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Isolation and Molecular Recognition of Heavy Metal and Antibiotic Resistant Bacteria in the Inlet and Outlet Sewage of Ahvaz Refinery, Iran

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Abstract

The influent of toxic heavy metals into aquatic environments has greatly increased and is considered a serious hazard for living organisms. In recent years, several technologies have been developed with the aim of reducing or removing heavy metals from the contaminated environment. Among these, technology developed based on microorganisms is more advantageous than other methods. In the present study, metal resistant bacteria (MRB) and antibiotic resistant bacteria (ARB) were first isolated from the sewage treatment plant of Ahvaz, Iran. Sampling was carried out from sewage. Then, biological oxygen demand (BOD), chemical oxygen demand (COD), pH, and concentration of heavy metals were detected in the samples by ICP-AES. Metal resistant bacteria were isolated by the agar diffusion method on lead (Pb) and cadmium (Cd) in a PHG II medium. The isolates were molecularly identified by genome sequencing. Next, the antibiotic resistant pattern of the potent metal resistant isolates (MIC > 1 mg/mL) was determined. Results showed that Pb and Cd concentrations in the sewage samples were above global standards (0.3 and 0.04 mg/mL, respectively). *Bacillus cereus* and *Salmonella enterica* subsp. enterica serovar typhi were found to be the most potent Pb resistant isolates (MIC = 5.5 mg/mL, MBC = 6 mg/mL on both isolates). The MIC and MBC on *Bacillus cereus* were 3.0 and 3.5 mg/mL, and the MIC and MBC on *Salmonella enterica* serovar typhi were 4.0 and 4.5 mg/mL, respectively. The isolated *Bacillus cereus* also showed high resistance to cefixime and penicillin.

1. Introduction

The development of various industries and the entrance of food, agricultural, medical, and industrial wastewaters into the environment, as well as the use of wastewater for irrigation of agricultural lands, have led to the pollution of ecosystems, such as surface and groundwater, as well as soils, by different metals. On the other hand, antibiotics have been shown to have a detrimental impact on plants and animals in both the short and long term (Kesari et al., 2010; Ali et al., 2019; Doyle et al., 2020). Research into the bioremediation of toxic heavy metals using bacteria has been gaining interest. It has received a great deal of attention due to its compatibility with the natural environment and safety for ecosystems, human beings, animals, and plants

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(Verma & Kuila, 2019; Deepa & Suresha, 2014). Nowadays, water resources are being exposed to different types of biological and chemical pollutions (Medfu Tarekegn et al., 2020). The abundance of pollutants from industrial waste hangs over vital human resources. The protection and maintenance of human health is the basic goal of environmental health, and recognition and elimination of potential threatening risks are essential (Briancesco et al., 2013).

Toxic heavy metals are considered one of the most durable and dangerous pollutants in the world and have harmful environmental impacts (Agoro et al., 2020). Heavy metals can enter the environment through several routes, including mines, agricultural drainage, metallurgy, and electronics and industrial wastes. For this reason, the removal of heavy metals from the environment is an important issue for public health (Gautam et al., 2016; Zhang et al., 2011; Medfu Tarekegn et al., 2020). Sources of heavy metals, especially cadmium and lead, result from human activities, such as fuel ignition, batteries waste, lead-acid batteries, smelting operations, and household as well as chemical sewages, toxins, insecticides and herbicides, industrial effluents, radioactive effluents, petroleum, and colored hydrocarbons (Medfu Tarekegn et al., 2020; Siddiquee et al., 2015). Humans are continual exposed to 35 toxic metals, among these, 23 metals are categorized as heavy metals. Since heavy metals do not break down, they gradually accumulate in the tissues of plants, animals, and humans and are one the most important water pollutants that lead to disorders in various chemical and biological processes (Zolfaghary et al., 2015.; Tchounwou et al., 2012).

