Characterization of heavy metal and antibiotic-resistant bacteria isolated from polluted localities in Egypt

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Objective
The aim of this study was to isolate and identify heavy metal-resistant and antibiotics-resistant bacteria from contaminated samples (wastewater and soil) collected from different industrial areas in Egypt and determine their role in heavy metal removing.
Materials and methods
Samples were collected from Helwan and 10th of Ramadan city areas and enriched in culture broth containing 200, 100, and 10 ppm of arsenic (As), lead (Pb), and cadmium (Cd) as $AsHNa_2O_4{\cdot}H_2O$, $Pb(NO_3)_2$, and $CdSO_4$, respectively. The highly resistant isolate (ST6) was selected and identified biochemically and also subjected to 16S rDNA sequencing. The growth parameters were optimized and the maximum tolerable concentration of the respective metals as well as the antibiotic resistance was determined.
Result and conclusion
After enrichment culture we isolated and purified 20 bacterial isolates resistant to the respective heavy metals As, Pb, and Cd. The morphological, biochemical, and phylogenetical characteristics of the most resistant bacterial isolates (ST6) were studied. The results showed that this isolate belongs to the species *Pseudomonas stutzeri.* The optimum temperature was 35°C, whereas the optimum pH was in the range of 6–7. Maximum tolerable concentration values for As, Pb, and Cd were 3500, 1750, and 50 ppm, respectively. Also, the isolate ST6 showed resistance against different antibiotics. The metal removal ability was 42.5, 57.1, and 52.9% of As, Pb, and Cd, respectively. It was concluded that the ST6 isolate was resistant and removed high concentrations of As, Pb, and Cd. Hence, this isolate may play a role in bioremediation processes of heavy metal in polluted areas.

Keywords:
antibiotics, arsenic, bioremediation, cadmium, heavy metal, lead, *Pseudomonas stutzeri*, resistance

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Introduction

Heavy metals are elements with a molecular weight greater than 53, a density greater than 6 g/cm^3, and anatomic number greater than 20 [1]. Heavy metal contamination in the environment has become a serious problem because of the increase in the addition of these metals to the environment [2]. Heavy metal is introduced into the environment through metalliferous mining, metal smelting, activities of metallurgical industries, waste disposal, corrosion of metals in use, and agriculture and petroleum exploration among others. The discharge of effluents containing heavy metals such as cadmium (Cd), lead (Pb), and arsenic (As) puts pressure on the ecosystem and consequently causes health hazards to plants, animals, aquatic life, and humans [3,4]. Microorganisms have various mechanisms to resist the heavy metal stress, including blockage by the permeability barrier, intracellular and extracellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions [5–9]. These mechanisms help in detoxification or cleaning-up of the metal from the environment. Metal tolerance reflects the ability of an organism to survive in high concentrations of metals or accumulate it without dying. Metal exposure also leads to the establishment of tolerant microbial populations, which are often represented by several Gram-positive bacteria belonging to the genus *Bacillus*, *Arthrobacter*, and *Corynebacterium*, as well as Gram-negative bacteria such as *Pseudomonas*, *Alcaligenes*, *Ralstonia*, and *Burkholderia* [2,10,11].

This study aims to isolate and identify heavy metal-resistant bacteria from different contaminated Egyptian localities. In addition, we also aimed to study the coresistance to different antibiotics to determine

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the resistance mechanism as well as possible use in bioremediation processes.

Materials and methods

Study area and sampling

The study sites encompassed metal-polluted sites at industrial areas. The first site was 10th of Ramadan city, Sharkeya governorate, Egypt (30°15'.54.51 N, 31°45'.39.95'E). The second site was Eltebeen industrial area, Helwan governorate, Egypt (29°46'.54.85 N, 31°18'.09.41'E). From the first site two wastewater samples were collected from the discharge point. From the second site two industrial sediments from 10–20 cm depth were collected. Samples were kept in sterile glass bottles and stored on ice at 4°C before being transported to the laboratory and processed. The collected samples were analyzed for physicochemical properties like pH, organic matter, total dissolved salts, and respective heavy metal contents. All analyses were performed in triplicate.

