

Antibacterial and Antioxidant Potential of Cadmium-Resistant Halotolerant Bacteria Isolated from Mangrove Rhizosphere Soils in Saudi Arabia

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Abstract

Objective: This study aimed to isolate halotolerant and halophilic bacteria and study their abilities to resist cadmium (Cd) and to produce antimicrobial and antioxidant agents.

Methods: Soil samples of mangroves were collected from four different sites in Saudi Arabia: three in Tarut Bay (Darin Safwa, and Syhat) and the fourth in the Farasan Islands (Sajid Island).

Results: Of the 125 isolates obtained on three different media containing 3 M NaCl and 100 ppm Cd (II), Luria-Bertani (LB), saline nutrient, and casein agar, LR40 and LD57 were the most resistant to cadmium metal. The previous isolates grew well for 5 days on LB medium at 37°C in the presence of Cd (II) metal (50-500 ppm at 3 M NaCl), with a minimum inhibitory concentration (MIC) of 600 ppm for both isolates. The two isolates were identified as *Vreelandella alkaliphila* strain LR40 (accession number PP767765.1) and *Halomonas smyrnensis* strain LD57 (accession number PP767765). The effect of several factors on the growth of isolates LR40 and LD57 in the presence of Cd (II) metal (300 ppm) was studied; the results showed that the best growth of both isolates in the presence of the metal was at pH 7, 3 M NaCl, 37°C, and after 2 days. The biosorption capacity of Cd (II) by the live biomass of isolates LR40 and LD57 in the solution with 500 ppm was 70.34% and 72.85%, respectively. The ethyl acetate extracts of the two isolates exhibited moderate antibacterial activity against uropathogenic bacteria, and the presence of cadmium in the growth medium enhanced this activity. Also, the cell and ethyl acetate extracts showed excellent antioxidant activity as determined by DPPH (1,1-Diphenyl-2-picrylhydrazyl), with antioxidant percentages ranging from 65-76%.

Conclusion: The coastal mangroves of Saudi Arabia are rich with new halotolerant bacteria that possess a specific combination of resistance to cadmium, tolerance to high salt, and, importantly, antimicrobial and antioxidant activities, making them highly promising candidates for many biotechnological applications

Keywords: Halotolerant, uropathogens, cadmium, *Halomonas*, *Vreelandella*, anti-bacterial agents, antioxidants

Introduction

Mangroves are one of the most diverse ecosystems on earth, with many organisms, including extremophiles, helping the ecosystem to grow and survive despite extreme environmental conditions. This wide mangrove environment is home to a great variety of extremophiles, including halophiles, halotolerant, thermophiles, and lots of other marine microorganisms.¹ The rhizosphere of mangroves involves highly functioning, varied microbial communities that contribute to nutrient cycling and the maintenance of ecosystem trophic networks. The toxicity of heavy metals may have harmful effects on these communities.²

Heavy metals get into mangrove ecosystems through anthropogenic or natural processes such as atmospheric deposition or soil erosion. Chemicals like these are considered among the most hazardous to the environment.³ Anthropogenic activities, including urbanization, oil spills, pollutants, industrial waste, wastewater treatment plants, and harmful waste disposal, strongly affect the mangrove habitats, and they are highly susceptible to heavy metal accumulation from these sources.⁴ Heavy metals in groundwater, soil, and plants have become a big problem that needs to be fixed.⁵ As these metals enter the food chain, they create a variety of health problems, including damage to the nervous system, endocrine system, malignancy, circulatory system, skin cancer, and benign prostatic hyperplasia. Humans may come into direct contact with heavy metals by consuming contaminated foods,

sea animals, and drinking water, inhaling polluted air as dust fumes, or by occupational exposure at work.⁶ The indiscriminate discharge of heavy metal-laden industrial effluents into bodies of water and soil is now causing life-threatening health risks to humans.⁷ Globally, about 160 million people consume untreated surface water and contaminated groundwater for drinking.⁵

In the current situation of large heavy metal contamination, microbial-aided remediation is a ray of hope. Conventional heavy metal remediation techniques are not only costly but are also ineffective at low metal concentrations.⁷ Therefore, it is high time to find alternative ways to remove toxicity from the environment, and bioremediation of heavy metals mediated by bacteria may be one of them.⁵ Bioremediation is an innovative and promising method for removing heavy metals from contaminated water and land. It is more appealing than physicochemical techniques because it is less expensive and more efficient at low metal concentrations.⁸ Heavy metals such as mercury, arsenic, cadmium, and other heavy metals are bioremediated by halophilic bacteria from wastewater because of their power, availability, and ease of cultivation.⁹ Many *Halomonas* species can detoxify various heavy metals via bio-adsorption, either directly with the cellular boundaries or via secreted molecules like extracellular polymeric substances (EPS).¹⁰

Cadmium (Cd) is a toxic heavy metal that, through different natural and anthropogenic sources, enters the environment and is a potential danger to most organisms, including

humans. By ingestion or inhalation, humans are exposed to cadmium. Overexposure to cadmium can lead to bone diseases, renal damage, and several forms of cancer.⁸ Many studies have been carried out in the past to isolate and identify cadmium-resistant bacterial strains, such as *Staphylococcus aureus*, *S. lugdunensis*, *Bacillus subtilis*, *B. thuringiensis*, *Lactobacillus plantarum*, *Ralstonia metallidurans*, *Alcaligenes eutrophus*, *Serratia liquefaciens*, *Pseudomonas putida*, *P. aeruginosa*, *P. fluorescens*, *E. coli*, *Comamonas testosteroni*, and *Klebsiella planticola*. There is a smaller number of reports showing bioremediation of cadmium by halotolerant bacteria.¹¹ Therefore, this study aimed to isolate halophilic and halotolerant bacteria from mangrove rhizosphere soils and study their ability to remove cadmium pollution at high salinity and production of antimicrobial and antioxidant agents.

Methodology

Sampling

Soil samples were collected in June 2021 from four locations within Tarut Bay on the Arabian Gulf coast and the Farasan Islands of the southern Red Sea, Saudi Arabia. Three locations (Darin, N26°32'56.03" E50°4'48.06", Safwa, N26°39'43.85" E49°59'17.07", and Syhat, N26°30'20.95" E50°2'38.11") were in Tarut Bay (Figure 1A), and one location, the Sajid Island in Farasan Islands (N16° 42' 12.59" E41° 58' 59.99") (Figure 1B). Figure 2 shows the four different sites where mangrove plants grow and they were used for rhizosphere soil collection. The soil samples (at a depth of 5 cm) were collected in labeled

containers, kept cool on ice, and then transported to the laboratory in a 4°C refrigerator for further analyses.

