Bioremediation Of Heavy Metals By Pseudomonas Putida Isolated From Groundwater In Egypt.

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ABSTRACT: In this present study total four bacterial isolates were obtained from 34 collected groundwater samples in 10th of Ramadan, Sharkia governorate, Egypt. These isolate were grown on nutrient agar supplemented with 1mg/l of iron, manganese and combination between them (V/V). Further testing of the bacterial isolates were grown on nutrient agar supplemented with different concentrations (2, 4, 5, 6, 7, 8 and 9 mg/l) of iron and manganese. Out of four isolates, one bacterial isolate no.83 has shown the resistance to heavy metals at maximum concentration of 8mg/l. Selected isolate no.S83 was identified as Pseudomonas putida S83 according to Bergey's manual depending on morphological and biochemical characteristics. Transmission electron microscopy study of P. putida isolate no. S83 showed accumulation of heavy metal salts within and external to bacterial cells. P. putida S83 have higher removal efficiency of Fe^{+2} 94.5% and Mn^{+2} 94% at concentration 2 mg/l and 96 hours.

Key words: Groundwater, heavy metal resistance bacteria, iron, manganese, transmission electron microscope, Pseudomonas putida, Bioremediation.

INTRODUCTION

Ground water has historically been assumed to be safer as a drinking water source than surface water. It is assumed that passage of groundwater through the soil would filter contaminants [1]. However, water borne diseases out breaks are known to have resulted from the use of untreated ground water for domestic purposes. The extent of ground water contamination depend on some factors such as, rainfall pattern, depth of water table, distance from the source of contamination and soil properties such as texture, structure, ions and infiltration rate [2]. Heavy metal is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 4000 kg m^{-3}, or 5 times more than water [3] and they are natural components of the earth's crust. The most toxic forms of these metals in their ionic species are the most stable oxidation states e.g. Cd^{2+}, Pb^{2+}, Hg^{2+}, Ag^{+} and As^{3+} in which, they react with the body's bio-molecules to form extremely stable biotoxic compounds which are difficult to dissociate [4]. Pseudomonas putida was able to remove 80% Cd^{+2}, Pb^{+2}, Zn^{+2}, and Cu^{+2} from aqueous solution [5]. Microbial populations in metal polluted environments adapt to different concentrations of heavy metals and become metal resistant [6].

MATERIALS AND METHODS

Sample collection and isolation of metals resistance bacteria.

Collected 34 groundwater samples are highly polluted with heavy metals from 10th of Ramadan city, Sharkia governorate, Egypt. The samples were placed into sterile bottles and transported to the microbiological laboratory of 10Th of Ramadan city, and they were stored in the refrigerator at 5ºC. Bacteria were isolated on nutrient agar supplemented with 1mg/l of iron and manganese. The plates were incubated for 24 hours at 35°C. Growing colonies were investigated for their morphological characteristics, purified and kept at 5°C as slant cultures.

Examination of bacterial tolerance to grow in increased concentration of heavy metal.

The bacterial tolerance isolates were inoculated on nutrient broth containing different concentrations of heavy metals (2, 3, 4, 5, 6, 7, 8 and 9 mg/L) of iron and manganese. Nutrient broth flasks containing 50 ml medium amended with different concentrations of heavy metal were inoculated with overnight bacterial culture and incubated at 35°C for 24 hours. Growth was confirmed as by measuring optical density at 600nm by Spectrophotometer (model Cecil 7400s). The most highly tolerant bacterium (with highest concentration of heavy metals) was selected for identification and further study.

Identification and characterization of heavy metal tolerant bacterial isolate.

The selected tolerated isolate was identified according to Bergey's Manual of Determinative Bacteriology [7] depending on morphological and biochemical characteristics.

Transmission Electron Microscope.

Bacterial culture (10^6 CFU/ml) of the selected bio-active bacteria was inoculated in three flasks containing 50ml nutrient broth supplemented with 4mg/L iron and manganese and incubated for an overnight. Drops of high titer filtered bacterial suspension were laid on formavar coated copper grid (400 meshes) with carbon coated colloid ion membrane, and then negatively stained by uranyl acetate (4 % aqueous). Excess fluids were withdrawn by a filter paper strep [8]. Prepared grids were washed 3 times by distilled water, dried by filter paper or air dried and viewed by electron microscopy. Morphological assessment of the tested bacteria was performed by Transmission electron microscopy (JEOL JEM.1010) at 80 KV and magnification range of 50X-500KV located at Electron microscopy unit, Faculty of Medicine, Zagazig University, Egypt).

Bioremediation of heavy metal solution at different influent concentration and time.

In this application used a pilot plant design consisted of cascaded aerator and down flow bio-filter. Pilot plant design used to examine efficiency of P. putida S83 removal Fe^{+2} and Mn^{+2} from polluted water at different concentration 2, 3, 4, 5, 6, 7 and 8mg/l of Fe^{+2} or/and Mn^{+2} solution at different time 24, 48, 72 and 96 hours. Each concentration has pilot plant design.

RESULTS

Sample collection and isolation of metals resistance bacteria.