Heavy metal-resistant bacteria isolation

Each sample (1.0 g of sediment sample or 1.0 ml of water samples) was enriched into a 100 ml flask containing 25 ml of nutrient broth (NB) medium [12]. In addition, each one of respective heavy metals Cd^{2+}, Pb^{2+}, and As^{5+} was also added separately as cadmium sulfate ($CdSO_4$), lead nitrate [$Pb(NO_3)_2$], and sodium arsenate ($AsHNa_2O_4 \cdot H_2O$), respectively, at a concentration of 10, 100, and 200 ppm, respectively. After incubation, flasks were incubated at 30°C and 120 rpm. Enriched cultures showing turbidity after 2 days of incubation were subcultured by streaking onto Petri dishes containing the same culture medium and heavy metal concentrations solidified with 1.6% of agar (Bacto-Agar, Difco, Detroit, MI, USA). Colonies appearing on inoculated plates and differing in shape, color, and margins were streak-purified at least three times on the nutrient agar medium in the presence of the same concentration of heavy metals, and kept at 5°C as agar slant for further studying [13].

Characterization and identification of isolate

After screening and selection of highly heavy metal-resistant bacterial isolates, the purified isolate was subjected to identification, which included culture characteristics, Gram stain, biochemical characterization according to *Bergey's manual of systematic bacteriology* [14], and finally at the molecular level using 16S rDNA sequencing.

Briefly, total DNA was extracted using the GeneJet genomic DNA extraction kit (Thermo K0721). PCR amplification of the 16S rRNA gene was performed using 10 ng of genomic DNA in 20 μl of 1× 'Amplitaq' (Perkin-Elmer, 940 Winter Street, Waltham, Massachusetts 02451, USA) buffer (10 mmol/l Tris-HCl, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 0.001% gelatin) with 150 ng each of primers, F: 5'-AGA GTT TGA TCC TGG CTC AG-3' and R: 5'-GGT TAC CTT GTT ACG ACT T-3', 250 μmol/l of dNTPs, and IU of 'Amplitaq' (Perkin-Elmer). The PCR reaction mixtures were incubated at 95°C for 3 min and then cycled 35 times as follows: at 95°C for 30 s, annealing temperature for 30 s, and at 72°C for 4 min. Annealing temperature was 60°C for the first five cycles, 55°C for the next five cycles, and 50°C for the last 25 cycles. Finally, the mixtures were incubated at 72°C for 10 min and at 60°C for 10 min; 2.0 μl of each amplification mixture was analyzed by agarose gel (1.0% w/v) electrophoresis in a TAE buffer (0.04 mol/l Tris-acetate, 0.001 MEDTA) containing 0.5 mg/ml (w/v) ethidium bromide.

Sequencing was carried out at Biomolecular Research Services (GATC Biotech, Konstanz, Germany) using ABI 3730 l× DNA sequencer with the same forward and reverse primers. A partial (first 500 bp) 16S rDNA sequence was determined for the most potent heavy metal-resistant isolate (ST6). The sequences obtained in this study were aligned to the GenBank database with the maximum similarity and identity using gene alignment database.

Optimization of growth parameters (temperature and pH)

The optimal growth conditions with reference to pH and temperature were studied. For studying the effect of pH, 0.1 ml of overnight broth culture ($OD_{620\,nm}$=0.8) of isolate was inoculated into NB medium with different pH values of 5, 6, 7, 8, and 9 using 1.0 mol/l NaOH or 1.0 mol/l HCl and incubated at 30°C for 24 h under shaking conditions (120 rpm/min). The effect of temperature was studied through inoculation of bacteria into NB medium and incubation at different temperatures of 25, 30, 35, 40, and 45°C for 24 h under shaking conditions (120 rpm/min). All experiments were performed in the presence of heavy metal separately at the following concentrations: 25, 100, and 200 ppm of Cd, Pb, and As, respectively. Also positive control (only medium inoculated with the bacteria) and negative control (only heavy metal-containing medium) were used. Bacterial growth was measured as optical density at 620 nm (UNICO 2100 UV Visible Spectrophotometer, Dickinson, Texas, USA).