Preparation of Cadmium Metal Solutions

The known amount of each analytical-grade cadmium chloride (CdCl_2) was dissolved in deionized distilled water to a concentration of 1000 ppm. The stock solution was diluted with deionized water to obtain the required concentrations.¹²

Isolation of Halophilic Bacteria

Isolation of halophilic bacteria was performed according to the method reported by Baati et al.¹³ with minor changes. Different types of media containing cadmium (II) metal (100 ppm) with high salt concentrations (3 M NaCl) were used for the specific isolation of halophilic bacteria resistant to cadmium in high salinity concentrations. The agar media used were Luria-Bertani (LB), which was prepared as described by Gan et al.,¹⁴ saline nutrient as termed by Elyasifar et al.,¹⁵ and casein agar as described by Nieto et al.¹⁶ Bacterial isolates on each medium were counted, purified, and maintained on the same medium until used.

Selection of Cadmium-Resistant Bacteria

The collected soil samples were air-dried, powdered, and 0.5 g of each sample was suspended in 10 mL of sterile water, after which serial dilutions were prepared. Petri dishes containing

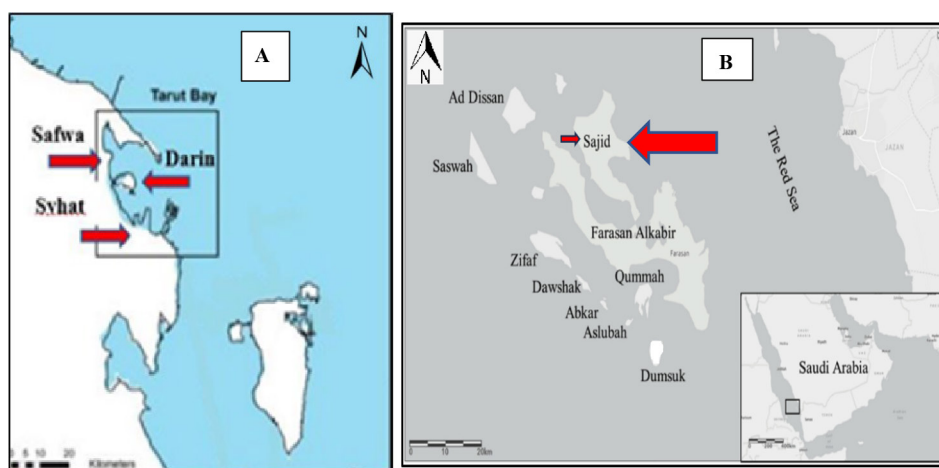


Fig. 1 Map showing the location of mangrove sites. A: Darin, Safwa, and Syhat Islands, and B: Sajid Island, Saudi Arabia. Red arrows show the studied mangrove Islands.

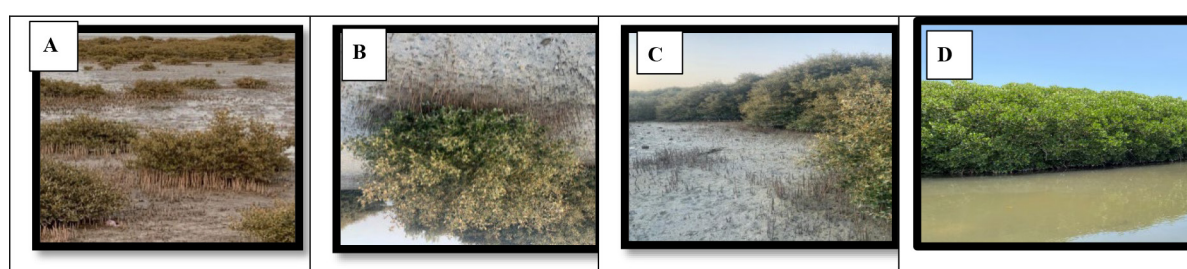


Fig. 2 Sampling sites used for mangrove rhizosphere soils collection, Saudi Arabia. A: Darin area (salinity, 4.29%), B: Safwa area (salinity, 2.02%), C: Syhat area (salinity, 2.55%), and D: Farasan area (salinity, 5.05%).

solidified media containing 100 ppm of CdCl_2 and 3 M NaCl were inoculated with 0.1 mL (100 μL) of the suitable dilution of soil suspension, which was spread on the surface of the medium using a glass spreader. All plates were incubated at 37°C. The growth of the appeared colonies on each surface plate was checked at 2-day intervals for at least one week. Agar plates without cadmium metal were used as controls. The growth of each isolate was detected as +++ (high growth), ++ (moderate growth), and + (low growth). After all, the most resistant isolates to cadmium metal were selected and screened on a medium containing increasing concentrations of the metal salts (100-600 ppm) to isolate strains resistant to high concentrations of the cadmium metal. For the highest cadmium resistance, isolates were chosen to analyze the minimum inhibitory concentration (MIC). The selected strains were grown on Cd-incorporated LB agar medium (50-800 ppm of Cd (II)) with a 3 M NaCl concentration. The lowest concentration of cadmium that completely inhibits visible bacterial growth on a Petri plate or a low to very little growth is known as the MIC dose.¹⁷

Halotolerant Bacteria Identification

The isolates LR40, LD39, LD57, and LD59 were grown in LB agar medium and examined after Gram staining using an 100X objective lens.¹⁸ The DNA of each isolate was extracted, purified, and amplified in PCR using universal primers (Macrogen, Korea): 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-TACGGCTACCTTGTACGACTT-3'). The obtained PCR products were sequenced and compared using the commercial service of Macrogen, Korea.

Antibacterial and Antioxidant Activities

The bacterial isolates LR40 and LD57 were grown in 1000 mL of LB broth medium containing 3 M NaCl, with or without 50 ppm of cadmium (II). About 50 mL of growth medium was distributed in a 250 mL Erlenmeyer flask and inoculated with 2 mL of the preculture of the selected bacterial isolate. All flasks were incubated at 37°C for 2 days. After growth, the culture filtrate was extracted with an equal volume of ethyl acetate, and the antimicrobial activity of the extract was detected against different uropathogenic bacteria using an agar well diffusion assay. Uninoculated LB broth containing 50 ppm Cd (II) was used as a control to ensure that pathogen growth inhibition was caused only by the tested isolates, not by the metal itself.

