 10^{5}$ cfu/mL) > H15 ($1.49 \times 10^{5}$ cfu/mL) > H28 ($1.47 \times 10^{5}$ cfu/mL). For all halophilic bacteria, a gradual increase in growth occurred when exposed to all concentrations (1%–5%) with a stationary phase observed on day 4 and a decline observed on day 5.

Halophilic isolates were found to show the lowest growth response when acetic acid was used as an external carbon source. Irrespective of the acetic acid concentrations, a gradual increase in halophilic bacterial growth was noted up to day 4 for H5 and H8, and up to day 3 for H12, H15, and H 28. However, an overall decline in bacterial counts occurred with an increase in acetic acid concentrations (Fig. 6c). The optimum acetic acid concentrations that triggered the highest bacterial growth rates were as follows: 1% for H5, H8, and H15 (0.477–1.451 $d^{-1}$); 1% and 2% for H12 (0.513–1.439 $d^{-1}$), finally

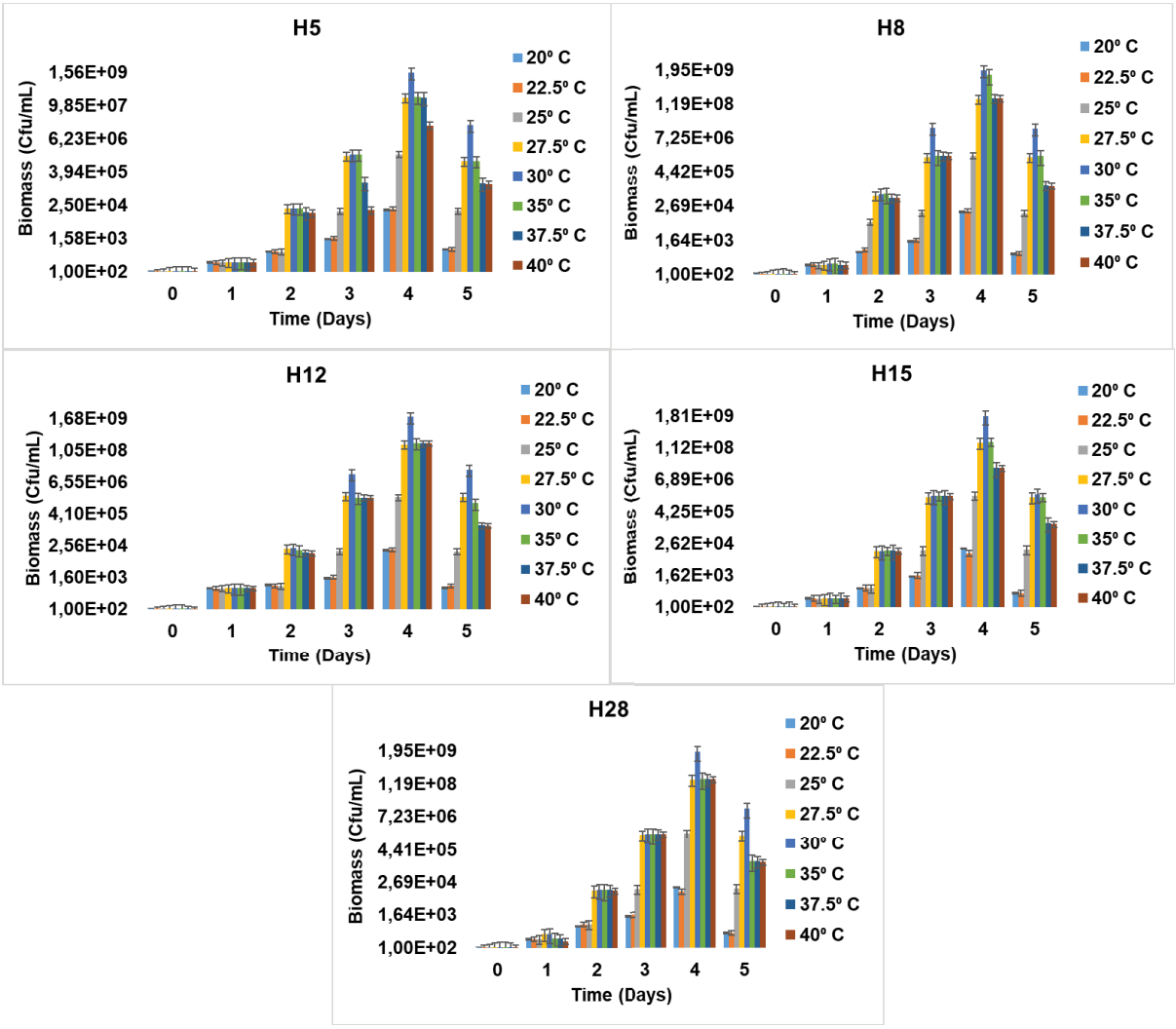


Fig. 5. Optimum growth conditions for halophilic bacteria with temperature at different ranges (20°C–40°C).

1%–4% for H28 (0.478–1.392 $d^{-1}$). For all halophilic bacteria, the lowest growth response occurred at 5% acetic acid as a source of carbon. The bacterial counts were observed in the following order, from highest to lowest: H15 ($6.88 \times 10^3$ cfu/mL) > H8 ($4.35 \times 10^3$ cfu/mL) > H12 ($4.18 \times 10^3$ cfu/mL) > H28 ($3.56 \times 10^3$ cfu/mL) > H5 ($2.38 \times 10^3$ cfu/mL).

Regardless of the suitability of sucrose compared to the other selected carbon sources, statistically, there were no significant differences in bacterial growth over the type of carbon sources in the culture media ($p > 0.05$). When comparing the effect of carbon source on individual bacterial isolates, isolates showed a significant difference ($p < 0.05$) in terms of growth between 1% carbon source and others for all types, except for the acetic acid where no significant difference was observed over variation of concentrations in the culture media ($p > 0.05$).

### 3.2.5. Effect of aeration on growth rate

The effect of aeration (oxygen level) on the growth rate was determined by increasing the agitation speed of the shaking incubator (Fig. 7). The moderately halophilic bacteria were able to grow at shaking speeds of between 40 and 120 rpm with optimum growth generally observed at 100 rpm on day 4, whereas a shaking speed of 40 rpm was found to result in the lowest growth rate. In other words, the growth rate of the halophilic isolate was directly proportional to the increase in the shaking speed of up to 100 rpm, while a decrease in growth was noted at a shaking speed of 120 rpm, regardless of the exposure period. The highest bacterial growth rates at a shaking speed of 100 rpm on day 4 were as follows: H5 (2.402 $d^{-1}$), H8 (2.401 $d^{-1}$), H12 (2.405 $d^{-1}$), H 15 (2.407 $d^{-1}$), and for H28 (2.403 $d^{-1}$). However, statistical analysis showed no significant difference ($p > 0.05$) in the bacterial growth at 100 rpm, but a significant difference was observed with the bacterial isolates at different exposure times ($p < 0.05$). After an exposure period of 4 d and at a shaking speed of 100 rpm, the growth of halophilic bacteria was observed to be in the following order, from highest to lowest: H5 ($1.96 \times 10^{10}$ cfu/mL) > H28 ($1.92 \times 10^{10}$ cfu/mL) > H12 ($1.92 \times 10^{10}$ cfu/mL) > H8 ($1.14 \times 10^{10}$ cfu/mL) > H15 ($1.94 \times 10^9$ cfu/mL). Moreover, on individual isolates, statistical analyses revealed significant