Metals enter water resources mostly by pollution from different industrial, medical, and domestic sewage. Also, agricultural fertilizers and pesticides introduce heavy metals to aquatic environments (Brahman et al., 2013; Agoro et al., 2020). In recent years, heavy metal pollution has become one of the most serious environmental problems. Environmental pollution by toxic heavy metals is widespread as a result of industrial progress all over the world. The presence of heavy metals has toxic and harmful effects on all living organisms (Doyle et al., 2020). Pollution of drinking water resources is particularly important and needs serious consideration. The origin of water pollution can be different from other pollutants; it may occur at the source of the water supply, as primary pollution in tanks, or as secondary contamination by exposure during storage. Secondary pollution is more severe where portable water supplies are provided. In general, heavy metals produce systemic toxicity, which specifically impacts the nervous system, kidney, and liver; and they can be carcinogenic to different tissues of the body, which can cause fatality (Genchi et al., 2020; Gautam et al., 2016). These metals also affect the immune system and cause cardiovascular impacts/diseases. In order to prevent the damage caused by heavy metals, it is necessary to prevent these metals from entering into the environment, including water resources (Zolfaghary et al., 2015). Along with the improvements of urbanization and industrial activities, environmental pollution, a variety of antibiotic resistant bacteria that are simultaneously resistant to heavy metals has become a global problem (Kraemer et al., 2019). While these bacteria are found in many environments, they are chiefly in aquatic environments (Uchida et al., 2016). Therefore, there is a direct link between human activities and the development of bacteria resistant to antibiotics and heavy metals (Di Cesare et al., 2016; Nguyen et al., 2019.; Tomova et al., 2015). In our research, isolation and molecular identification of heavy metal resistant bacteria (HMRB) and antibiotic resistant bacteria (ARB) was done on the inlet and outlet of a sewage treatment plant in Ahvaz, Iran. Additionally, the relationship between heavy metals and antibiotics resistance was studied.

2. Materials and methods

2.1. Sampling area and biochemical testing for bacterial identification

Samples were aseptically obtained from the influent and effluent sewage treatment plant No. 2 of Ahvaz (Iran). Sampling was performed in sterilized flat plastic bottles. The samples were transferred cold to the research laboratory at the Islamic Azad University, Falavarjan branch, to perform various experiments. The samples were transferred to the laboratory on ice within 5 to 6

hours. The research community in this study included a number of bacteria entering and living in that specific sewage treatment plant. During this study, a total of about 23 bacteria were isolated. In order to identify the phenotypic properties of the bacteria, bacterial colonies were gram stained and then evaluated in terms of shape, color, margin, surface, and consistency of the colony. Also, biochemical tests including catalase, urease, coagulase, hemolysis in blood agar, mannitol salt agar, and novobiocin and bacitracin resistant tests were performed on the gram-positive cocci. A catalase test, starch hydrolysis, gelatin hydrolysis, motility, hemolysis, growth in the presence of 7% NaCl, and VP test were performed on the gram-positive bacilli. Oxidase, urease, MR-VP, citrate, TSI, SIM, glucose broth, lactose broth, and sucrose broth and mannitol broth tests were used to identify the gram-negative bacilli (Rania et al., 2019).

2.2. Antibiotic susceptibility testing

Each of the isolated bacteria was evaluated for resistance to heavy metals and antibiotics. One of the methods used to determine antibiotic resistance using the Kirby Bauer disc agar diffusion technique, which was used in this study. Antibiotic susceptibility testing was done by the disc diffusion method. Bacterial suspension with a count of 1.5×10^8 CFU/mL was prepared and inoculated on the surface of Mueller-Hinton agar plates. Cultures of the selected bacteria were prepared on an MHA plate. The antibiotic discs were placed on the medium, and after 18-24 h incubation, the diameter of the growth inhibition zone was measured in millimeters and then compared with the CLSI standard, based on sensitivity or resistance (Tomova et al., 2015).

2.3. Determination of BOD

Manganese sulfate (2 mL) and 2 mL of alkaline iodine and sodium azide (NaN_3) were added to each sample, and then the sealed bottle was mixed upside down at least 15 times until a manganese hydroxide precipitate appeared. Next, 2 mL of concentrated sulfuric acid was added and mixed gently until the precipitate was completely dissolved, then 1-2 mL of starch was added to make it blue. The titration was continued until the solution was discolored, and then the volume of thiosulfate used was measured (Marzan et al., 2017.; Iloms et al., 2010). Finally, the BOD of the samples was measured using a BOD meter (Metrohm, 857).

2.4. Determination of COD

First, 0.4 g of $HgSO_4$ was placed in a 250 mL flask. Then, 20 mL of the sample was added to the flask and mixed. Next, 2 mL of concentrated sulfuric acid were added, and the balloon was shaken to mix the contents well. Using a pipette, 10 mL of 0.25 N potassium dichromate ($K_2Cr_2O_7$) solution was added to the flask, followed by 30 mL of silver sulfuric acid-sulfate solution. A control solution was prepared by repeating the above steps and replacing the sample with distilled water. Then, a Ferric ammonium sulfate (Ammonium iron III sulfate) solution was added. Next, 30 mL of concentrated sulfuric acid was added and then titrated with a ferric ammonium sulfate solution. Finally, the COD of the samples was measured using a COD meter (Marzan et al., 2017).