Also, cells of isolate LR40, grown in normal medium or in Cd-containing medium (100 ppm) (due to being susceptible to cadmium chloride), were collected, prepared for scanning electron microscope (SEM), and examined at King Fahad Center for Medical Research in Jeddah using a FEI Quanta 250 SEM.

Similarly, flasks containing the same medium without Cd (II) addition were prepared, and the antioxidant activities of the filtrate, ethyl acetate extract, and cell extract were detected after growth in LB broth for 2 days at 37°C. After centrifugation at 5000 rpm for 10 min at 4°C, cells were collected, lyophilized for 24 hr. and disrupted in liquid nitrogen. The obtained cell lysate was extracted with acetone in the dark. Then, acetone extracts were centrifuged at 5000 rpm, 4°C for

10 min, and the resulting supernatant, containing carotenoids and other lipophilic compounds, was dried and dissolved in phosphate-buffered saline (PBS). The crude filtrate was sterilized by filtration or extracted with ethyl acetate as described previously and dried using a lyophilizer and a rotary evaporator, respectively. Then, they were dissolved in DMSO and tested at a concentration of 1 mg/mL for antioxidant activity. Butylated hydroxytoluene (BHT, 1 mg/mL) was used as a positive control in the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method. The extract solvents (DMSO or PBS) were used as a negative control. All measurements were taken three times, and the mean was calculated. The percent of inhibition relative to the negative control (radical scavenging activity) was measured from the formula:

$$\% \text{ Scavenging activity} = 100 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The radical scavenging assay was performed as described by Sachindra et al.¹⁹ with some modifications. A 0.12 mM DPPH solution was prepared in methanol, and 200 μL of this solution was mixed with 22 μL of each extract solution. The absorbance decrease at 517 nm was recorded in a microplate reader after incubation at 25°C for 30 min in the dark. A color control prepared with 200 μL of methanol and 22 μL of each extract sample was used as a control. When the activity of a sample exceeded 65%, the concentration of the extract providing 50% reduction of the radical scavenging activity (EC50) was calculated from a graph built by plotting the log of the radical scavenging activity (%) against the different sample concentrations.

Determination of the Effect of Cadmium Ions on Bacterial Growth in a Liquid Medium

Bacterial growth patterns under cadmium (II) stress were studied by growing the selected resistant bacterial strains (LR40 and LD57) in the absence or presence of varying Cd (II) concentrations. During this study, fresh cultures of LR40 and LD57 were grown in flasks containing 25 mL of LB broth medium (pH ~7.0; 3 M NaCl) supplemented with Cd (II) metal (ranging from 100-1000 ppm) or without the metal (control). This experiment was carried out in an orbital shaker incubator at 37°C and 150 rpm agitation speed for 48 hr. The bacterial growth in different Cd (II) concentrations was measured by taking optical density (OD) at 600 nm using a UV-Visible Spectrophotometer (Shimadzu UV-1650PC).²⁰

The Effect of Different Physiological Factors on the Growth of The Two Tested Isolates

The effect of different incubation periods (1 to 5 days), various NaCl concentrations (0, 1, 2, 3, 4, and 5 NaCl), varied pH (4, 5, 6, 7, 8, 9, and 10), and different temperatures (24, 30, 35, 37, 40, 45, and 50°C) on growth of the two tested isolates was performed according to the method reported before.²¹⁻²³ The selected isolates (2 mL of 24-hr culture) were inoculated into 25 mL of sterile LB broth containing 3 M NaCl, then incubated at the appropriate temperature. After incubation, the OD of each sample was measured at 600 nm with a UV spectrophotometer.

Preparation of Live Bacterial Biomass for the Biosorbent Process

Biomass of isolates LR40 and LD57 (biosorbent) was prepared by inoculating the bacterial strains into LB broth containing 3 M NaCl, with the media shaken in an orbital rotary shaker (JSSI-I00T from JSR) at 150 rpm for 72-96 hr. at 37°C. Live bacterial biomass was separated from the culture by centrifuging at 4500 rpm for 10 min at 4°C and then washed twice with sterile saline (0.9 g NaCl/100 mL distilled water). The collected live bacterial biomass was dried at room temperature in a laminar flow hood and then stored at 4°C in the refrigerator until use.²⁰ In the stoppered conical flasks (100 mL), 25 mL of LB broth medium containing 3 M NaCl and cadmium (II) metal solution at different concentrations (100-500 ppm) was prepared, and the pH was adjusted to 7.0. About 0.25 g of live LR40 and LD57 bacterial biomass was resuspended, then the flasks were kept shaking in an orbital rotary shaker at 150 rpm for 144 hr. For cadmium analysis, a fixed volume of culture supernatant was filtered through Whatman No.1 filter paper, and the filtrates were analyzed for cadmium concentration by Avio 200 ICP-OES. Using the following equation, the metal removal efficiency (MRE) was obtained:

$$\% \text{ of MRE} = C_i - C_f / C_i \times 100$$

Where C_i represents the initial cadmium metal ion concentration, and C_f represents the final cadmium metal ion concentration.

Statistical Analysis

All values were presented as the mean of three readings (mean $\pm$ standard deviation (SD)). Statistical analyses were conducted using one-way ANOVA for parametric data and the Kruskal-Wallis test for non-parametric data, utilizing the Statistical Package for Social Sciences (SPSS) program (Version 26.0). The one-way ANOVA was employed to assess variations in the primary research variable based on the secondary variables. Results with a p -value ≤ 0.05 were considered statistically significant.²⁴

Results

Soil samples from mangrove rhizosphere soils along the Red Sea coasts of Saudi Arabia were collected to isolate halotolerant bacteria. The four locations were Darin, Safwa, and Syhat in Tarut Bay and Sajid Island in the Farasan Islands

(Figures 1 and 2). A range of agar media (Luria-Bertani (LB), saline nutrient, and casein agar) containing 3 M NaCl and 100 ppm cadmium (II) were used to isolate halophilic bacteria resistant to cadmium under high-salinity conditions. This technique resulted in the isolation of 125 Cd (II)-resistant bacteria. The majority of resistant isolates were obtained on casein agar > saline nutrient agar > Luria-Bertani agar (Figure 3A). It can also be concluded that most resistant bacteria isolated from all agar media were obtained from Darin > Farasan Island > Syhat > Safwa (Figure 3B).