A total of 34 groundwater samples are highly polluted with heavy metals collected from 10th of Ramadan city, Sharkia, governorate, Egypt were tested on nutrient agar supplemented with 1mg/l of iron and manganese. Out of 34 bacterial isolates, only 4 bacterial isolates no. (S75, S84, S85 and S83) were resistant to heavy metals and selected for further studies.

Examination of bacterial tolerance to grow in increased concentration of heavy metal.

The results of screening of isolates for grow in broth medium containing different concentrations of heavy metals (2, 3, 4, 5, 6, 7, 8 and 9 mg/L) of Fe^{+2} and Mn^{+2}. At concentration 4 mg/L the four isolates able to resist the heavy metals were observed in table (1). At concentration 5 and 6 mg/L the two isolates no. (S83 and S84) able to resist the heavy metals. While two isolates (S75 and S85) were sensitive to heavy metals and at concentration 7 and 8 mg/L isolate no. (S83) able to resist the heavy metals. While three isolates (S75, S84 and S85) were sensitive to heavy metals. Obviously, at concentration 9 mg/L in growth medium were the inhibitory concentration for all four isolates.

Table (1): *Tolerance of selected bacteria to increasing concentrations of Iron and Manganese.*

Isolate number	Fe^{+2} and Mn^{+2} Concentration mg/l							
	2	3	4	5	6	7	8	9
S75	+	+	+	-	-	-	-	-
S83	+	+	+	+	+	+	+	-
S84	+	+	+	+	+	-	-	-
S85	+	+	+	-	-	-	-	-

Identification and characterization of heavy metal tolerant bacterial isolate.

The morphological and biochemical tests were carried out for the identification the selected tolerant bacterial isolate no. 83 as in table (2). These tests were identified according to Bergey's Manual. Selected bacterial isolate (S83) was identified as Pseudomonas putida.

Table (2): *Morphological and biochemical characteristics of isolated no. S83.*

Characters	Isolate no. S83
Shape	Rod
Staining	Negative
Arrangement	Single
Gram staining	-ve
Catalase test	+ve
Oxidase test	+ve
Methyl red test	-ve
Indole test	-ve
Vogas-proskauer	-ve
Motility test	+ve
Gelatin test	-ve
Urease test	-ve
Citrate utilization	+ve
H_2S production	-ve
Coagulase test	+ve
glycerol test	-ve
D-sorbitol test	-ve
D-glucose test	+ve
D-Mannitol test	-ve
D- Maltose test	-ve
D- Sucrose test	-ve
Mannose test	-ve

Transmission Electron Microscope.

P. putida no. S83 was chosen to study the mechanism of heavy metal removal using Transmission Electron Microscope (TEM) as in fig. (1, 2 and 3). Overnight P. putida cultures of grown in nutrient broth medium containing 4 mg/L of Fe^{+2} and Mn^{+2} were loaded on copper grids then stained by uranyl acetate and investigated using TEM. Fig. (1, 2 and 3) showed the electron photographs of the untreated and metal treated P. putida cells. Regarding to the changes in the cell structure and morphology, the results showed that, fig. (2 and 3) there was a dramatic changes in cell shape and formation of amorphous irregular cellular structure as a result of Fe^{+2} and Mn^{+2} salts treatment of P. putida S83. Whereas Fe^{+2} and Mn^{+2} salts were accumulated inside the vacuoles of cells P. putida isolate no. S83 and the deposited metal salts were appeared as dark precipitates fig. (2). Compared with fig. (1) Non treated cells. As well as the cells of P. putida no. S83 which accumulated Fe^{+2} and Mn^{+2} salts in vacuoles were degradation of the cells due to high salt concentration fig. (3).

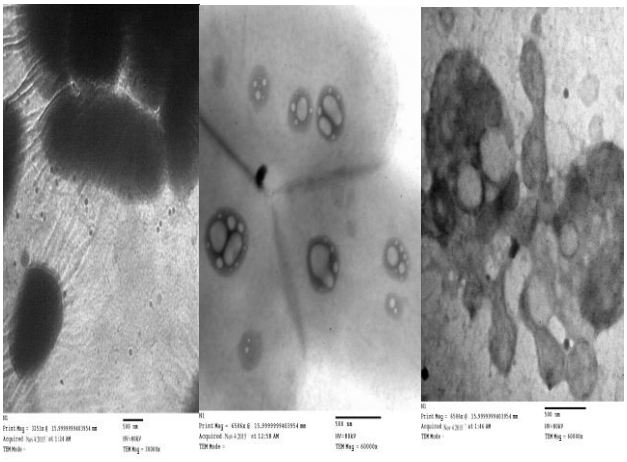

Fig. (1): Electrophotogram, showing normal surface structure cells of *P. Putida* S83 sheath untreated cells. Magnification powers 30000 X.

Fig. (2): Electrophotogram showing accumulation of metal Fe^{+2} and Mn^{+2} salts in vacuoles inside the celles of *P. Putida* S83. Magnification powers 60000X.

Fig. (3): Electrophotogram showing the degradation and accumulation of metal Fe^{+2} and Mn^{+2} salts in vacuoles inside the celles of *P. