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Biophysical Removal of Some Toxic Heavy Metals by *Aeromonas* Strains

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Some novel approaches have been developed to overcome pollution with the toxic heavy metals as alternatives to physical methods. In this study, bacterial bioremediation using four different recently isolated *Aeromonas* strains, *A. salmonicida, A. piscicola, A. hydrophila,* and *Aeromonas sp.*, was applied to overcome pollution with copper sulphate, cadmium sulphate, arsenic oxychloride, and vanadium pentoxide. *Aeromonas* isolates have been identified by partial sequencing of the 16S rDNA (approx. 1000bp) and subjected to phylogenetic analysis. *Aeromonas* strains are strongly resistant to high concentration (15mg/l) of the heavy metals under test, Cu, Cd, As and V, with different degrees. *Aeromonas sp.* strain E4 is less resistant to As and V. Its maximum resistance was till 8 and 10 mg/l to As and V, respectively. *Aeromonas salmonicida* was capable to remove 90.5%, 49.3% and around 36.7% of 15 mg/l Cu, As, and V solutions, respectively, in a contact time of 4 h under shaking conditions. On the other hand, *Aeromonas sp.* was promising in removing 86% of Cd solution under the same conditions. Besides, 81.3% of 15 mg/l Cd solution was efficiently removed by *Aeromonas hydrophila*.

Keywords: *Aeromonas*; Cu; Cd; As; V; biophysical remediation.

Environmental pollution is increasing significantly due to industrialization, urbanization, and natural sources[1-2]. Among various pollutants, heavy metals are released to the environment mainly as a result of industrial operations[3] and agricultural activities[4]. Some novel approaches have been developed to overcome pollution as alternatives to physical methods which are comparatively too costly[5]. Hence, the ideal solution for environmental pollutions is the bioremediation which is the most efficient strategy to manage and recover the contaminated environment[6]. Bioremediation using microorganisms such as bacteria is very efficient and promising as reported by many researchers[7-9].

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Aeromonas is a bacterium of an environmental interest. For instance, *Aeromonas sp.* strain Etl-1949 [10] is an isolate from a textile wastewater treatment plant in India. It could decolorize and degrade an azo dye, orang 16, under static conditions. In addition, *Aeromonas hydrophila* was successfully used to remove toxic heavy metals from polluted aquaculture ponds in Point Calimer, India[11]. Moreover, Goswami et al, 2014[12], have studied the effect of temperature and arsenic on *A. hydrophila* growth. In parallel, Odokuma (2009)[13] has also studied the effect of culture age and biomass concentration on heavy metal uptake by *Bacillus sp*., *Pseudomonas sp*., and *Aeromonas sp*.

The walls of bacteria are efficient metal chelators though a wide spectrum of uptake capacities may be exhibited. Therefore, metabolism-independent biosorption may be may be the most significant proportion of total uptake[14].

For example, *A. cavae* biomass was successfully used for the removal of cadmium and chromium ions from aqueous solutions[15] and exhibited particular tolerance to heavy metals[16].

Therefore, the current study focuses in the uptake/biosorption of four significant heavy metals, Cd, Cu, As, and V, by some recently isolated *Aeromonas* strains, *A. salmonicida* B2*, A. piscicola* C3*, A. hydrophila* D4*,* and *Aeromonas sp*. E5. Besides, a phylogenetic study was performed for accurate identification of the new isolates using partial sequences of the 16S rDNA (approx 1000bp) gene and their resistance to heavy metals and some antibiotics were also investigated.

MATERIALS AND METHODS

Source of Sample and Water Chemical Analysis

The industrial waste water used in this study was collected in February- 2014 from a heavy metal polluted lake, Riyadh city, KSA. Bacteria were isolated, purified and maintained in glycerol. Heavy metal analysis of the water sample was performed at Soil, Water and Environment Research Institution (SWERI), Giza, Egypt.

Cell Morphology and Antibiotic Sensitivity Test

Routine Gram stain reaction was performed and cells were examined using bright field microscopy at 100X magnification. Sensitivity of the isolate toward some commercially available antibiotic discs was tested in Muller Hinton agar plates. The plates were incubated at 30 f C for 24h. The antibiotics were as follows (µg): Ampicillin (20), Penicillin G (10), Cephalothin (30), Amoxycillin (10), and Sulphamethoxazole (25).