Determination of the effect of metals on bacterial growth [15]

Isolate was grown in sterile NB medium without (positive control sample) or with heavy metals at 25,

100, and 200 ppm of Cd, Pb, and As, respectively. Erlenmeyer flasks were placed in a rotary shaker (120 rpm) at optimal conditions obtained from the previous experiment. NB medium was inoculated 1 : 100 (v/v) with an overnight culture, and each sterile heavy metal was added immediately before inoculation of bacterial isolate. Growth was monitored as a function of biomass by measuring the optical density (UNICO 2100 UV Visible Spectrophotometer).

Determination of maximum tolerable concentrations of heavy metals on nutrient broth medium [16]

Heavy metal ion resistance was studied using the maximum tolerable concentrations (MTCs) of the metal ions in NB media. Analytical-grade $CdSO_4$, $Pb(NO_3)_2$, and $AsHNa_2O_4{\cdot}H_2O$ were dissolved in sterilized deionized water to form desired stock solutions (1000, 5000, and 10 000 ppm of Cd^{2+}, Pb^{2+}, and As^{5+}, respectively). A volume of 0.1 ml of overnight broth culture ($OD_{620\ nm}$=0.8) of isolate was inoculated in 10 ml sterile NB containing 25, 50, 75, 100, and 125 ppm of Cd; 1000, 1250, 1500, 1750, and 2000 of Pb; or 2000, 2500, 3000, 3500, and 4000 of As individually. The inoculated culture was incubated at 30°C for 48 h in addition to negative control (culture media containing the same concentration of metals without inoculation) and blank (culture media neither inoculated with bacteria nor with heavy metal addition). After 48 h, bacterial growth was measured as optical density values at a wavelength of 620 nm using UNICO 2100 UV Visible Spectrophotometer. Experiments were carried out in triplicate.

Determination of the coresistance to antibiotics [17]

Antibiotic resistance behavior of the isolated strain was determined by the standardized Kirby–Bauer disc-diffusion method on Mueller–Hinton agar using the following antibiotics: azactam, ampicillin, tetracycline, vancomycin, clindamycin, trimethoprim/sulfamethoxazole, ciprofloxacin, chloramphenicol, bacitracin, erythromycin, rifampicin, tobramycin, amikacin, and flucloxacillin. The concentration of each disc of used antibiotic was 10, 10, 30, 30, 2, 25, 5, 30, 10, 15, 30, 10, 30, and 5 μg/ml, respectively. Mueller–Hinton agar plates were prepared by pouring sterile medium into Petri dishes. After the solidification of the medium, plates were incubated overnight at 35°C to remove excess moisture from the surface and to check contamination of the plates. A 0.1 ml of overnight broth culture appropriately diluted in normal saline solution ($OD_{620\ nm}$=0.1–0.08) was spread evenly on the Mueller–Hinton agar plates. Plates were kept at room temperature for 10 min. Antibiotic discs were mounted and plates were placed at 4°C for 2 h to allow the diffusion of antibiotics; thereafter, the plates were incubated overnight at 35°C. The plates were scored for resistance or sensitivity after 18 h by comparing the chart on the inhibitory zone diameter as given by the disc manufacturer. Control plates were incubated without antibiotic discs.

Determination of heavy metal removing by bacterial cultures [1]

Liquid culture was preincubated in 100 ml of metal-deficient NB until it reached mid-log phase, and 1 ml bacterial sample ($OD_{620\ nm}$=0.8) was transferred into 50 ml NB supplemented with heavy metal ions (Cd, Pb, and As at the 0.25 MTC values of each isolate) in 250 ml Erlenmeyer flasks. The culture was incubated at optimum conditions of each isolate on an environmental rotary shaker (New Brunswick, New Jersey, USA) at 120 rpm for 48 h. The samples were centrifuged for 10 min at 6000 rpm using centrifuge (Sigma-Aldrich, Germany) and the supernatant was used for residual metal analysis by using an atomic absorption spectrophotometer (Analyst 400; Perkin-Elmer). The amount of metal ion removed by the bacterial strain was determined by the difference between the initial and residual concentrations. All experiments were performed in triplicate and the average values were determined.

Result and discussion

Soil analysis

Rapid industrialization and economic development in the last decades has resulted in increased pollution of heavy metal. This issue has been the focus of numerous studies [18]. Heavy metal contamination has attracted the attention of environmental researchers because of its increasing input in coastal waters, especially in developing countries [19]. The collected industrial samples were assessed for its physicochemical properties and toxic metal levels (Table 1).