All bacterial isolates grown on the three media types containing 100 ppm Cd (II) and 3 M NaCl produced a range of colony colors, including creamy, white, or yellow. They were either Gram-positive or Gram-negative bacteria, with the shape of bacilli, coccobacilli, or streptobacilli. The characters of seven of these isolates were summarized in Table 1. The LR40, LD47, LD57, and LD59 isolates grew well on/in LB medium and were the most resistant isolates to Cd (II). The LD47 and LD59 isolates showed good growth in the presence of 100 ppm Cd (II), with an MIC of 300 ppm. However, the two other isolates, LR40 and LD57, could grow at higher metal concentrations, with excellent growth (++++) at 100 ppm Cd(II), and the growth decreased with increasing metal concentration up to 500 ppm. Their growth was very weak or disappeared at 600 ppm, and the MIC of Cd (II) for them was 600 ppm (Table 2 and Figure 4); thus, these two Cd (II)-resistant bacteria were selected for further investigation.

The 16S rRNA sequence of metal-tolerant bacterial isolates showed the highest (97-99%) similarity to the genera *Vreelandella*, *Halomonas*, and *Halobacillus*. They were identified as *Vreelandella alkaliphila* strain LR40 (accession number PP767765.1), *Halomonas smyrnensis* strain LD57 (accession number PP767765), *Halomonas maris* strain LD47, and *Halobacillus faecis* strain LD59 (Table 3 and Figure 5).

Figure 6A illustrates the growth charts of the isolated LR40 and LD57 grown in LB broth at various concentrations of Cd (II) (ranging from 100-1000 ppm). The results showed that, in comparison to the control, the growth of the LD57 isolates decreased significantly at 600 ppm Cd (II), while the growth of LR40 was much higher than that of the LD57 isolate at this metal concentration. The effect of the incubation period on the growth of LR40 and LD57 isolates in the presence or absence of Cd (II) metal was studied. The bacterial growth gradually improved as the incubation period increased from 1 to 2 days, and the best growth of both isolates in the presence of Cd (II) was for a period of 2 to 4 days (Figure 6b).

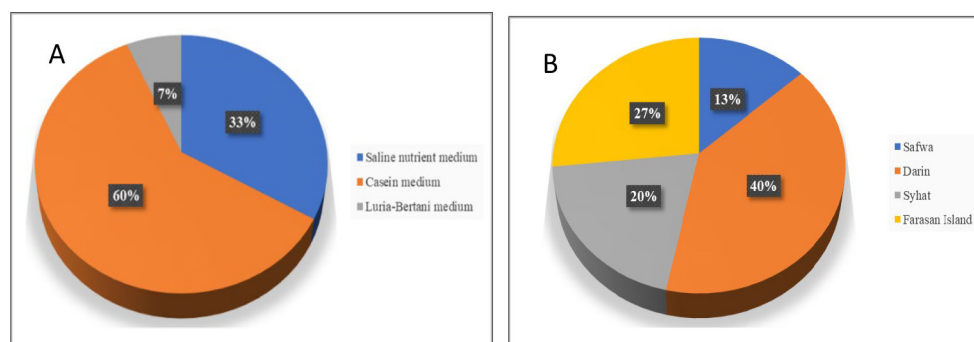


Fig. 3 Isolation of cadmium-resistant halophilic bacteria. A: The used media with 3 M NaCl and 100 ppm Cd (II), and B: The isolation areas.

Table 1. **Growth of the most cadmium-resistant bacterial isolates on modified Luria-Bertani (LB) medium containing 3 M NaCl and 100 ppm Cd(II)**

No.	Bacterial isolate	Source of soil	Color of the colony	Shape of bacterial cell	Gram's reaction	Growth on LB agar	Growth on LB agar with Cd(II) (100 ppm)
1	LR39	Farasan Island	Creamy				++
2	LR40	N16°42'12.59" E41°58'59.99"	Creamy				+++
3	LD47	Darin	White				+++
4	LD57	N26°32'56.03" E50°4'48.06"	Creamy	Bacilli	Gram-negative	+++	+++
5	LD59		Creamy				+++
6	LF57	Safwa	Yellow				++
7	LF58	N26°39'43.85" E49°59'17.07"	Creamy				++

+++; High growth; ++, Moderate growth.

Table 2. **Screening of four bacterial isolates for tolerance to Cadmium (0-600 ppm) using Luria-Bertani (LB) agar containing 3 M NaCl**

Cd(II) concentration	Tested isolate			
	LR40	LD47	LD57	LD59
Control	+++	+++	+++	+++
50 ppm	+++	+++	+++	++
100 ppm	+++	++	+++	++
200 ppm	++	+/-	++	+
300 ppm	++	+/-	++	-
400 ppm	+	-	+	-
500 ppm	+	-	+	-
600 ppm	+/-	-	+/-	-

+++; High growth; ++, Moderate growth; +, Low growth; +/-, Very low growth; -, No growth.

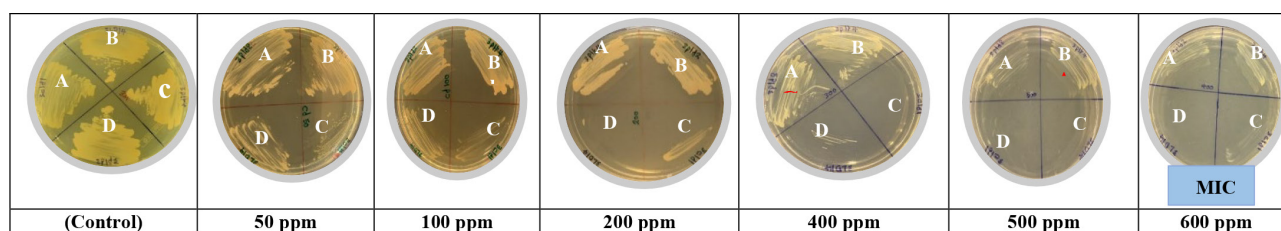


Fig. 4 **Growth of some bacterial isolates on Luria-Bertani (LB) amended with varying concentrations of cadmium(II). A: LD57, B: LR40, C: LD47, and D: LD59, and a minimum inhibitory concentration (MIC) of 600 ppm of the metal for LR40 and LD57.**