Bacterial Resistance to heavy metals

The pure bacterial cells were cultured in nutrient agar plates supplemented with different and separate concentrations of copper sulphate, cadmium sulphate, arsenic oxychloride, and vanadium pentoxide, 2 to 15 mg/l. The plates were incubated at 30 f C for 24h.

16S rDNA Partial Sequencing

DNA was extracted using Insta Gene Matrix (BIO-RAD). Amplification of the 16S rDNA (primers are shown in Table 1) was done using 20 ng DNA in 30 µl reaction mixture using EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95 f C for 2 min, 35 cycles of 95 f C for 1 min, 55 f C and 72 f C for 1 min, and 10 min step at 72 f C. Amplicons were purified using multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing (approx 1000bp) was performed at Macrogene incorporation, Seoul, Korea using PRISM Big Dye Terminator V3.1 cycle sequencing kit. Amplification products were analyzed by ABI PRISM 3730XL DNA analyzer (applied Biosystems, Foster city, CA). The sequences were compared with those in the GenBank data base using BLAST search [17]. The sequences were finally deposited in the GenBank and accession numbers were obtained as will be indicated in the results. Forward and reverse primers (Macrogene incorporation, Seoul, Korea) used in PCR reactions and sequencing are illustrated in Table 1.

Phylogeny and data analysis

Homology search for the obtained sequences was performed against DDBJ (DNA Data Bank of Japan) using Blast program to find the sequences producing significant alignment with the obtained ones. Similarity percentages among the sequences were obtained using Biology WorkBench software version 3.2. Multisequence alignment and molecular phylogeny were performed using ClustalW (a distance-based analysis program at http://www.ddbj.nig.ac.jp/) program[18]. The tree topology was evaluated using the neighbor-joining method based on 1000 resamplings[18; 19].

Heavy metals biophysical removal

Bacterial cells obtained from nutrient broth cultures grown at 30 f C for 24h with agitation at 100 rpm were harvested by centrifugation. Harvested cells were then weighed and 0.1 gm wet cells were used for inoculation of Cu, Cd, As, and V solutions (15mg/l). Each strain was inoculated separately to each metal solution. The total volume of each metal solution was 20 ml in a 100ml conical flask and the pH was adjusted to 7. Cells were suspended in heavy metal solutions for 4h at 30 f C with agitation rate of 100 rpm. Finally, cells were centrifuged and clear supernatant was isolated in clean tubes for quantitative analysis of arsenic using atomic absorption spectrometer, Conter AA700 Graphite, France Analytik Jena AG. Measurements were performed twice and obtained values were in the range of ±3% from average values (results are given as average values).

RESULTS AND DISCUSSION

The wastewater sample used in this study contains different heavy metals (data not shown). Cu, Cd, As, and V were chosen due to their high concentrations in the environmental sample. The bioremediation experiment was performed using the recently isolated and identified *Aeromonas* strains. The isolates were identified at the molecular level by partial sequencing of the 16S rRNA gene (approx 1000bp). Primers used for the PCR reaction and sequencing were listed in Table 1. The sequencing and BLAST search results revealed that the isolates are belonging to *Aeromonas*, with high similarity percentages (Table 2). The sequences of the 16S rRNA gene were deposited in the GenBank and the accession numbers were illustrated in Table 3. The phylogenetic analysis of the resulted sequences is summarized in Fig. 1. From the tree topology, it can be deduced that there are four different bacterial groups belonging to *Aeromonas*. Therefore, the unrooted tree showed 4 different *Aeromonas* clades, *A. salmonicida, A. piscicola, A. hydrophila,* and *Aeromonas sp*. The 5‘ hypervariant region of the 16S rDNA has been successfully used in many studies to identify bacterial species[20-21]. In this study, the 16S rDNA hyper variant region not only located the strains in its precise taxonomic position, but also exhibited a discriminating tool among the closely related sequences[20].

The antibiotic sensitivity test results are illustrated in Table 4. *Aeromonas salmonicida* is sensitive to penicillin (10 µg) and cephalothin (30 µg). *A. piscicola* is also sensitive to penicillin (10 µg), ampicillin (20 µg), and sulphamethoxazole (25 µg). On the other hand, *Aeromonas sp.* is resistant

Table 1. Forward and reverse primers used in PCR reactions and sequencing.