The normal pH of water should range between 6.0 and 8.0 [20]. The collected samples had a high pH when compared with normal water, indicating the

Table 1 Concentrations of heavy metals and metalloids in collected samples

Sites	Sample form	OM (%)	pH	TDS (ppm)	Heavy metal contents (mg/kg)		
					Arsenic	Lead	Cadmium
1	Water	0.04	9.3	1780	12.7	4.1	0.9
1	Water	0.057	7.8	1610	14.2	7.5	0.7
2	Soil	0.84	9.7	1840	32.3	10.3	2.1
2	Soil	0.73	7.9	4380	39.1	13.1	3.3

Values are reported as mg/kg (dry weight); OM, organic matter; TDS, total dissolved salt.

alkaline nature of the effluent due to the presence of high concentrations of salts of sodium, potassium, carbonate, etc. The presence of a higher level of total dissolved salts in the effluent might be due to the presence of insoluble organic matter and unused inorganic salts. As, Pb, and Cd are among the most hazardous components of the industrial effluents. The use of excessive amounts of these chemicals in industrial processes leads to their high concentrations in effluents, causing the pollution of the environment with heavy metals that lead to the appearance of heavy metal-resistant microorganisms in the soil and water of industrial regions.

Isolation and identification of heavy metal-resistant bacterial strains

This study resulted in the isolation and purification of 20 bacterial isolates from heavy metal-contaminated sites according to morphological characteristics. These isolates have the ability to resist one or more metals; only one isolate, named ST6, was able to resist all metals. This isolate was identified on the basis of morphological and biochemical characteristics and through 16S rDNA sequencing.

Morphologically, the colonies of the ST6 strain were small, rough, wrinkled, adherent, and irregular with undulating margin and produced diffusible brown pigment. The bacterial cells were Gram-negative thin rods in single straight cells (Fig. 1).

The biochemical characteristics of the selected strain are listed in Table 2.

According to the morphological and biochemical analysis, the isolate was belonged to *Pseudomonas stutzeri* and the phylogenetic analysis confirmed the 16S rDNA gene sequence of a single band was confirmed these results by comparison with those retrieved from GenBank database. Thus, it was considered a variation from this genus and named *P. stutzeri* ST6 (Fig. 2).

According to Fig. 3 the sequences have high similarity to *P. stutzeri*.

Optimization of growth parameters (temperature and pH)

The bacterial resistance to heavy metal ions was affected not only by the surface properties of the organism but also by environmental conditions like temperature and pH. The bacterial strain was able to grow at a wide range of temperatures, with high growth rates (Fig. 5). The range of growth temperatures helped to characterize our heavy metal-resistant bacterial strain as a potential agent for use in bioremediation processes under a wide range of temperatures. This is an important aspect, considering that temperature control may not be possible during some bioremediation processes [21].

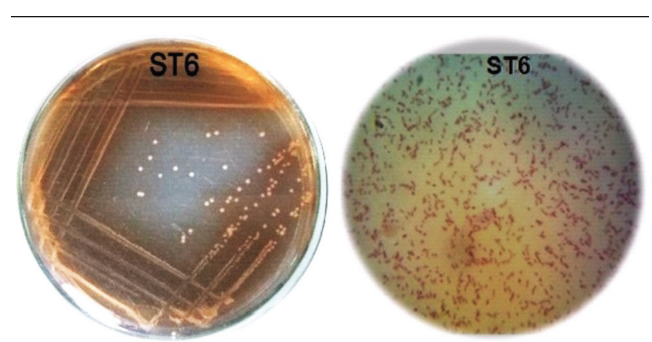
The pH value is one of the main factors affecting the growth of heavy metal-resistant bacteria [22–25]. pH plays a very critical role in microbial metal resistance and uptake by influencing the metal speciation and solution chemistry as well as surface properties of bacterial cells. pH was evaluated as it affects the