2.5. pH measurement

The pH meter was calibrated with the buffer solutions, and the pH of the samples was measured. The pH value is the most important parameter for water and wastewater analysis. The pH of a neutral solution is normally 7 at 25 ˚C, but at a temperature of 0˚C, it is set to 7.5 and at 60 ˚C to 6.5. Most metal compounds dissolve at a low pH, and their concentration influences the degree of metal resistance by microorganisms (Kuhn & Pfizer, 1990). Most bacteria prefer a neutral pH to measure the pH, about 50 mL of the sample was first transferred to a 200 mL balloon. Then, 5 mL of concentrated nitric acid was added to the sample, and it was slowly evaporated over a steam bath. We continued until a clear solution was reached, and the sample was further dried during the digestion step. When the sample size was reduced to 10 to 20 mL, the amount of heavy metals and non-metallic materials was read by ICP-AES (Azad et al., 2020).

2.6. Measurement of metals and non-metals concentration

To measure the metal and non-metal concentrations, about 50 mL of the sample was first transferred to a 200 mL balloon. Then, 5 mL of concentrated nitric acid was added to the sample and slowly evaporated over a steam bath. When the sample size was reduced to 10 to 20 mL, the amount of heavy metals and non-metallic materials was read by ICP-AES.

2.7. Preparing samples for microbial counting

To determine the microbial population of the samples, the pour plate method was used to count the heterotrophic bacteria in the inlet and outlet water samples of the treatment plant. To reduce the experimental error, this method was repeated three times for each sample.

2.8. Studying of heavy metal resistant strains/bacteria

There are two practical methods for studying metal-resistant microbes, the agar dilution diffusion method and the replica plating method. The agar diffusion method was used in this study. In this method, a sterile PHG II culture medium (Peptone: 4 g, yeast extract 1 g, glucose 2 g, agar 15 g) was first prepared, its temperature was adjusted to 55°C, and the metal solution was added Next, the pH of the culture medium was adjusted to 7. The plates were then placed in an oven at 37 °C for 30 minutes to rid the surface of the plate of any moisture. In the next step, 0.5-0.1 mL of different dilutions of effluent from 1-10 to 6-10 was spread on the surface of a PHG II metal culture medium with a sterile pipette, and then the plates were incubated at 30 °C for up to 5 days. Two plates were also used as the sterile control. After the incubation period, the number of metal-resistant colonies was calculated according to the dilution and the sample size. It should be noted that for each metal and each dilution, three experiments were performed. After the growth of resistant colonies and counting, those that differed in appearance (color, consistency, and surface) were selected and enriched in tubes containing PHG II culture medium with the same metal and the same concentration of the original medium. These culture tubes were incubated for 18 to 24 hours to purify the resistant bacteria. To store the bacteria, a loop of each was added to 1 mL of a PHG broth medium containing 10% glycerol and was transferred to -70ºC (Marzan et al., 2017; Tomova et al., 2015).

2.9. Determination of minimum inhibitory concentration (MIC) of heavy metals

The MIC is generally used to verify unusual resistances. The range of heavy metal concentrations used to determine the MIC is generally accepted to be twice as high and below 1 mg/L. MIC was considered as the minimum concentration of antimicrobial agent (here heavy metal), which had the ability to inhibit bacterial growth after 24 hours of incubation (Andrews, 2001). The MBC was the minimum concentration of the antimicrobial substance (heavy metal) that has the ability to kill 99.9% of bacteria. The MBC is determined after the MIC. The resistance and tolerance of microorganisms to heavy metals can be evaluated in three ways: the agar diffusion method, using sterile paper discs, and the method based on preparing consecutive dilutions in test tubes. In this study, the agar diffusion method was used. Plates containing PHG II agar medium with different concentrations of metal were prepared; each plate contained one concentration of metal. The plates were placed at 30°C for 48-96 hours, and a blank metal plate was used as a sterilization control (Marzan et al., 2017; Tomova et al., 2015; Shahsanaei Goneirani et al., 2016).

2.10. Assessment of multi-resistance to heavy metals

To determine the resistance to multiple metals, a PHG II media salted with different metals was used. In this study, lead and cadmium were the main targets for the study of resistance to heavy metals (Marzan et al., 2017). Twenty-three isolates of heavy metal resistant bacteria were isolated (codes 1 to 23) and re-cultured on PHG II agar medium containing the heavy metals lead and cadmium and then incubated in a 30°C incubator for 1-3 days (depending on the strain) (Kermanshahi et al., 2007). Bacterial colonies appeared on the above medium. These pure colonies of the samples that had the highest resistance to these two metals were used for PCR.