Primer name	Primer direction	primer sequence	Purpose
27F	Forward	5‘-AGAGTTTGATCMTGGCTCAG-3‘	For PCR
1492R	Reverse	5‘-TAGGGYTACCTTGTTACGACTT-3‘	
518F	Forward	5‘-CCAGCAGCCGCGGTAATACG-3‘	For sequencing
800R	Reverse	5‘-TACCAGGGTATCTAATCC-3‘	

Table 2. Similarity matrix of *Aeromonas* strains 16S rDNA and other homologous sequences

	Hydro.	*Aeromonas sp.*	*Piscic.*	*Salmon.*	***Hydro.* E5**	***Aeromonas* sp. D4**	***Piscic.* C3**	***Salmon.* B2**
Hydro.								
Aeromonas sp.	97							
Piscic.	97	96						
Salmon.	97	97	97					
***Hydro.* E5**	99	97	97	97				
***Aeromonas sp.* D4**	97	99	97	97	97			
***Piscic.* C3**	97	97	100	97	97	97		
***Salmon.* B2**	97	97	97	100	97	97	97	

Strains under study are illustrated in bold. *Hydro*. E5: *Aeromonas hydrophila* strain E5; *Piscic.* C3: *Aeromonas piscicola* strain

Table 3. Accession numbers of the newly isolated *Aeromonas* strains

Code	Strain	Accession number
B2	*Aeromonas salmonicida* strain E2	KJ781362
C3	*Aeromonas piscicola* strain E3	KJ781363
D4	*Aeromonas sp.* strain E4	KJ781364
E5	*Aeromonas hydrophila* strain E5	KJ781365

to all antibiotics under test and *A. hydrophila* is sensitive only to sulphamethoxazole (25 µg) (Table 4). The antibiotics sensitivity test successfully distinguished among the different *Aeromonas* strains under test. Gram stain reaction showed Gram- negative rods for the four tested strains.

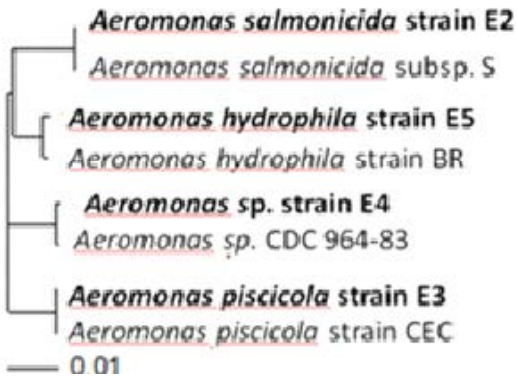

Fig. 1. Phylogenetic position of the new *Aeromonas* strains, based on partial sequences (approx 1000 bp) of the 16S rRNA gene. The tree was constructed by neighbor-joining method using ClustalW software. The scale indicates substitutions per site

The results of heavy metals resistance test are presented in Table 5. *Aeromonas* strains are strongly resistant to high concentration (1.5mg/l) of the heavy metals under test, Cu, Cd, As and V. *Aeromonas sp.* strain E4 is less resistant to As and V. Its maximum resistance was till 8 and 10 mg/l to As and V, respectively. In a parallel study of Odokuma and Akponah, 2010[22], they found that *Aeromonas* was resistant to the toxicity of all heavy metals under test, Fe, Zn, Cd, Cu, Ni, and Pb, within

Table 4. Resistance and sensitivity of *Aeromonas* strains to different antibiotics

Antibiotics discs (µg)	Isolates resistance			
	B2	C3	D4	E5
Ampicillin (20)	r	s	r	r
Penicillin G (10)	s	s	r	r
Cephalothin (30)	s	r	r	r
Amoxycillin (10)	r	r	r	r
Sulphamethoxazole(25)	r	s	r	s

B2: *Aeromonas salmonicida* strain E2; C3: *Aeromonas piscicola* strain E3; D4: *Aeromonas sp.* strain E4; *Aeromonas hydrophila* strain E5.