Table 2 Biochemical characterization isolates of *Pseudomonas stutzeri* ST6

Biochemical tests	*Pseudomonas stutzeri* ST6
Gram stain	–
Cell shape	Short rod
Spore formation	–
O_2 requirement	Facultative anaerobic
Pigment	Brown
Motility	+
Catalase	+
Oxidase	+
H_2S production	–
Urease	–
Vogues proskauer	–
Methyle red	+
Indol production	-
Nitrate reduction	+
Starch hydrolysis	+
Lipid hydrolysis	-
Gelatin hydrolysis	+
Esculin hydrolysis	–
MacConky agar	+
Growth at 45°C	+
Growth at pH 5.5–6	+
Citrate utilization	+
d-Mannose	–
d-Mannitol	+
d-Glucose	+
l-Arabinose	–
Potassium gluconate	+
N-acetyl-glucosamine	–
d-Maltose	–

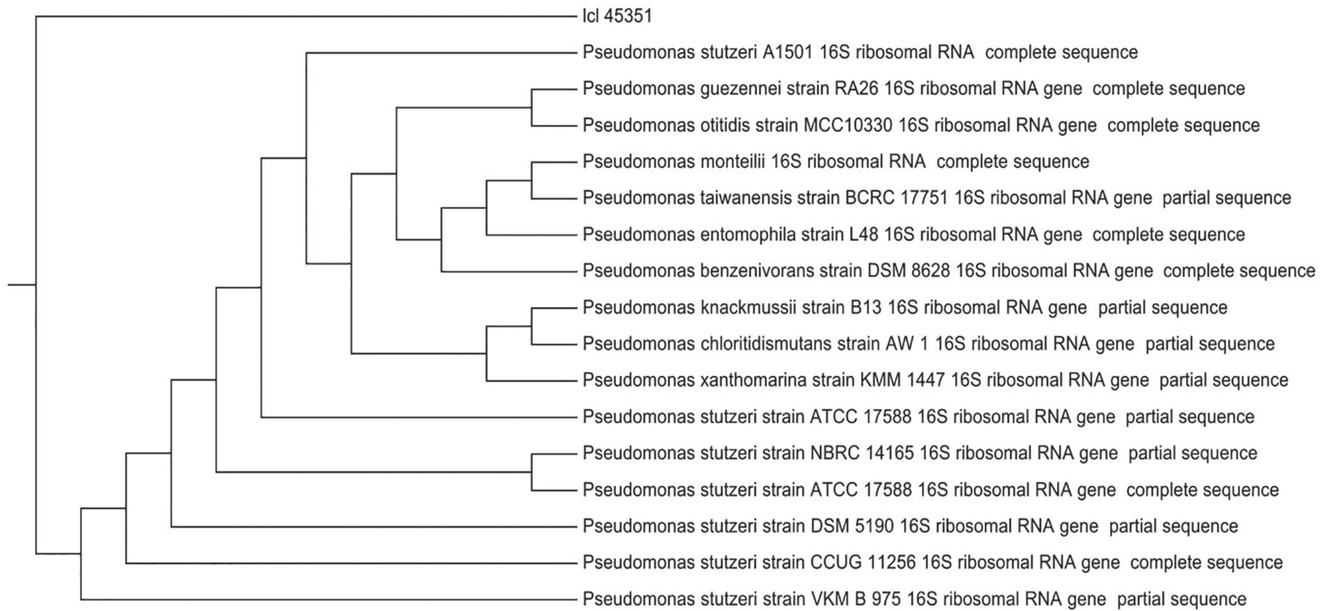
+, positive result; -, negative result.

Figure 1



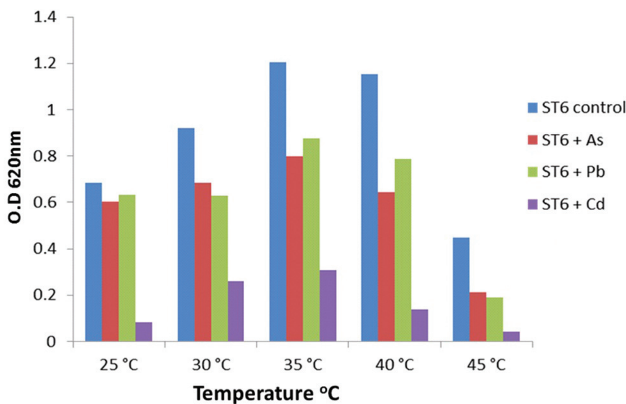
Culture characteristics and Gram stain reaction indicating the type of cells.