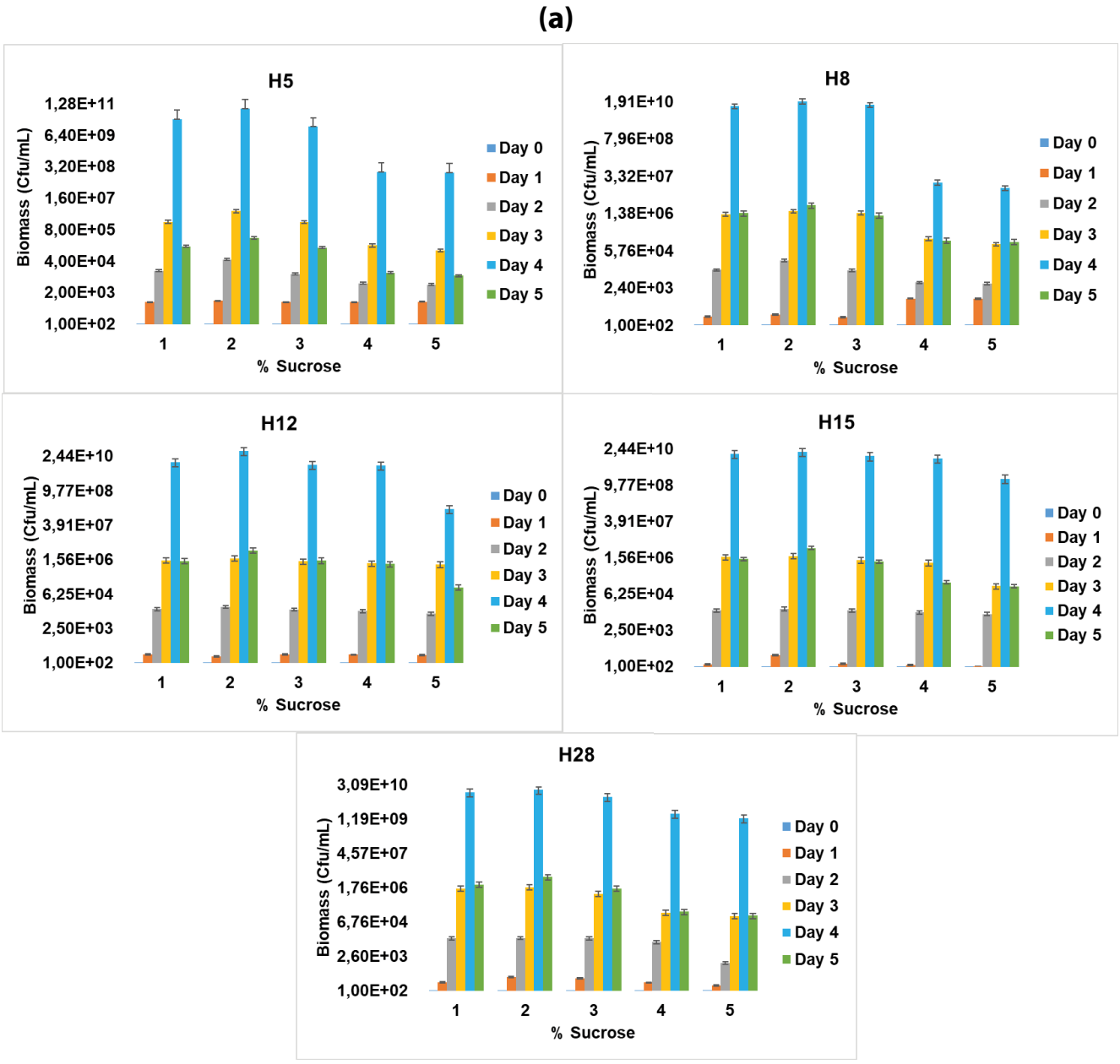


Fig. 6. *Continued*

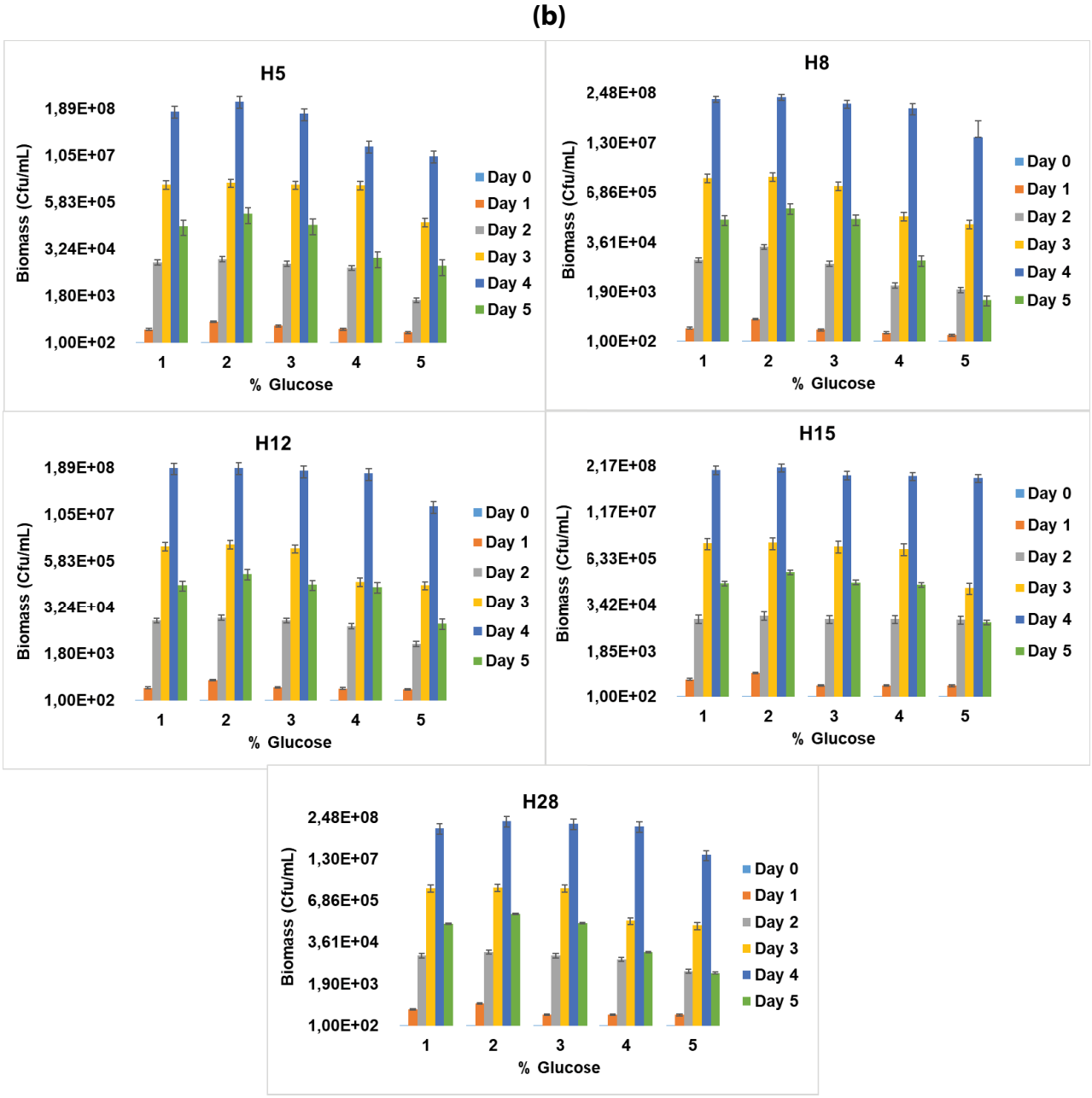


Fig. 6. *Continued*

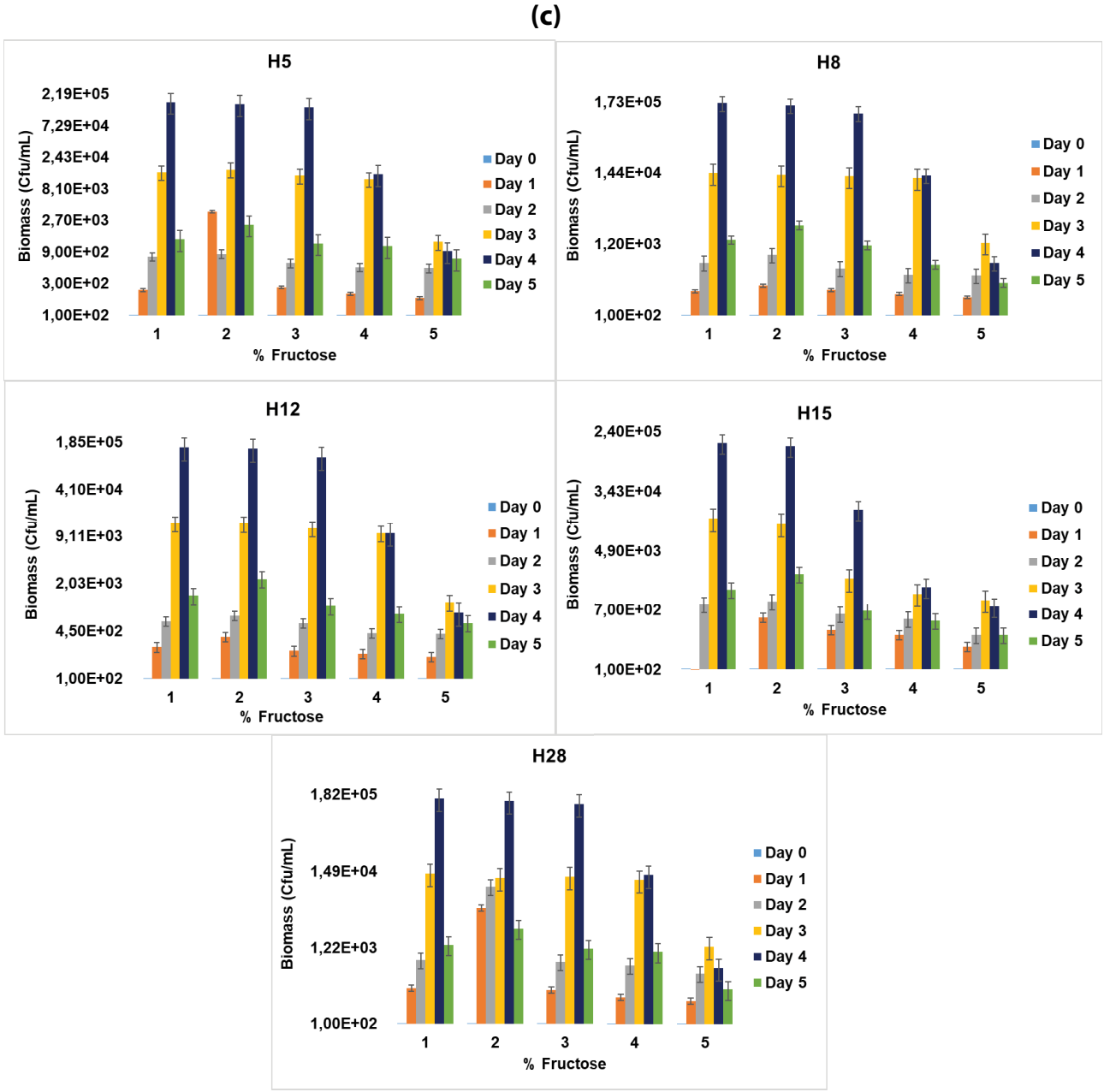


Fig. 6. *Continued*

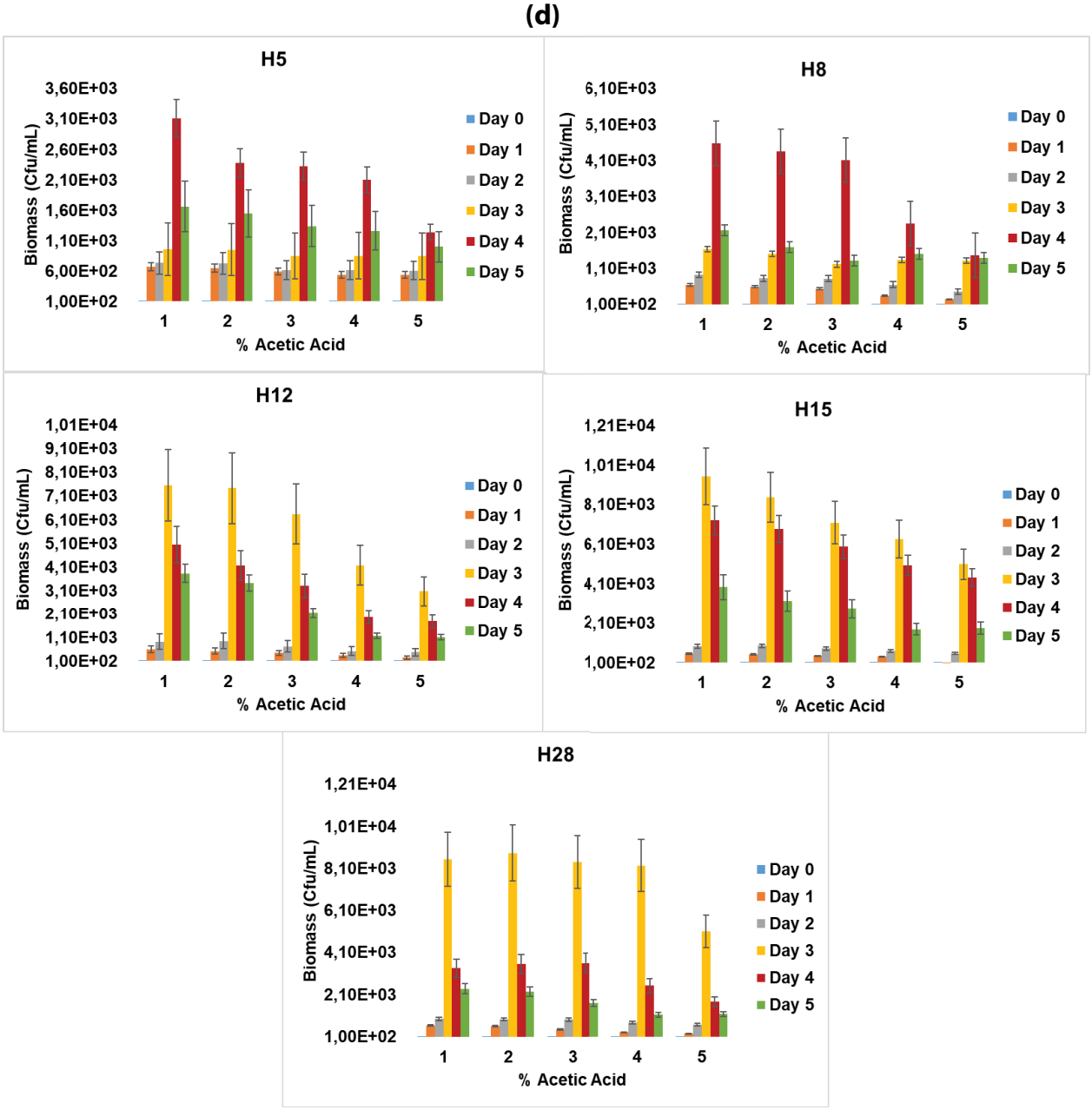


Fig. 6. Optimum growth conditions for target halophilic bacteria in (a) sucrose, (b) glucose, (c) fructose, and (d) acetic acid (1%–5%) carbon source.

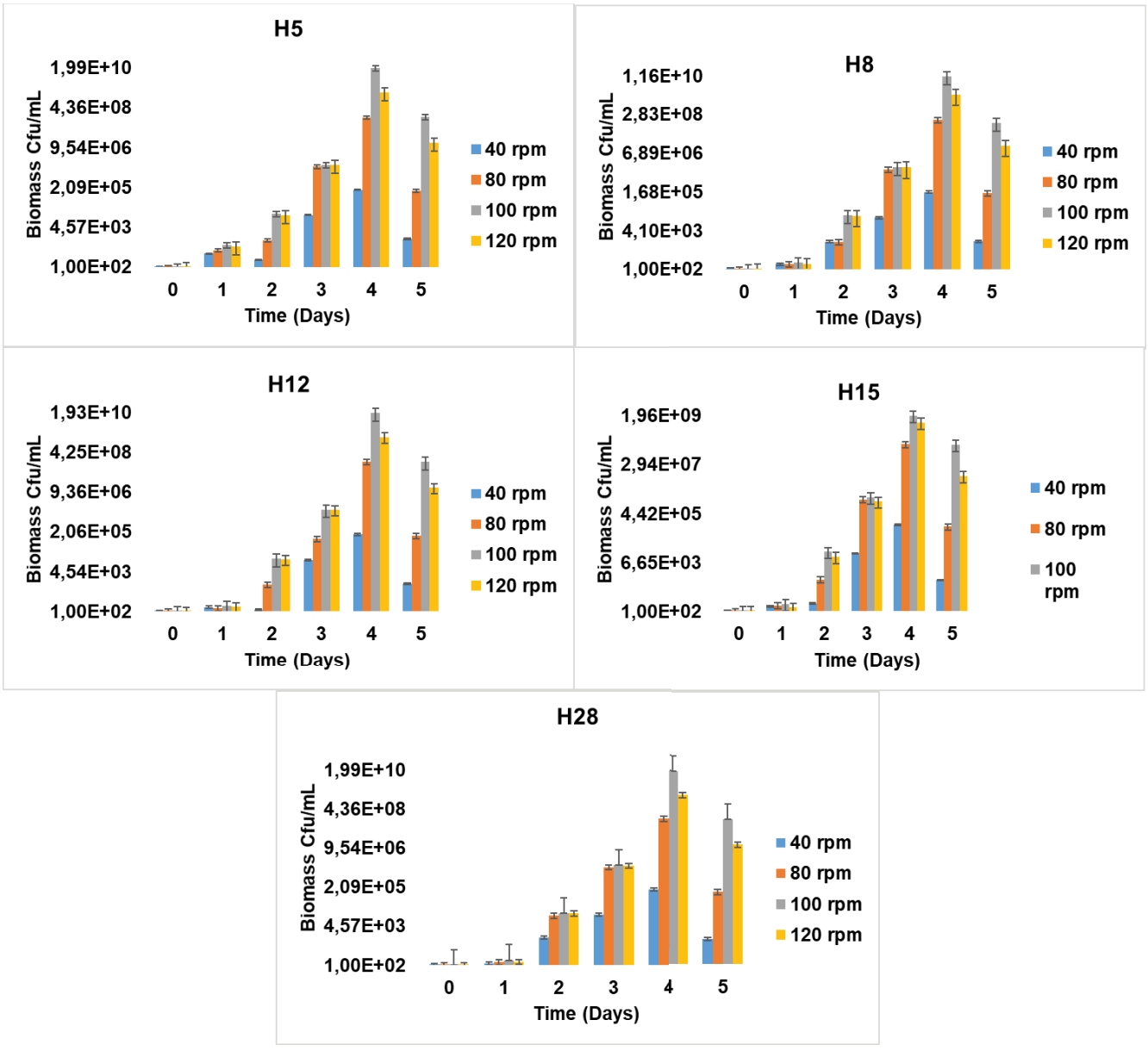


Fig. 7. Optimum growth conditions for the halophilic bacteria with varying shaker agitation speeds (40–120 rpm).

differences between shaking speeds ($p < 0.05$) except isolate H15 that showed no significant difference ($p > 0.05$) between growth in culture media at a shaking speed of 80 and 100 rpm.

### 3.3. Assessment of percentage removal nZnO, $nTiO_2$, and other chemical constituents

Regardless of the toxic effect of both nZnO and $nTiO_2$ on selected halophilic bacteria, the present study further investigated the removal of these metal oxide nanoparticles (Figs. 8a and b) as well as other chemical constituents from wastewater mixed liquor under optimum conditions (4% NaCl, pH 7, 30°C, 2% sucrose, 100 rpm). In general, the initial concentrations of the Zn and Ti in wastewater from ICP results were very low and approximately 0.417 and 0.987 mg/L, respectively (Tables 4 and 5). The overall efficiency of the isolates in removing metal oxide nanoparticles from wastewater mixed liquor decreased as the concentrations of either nZnO or $nTiO_2$ increased over the time of exposure.