Table 3. **Identification of the four cadmium-resistant bacteria based on 16S rRNA gene sequences**

The isolate	The recommended species	Similarity (%)	GC content (%)
LR40	<i>Vreelandella alkaliphila</i> strain LR40	99	54.0
LD47	<i>Halomonas maris</i> strain LD47	97	48.0
LD57	<i>Halomonas smyrnensis</i> strain LD57	99	39.0
LD59	<i>Halobacillus faecis</i> strain LD59	99	44.0

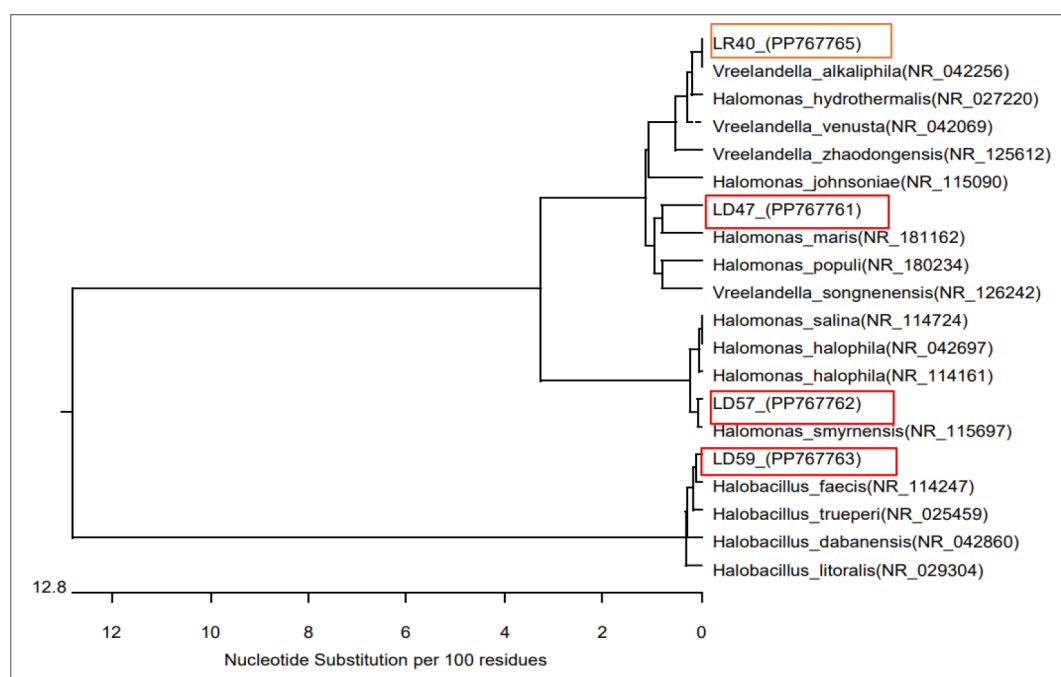


Fig. 5 The phylogenetic tree of the four isolated bacteria based on 16S rRNA gene sequences and the most related genera.

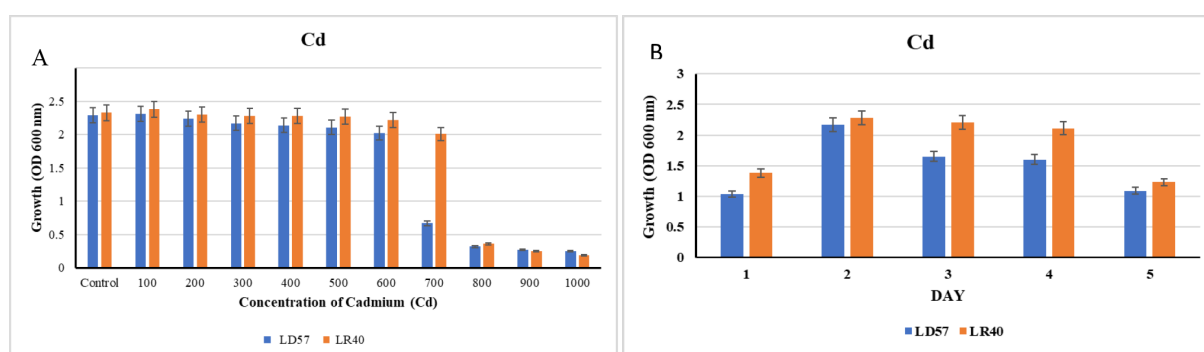


Fig. 6 The growth of LR40 and LD57 isolates in Luria-Bertani (LB) broth with 3 M NaCl. A: LB containing different concentrations of Cd (II) metal, and B: Different incubation periods for the media. (I) refers to standard error (SE).

Based on the salt range, isolates LR40 and LD57 grew well and tolerated 2 and 3 M NaCl in LB broth with 300 ppm Cd (II), with the optimal salt concentration being at 3 M NaCl. With increasing NaCl concentration, the growth gradually decreased, and very weak growth was recorded at 5 M NaCl (Figure 7A). Because the two strains could grow in the absence of salt, they are characterized as halotolerant. The growth of LR40 and LD57 isolates in LB medium containing 3 M NaCl and 300 ppm Cd (II) was affected by various pH levels, as shown in Figure 7B. The results showed that LR40 and LD57 isolates grew at different pH values, where weak growth was noted at very acidic (4 and 5) and alkaline (9 and 10) conditions. However, well-developed growth was recorded at pH 6 and 7, while at pH 8, the growth was moderate. Thus, it could be said that very weak acid or alkaline conditions were optimal for the growth and survival of LR40 and LD57 strains.

The effects of varying temperatures on the growth of isolates LR40 and LD57 were depicted in Figure 8A. The outcome demonstrated that isolates LR40 and LD57 could grow in LB broth medium (with 3 M NaCl and 300 ppm cadmium(II)) for 48 hr at 24°C to 45°C, with 37°C being the optimum

temperature. Figure 8B shows the amount of Cd (II) metal uptake by the live biomass of isolates LR40 and LD57 at different metal concentrations (100-500 ppm). The cadmium adsorption capacity of the live biomass of isolate LR40 ranged from 20.62 to 70.34%, with a maximum of 71.56% at 400 ppm Cd (II). In comparison, the capacity of isolate LD57 ranged from 45.75 to 72.85%, with a maximum adsorption of 72.85% at 500 ppm.