2.11. Molecular identification of the isolated bacteria based on 16S rDNA sequence

Bacterial genome extraction was performed using Qiagen DNA extraction kit (QIAamp DNA Mini Kit). PCR was carried out for amplification of 16S rDNA using a pair of universal primers, including 27F (5'- AGA GTT TGA TCC TGG CTC AC-3') and 1492R (5'- CGG TTA CCT TGT TAC GAC TT-3'). The PCR products were sequenced following agarose gel electrophoresis and the purification of single bands; then, the sequences were analyzed, proofed by Chromas 2.1 software, and detected by a BLAST server. For this purpose, after evaluating the quality of the PCR product resulting from the amplification of the desired fragment related to the 16S rDNA gene in terms of being single-band, 30 microliters of the PCR product were sent to the relevant companies for sequencing. The results were checked, and the relevant or close species were identified in the NCBI database/gene bank using the BLAST database according to the percentage of coverage and similarity (Rania et al., 2019; Momtaz et al., 2013).

2.12. Statistical analysis of data

ANOVA statistical analysis and SPSS software version 20 were used to compare the mean populations of isolated heterotrophic and resistant bacteria. Also, Excel software was used to compare the results and determine the agreement confidence interval with standard error.

3. Results and Discussion

In the present study, two species of Pb and Cd resistant bacteria were isolated. The MIC of both heavy metals was determined on the two isolates. MIC=5.5 mg/mL and MBC=6 mg/mL were detected on both isolates. Both isolates were also resistant to Cd; MIC=3.0 mg/mL and MBC=3.5 were detected on *Bacillus cereus*, and MIC=4.0 mg/mL and MBC=4.5 mg/mL were detected on *Salmonella enterica*. In Roan and Kellogg's study (1995), the MIC of Pb on Bacillus sp., Corynebacterium sp., Staphylococcus aureus, and *Pseudomonas aeruginosa* was reported as 2.5-6.5 mg/mL. This high level of resistance was probably due to the large amounts of Pb in the water and soil of the sampling area and the long-term Pb presence in the region. Multiple tolerances to metals are common among resistant bacteria and are transmitted by means of different genetic paths such as transformation, conjugation, transduction, and by transposons (Allocati et al., 2013; Abskharon et al., 2018). In the present study, heavy metal concentrations for the isolation of resistant bacteria were selected based on the results of previous reports (Hussein et al., 2004). In these studies, the metal resistance of bacteria was determined based on the tolerance to the concentrations of 1 mM of CU, Zn, and Pb and 0.1 mg/mL of Cd. Raja and Selvam (2009) also considered the isolates that grow in 0.5 mM Ni, Cd, and Pb as resistant to these heavy metals.

In the present study, showed high sensitivity against ordinary antibiotics, although the isolated *Bacillus cereus* showed high resistance to Cefixime and Penicillin. The BOD and COD changes depend not only on the concentration of organic matter but on the potential of the bacteria and the temperature of the wastewaters (Burgohain et al. 2010). It has been shown that a low BOD amount represents clean water and the loss of microorganisms (Ziv et al., 2011). When the amount of BOD reaches 5, the purity of the water is questionable, and when it exceeds 20, it is considered threatening. The higher COD concentration in comparison to BOD may be due to the presence of toxic compounds, such as heavy metals in the water that prevents the activity of degrading microorganisms. The activity of these organisms determine the BOD amount (Hussein et al., 2004). Amplification of the 16S rDNA gene resulted in the production of a 370 bp fragment in isolate 7 and a 540 bp fragment in isolate 23 (Fig. 4). Phylogenetic analysis of the isolates showed that the Gram-positive rod (isolate 7) was related to *Bacillus cereus* strain 18A-B5 (99% homology), and the Gram-negative rod (isolate 23) was related to *Salmonella enterica* sub sp. enterica serovar typhi strain ERL082356 (100% homology). As the isolated bacteria in the present study were highly resistant to Pb and Cd, they can be selected in the future to eliminate these heavy metals from industrial wastewaters after removing the genes responsible for antibiotic resistance. The location of studied area was shown in fig. 1.

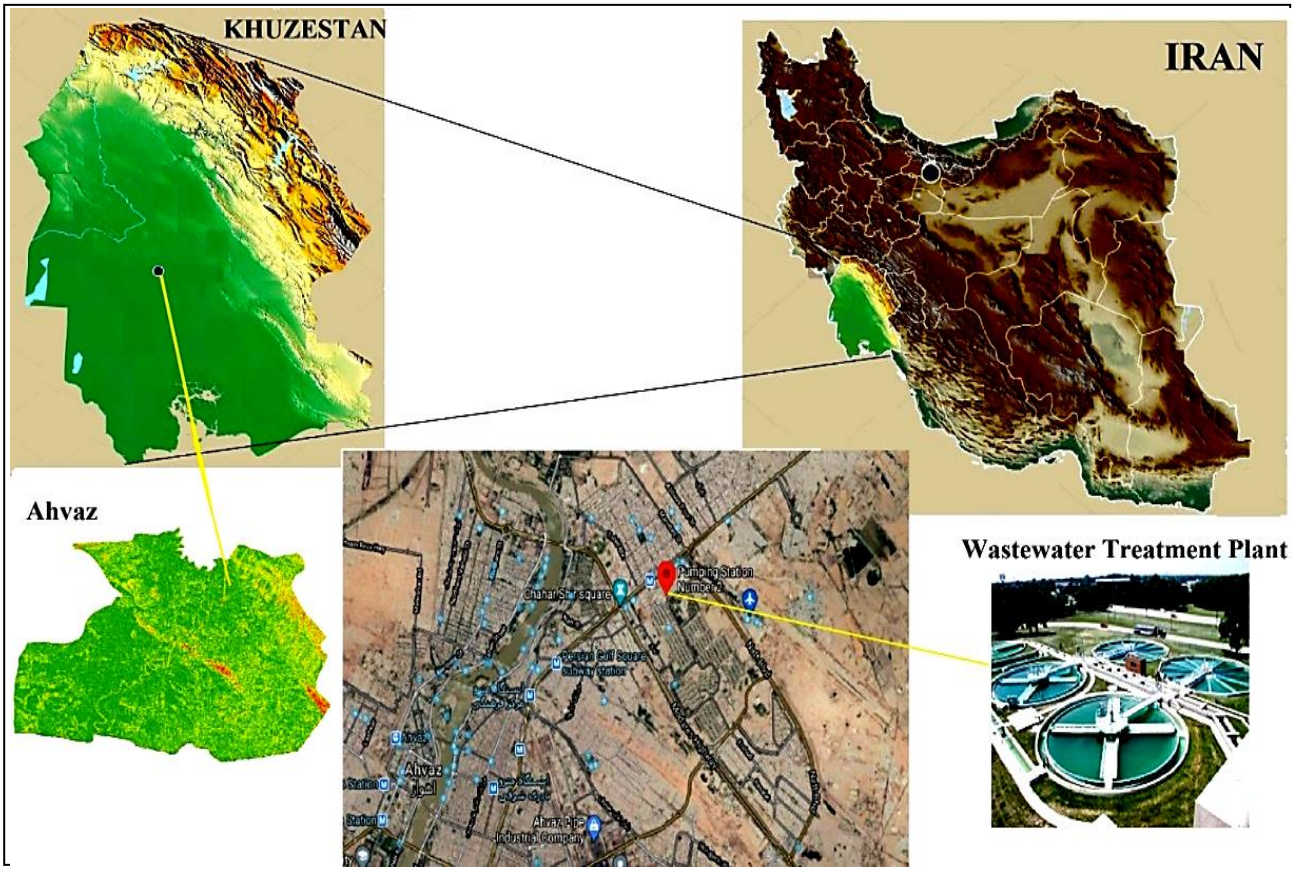

Fig. 1. Location of studied area; Sewage treatment plant No. 2 in Ahvaz city, Iran.

3.1. Biochemical tests of isolates

The biochemical tests of isolated bacteria to heavy metals were detected after gram-staining, and were used to identify the morphology and properties of bacteria (Table 1).

Table 1. Biochemical tests to identify Pb and Cd resistant isolates

Isolate 7 (Gram-positive rod)							
Motility Test		Hemolysis	VP	Growth in 7% Nacl	Starch Hydrolysis	Catalase Test	Starch Hydrolysis Test
+		β	+	-	+	+	+
Isolate 23 (Gram-negative rod)							
Indole	Citrate	Carbohydrate Fermentation Test (Mannitol, Sucrose, Lactose, Glucose)	MR/VP	TSI	Oxidase	Urease	Motility Test
-	-	+---	+/-	K/A/H2S+	-	-	+

Explanation of the signs of the table: The sign (-) in the table indicates a negative answer to the relevant test, the sign (+) in the table indicates a positive answer to the relevant test, Symbol (A) means bacteria produced in an acidic environment, and Symbol (K) means that the bacteria has produced in the open.

3.2. Antibiotic resistance of isolates

The antibiogram results can be seen in Table 2 and Figs. 2 and 3. Among 23 isolated bacteria, the highest resistance against Pb and Cd was found by isolates 7 and 23. Isolate 7 showed the highest resistance to cefixime and penicillin antibiotics (Table 2).

Table 2. The results of antibiotic resistance pattern analysis of Pb and Cd resistant isolates

Isolate 7 (Gram-positive rod)					
CP (5 µg/disc) S (33mm)	NOR (10 µg/disc) S (31 mm)	P (10 µg/disc) R	V (30 µg/disc) S (18 mm)	CFM (5 µg/disc) R	TE (30 µg/disc) S (28 mm)
Isolate 23 (Gram-negative rod)					
CP (5 µg/disc) S (38mm)	CFM (5 µg/disc) S (32 mm)	GM (10 µg/disc) S (38mm)	FM (300 µg/disc) S (23 mm)	C (30 µg/disc) S (26 mm)	NA 30 (µg/disc) S (25 mm)

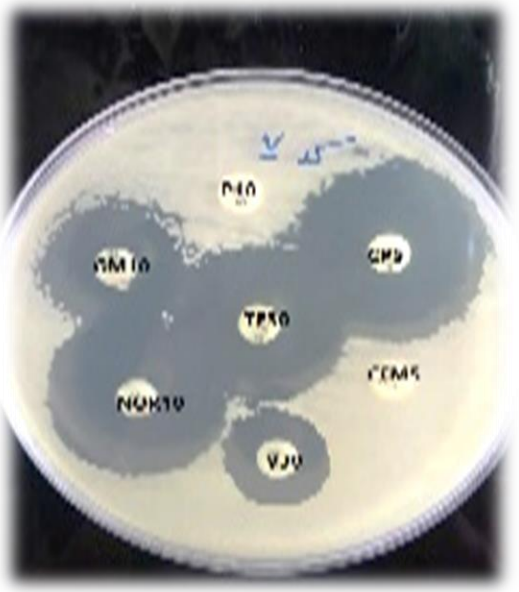

Fig. 2. Isolated antibiogram test, code 7 (*Bacillus cereus* strain 18A-B5).

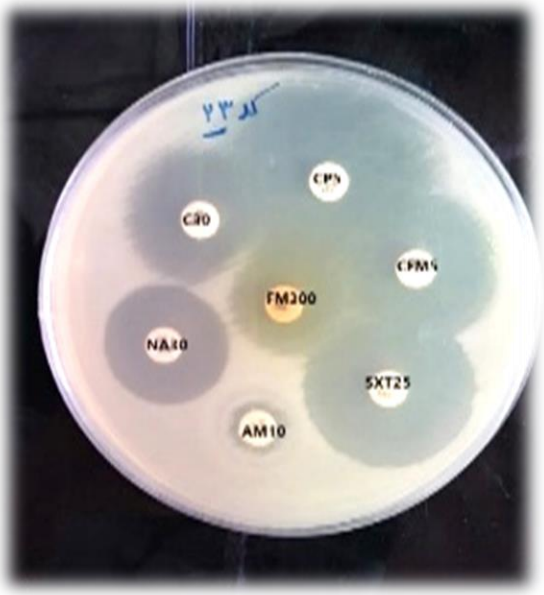

Fig. 3. Isolated antibiogram test, code 23 (*Salmonella enterica* subsp. *enterica* serovar *typhi* strain ERL082356).

3.3. The pH, BOD, and COD results

The amounts of pH, BOD, and COD of the influent and effluent samples are shown in Table 3. The pH of wastewaters in the present study was near neutral, although the population of heterotrophic bacteria was different. Therefore, this difference cannot be considered to be due to the different pH of the wastewaters. The BOD of wastewaters in the present study were between 2-4 mg/L for influents (average = 3 mg/L) and 0.5-1 mg/L for effluents (average = 0.75 mg/L). The COD of wastewaters were between the range of 3.2-4.8 mg/L (average = 4.5 mg/L) for influents and 0.9-2.8 mg/L (average = 1.7 mg/L) for effluents. According to the global standard, industrial wastes should fall in the range of 20 to 100 mg/L for BOD, and the maximum of 120 mg/L for COD, the maximum amount of Cu, Pb, Cd, and Ni should be 1, 0.2, 0.03, and 0.2 mg\L, respectively.

Table 3. Physicochemical analysis of the sewage samples obtained from Ahvaz treatment plant No. 2 influent and effluent.

	pH		**BOD (mg/L)**		**COD (mg/L)**	
	Infl.	Effl.	Infl.	Effl.	Infl.	Effl.
Min	7.5	7.3	2.0	0.5	3.2	0.9
Max	8.1	8.0	4.0	1.0	4.8	2.8
Mean	7.9	7.6	3.0	0.75	4.5	1.7

Infl: Influent; Effl: Effluent; Min: Minimum; Max:Maximum

3.4. The concentrations of heavy metals

The heavy metals concentrations in the samples are shown in Table 4. According to the wastewater analysis in this study, the amount of Pb and Cd in the influent and effluent of the Ahvaz sewage treatment plant were above the global standard, for Pb 0.3, 0.04 mg/L and for Cd 0.2, 0.04 mg/L (which had high concentrations). Andrews et al. (2005) previously isolated heavy metal resistant bacteria from highly contaminated wastewater. They reported high concentrations of Cu, Cd, and Pb in wastewaters to be 0.176, 0.250, and 1.575 mg/mL, respectively.

Table 4. The concentrations of measured heavy metals in Ahvaz sewage treatment plant No. 2 influent and effluent.

Waste water	Cu (mg/mL)	Ni (mg/mL)	Pb (mg/mL)	Cd (mg/mL)
Influent	<0.05	<0.05	0.3	0.04
Effluent	<0.05	<0.05	0.2	0.04

3.5. The number of heavy metals resistant bacteria The average population of heterotrophic bacteria in the inlet and outlet of Ahvaz sewage treatment plant No. 2 treatment plant showed that there was a significant difference between the population of heterotrophic bacteria entering and leaving the treatment plant ($p \leq 0.005$). Table 5 shows the average population of heterotrophic bacteria in this treatment plant in both water inlet and outlet modes.

Table 5. Total heterotrophic bacteria count and the number of heavy metals (Pb and Cd) resistant heterotrophic bacteria.

Wastewater	Total heterotrophic bacteria (Cfu/mL)	Pb and Cd resistant heterotrophic bacteria (Cfu/mL)
Influent	1.02×10^4	4.3×10^2
Effluent	4.8×10^2	1.03×10^1

3.6. MIC of heavy metals To determine the minimum concentration of heavy metals that inhibited the growth of bacteria, culture medium containing 0.5, 1, 2, 4, 8, 16, 24, and 32 mmol of cadmium (Cd) and lead (Pb) were used. Table (6) show the MIC and MBC values for the heavy metal resistant bacteria mentioned in the inlet and outlet of Ahvaz sewage treatment plant No. 2 treatment plant. The MIC and MBC on *Bacillus cereus* were 3.0 and 3.5 mg/mL, and MIC and MBC on *Salmonella enterica* serovar *typhi* were 4.0 and 4.5 mg/mL.

Table 6. The results from the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Pb and Cd against metal resistant isolates.

Bacteria	Pb		Cd	
	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)
Isolate 7	5.5	6.0	3.0	3.5
Isolate 23	5.5	6.0	4.0	4.5

3.7. Molecular characterization of isolates Molecular identification of the highly resistant bacteria to Pb and Cd revealed two strains. A thermocycler was optimized to amplify the 16S rDNA fragment as follows: The initial denaturation stage was considered at 95 °C for 1 minute, and another denaturation at 94°C for 5 min, then annealing at 58°C for 30 s, an extension stage at 72 ° for 1 min, and finally the 72°C elongation for 5 minutes. *Bacillus cereus* and a *Salmonella enterica* subsp. enterica serovar *typhi* are displayed in Fig. 4.

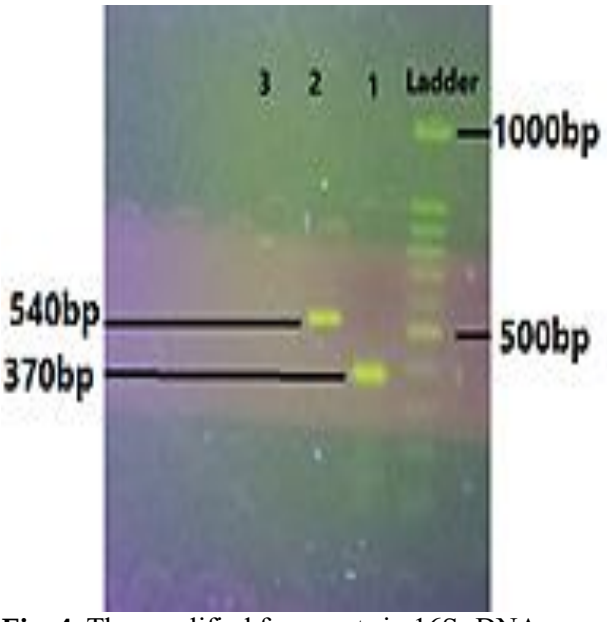

Fig. 4. The amplified fragments in 16S rDNA genes of Pb and Cd resistant isolates according to the 100 bp DNA ladder. No. (1) is the isolate 7, (2) is the isolate 23, and (3) is the negative control.

3.8. The phylogenetic trees

The phylogenetic trees are illustrated in Figs. 5 and 6. At this stage, bacterial genotypes were identified after molecular analysis with blast software. After sequencing, code (7) had the closest resemblance to *Bacillus cereus* strain 18A-B5, showing a 99% similarity. The phylogenetic tree of this bacterium is also shown in Fig. 5. After sequencing, the isolates (23) showed closeness to *Salmonella enterica* subsp. enterica serovar typhi ERL082356, with a 100% similarity. The phylogenetic tree of this bacterium is also shown in Fig. 6.

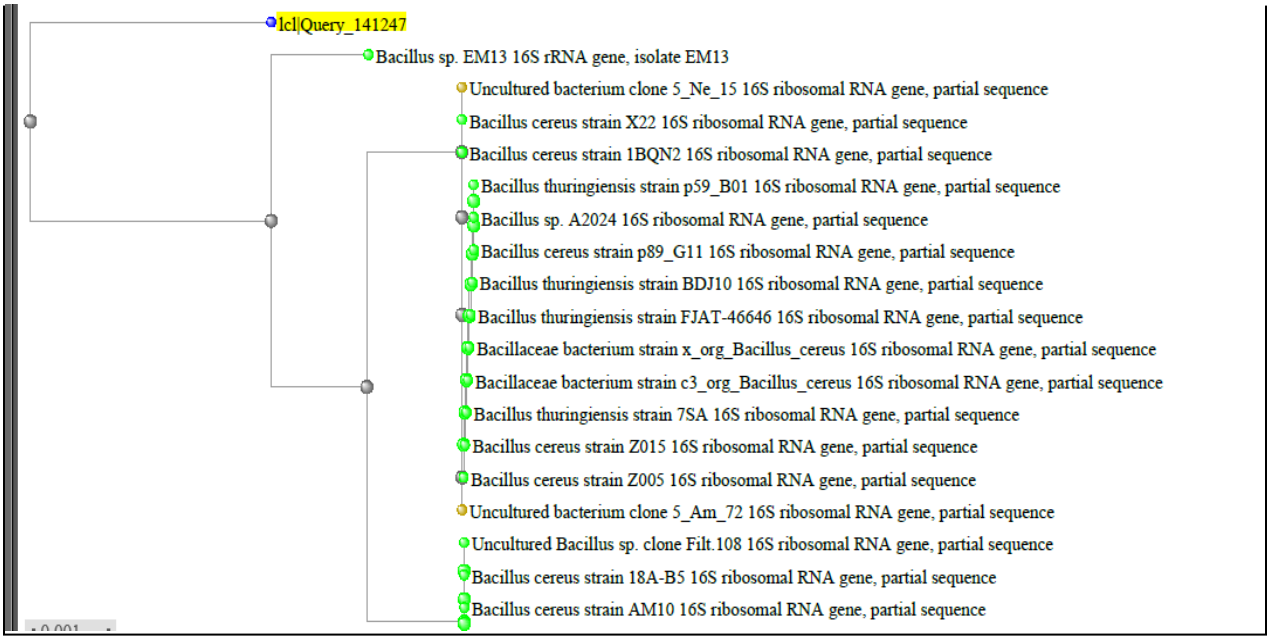

Fig. 5. Phylogenetic tree isolate code (7) with *Bacillus cereus* strain 18A-B5 (used in this study).

Fig. 6. Phylogenetic tree isolate code (23) with *Salmonella enterica* subsp. *enterica* serovar *typhi* ERL082356 (in this study)

4. Conclusions

According to international standards, the amount of most metals and Pseudo-metals found in the inlet and outlet of Ahvaz sewage treatment plant No. 2 were within the global standard, with the exception of lead and cadmium, which were reported to be higher than normal in both inlet and outlet. In this study, *Staphylococcus aureus, Staphylococcus epidermidis, Bacillus cereus, Bacillus subtilis, Bacillus polymyxa, Pseudomonas aeruginosa, Escherichia coli, Citrobacter freundi, Shigella sonei, S. salmonella typhi, and Salmonella typhi,* which were more or less resistant to lead and cadmium, were isolated from the Ahvaz sewage treatment plant. In our research, two species of Pb and Cd resistant bacteria from the 23 strains isolated from the Ahvaz (Iran) sewage treatment plant No. 2, were isolated and considered as water pollution (biological pollutants). The *Bacillus cereus* strain 18A-B5 and *Salmonella enterica* subtype enterica serovar typhi strain ERL082356 both had high resistance to lead and cadmium. The *Bacillus cereus* strain 18A-B5 was also greatly resistant to the antibiotics Cefixime (CFM5) and penicillin (P10). The heavy metal resistant bacteria in the present study showed high sensitivity against ordinary antibiotics. Potential Pb and Cd resistant bacteria were detected in this study; moreover, the antibiotic resistance of some of the other isolates is also a major disadvantage and should be considered for environmental metal bioremediation. In this study, the colony-PCR technique and 16S rDNA gene were used for the molecular identification of isolates. This research provided useful information for the management and control of these pollutants (toxic heavy metals) in the future.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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