In the presence of nZnO, a decrease in bacterial removal efficiency ranging from 100% to 43% was observed with an increased concentration of nZnO in the medium during the exposure time. For individual bacteria, *Bacillus* sp. (100%–60%) appeared to have the highest nZnO-removal range, followed by *Serratia* sp. (100%–58%), *Morganella* (100%–50%) and, *Citrobacter freundii* (97%–49%). Irrespective of the highest removal observed with individual isolates, the lowest nZnO removal was observed with *Lysinibacillus* sp. (99%–43%). The consortium of halophilic bacteria was found to have the highest efficiency in removing nZnO from the mixed liquor (100%–63%) (Fig. 9a).

The test bacterial isolates were able to remove the chemical constituents relative to their concentrations (Tables 4 and 5). In the presence of nZnO, an increasing concentration of nZnO in wastewater decreased the removal efficiency of moderately halophilic bacteria after a 5 d exposure period and this was also found to be species-specific. *Serratia* sp., was able to completely (100%) remove Fe and Ba when exposed to all nZnO concentrations excepted for 200 mg/L, while Ag, Co, and Li were completely removed in presence of 1–10 mg/L nZnO and again Ag in wastewater with 20 mg/L. For other chemical compounds, the overall removal efficiency of *Serratia* sp. ranged between 12% and 97%.

*Bacillus* sp. was able to completely remove (100%) Ag, Co, and Cu when exposed to 1–5 mg/L and was also able to completely remove Ba and Li when exposed to 1–10 mg/L nZnO concentrations. The overall removal efficiency of *Bacillus* sp. for other chemical compounds ranged from 2% to 97% upon exposure to nZnO over time. *Morganella* sp. completely and efficiently removed only Ag (100%) in

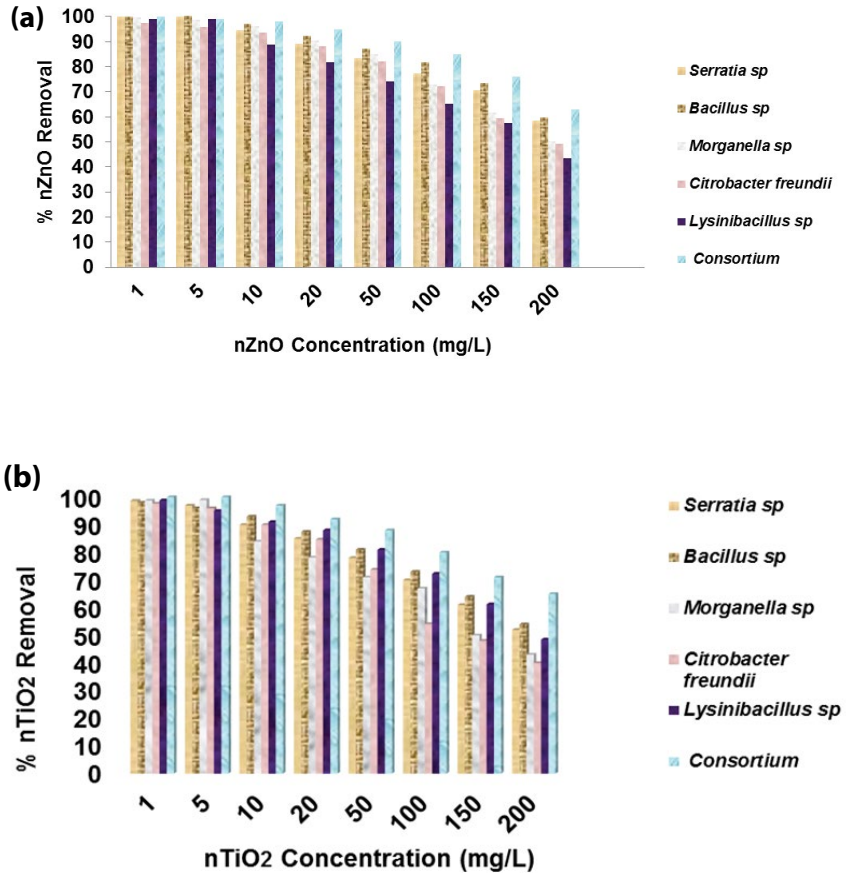


Fig. 8. Removal of (a) zinc oxide and (b) titanium dioxide nanoparticles (percentage) by moderately halophilic bacteria after a 4 d exposure period.