A scanning electron microscope (SEM) was used to analyze the morphological variations in *Vreelandella alkaliphila* strain LR40 in the presence and absence of cadmium ions (Figure 9). It demonstrated that bacteria grew in the absence of Cd (II), aggregated with an intact and well-defined membrane, and maintained morphological features (Figure 9A). In the presence of Cd (II), cells became twisted with stretched cell size, and precipitates were created, as seen in Figure 9B. The various abnormalities in shape and deformation of all these results showed cadmium toxicity to bacteria, which causes varying degrees of harm.

Uropathogenic bacteria are specialized microorganisms, primarily strains of *E. coli* cause urinary tract diseases by

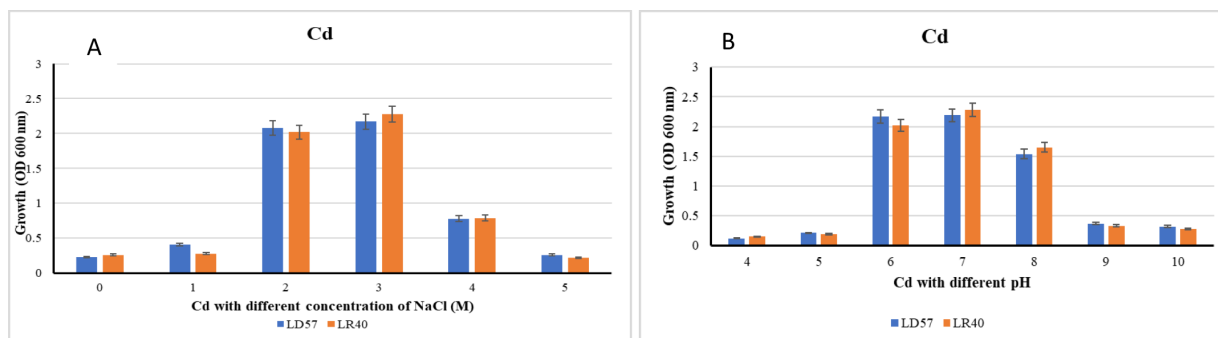


Fig. 7 The growth of isolates LR40 and LD57 in Luria-Bertani (LB) containing cadmium(II) (300 ppm). A: LB medium with different NaCl concentrations (0-5 M), and B: LB medium with varied pH (4-10) values. (I) refers to standard error (SE).

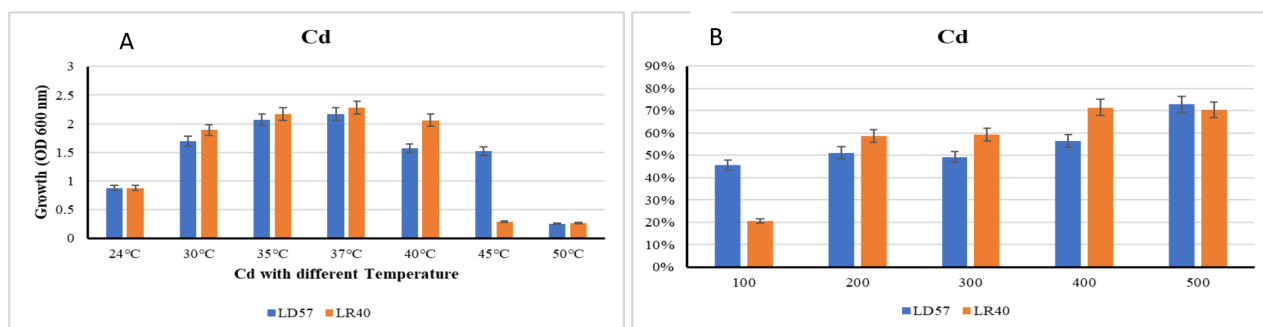


Fig. 8 A: Effect of different temperatures on the isolates LR40 and LD57 grown in LB broth with 300 ppm cadmium(II), and B: The capacity of Cd(II) removal by isolates LR40 and LD57 grown in different concentrations of the metal.

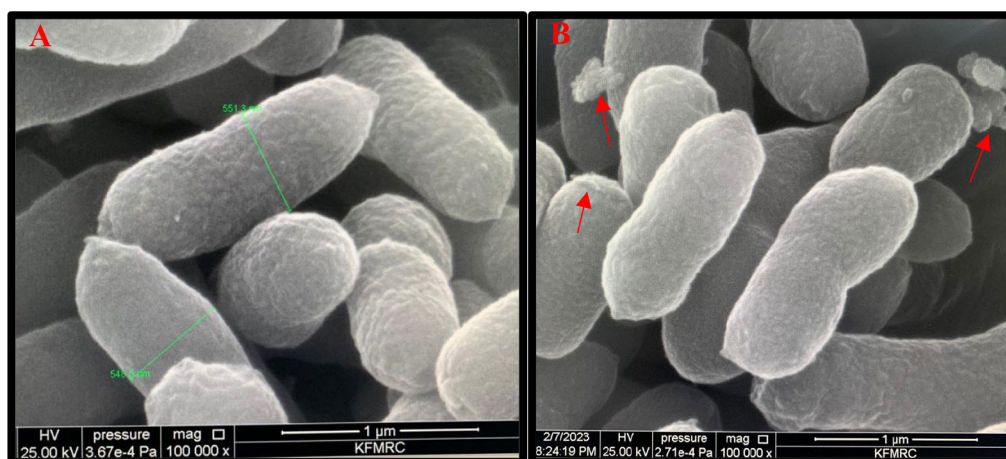


Fig. 9 Scanning electron microscopy (100,000X magnification) of *Vreelandella alkaliphila* strain LR40 grown in Luria-Bertani (LB) medium with 3 M NaCl. A: LB without cadmium (II) metal, and B: LB with 100 ppm Cd (II). The arrows show the precipitates of metal ions.

colonizing the urinary tract, adhering to uroepithelial cells, and invading tissue. They are the main cause of community-acquired infections, often leading to cystitis (bladder infection) or pyelonephritis (kidney infection). In the screening experiment for antimicrobial property, the control (50 ppm cadmium-supplemented media without isolates) showed no antibacterial activity against all tested pathogens. The isolates *V. alkaliphila* strain LR40 and *H. smyrnensis* strain LD57 had moderate antibacterial activities with inhibition zone diameters ranging from 10 to 16 mm. The highest activities of the two isolates grown in LB without cadmium were against Gram-positive bacteria (*Staphylococcus aureus* and

Enterococcus faecalis) and Gram-negative *Klebsiella aerogenes*. However, the presence of Cd (II) significantly enhanced the antibacterial activity of the two isolates against *E. coli* and *Klebsiella aerogenes* and the activity of *H. smyrnensis* strain LD57 against *Pseudomonas aeruginosa* and *Proteus mirabilis*. While no clear inhibition was recorded by the *V. alkaliphila* strain LR40 against the two previous pathogens (Table 4).