Figure 2



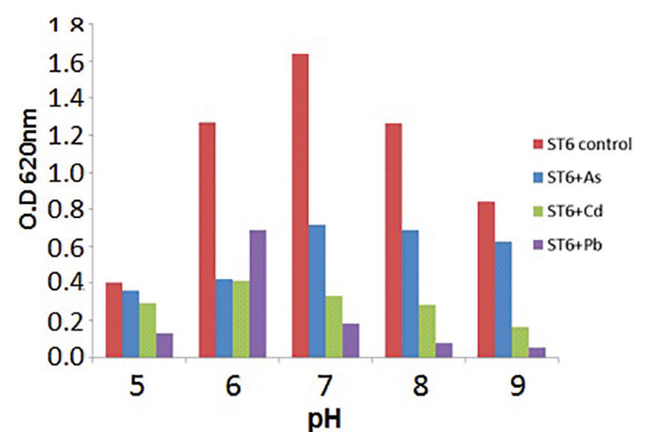
Phylogenetic tree based on 16S rRNA gene partial sequences obtained from the respective band of isolate ST6 with code no. lcl 45351 matched to the National Center for Biotechnology Information nucleotide sequence database (GenBank).

Figure 3



Effect of different temperatures on the growth of the ST6 isolate. As, arsenic; Cd, cadmium; Pb, lead.

Figure 4



Effect of different pH values on the growth of the *Pseudomonas stutzeri* ST6 isolate. As, arsenic; Cd, cadmium; Pb, lead.

number of cellular surface sites available to bind cations, as well as metal speciation [26]. Results indicated that the optimum pH for the ST6 strain was 7.0 using the controlled medium (no metal) and with As, whereas the optimum pH with Pb and Cd was 6 (Fig. 4). These results were in agreement with those of Congeevaram *et al.* [27], who found that optimal pH for growth and bioaccumulations of Cr^{6+} and Ni^{2+} by the heavy metal-resistant bacteria *Micrococcus* spp. was pH 7. The selected metal-resistant strain showed that their growth was only slightly affected with different pH values. Therefore, it is clear that growth of the ST6 isolate is not inhibited with different pH values and this fact makes them a strong candidate for future application in metal bioremediation.

Determination of the effect of metals on bacterial growth

This isolate exhibited different growth patterns in the presence of different heavy metals. It was observed that growth of ST6 was affected by the presence of Pb and Cd during the incubation period, whereas As did not affect the bacterial growth. Thus, the growth pattern in the presence of As was similar to the growth pattern without metal (positive control) (Fig. 5). The result showed that the maximum growth of the isolate ST6 against Pb occurred after 24 h, whereas in case of Cd the maximum growth appeared after 40 h. However, in case of the control (no metal) and As, the maximum growth rate occurred after 32 h. Similar results were reported earlier [28,29].

Determination of maximum tolerable concentrations of heavy metals by nutrient broth medium

The MTC of heavy metals was designated as the highest concentration of heavy metals that allowed growth after 24 h [30]. The *P. stutzeri* ST6 isolate showed a high degree of resistance to As and Pb, whereas Cd was highly toxic (Table 3). This variation in response might be due to the difference in resistance mechanisms [1].

Toxicity testing in liquid medium facilitates a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates [31]. Liquid medium toxicity testing is different from toxicity testing on solid medium, where the conditions of diffusion, complexation, and availability of metals are different from those in solid medium [31].

Determination of the coresistance to antibiotics

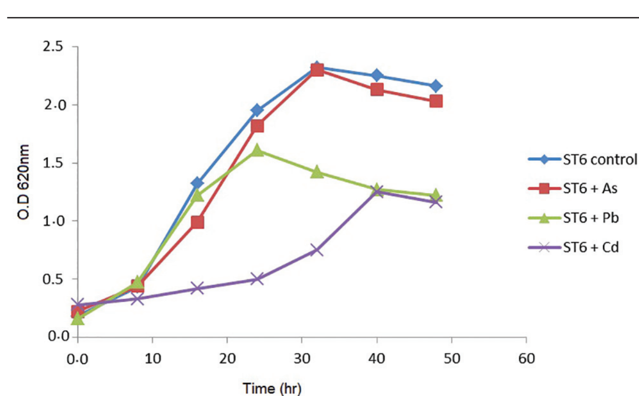
In this study, the isolate *P. stutzeri* ST6 exhibited resistance against more than one antibiotic. The results are summarized in Table 4.