Table 4
Metal removal (%) by moderately halophilic bacteria in the presence of different nZnO concentrations (ranging between 1 and 200 mg/L) after a 4 d exposure period

| *Serratia* sp. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Metals | Initial conc. (mg/L) | % Removal | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 100 | 95 | 90 | 84 | 78 |
| Co | 0.366 | 100 | 100 | 100 | 99 | 94 | 90 | 86 | 80 |
| Cu | 0.742 | 100 | 100 | 96 | 93 | 88 | 83 | 79 | 76 |
| *Bacillus* sp. | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 98 | 95 | 82 | 77 | 73 | 67 |
| Co | 0.366 | 100 | 100 | 99 | 96 | 90 | 86 | 80 | 75 |
| Cu | 0.742 | 100 | 100 | 94 | 90 | 86 | 81 | 77 | 72 |
| *Morganella* sp. | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 100 | 99 | 96 | 91 | 87 | 84 |
| Co | 0.366 | 100 | 100 | 99 | 97 | 93 | 90 | 86 | 80 |
| Cu | 0.742 | 100 | 100 | 98 | 96 | 92 | 87 | 82 | 75 |
| *Citrobacter freundii* | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 99 | 95 | 90 | 87 | 83 | 71 |
| Co | 0.366 | 100 | 100 | 100 | 98 | 94 | 80 | 75 | 70 |
| Cu | 0.742 | 100 | 100 | 98 | 92 | 85 | 78 | 71 | 65 |
| *Lysinibacillus* sp. | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 97 | 91 | 85 | 80 | 72 | 65 |
| Co | 0.366 | 100 | 100 | 100 | 98 | 92 | 85 | 80 | 70 |
| Cu | 0.742 | 100 | 100 | 98 | 94 | 86 | 74 | 69 | 60 |
| Consortium of halophilic bacteria | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 100 | 100 | 95 | 91 | 87 | 82 |
| Co | 0.366 | 100 | 100 | 100 | 100 | 97 | 92 | 88 | 85 |
| Cu | 0.742 | 100 | 100 | 100 | 100 | 96 | 93 | 90 | 84 |

the presence of 1–10 mg/L nZnO, while Co, Cu, Ba, and Li were completely removed in the presence of 1–5 mg/L nZnO concentrations. For other chemical compounds, the overall removal efficiency of *Morganella* sp. showed a range between ranged from 2% and 97% upon exposure to nZnO over time. *Citrobacter freundii* was able to completely (100%) remove Ag, Cu, and Ba when exposed to 1–5 mg/L nZnO and again Co was completely removed when exposed to 1–10 mg/L, while Li was completely removed when exposed to 20 mg/L nZnO concentrations. For the removal of the other chemical compounds, the overall removal efficiency of *Citrobacter freundii* ranged between 1% and 97%. *Lysinibacillus* sp. was able to completely remove (100%) Ag, Cu, and Ba when exposed to1–5 mg/L nZnO concentrations, and Co when exposed to 1–10 mg/L while and Li was completely removed when exposed to 1–20 mg/L nZnO concentrations. The overall removal efficiency of *Lysinibacillus* sp. for other chemical compounds ranged from 3% to 97% upon exposure to nZnO over time. Compared to the individual isolates, the consortium of bacteria demonstrated the highest complete removal efficiency for Ag, Co, Cu, Ba, and Li when exposed to 20 mg/L nZnO concentrations, while Fe was only completely removed when exposed to a concentration of 1 mg/L nZnO. The efficiency of the bacterial consortium to remove the remainder of the chemical compounds ranged from 5% to 98% upon exposure to nZnO over time.

In the presence of $nTiO_2$, a progressive decrease in bacterial removal efficiency was observed ranging from 100% to 40%, with an increasing $nTiO_2$ concentration over the exposure time. For individual isolates *Bacillus* sp. (98%–54%) appeared to have the highest $nTiO_2$-removal range, followed by *Serratia* sp., (99%–52%), *Lysinibacillus* sp. (99%–50%) and the lowest removal efficiency was observed for *Morganella* sp., (99%–43%) and *Citrobacter freundii* (99%–40%). The consortium of halophilic bacteria was found to remove the highest amount of $nTiO_2$ from the mixed liquor (99%–65%).

The halophilic bacterial isolates were able to remove chemical compounds relative to their concentration (Tables 4 and S1). In the presence of $nTiO_2$, an increasing concentration of $nTiO_2$ in wastewater decreased the removal efficiency of moderately halophilic bacteria after a 5 d exposure period and this was also found to be species-specific. *Serratia* sp. was

Table 5
Metal removal (%) by moderately halophilic bacteria in the presence of different $nTiO_2$ concentrations (ranging between 1 and 200 mg/L) after a 4 d exposure period

| Metal removal (%) in presence of $nTiO_2$ at different concentrations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Serratia* sp. | | | | | | | | | |
| Metals | Initial concentration (mg/L) | % Removal | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 98 | 92 | 86 | 81 | 75 |
| Co | 0.366 | 100 | 96 | 93 | 90 | 83 | 79 | 75 | 68 |
| Cu | 0.742 | 99 | 99 | 98 | 93 | 87 | 83 | 78 | 72 |
| *Bacillus* sp. | | | | | | | | | |
| Ag | 1.127 | 100 | 100 | 99 | 96 | 90 | 85 | 70 | 73 |
| Co | 0.366 | 100 | 98 | 95 | 92 | 89 | 85 | 79 | 75 |
| Cu | 0.742 | 100 | 99 | 97 | 90 | 87 | 82 | 77 | 72 |
| *Morganella* sp. | | | | | | | | | |
| Ag | 1.127 | 99 | 97 | 91 | 84 | 78 | 71 | 63 | 53 |
| Co | 0.366 | 100 | 100 | 100 | 97 | 90 | 84 | 76 | 69 |
| Cu | 0.742 | 100 | 100 | 95 | 89 | 81 | 70 | 59 | 48 |
| *Citrobacter freundii* | | | | | | | | | |
| Ag | 1.127 | 99 | 96 | 91 | 85 | 80 | 74 | 66 | 60 |
| Co | 0.366 | 99 | 97 | 93 | 87 | 82 | 76 | 70 | 64 |
| Cu | 0.742 | 100 | 100 | 100 | 97 | 94 | 88 | 80 | 73 |
| *Lysinibacillus* sp. | | | | | | | | | |
| Ag | 1.127 | 99 | 99 | 96 | 90 | 86 | 76 | 70 | 61 |
| Co | 0.366 | 99 | 98 | 94 | 89 | 76 | 69 | 55 | 44 |
| Cu | 0.742 | 100 | 100 | 96 | 92 | 85 | 79 | 65 | 53 |
| Consortium of halophilic bacteria | | | | | | | | | |
| Ag | 1.127 | 99 | 97 | 91 | 86 | 79 | 72 | 68 | 60 |
| Co | 0.366 | 99 | 99 | 92 | 85 | 79 | 71 | 66 | 58 |
| Cu | 0.742 | 100 | 100 | 97 | 91 | 87 | 80 | 72 | 60 |

able to completely (100%) remove Ag and Li when exposed to 1–10 mg/L $nTiO_2$ concentrations, while the overall removal efficiency of other chemical compounds in the medium in the presence of $nTiO_2$ ranged between 5% and 99%.

*Bacillus* sp. was able to completely remove Ag and Li (100%) when exposed to 1–5 mg/L followed by Ba, Co, and Cu, which were completely removed in the presence of only 1 mg/L $nTiO_2$ concentrations. For the overall removal efficiency of other chemical compounds in the medium containing $nTiO_2$, *Bacillus* sp. showed a removal range of between 4% and 99%.

*Morganella* sp. was able to completely and efficiently remove only Cu (100%) in the presence of 1–5 mg/L $nTiO_2$, while Co and Li were completely removed when exposed to 1–10 mg/L under $nTiO_2$ concentrations. For the overall removal of other chemical compounds in the medium containing $nTiO_2$, *Morganella* sp. showed a removal range of between 7% and 99%. *Citrobacter freundii* was able to completely remove Li and Cu (100%) at concentrations of 1–10 mg/L when exposed to varying $nTiO_2$ concentrations. For the removal of the other chemical compounds present in the medium containing $nTiO_2$, *Citrobacter freundii* was efficient at a range of between 2% and 99%. *Lysinibacillus* sp. was able to completely remove Cu (100%) when exposed to 1–5 mg/L $nTiO_2$ concentrations and Li when exposed to 1–15 mg/L $nTiO_2$ concentrations. The overall removal of other chemical compounds present in the medium containing $nTiO_2$ ranged from 1% to 99% over time.