The antioxidant potential of *V. alkaliphila* strain LR40 and *H. smyrnensis* strain LD57 extracts was tested in radical scavenging assays, based on the DPPH (1,1-Diphenyl-2-picrylhydrazyl) method. The half maximal inhibitory concentration IC50 was calculated for the extracts that exhibited antioxidant

activities higher than 65%. The antioxidant activity (%) and the half effective concentration (EC₅₀) were calculated for aqueous and ethyl acetate extracts in addition to acetone extracts of cells (1 mg/mL) obtained from *V. alkaliphila* strain LR40 and *H. smyrnensis* strain LD57 and determined by DPPH. No clear antioxidant activity was recorded for the filtrates of the two tested isolates, while excellent activity was observed in the ethyl acetate and cell extracts of the tested bacterial isolates. The highest antioxidant activity was recorded for the cell extract of *H. smyrnensis* strain LD57 (76%, EC: 11.4), followed by the cell extract of *V. alkaliphila* strain LR40 (72.6%, EC: 10.9). The data were submitted to one-way analysis of variance (ANOVA), and the same letters denote groups with no significant differences ($P < 0.05$) (Table 5).

Discussion

Mangrove rhizosphere soil harbors a great diversity of halotolerant or halophilic bacteria. In this study, the four selected sites were rich with plants associated with salt-tolerant bacteria, which showed good growth on the three selected media containing 100 ppm of Cd (II). Chang et al.²⁵ found that the salinity of the rhizosphere soil of mangroves in Saudi Arabia was lower compared to that of the mangrove forest in Al-Thakira, Qatar (15.48 ppt). The addition of Cd (II) to the isolation media was used for the specific isolation of Cd-resistant bacteria that live at high salinity concentrations. Casein agar was the best medium for isolation of halotolerant Cd-resistant bacteria compared to saline nutrient agar and LB media, which may be due to the suitable compounds and pH value. Also, the highest bacterial counts were recorded from Darin Island, which may be due to the moderate salinity of its soil compared to the other

studied saline sites of Tarut Bay (Syhat and Safwa) and Farasan Island. Out of 125 isolates, 7 (5.6%) showed good growth in the presence of Cd (II). Out of them, isolates LR40 and LD57 were the most tolerant to Cd (II) with MIC of 600 ppm. Examination of these isolates under the light microscope showed that the cells of isolates LR40 and LD57 from an overnight culture were Gram-negative and rod-shaped (bacilli). Similar to these results, Orji et al.¹⁸ reported that three unique halotolerant and metal-tolerant aerobic bacterial strains were isolated from brine and sediment samples of Uburu Salt Lake, Ebonyi State, Nigeria. They were all Gram-negative and rod-shaped. In addition, Amasha and Alhazzani²⁶ isolated halophilic bacteria from mangrove rhizosphere soils of Saudi Arabia that were active in lead removal.

Moreover, LR40 and LD57 strains could grow to a maximum concentration of NaCl (5 M) and also grow in the absence of salt; they were classified as halotolerant. It is worth mentioning that halotolerant bacteria grow best at <1% w/v salt and can tolerate high salt, whereas halophile bacteria require high salt for growth.²⁷ These strains represent adequate characteristics to resist the harsh conditions of NaCl and heavy metals, which is consistent with the findings of other researchers, such as the study results of Chaïda et al.,²⁸ which demonstrated that *Bacillus aquimaris* could grow against a wide range of NaCl concentrations (between 0-12% w/v) and pH 5.5-12.

According to the results of the 16S rRNA analysis, LR40 and LD57 strains were identified as *Vreelandella alkaliphila* strain LR40 and *Halomonas smyrnensis* strain LD57. Zmorrod et al.¹² isolated, from solar salterns in Lebanon, a species *Halomonas venusta* H9, which showed high tolerance to both heavy metals (lead and cadmium). The cadmium resistance of

Table 4. Inhibition zones diameters produced by extracts of the two tested isolates grown with or without 50 ppm cadmium(II), against seven uropathogenic bacteria

Tested bacterial pathogen	50 ppm Cd (II) (control)	<i>V. alkaliphila</i> LR40	<i>V. alkaliphila</i> LR40 + Cd (II)	<i>H. smyrnensis</i> LD57	<i>H. smyrnensis</i> LD57 + Cd (II)
<i>E. coli</i>		**13.5 ± 0.55	15.1 ± 0.45*	11.5 ± 0.25	13.1 ± 0.13*
<i>Klebsiella pneumoniae</i>		15.5 ± 0.01	16.1 ± 0.16	13.0 ± 0.15	12.4 ± 0.13
<i>Klebsiella aerogenes</i>		11.0 ± 0.11	15.1 ± 0.43*	16.1 ± 0.13	16.4 ± 0.15
<i>Pseudomonas aeruginosa</i>	ND	ND	ND	11.1 ± 0.11	14.1 ± 0.18*
<i>Proteus mirabilis</i>		ND	ND	11.4 ± 0.18	13.1 ± 0.33*
<i>Staphylococcus aureus</i>		16.4 ± 0.18	16.1 ± 0.13	10.1 ± 0.23	11.1 ± 0.31
<i>Enterococcus faecalis</i>		16.1 ± 0.63	16.1 ± 0.13	10.1 ± 0.44	10.1 ± 0.21

ND, No inhibition was recorded; **, Mean value of inhibition zone diameter (mm) ± SD; *, Significant differences compared to control at $P < 0.05$.

Table 5. Determination of the antioxidant activity (%) and IC₅₀ (µg/mL) of the filtrate, ethyl acetate extracts, and cell lysate acetone extracts (1 mg/mL) obtained from the two tested isolates by DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay

<i>V. alkaliphila</i> strain LR40			<i>H. smyrnensis</i> strain LD57		
Filtrate	Ethyl acetate extract	Cell extract	Filtrate	Ethyl acetate extract	Cell extract
ND	9.8b (65.3%)	10.9a (72.6%)	ND	10.4a (69.3%)	11.4a (76.0%)

ND, Not detectable.

the two studied isolates, LR40 and LD57, was similar to that of the halophilic bacterium *H. venusta* H9. The *Halomonas* genus has been extensively studied for bioremediation and the synthesis of desired compounds. It is known to have strains that can tolerate high lead (Pb^{+2}) and cadmium (Cd^{+2}) concentrations above 1000 and 500 mg/L, respectively.²⁹

The effect of various Cd (II) concentrations on bacterial growth was studied. The results showed that as Cd (II) concentrations increased, growth across all isolates decreased relative to the control. The greatest effect of salt concentration on the growth of isolates LR40 and LD57 with the presence of Cd (II) was observed when the NaCl concentration was increased from 1 to 3 M, which raised growth. We concluded that elevating the NaCl concentration increased cadmium tolerance, which agrees with the findings of Sowmya et al.²¹ Sodium and potassium are necessary elements for the activity of enzymes and pumps in halophiles; therefore, it seems that these metals enhance tolerance to toxic metals.³⁰ The effect of different NaCl concentrations on heavy metal tolerance and removal should be considered in future studies as a significant factor.²⁹ The selective pressure imposed by high salinity and metal concentrations allows the enrichment of microorganisms with metabolic capabilities for simultaneous tolerance of salts and heavy metals.²

The results also showed that there is no growth for both isolates in the presence of Cd ions at pH 4 and 5, which is similar to the findings of Divakar et al.²² Also, the optimum growth rates for LR40 and LD57 isolates were generally observed between 30–37°C, regardless of the cadmium present. A drastic drop in growth occurred when the temperature increased to 45°C, followed by a rapid decline in growth at temperatures above 45°C, which agrees with the findings of Mgbodile et al.²³ The result showed that when the concentration of Cd (II) metal increased from 100 to 500 ppm, it was observed that the removal % of Cd (II) increased as well, which is consistent with the findings of Zmorrod et al.¹² The present study found a Cd (II) removal rate of approximately 21 to 70% by *Vreelandella alkaliphila* LR40 and 46 to 73% by *Halomonas smyrnensis* LD57. These results corroborate the work of Castillo-Zacarias et al.³¹, who isolated phenol-resistant bacteria in Monterrey, México, from industrial polluted effluents, showing Cd^{2+} removal rate of 23 to 78% by *Enterobacter cloacae*, 23 to 64% by *Pseudomonas aeruginosa*, and 24 to 64% by *Klebsiella pneumoniae*.³²

Vreelandella alkaliphila strain LR40 in the presence and absence of cadmium was examined under the SEM. The cells' morphology appeared normal when grown in medium without Cd(II) metal. In contrast, in the presence of the metal, cells became abnormal in shape and size and precipitated metal ions. Similarly, Mohapatra et al.³³ studied the interaction of metal ions on biomass surfaces using scanning electron microscopy.

Halophilic and halotolerant bacteria exhibit antibacterial activity primarily through the production of halocins that inhibit the growth of competing microorganisms. They are considered potent probiotics due to their ability to modulate

immune responses, detoxify heavy metals, and enhance bactericidal activity in aquatic culture. These isolated bacteria from mangrove rhizosphere soils live in hypersaline environments and possess excellent characteristics to survive.

Their significant antimicrobial activity is due to the production of compounds like bioactive peptides, biosurfactants, and enzymes that inhibit pathogens in food and clinical settings. They can be promising agents in food preservation, and biocontrol against some pathogens.^{34–36} The halotolerant bacterium *Halomonas* sp. produces a range of secondary metabolites with antimicrobial qualities. These extracellular metabolites (filtrate) were secreted into the medium and exhibited moderate to good inhibition zones against pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*, with MICs ranging from 128 to 256 µg/mL. These metabolites showed antioxidant properties with 100% scavenging at 512 µg/mL.³⁷ Bahamdain and Aly³⁸ reported that the presence of copper (Cu) or nickel (Ni) (50 mg/L) in the growth medium significantly enhanced the antibacterial activity of *Streptomyces vinaceusdrappus* and *S. huasconensis* against *Staphylococcus hominis*.

Gómez-Villegas et al.³⁹ reported that none of the extracellular extracts obtained from the culture media of *Halococcus hispanicus* and *Halobacterium salinarum* exhibited antioxidant activity. However, cellular extracts of LR40 and LD57 isolates showed considerable antioxidant activity, effectively scavenging DPPH radicals at rates of 72.6 and 76%, respectively. The antioxidant capacity of halobacteria has been documented in several studies.^{40,41} Supporting the previous findings, *Halogeometricum rufum*, *Halogeometricum limi*, and *Haladaptatus litoreus* exhibited antioxidant activity with EC50 values ranging from 2.5 to 10 µg/mL,^{40,41} which may be due to the presence of antioxidant compounds such as polyphenols and carotenoid pigments. These results indicated that halotolerant bacterial extracts could be a promising source of antioxidant compounds with various applications, including natural food preservatives, colorants, and supplements aimed at preventing oxidative damage.^{42,43}

In conclusion, the bioremediation of toxic cadmium metal in hypersaline environments can be achieved using *V. alkaliphila* strain LR40 and *H. smyrnensis* strain LD57, which were discovered to be resistant to Cd (II) under extreme conditions at 3 M NaCl concentration. Because of their ability to treat metal under elevated salt conditions, this is an advantage over other current remediation techniques. Where they can remove cadmium metal from saline and non-saline environments. This was the first study that investigated cadmium-resistant bacteria in mangrove soil. Several other microorganisms with the potential for bioremediation can be isolated using the mangrove ecosystem. Bioremediation using extremophilic microbes is a powerful technology because these microbes can thrive in unfavorable environmental conditions.

Conflict of Interest

None. ■

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