Table 5. Resistance of *Aeromonas* strains to different concentrations of Cd, Cu, As, and V.

Isolate resistance	Heavy metal concentration (mg/l)															
	Cd				Cu				As				V			
	6	8	10	15	6	8	10	15	6	8	10	15	6	8	10	15
B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D4	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-
E5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

B2: *Aeromonas salmonicida* strain E2; C3: *Aeromonas piscicola* strain E3; D4: *Aeromonas sp.* strain E4; *Aeromonas hydrophila*

Table 6. Arsenic removal by different *Aeromonas* strains

Strains	Remaining heavy metal concentration (mg/l)				% Heavy metal removed			
	Cd	Cu	As	V	Cd	Cu	As	V
Aeromonas salmonicida strain E2	0.41	0.142	0.76	0.95	72.7	90.5	49.3	36.7
Aeromonas piscicola strain E3	0.35	0.76	0.78	1.3	76.7	49.3	48	13.3
Aeromonas sp. strain E4	0.21	0.37	1.28	1.31	86	75.3	14.7	12.7
Aeromonas hydrophila strain E5	0.28	0.61	0.83	1.1	81.3	59.3	44.7	26.7
Control solution	15	0						

24h exposure period. The persistence of *Aeromonas* strains in the presence of heavy metals may be as a result of the possession of heavy metal resistant plasmids[23]. Moreover, *Aeromonas hydrophila* is frequently reported from arsenic affected areas and was found to resist high As concentrations according to different temperatures[12] .

Aeromonas strains demonstrated different and promising levels of heavy metal removal (Table 6). *Aeromonas salmonicida* was capable to remove 90.5%, 49.3% and around 36.7% of 1.5 mg/l Cu, As, and V solutions, respectively, in a contact time of 4 h under shaking conditions. On the other hand, *Aeromonas sp.* was promising in removing 86% of Cd solution under the same conditions. Besides, 81.3% of 1.5 mg/l Cd solution was efficiently removed by *Aeromonas hydrophila*. Therefore, these promising strains can be used efficiently in the bioremediation of toxic heavy metals in aqueous solutions. It is obvious that the removal ability of heavy metals from aqueous solutions by *Aeromonas* strains under test was generally maximum for Cd, followed by Cu, then As, and finally V. The lowest removal ability can be noticed clearly for strain *Aeromonas sp.* in case of As and V. These results are in parallel with those of sensitivity against As and V.

Odokuma, 2009[13], studied the effect of culture age and biomass concentrations of *Bacillus sp*., *Pseudomonas sp*., and *Aeromonas sp*. on their capabilities of bioconcentrating various heavy metals (Fe, Zn, Cu, Cd, Pb, and Ni) associated with crude oil. He found that *Aeromonas sp*. uptake was increasing with time. He also stated that, Gram-negative organisms had a higher metal accumulation capacity than the Gram-positive isolates. This may be due to the cell wall structural differences[13]. Moreover, Odokuma and Akponah, 2010[22], also demonstrated that the ability of *Aeromonas* to uptake Fe, Zn, Cd, Cu, Ni, and Pb. They showed that the uptake level depends on heavy metal concentration and contact time. In addition, Umamaheswari et al, 2010[11], have used *Aeromonas hydrophila* in concentrating heavy metals from shrimp cultures.

Aeromonas strains under study were initially viable, but because they were transferred into solutions of different heavy metals, they may act like a trap for these heavy metals throughout the different negatively charged groups (carboxyl, hydroxyl, and phosphoryl) of their cell walls[24]. The interest of using non metabolic cells over living biomass is due to the complexity of the later. This is because the cell response to ambient conditions including the presence of toxic heavy metals[25]. In addition, Al-Daghistani, 2012[26], has stated that the dead cells exhibited higher adsorption potential for heavy metals over living cells. Besides, living cells require the addition of nutrients and this increases the biological and chemical oxygen demands of the treated water[26].

In this study, four different recently isolated and molecularly identified *Aeromonas* strains were successfully used for removal of some toxic heavy metals. Biophysical removal instead of metabolic dependent bioremediation was applied. Higher removal levels may be obtained by optimizing the environmental conditions for the various strains. However, I strongly recommend strains to be used in toxic heavy metals absorption and for more optimization studies.

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