### 3.4. Interaction between halophilic bacteria and metal oxide nanoparticles

The present study also investigated the possible interaction between bacterial isolates and the test metal oxide nanoparticles (nZnO and $nTiO_2$). The results of the TEM analysis indicated that there was a significant interaction between the isolates and the titanium dioxide nanoparticles as revealed in Fig. 9. The highest activity was exhibited by

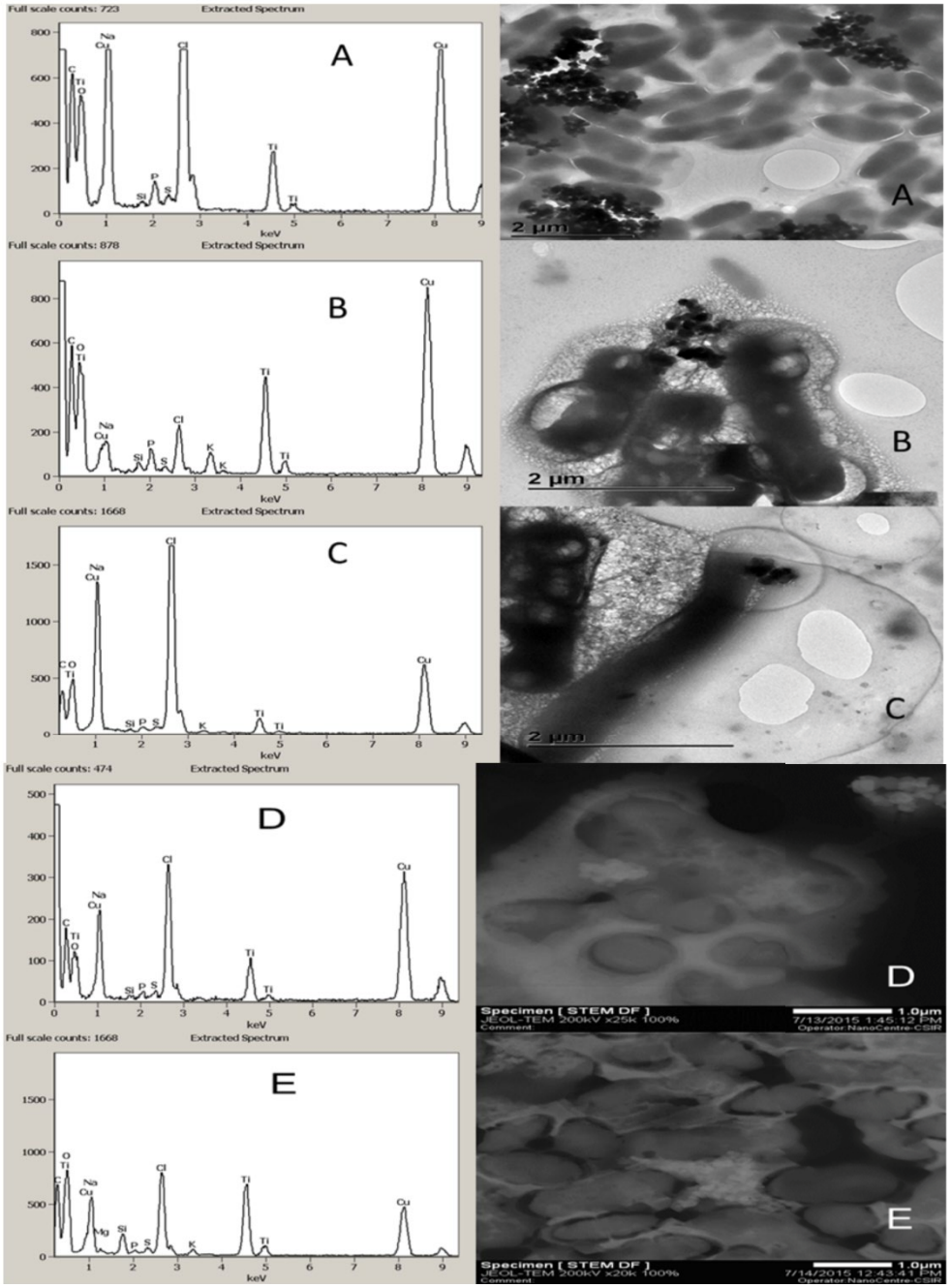


Fig. 9. Representation of the interaction between halophilic bacterial isolates and $nTiO_2$ at 50 mg/L. Elemental mapping using TEM (2 µm): (A) *Serratia* sp., (B) *Bacillus* sp., and (C) *Morganella* sp. Elemental mapping using scanning transmission electron microscopy (1 µm): (D) *Citrobacter freundii* and (E) *Lysinibacillus* sp.

*Serratia* sp. compared to the rest of the isolates. However, no nZnO was detected during TEM analysis, which could be due to a different mechanism of bioremediation taking place. To show the change in size and shape of the halophilic bacteria in the presence of nZnO and $nTiO_2$, SEM analysis was conducted. The results revealed a dramatic change in the shape of the target bacteria when compared to the initial shape of the bacteria prior to being exposed to the metal oxide nanoparticles.

## 4. Discussion

As clean freshwater is diminishing around the world especially in arid regions, unconventional water resources, such as desalinated water have been proposed to fill the water demand gap [20]. To date, there are approximately 16,000 desalination plants either active or under construction worldwide. Despite the ability of these plants to generate freshwater destined for drinking, they are also pumping

out a very large amount of highly polluted, hyper-salty brine water into the environment [20]. Therefore, there is a need to improve brine water management to mitigate environmental concerns associated with desalination plants. Pérez-González et al. [21] state that a combination of different technologies, such as RO with biological treatment, is needed to reduce the problem. According to [22], biological treatment with microorganisms offers a safe, effective and practical process. In this context, the halophilic microorganisms may be suitable for the treatment of such environments, since a high concentration of metal salts is needed for their growth [23].

Prior to investigating the bioremediation ability, identity and growth conditions of the isolated halophilic bacteria were assessed. Isolated halophilic bacteria namely H5, H8, H12, H15, and H28 were found to be closely related to *Serratia* sp. INBio 4041 (KM242484.1), *Bacillus cereus* strain CASA51-1 (HQ179148.1), *Morganella morganii* strain AP28 (DQ358125.1), *Citrobacter freundii* strain C09 (KM222617.1) and *Lysinibacillus* sp. NOSK (KM241862.1), respectively. The selected bacterial species exhibited growth in salinity levels ranging from 0.8% to 25% from day 1 to day 5. This requirement classified them as moderately halophilic bacteria as defined by previous investigators [24–26]. It is also important to note that these bacterial strains had an optimal salinity ranging between 4% and 10%.

Ventosa et al. [13] pointed out that the salt requirements and tolerance of halophilic species vary according to growth conditions, which include temperature and medium composition. In the present study, temperature (20°C–40°C), pH (5–10) and carbon sources (1%–5%) were taken into consideration. It was very important to specify each of these conditions to establish the ranges enabling growth of the isolates. Results revealed that moderately halophilic bacterial isolates had the ability to progressively grow over a wide range of pH values (5–8) (Fig. 4), temperatures (20°C–40°C) (Fig. 5) and carbon sources such as sucrose (1%–5%), glucose (1%–5%), fructose (1%–3%) and acetic acid (1%–4%), (Figs. 6a–d, respectively), with the best growth occurring on day 4, irrespective of the parameters. These isolates were also able to grow in the presence of varying shaking speeds (40–120 rpm) with a general optimum growth observed at 100 rpm on day 4, and 40 rpm with the lowest growth rate (Fig. 7).

In terms of pH conditions, Roohi et al. and Mormile et al. [27,28] who stated that pH optimum conditions for moderately halophilic bacteria ranged between 6 to 8 reported similar findings. Amoozegar et al. [29] demonstrated that the growth of *Halobacillus karajensis* sp. nov., a novel moderately halophilic, Gram-positive, spore-forming bacterium isolated from saline surface soil of the Karaj region (Iran) occurred at 10°C–49°C and in a pH range of 6.0–9.6. The results of the present study also corroborate the findings of [24] who reported similar observations.

It is important to point out that findings also showed that sucrose remained the preferred carbon source although the selected moderately halophilic bacteria isolated from South African brines were able to survive in various carbon sources such as glucose, fructose, and acetic acid. Amoozegar et al. [30] experimented to determine the production of an extracellular protease under stress conditions of high temperature and high salinity by a newly isolated, moderately halophilic, *Salinivibrio* sp. strain AF-2004 in a basal medium containing peptone, beef extract, glucose, and NaCl. The protease activity was determined at various temperatures (10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C) and the optimum temperature range was found to be 30°C–32°C.

As the growth and metabolism of aerobic and facultative microorganisms are strictly dependent upon the amount of DO available in their environment, oxygen demand of each of the isolates was found to be a crucial parameter for the growth of the moderately halophilic bacterial strains [31]. In the present study, the increase in microbial growth was associated with the increase in the shaking speed, which translated into increased aeration rates, and all bacterial species appeared to grow best when at an agitation speed of 100 rpm. Finally, a growth curve was plotted to validate the growth trend of the optimized halophilic bacterial isolates growth conditions in terms of NaCl, pH, agitation speed, and temperature and carbon source required. Using the best growth response for each of these conditions (4% NaCl, pH 7, 30°C, 2% sucrose, 100 rpm), a progressive increase in bacterial growth was observed up to day 4, with H5 and H15 taking the lead with the highest growth and H12 exhibiting the lowest growth. In spite of exhibiting the highest growth under these conditions compared to other targeted isolates, there was no significant difference between the growth of the two isolates (H5 and H15) and that of the rest of moderately halophilic bacteria ($p > 0.05$), therefore making all of them suitable for biotechnological use. The results of this experimental study were in agreement with those of [13], who conducted a detailed study of the moderately halophilic heterotrophic aerobic bacteria within a diverse group of microorganisms. These microorganisms have the potential to be used for the treatment of wastewater due to their salt tolerance as well as their mechanisms of adaptability. Although halophilic microorganisms have received increasing interest in recent years, most studies have been performed on extreme halophiles. However, moderately halophilic bacteria represent an excellent model of adaptation to frequent changes in extracellular osmolality and constitute an interesting group of microorganisms from a biotechnological point of view. The ability of the moderately halophilic bacteria to grow under harsh conditions over a very wide range of salinities makes them very attractive for research. The moderately halophilic bacteria also reveal a high potential for use in industrial applications.

The present study further investigated the ability of bacterial isolates in removing nZnO, $nTiO_2$ and other metal and metalloid constituents from synthetic wastewater containing a varying concentration of nZnO or $nTiO_2$ between 1 and 200 mg/L. The test bacterial isolates were able to remove the chemical constituents relative to their concentrations (Tables 4, 5, S1, and S2). Significant removal efficiencies of up to 100% for nZnO and 100% for $nTiO_2$ were observed in the synthetic wastewater containing NPs ranging between 1 to 5 mg/L. The highest removal efficiency was noted in the media inoculated with *Bacillus* (100%–60% for nZnO) and *Serratia* sp. (99%–52% for $nTiO_2$) as individual organisms and with the consortium of halophilic bacteria (100%–63% for nZnO, 100%–65% for $nTiO_2$). *Lysinibacillus* sp. and *Citrobacter freundii* (97%–50% for nZnO and 99%–43% for $nTiO_2$, respectively) were the isolates with the lowest removal efficiency.

However, a drastic decrease in NP removal was noted as their concentrations increased to 10 and 200 mg/L. Despite the fact that higher concentrations of NPs were found to affect the growth of test halophilic bacterial isolates, the present results revealed that moderately halophilic bacteria could still remove NPs from polluted wastewater [32–34]. The findings of this study are in agreement with those of [35], who demonstrated the bioremediation of both minimal and complex media in the presence of nZnO up to 2.0 mM by four halophilic bacteria, and [36] who also reported the bioremediation of nZnO by halophilic bacteria. Maurer-Jones et al. [37] pointed out that bacteria especially halophiles are capable of the removal of $TiO_2$ nanoparticles. Megharaj et al. [38] further stated that halophilic bacteria and microalgae are good candidates for the treatment of metal-polluted saline and alkaline effluents. Dönmez and Aksu [39] also reported that the biosorption of chromium (VI) by *Dunaliella*, a halophilic microalga, increased with increasing chromium (VI) concentrations up to 250–300 mg/L but at low salt concentrations. In another study by Dönmez and Koçberber [40] the bioaccumulation capacities of a mixed culture isolated from industrial saline effluents contaminated with chromium (VI) were also observed. In their studies, Amoozegar et al. [30] reported that a moderately halophilic chromate-reducing bacterial strain, *Nesterenkonia* sp. strain MF2 was able to completely reduce 0.2 mM of highly toxic and soluble Cr(VI) into less toxic Cr(III).

The findings for the consortium of halophilic bacteria in this study are in agreement with those of Sheng and Liu [41] who studied the effects of silver nanoparticles on wastewater biofilms to understand the potential antibacterial effect of silver nanoparticles (Ag-NPs) on biological wastewater treatment processes. These authors pointed out that extracellular polymeric substances and microbial community interactions in biofilms play important roles in controlling the antimicrobial effects of Ag-NPs. In addition, slow growth rates may enhance the tolerance of certain bacteria to Ag-NPs. Woolard and Irvine [23] reported the removal of more than 99% of phenol from a waste stream containing 15% salt, using a batch biofilm reactor for the treatment of hypersaline wastewaters containing phenol. Similar observations were also reported by other investigators [42,43].

## 5. Conclusion

Water pollution is currently considered as one of the most important areas of environmental concern, impacting most of the existing freshwater resources, whether surface water sources or groundwater sources. As a result, an interest in the use of unconventional water resources has been proposed. The study revealed that moderately halophilic bacteria were able to remove nZnO and $nTiO_2$ NPs, and other chemical pollutants from polluted wastewater. The study further revealed that in consortium, bacterial isolates removed high percentage removal efficient than when individually.

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## References

[1] B.M. Peyton, T. Wilson, D.R. Yonge, Kinetics of phenol biodegradation in high salt solutions, Water Res., 36 (2002) 4811–4820.
[2] K. Bakker, Water security: research challenges and opportunities, Science, 337 (2012) 914–915.
[3] I. Kamika, M.N.B. Momba, Comparing the tolerance limits of selected bacterial and protozoan species to nickel in wastewater systems, Sci. Total Environ., 410 (2011) 172–181.
[4] I. Kamika, M.N.B. Momba, Assessing the resistance and bioremediation ability of selected bacterial and protozoan species to heavy metals in metal-rich industrial wastewater, BMC Microbiol., 13 (2013) 28.
[5] 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) 86189.
[6] E. Corcoran, C. Nelleman, E. Baker, R. Bos, D. Osborn, H. Savelli, Eds., Sick Water? The Central Role of Wastewater Management in Sustainable Development: A Rapid Response Assessment, UNEP (United Nations Environment Programme), UN-HABITAT, GRID-Arendal, 2010.
[7] J.E. Hardoy, D. Mitlin, D. Satterthwaite, Environmental Problems in an Urbanizing World: Finding Solutions in Cities in Africa, Asia and Latin America, Routledge, New York, USA, 2013.
[8] M.C. Ncibi, B. Mahjoub, O. Mahjoub, M. Sillanpää, Remediation of emerging pollutants in contaminated wastewater and aquatic environments: biomass-based technologies, Clean Soil Air Water, 45 (2017), https://doi.org/10.1002/clen.201700101.
[9] C. Hui, C.M. Shen, J. Tian, L.H. Bao, H. Ding, C. Li, Y. Tian, X.Z. Shi, H.-J. Gao, Core-shell $Fe_3O_4$@$SiO_2$ nanoparticles synthesized with well-dispersed hydrophilic $Fe_3O_4$ seeds, Nanoscale, 3 (2011) 701–705.
[10] M.R. Wiesner, G.V. Lowry, K.L. Jones, M.F. Hochella Jr., R.T. Di Giulio, E. Casman, E.S. Bernhardt, Decreasing uncertainties in assessing environmental exposure, risk, and ecological implications of nanomaterials, Environ. Sci. Technol., 43 (2009) 6458–6462.
[11] R.D. Handy, B.J. Shaw, Toxic effects of nanoparticles and nanomaterials: implications for public health, risk assessment and the public perception of nanotechnology, Health Risk Soc., 9 (2007) 125–144.
[12] H. Shi, R. Magaye, V. Castranova, J. Zhao, Titanium dioxide nanoparticles: a review of current toxicological data, Part. Fibre Toxicol., 10 (2013) 1.
[13] A. Ventosa, J.J. Nieto, A. Oren, Biology of moderately halophilic aerobic bacteria, Microbiol. Mol. Biol. Rev., 62 (1998) 504–544.
[14] K.H. Lee, C.K. Chiang, Z.H. Lin, H.T. Chang, Determining enediol compounds in tea using surface-assisted laser desorption/ionization mass spectrometry with titanium dioxide nanoparticle matrices, Rapid Commun. Mass Spectrom., 21 (2007) 2023–2030.
[15] R. Chen, L.L. Huo, X.F. Shi, R. Bai, Z.J. Zhang, Y.L. Zhao, Y.Z. Chang, C.Y. Chen, Endoplasmic reticulum stress induced by zinc oxide nanoparticles is an earlier biomarker for nanotoxicological evaluation, ACS Nano, 8 (2014) 2562–2574.
[16] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, R.A. Lesniewski, B.B. Oakley, D.H. Parks, C.J. Robinson, J.W. Sahl, B. Stres, G.G. Thallinger, D.J. Van Horn, C.F. Weber, Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities, Appl. Environ. Microbiol., 75 (2009) 7537–7541.
[17] 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, Microb. Ecol., 48 (2004) 449–462.
[18] J.M. Lim, C.O. Jeon, S.M. Song, C.J. Kim, *Pontibacillus chungwhensis* gen. nov., sp. nov., a moderately halophilic Gram-positive bacterium from a solar saltern in Korea, Int. J. Syst. Evol. Microbiol., 55 (2005) 165–170.
[19] L.O. Kachieng'a, M.N.B. Momba, Bioremediation of fats and oils in domestic wastewater by selected protozoan isolates, Water Air Soil Pollut., 226 (2015) 1–15.
[20] E. Jones, M. Qadir, M.T.H. van Vliet, V. Smakhtin, S.-m. Kang, The state of desalination and brine production: a global outlook, Sci. Total Environ., 657 (2019) 1343–1356.
[21] A. Pérez-González, A.M. Urtiaga, IbáñezI, R. Ortiz, State of the art and review on the treatment technologies of water reverse osmosis concentrates. Water Res. 46(2012) 267–283.
[22] C.A. Santos, A.M. Vieira, H.L. Fernandes, J.A. Empis, J.M. Novais, Optimisation of the biological treatment of hypersaline wastewater from *Dunaliella salina* carotenogenesis, J. Chem. Technol. Biotechnol., 76 (2001) 1147–1153.
[23] C.R. Woolard, R.L. Irvine, Biological treatment of hypersaline wastewater by a biofilm of halophilic bacteria, Water Environ. Res., 66 (1994) 230–235.
[24] C.R. Tsai, J.L. Garcia, B.K. Patel, J.L. Cayol, L. Baresi, R.A. Mah, *Haloanaerobium alcaliphilum* sp. nov., an anaerobic moderate halophile from the sediments of Great Salt Lake, Utah, Int. J. Syst. Evol. Microbiol., 45 (1995) 301–307.
[25] A. Poli, E. Esposito, P. Orlando, L. Lama, A. Giordano, F. de Appolonia, B. Nicolaus, A. Gambacorta, *Halomonas alkaliantarctica* sp. nov., isolated from saline lake Cape Russell in Antarctica, an alkalophilic moderately halophilic, exopolysaccharide-producing bacterium, Syst. Appl. Microbiol., 30 (2007) 31–38.
[26] S. Dassarma, P. Dassarma, In: eLS, Halophiles, John Wiley & Sons Ltd., Chichester, 2012, doi: 10.1002/9780470015902.a0000394.pub3.
[27] A. Roohi, I. Ahmed, M. Iqbal, M. Jamil, Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan, Pak. J. Bot., 44 (2012) 365–370.
[28] M.R. Mormile, M.F. Romine, M.T. Garcia, A. Ventosa, T.J. Bailey, B.M. Peyton, *Halomonas campisalis* sp. nov., a denitrifying, moderately haloalkaliphilic bacterium, Syst. Appl. Microbiol., 22 (1999) 551–558.
[29] M.A. Amoozegar, F. Malekzadeh, K.A. Malik, P. Schumann, C. Spröer, *Halobacillus karajensis* sp. nov., a novel moderate halophile, Int. J. Syst. Evol. Microbiol., 153 (2003) 1059–1063.
[30] M.A. Amoozegar, A.Z. Fatemi, H.R. Karbalaei-Heidari, M.R. Razavi, Production of an extracellular alkaline metalloprotease from a newly isolated, moderately halophile, *Salinivibrio* sp. strain AF-2004, J. Microbiol. Res., 162 (2007) 369–377.
[31] G.A. Somerville, R.A. Proctor, Cultivation conditions and the diffusion of oxygen into culture media: the rationale for the flask-to-medium ratio in microbiology, BMC Microbiol., 13 (2013) 9.
[32] R.H. Vreeland, C.D. Litchfield, E.L. Martin, E. Elliot, *Halomonas elongata*, a new genus and species of extremely salt-tolerant bacteria, Int. J. Syst. Evol. Microbiol., 30 (1980) 485–495.
[33] A. Uygur, F. Kargı, Salt inhibition on biological nutrient removal from saline wastewater in a sequencing batch reactor, Enzyme Microb. Technol., 34 (2004) 313–318.
[34] H. Mu, Y. Chen, N. Xiao, Effects of metal oxide nanoparticles ($TiO_2$, $Al_2O_3$, $SiO_2$ and ZnO) on waste activated sludge anaerobic digestion, Bioresour. Technol., 102 (2011) 10305–10311.
[35] R. Sinha, R. Karan, A. Sinha, S.K. Khare, Interaction and nanotoxic effect of ZnO and Ag nanoparticles on mesophilic and halophilic bacterial cells, Bioresour. Technol., 102 (2011) 1516–1520.
[36] R. Sinha, S.K. Khare, Differential interactions of halophilic and non-halophilic proteases with nanoparticles, Sustainable Chem. Processes, 2 (2014) 1–8.
[37] M.A. Maurer-Jones, I.L. Gunsolus, C.J. Murphy, C.L. Haynes, Toxicity of engineered nanoparticles in the environment, Anal. Chem., 85 (2013) 3036–3049.
[38] M. Megharaj, S. Avudainayagam, R. Naidu, Toxicity of hexavalent chromium and its reduction by bacteria isolated from soil contaminated with tannery waste, Curr. Microbiol., 47 (2003) 51–54.
[39] G. Dönmez, Z. Aksu, Removal of chromium(VI) from saline wastewaters by *Dunaliella* species, Process Biochem., 38 (2002) 751–762.
[40] G. Dönmez, N. Koçberber, Bioaccumulation of hexavalent chromium by enriched microbial cultures obtained from molasses and NaCl containing media, Process Biochem., 40 (2005) 2493–2498.
[41] Z. Sheng, Y. Liu, Effects of silver nanoparticles on wastewater biofilms, Water Res., 45 (2011) 6039–6050.
[42] B.K. Singh, A. Walker, Microbial degradation of organophosphorus compounds, FEMS Microbiol. Rev., 30 (2006) 428–471.
[43] S. le Borgne, D. Paniagua, R. Vazquez-Duhalt, Biodegradation of organic pollutants by halophilic bacteria and archaea, J. Mol. Microbiol. Biotechnol., 15 (2008) 74–92.

**Supplementary information**

Table S1
Metal removal (%) by moderately halophilic bacteria in the presence of different nZnO concentrations (ranging between 1 and 200 mg/L) after a 4 d exposure period

| *Serratia* sp. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Metals | Initial concentration (mg/L) | % Removal | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 100 | 95 | 90 | 84 | 78 |
| Co | 0.366 | 100 | 100 | 100 | 99 | 94 | 90 | 86 | 80 |
| Cu | 0.742 | 100 | 100 | 96 | 93 | 88 | 83 | 79 | 76 |
| Fe | 0.956 | 100 | 100 | 97 | 100 | 100 | 100 | 100 | 81 |
| Na | 54.588 | 27 | 25 | 21 | 19 | 16 | 11 | 7 | 4 |
| S | 25.221 | 40 | 39 | 37 | 32 | 30 | 25 | 20 | 12 |
| As | 0.637 | 89 | 89 | 87 | 83 | 70 | 76 | 67 | 57 |
| Ba | 0.113 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 70 |
| Ca | 63.945 | 96 | 95 | 95 | 90 | 85 | 80 | 75 | 65 |
| K | 15.654 | 89 | 88 | 88 | 78 | 66 | 56 | 47 | 36 |
| Li | 0.096 | 100 | 100 | 100 | 96 | 93 | 90 | 87 | 80 |
| Mg | 32.107 | 77 | 76 | 74 | 66 | 56 | 50 | 47 | 37 |
| Pb | 1.949 | 80 | 78 | 77 | 76 | 71 | 66 | 56 | 45 |
| Sr | 0.227 | 97 | 96 | 92 | 88 | 77 | 67 | 61 | 57 |
| *Bacillus* sp. | | | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 98 | 95 | 82 | 77 | 73 | 67 |
| Co | 0.366 | 100 | 100 | 99 | 96 | 90 | 86 | 80 | 75 |
| Cu | 0.742 | 100 | 100 | 94 | 90 | 86 | 81 | 77 | 72 |
| Fe | 0.956 | 97 | 96 | 96 | 91 | 86 | 83 | 76 | 70 |
| Na | 54.588 | 38 | 37 | 351 | 31 | 27 | 24 | 20 | 11 |
| S | 25.221 | 26 | 26 | 23 | 20 | 17 | 15 | 11 | 9 |
| As | 0.637 | 89 | 88 | 87 | 84 | 75 | 71 | 65 | 56 |
| Ba | 0.113 | 100 | 100 | 100 | 94 | 90 | 86 | 83 | 79 |
| Ca | 63.945 | 95 | 90 | 83 | 78 | 70 | 95 | 95 | 95 |
| K | 15.654 | 89 | 84 | 80 | 76 | 70 | 63 | 50 | 44 |
| Li | 0.096 | 100 | 100 | 100 | 96 | 90 | 85 | 81 | 78 |
| Mg | 32.107 | 74 | 71 | 68 | 54 | 50 | 45 | 35 | 25 |
| Pb | 1.949 | 73 | 70 | 68 | 65 | 61 | 56 | 51 | 44 |
| Sr | 0.227 | 97 | 95 | 91 | 88 | 86 | 77 | 75 | 71 |
| *Morganella* sp. | | | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 99 | 96 | 91 | 87 | 84 |
| Co | 0.366 | 100 | 100 | 99 | 97 | 93 | 90 | 86 | 80 |
| Cu | 0.742 | 100 | 100 | 98 | 96 | 92 | 87 | 82 | 75 |
| Fe | 0.956 | 100 | 98 | 95 | 92 | 87 | 86 | 66 | 60 |
| Na | 54.588 | 34 | 33 | 31 | 27 | 24 | 18 | 12 | 6 |

*(Continued)*

Table S1 Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | *Morganella* sp. | | | | |
| S | 25.221 | 34 | 28 | 25 | 22 | 19 | 16 | 11 | 8 |
| As | 0.637 | 89 | 87 | 85 | 81 | 77 | 68 | 60 | 56 |
| Ba | 0.113 | 100 | 100 | 99 | 94 | 86 | 81 | 76 | 71 |
| Ca | 63.945 | 95 | 93 | 89 | 85 | 80 | 74 | 67 | 55 |
| K | 15.654 | 96 | 91 | 86 | 76 | 70 | 66 | 54 | 36 |
| Li | 0.096 | 100 | 100 | 99 | 97 | 92 | 85 | 80 | 75 |
| Mg | 32.107 | 75 | 74 | 70 | 66 | 60 | 54 | 45 | 35 |
| Pb | 1.949 | 75 | 73 | 71 | 65 | 60 | 52 | 45 | 39 |
| Sr | 0.227 | 97 | 94 | 90 | 87 | 75 | 65 | 57 | 47 |
| | | | | | *Citrobacter freundii* | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 99 | 95 | 90 | 87 | 83 | 71 |
| Co | 0.366 | 100 | 100 | 100 | 98 | 94 | 80 | 75 | 70 |
| Cu | 0.742 | 100 | 100 | 98 | 92 | 85 | 78 | 71 | 65 |
| Fe | 0.956 | 97 | 96 | 91 | 86 | 81 | 66 | 57 | 46 |
| Na | 54.588 | 22 | 20 | 17 | 14 | 11 | 7 | 4 | 1 |
| S | 25.221 | 38 | 37 | 35 | 31 | 27 | 25 | 21 | 15 |
| As | 0.637 | 87 | 87 | 84 | 80 | 74 | 70 | 65 | 64 |
| Ba | 0.113 | 100 | 100 | 100 | 97 | 94 | 90 | 88 | 85 |
| Ca | 63.945 | 95 | 92 | 86 | 80 | 75 | 65 | 53 | 45 |
| K | 15.654 | 95 | 91 | 86 | 83 | 77 | 70 | 64 | 52 |
| Li | 0.096 | 100 | 100 | 100 | 100 | 94 | 90 | 87 | 81 |
| Mg | 32.107 | 75 | 74 | 71 | 67 | 61 | 53 | 45 | 35 |
| Pb | 1.949 | 91 | 90 | 85 | 79 | 75 | 70 | 68 | 63 |
| Sr | 0.227 | 97 | 97 | 95 | 91 | 87 | 83 | 76 | 70 |
| | | | | | *Lysinibacillus* sp. | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 97 | 91 | 85 | 80 | 72 | 65 |
| Co | 0.366 | 100 | 100 | 100 | 98 | 92 | 85 | 80 | 70 |
| Cu | 0.742 | 100 | 100 | 98 | 94 | 86 | 74 | 69 | 60 |
| Fe | 0.956 | 96 | 94 | 90 | 85 | 78 | 70 | 66 | 56 |
| Na | 54.588 | 24 | 23 | 20 | 17 | 13 | 10 | 7 | 3 |
| S | 25.221 | 38 | 36 | 31 | 24 | 19 | 12 | 7 | 3 |
| As | 0.637 | 88 | 87 | 82 | 76 | 70 | 65 | 60 | 54 |
| Ba | 0.113 | 100 | 100 | 98 | 94 | 90 | 84 | 79 | 70 |
| Ca | 63.945 | 96 | 94 | 90 | 86 | 81 | 75 | 61 | 55 |
| K | 15.654 | 98 | 98 | 96 | 96 | 96 | 96 | 96 | 96 |
| Li | 0.096 | 100 | 100 | 100 | 100 | 97 | 92 | 87 | 80 |
| Mg | 32.107 | 80 | 79 | 74 | 69 | 64 | 59 | 52 | 44 |
| Pb | 1.949 | 78 | 75 | 69 | 77 | 73 | 72 | 76 | 75 |
| Sr | 0.227 | 97 | 95 | 91 | 88 | 84 | 79 | 75 | 69 |

| Consortium of halophilic bacteria | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 100 | 95 | 91 | 87 | 82 |
| Co | 0.366 | 100 | 100 | 100 | 100 | 97 | 92 | 88 | 85 |
| Cu | 0.742 | 100 | 100 | 100 | 100 | 96 | 93 | 90 | 84 |
| Fe | 0.956 | 100 | 98 | 95 | 88 | 84 | 80 | 76 | 70 |
| Na | 54.588 | 39 | 38 | 36 | 32 | 29 | 25 | 21 | 13 |
| S | 25.221 | 97 | 95 | 91 | 87 | 82 | 78 | 72 | 67 |
| As | 0.637 | 90 | 88 | 85 | 81 | 77 | 71 | 66 | 54 |
| Ba | 0.113 | 100 | 100 | 100 | 100 | 97 | 94 | 90 | 87 |
| Ca | 63.945 | 96 | 94 | 90 | 86 | 80 | 74 | 67 | 95 |
| K | 15.654 | 96 | 94 | 91 | 87 | 82 | 78 | 75 | 70 |
| Li | 0.096 | 100 | 100 | 100 | 100 | 99 | 95 | 90 | 83 |
| Mg | 32.107 | 66 | 64 | 60 | 56 | 52 | 45 | 36 | 30 |
| Pb | 1.949 | 75 | 73 | 69 | 65 | 60 | 53 | 49 | 30 |
| Sr | 0.227 | 98 | 97 | 96 | 91 | 95 | 87 | 82 | 77 |

Table S2
Metal removal (%) by moderately halophilic bacteria in the presence of different $nTiO_2$ concentrations (ranging between 1 and 200 mg/L) after a 4 d exposure period

| Metal removal (%) in presence of $nTiO_2$ at different concentrations | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *Serratia* sp. | | | | | |
| Metals | Initial concentration (mg/L) | % Removal | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 100 | 98 | 92 | 86 | 81 | 75 |
| Co | 0.366 | 100 | 96 | 93 | 90 | 83 | 79 | 75 | 68 |
| Cu | 0.742 | 99 | 99 | 98 | 93 | 87 | 83 | 78 | 72 |
| Fe | 0.956 | 97 | 96 | 92 | 88 | 82 | 76 | 71 | 65 |
| Na | 54.588 | 30 | 30 | 27 | 23 | 20 | 16 | 11 | 6 |
| S | 25.221 | 65 | 62 | 55 | 50 | 46 | 40 | 36 | 31 |
| As | 0.637 | 89 | 87 | 84 | 80 | 77 | 64 | 56 | 50 |
| Ba | 0.113 | 98 | 96 | 92 | 87 | 78 | 75 | 66 | 53 |
| Ca | 63.945 | 95 | 94 | 91 | 86 | 81 | 76 | 64 | 52 |
| K | 15.654 | 71 | 67 | 60 | 57 | 54 | 42 | 35 | 31 |
| Li | 0.096 | 100 | 100 | 100 | 98 | 93 | 86 | 80 | 75 |
| Mg | 32.107 | 64 | 61 | 58 | 53 | 48 | 42 | 36 | 26 |
| Pb | 1.949 | 62 | 58 | 52 | 47 | 36 | 28 | 21 | 17 |
| Sr | 0.227 | 96 | 94 | 90 | 85 | 79 | 74 | 68 | 55 |
| | | | | *Bacillus* sp. | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 100 | 100 | 99 | 96 | 90 | 85 | 70 | 73 |
| Co | 0.366 | 100 | 98 | 95 | 92 | 89 | 85 | 79 | 75 |
| Cu | 0.742 | 100 | 99 | 97 | 90 | 87 | 82 | 77 | 72 |
| Fe | 0.956 | 96 | 93 | 88 | 83 | 65 | 57 | 51 | 45 |
| Na | 54.588 | 32 | 31 | 28 | 25 | 23 | 20 | 17 | 12 |
| S | 25.221 | 53 | 50 | 46 | 41 | 35 | 28 | 21 | 16 |

*(Continued)*

Table S2 Continued

| | | | | *Bacillus* sp. | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| As | 0.637 | 91 | 88 | 85 | 79 | 71 | 67 | 61 | 55 |
| Ba | 0.113 | 100 | 98 | 93 | 86 | 79 | 72 | 68 | 60 |
| Ca | 63.945 | 95 | 92 | 87 | 80 | 75 | 69 | 58 | 42 |
| K | 15.654 | 93 | 89 | 82 | 75 | 69 | 60 | 53 | 45 |
| Li | 0.096 | 100 | 100 | 96 | 93 | 87 | 80 | 73 | 60 |
| Mg | 32.107 | 67 | 65 | 60 | 53 | 46 | 40 | 35 | 28 |
| Pb | 1.949 | 65 | 62 | 57 | 45 | 37 | 30 | 25 | 16 |
| Sr | 0.227 | 97 | 95 | 80 | 76 | 71 | 64 | 53 | 41 |
| | | | | *Morganella* sp. | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 99 | 97 | 91 | 84 | 78 | 71 | 63 | 53 |
| Co | 0.366 | 100 | 100 | 100 | 97 | 90 | 84 | 76 | 69 |
| Cu | 0.742 | 100 | 100 | 95 | 89 | 81 | 70 | 59 | 48 |
| Fe | 0.956 | 96 | 93 | 90 | 87 | 82 | 75 | 68 | 60 |
| Na | 54.588 | 27 | 26 | 23 | 20 | 16 | 11 | 7 | 5 |
| S | 25.221 | 41 | 39 | 35 | 32 | 30 | 28 | 25 | 20 |
| As | 0.637 | 90 | 88 | 83 | 75 | 68 | 52 | 41 | 29 |
| Ba | 0.113 | 98 | 96 | 92 | 87 | 82 | 73 | 68 | 55 |
| Ca | 63.945 | 95 | 93 | 89 | 82 | 74 | 64 | 55 | 45 |
| K | 15.654 | 99 | 98 | 94 | 69 | 62 | 74 | 65 | 54 |
| Li | 0.096 | 100 | 100 | 100 | 97 | 93 | 89 | 82 | 77 |
| Mg | 32.107 | 67 | 64 | 59 | 53 | 46 | 38 | 30 | 23 |
| Pb | 1.949 | 69 | 66 | 60 | 54 | 46 | 39 | 21 | 14 |
| Sr | 0.227 | 96 | 94 | 90 | 76 | 71 | 66 | 59 | 50 |
| | | | | *Citrobacter freundii* | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 99 | 96 | 91 | 85 | 80 | 74 | 66 | 60 |
| Co | 0.366 | 99 | 97 | 93 | 87 | 82 | 76 | 70 | 64 |
| Cu | 0.742 | 100 | 100 | 100 | 97 | 94 | 88 | 80 | 73 |
| Fe | 0.956 | 97 | 96 | 94 | 90 | 84 | 72 | 64 | 53 |
| Na | 54.588 | 25 | 25 | 24 | 19 | 15 | 10 | 4 | 1 |
| S | 25.221 | 43 | 40 | 36 | 29 | 22 | 17 | 12 | 7 |
| As | 0.637 | 88 | 84 | 80 | 77 | 71 | 65 | 53 | 45 |
| Ba | 0.113 | 98 | 95 | 88 | 83 | 76 | 67 | 53 | 44 |
| Ca | 63.945 | 96 | 92 | 85 | 80 | 75 | 66 | 96 | 95 |
| K | 15.654 | 99 | 96 | 92 | 86 | 81 | 75 | 95 | 96 |
| Li | 0.096 | 100 | 100 | 100 | 98 | 93 | 85 | 76 | 70 |
| Mg | 32.107 | 25 | 22 | 18 | 16 | 14 | 11 | 9 | 4 |
| Pb | 1.949 | 59 | 57 | 52 | 46 | 31 | 26 | 19 | 11 |
| Sr | 0.227 | 97 | 95 | 90 | 86 | 75 | 65 | 55 | 46 |
| | | | | *Lysinibacillus* sp. | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 99 | 99 | 96 | 90 | 86 | 76 | 70 | 61 |
| Co | 0.366 | 99 | 98 | 94 | 89 | 76 | 69 | 55 | 44 |
| Cu | 0.742 | 100 | 100 | 96 | 92 | 85 | 79 | 65 | 53 |
| Fe | 0.956 | 97 | 95 | 91 | 87 | 77 | 67 | 54 | 41 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Na | 54.588 | 28 | 27 | 25 | 22 | 19 | 15 | 10 | 7 |
| S | 25.221 | 23 | 21 | 19 | 14 | 10 | 7 | 4 | 1 |
| As | 0.637 | 88 | 86 | 81 | 75 | 66 | 57 | 46 | 35 |
| Ba | 0.113 | 99 | 97 | 91 | 87 | 80 | 72 | 63 | 52 |
| Ca | 63.945 | 95 | 94 | 90 | 86 | 74 | 65 | 56 | 45 |
| K | 15.654 | 98 | 96 | 91 | 86 | 77 | 68 | 59 | 39 |
| Li | 0.096 | 100 | 100 | 97 | 92 | 88 | 81 | 70 | 69 |
| Mg | 32.107 | 54 | 52 | 50 | 46 | 35 | 26 | 20 | 12 |
| Pb | 1.949 | 61 | 60 | 56 | 50 | 44 | 38 | 30 | 21 |
| Sr | 0.227 | 97 | 96 | 92 | 87 | 76 | 64 | 56 | 46 |
| Consortium of halophilic bacteria | | | | | | | | | |
| | | 1 | 5 | 10 | 20 | 50 | 100 | 150 | 200 |
| Ag | 1.127 | 99 | 97 | 91 | 86 | 79 | 72 | 68 | 60 |
| Co | 0.366 | 99 | 99 | 92 | 85 | 79 | 71 | 66 | 58 |
| Cu | 0.742 | 100 | 100 | 97 | 91 | 87 | 80 | 72 | 60 |
| Fe | 0.956 | 96 | 94 | 88 | 83 | 76 | 70 | 63 | 54 |
| Na | 54.588 | 38 | 37 | 36 | 32 | 29 | 26 | 20 | 14 |
| S | 25.221 | 23 | 22 | 20 | 18 | 15 | 11 | 7 | 3 |
| As | 0.637 | 86 | 85 | 81 | 77 | 71 | 65 | 53 | 40 |
| Ba | 0.113 | 98 | 97 | 93 | 87 | 81 | 76 | 67 | 60 |
| Ca | 63.945 | 95 | 93 | 90 | 85 | 79 | 65 | 54 | 43 |
| K | 15.654 | 94 | 91 | 87 | 81 | 74 | 65 | 59 | 48 |
| Li | 0.096 | 100 | 100 | 98 | 93 | 87 | 80 | 73 | 62 |
| Mg | 32.107 | 59 | 54 | 50 | 46 | 40 | 34 | 21 | 12 |
| Pb | 1.949 | 59 | 58 | 52 | 47 | 39 | 32 | 23 | 12 |
| Sr | 0.227 | 97 | 96 | 91 | 86 | 80 | 71 | 62 | 51 |