Many earlier studies observed that heavy metal-resistant bacteria are also resistant to many antibiotics and other toxic chemicals [32], by carrying plasmids and or transposons encoding genetically linked metal and antibiotic resistance. Several studies reported that there would be some association between resistance to heavy metals and antibiotics, which was demonstrated by the analysis. In fact, under conditions of metal stress, resistance to these two types of compounds would help the microorganisms to adapt faster by the spread of resistance factors than by mutation and natural selection [33]. Multiple tolerances occur only to toxic compounds that have similar mechanisms underlying their toxicity. As heavy metals are all similar in their toxic mechanism, multiple tolerances are common among heavy metal-resistant bacteria. In wastewater, there are some substances that have the potential to select for antibiotic resistance even though they are not antibiotics themselves. Heavy metals and biocides are two of them. The exposure to heavy metals or biocides results in the selection of a bacterial strain that is also able to resist antibiotics. This happens because genes encoding heavy metals and biocides are located together with antibiotic resistance genes, or alternatively because bacteria can have unspecific mechanism of resistance common to different substances including heavy metals, biocides, and antibiotics [34].

Determination of heavy metal removing by bacterial cultures

The residual heavy metal concentration was determined by the use of an atomic absorption spectrophotometer. The metal bioaccumulation capacity of the three metals by *P. stutzeri* ST6 was found to be in the following order: $Pb^{2+} > Cd^{2+} > As^{5+}$ (Table 5).

Figure 5



Growth curve of two bacterial isolates of *Pseudomonas stutzeri* ST6 with different heavy metals. As, arsenic; Cd, cadmium; Pb, lead.

Table 3 Maximum tolerable concentration of different heavy metals for bacterial *Pseudomonas stutzeri* ST6 isolate

Bacterial strain	Arsenic (ppm)	Lead (ppm)	Cadmium (ppm)
ST6	3500	1750	50

Table 4 The antibiotic profile of isolate *P. stutzeri* ST6

Antibiotics	Abbreviation of antibiotics	diameter (mm)	Interpretation
Azactam	ATM	0	R
Ampicillin	AM	0	R
Tetracycline	TE	1.4	R
Vancomycin	VA	2.7	S
Clindamycin	DA	2.4	S
Trimethoprim/ sulfamethoxazole	SXT	0	R
Ciprofloxacin	CIP	2.4	S
Chloramphenicol	C	3	S
Bacitracin	B	2.5	S
Rifampicin	RF	1.6	R
Tobramycin	TOB	0.7	R
Amikacin	AK	1.5	INT
Flucloxacillin	FL	2	R

INT, intermediate; R, resistant; S, sensitive.

Table 5 Concentration of metals (ppm) in samples after atomic absorption spectroscopy

Isolate	Cadmium				Lead				Arsenic			
	I	R	A	%	I	R	A	%	I	R	A	%
ST6	11.9	5.6	6.3	52.9	331.9	142.1	189.8	57.1	855.4	491.6	363.8	42.5

%, removing percentage; A, accumulated metal (ppm); I, initial concentration (ppm); R, residual concentration (ppm).

This resistance can be accomplished by using a biosorption and/or bioaccumulation mechanism [35]. The biosorption mechanism is associated with the availability of exopolysaccharide on the dead bacterial cell wall that functions as the heavy metal chelating agent on the surface of the cell [36], while bioaccumulation mechanism in living cells is associated with the availability of the operon gene in accordance with the accumulated metal [37].

Conclusion

Out of 20 isolates from four contaminated samples, ST6 was found to show tolerance to As (3500 ppm), Pb (1750 ppm), Cd (50 ppm) and more than one antibiotic. The ST6 was identified as *P. stutzeri* ST6. It is concluded that the minimum inhibition concentration (MIC) of each heavy metal was different but the general order of resistance to the metals was found to be As > Pb > Cd. The toxic effects of the metals increased with their high concentrations. All of these results suggest that the isolate can survive in heavy metal-contaminated sediments. Therefore, the isolate may be useful as an indicator of potential toxicity of heavy metals in industrial effluents and could be designed as a bioremediation tool by advanced studies.

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Conflicts of interest

There are no conflicts of interest.

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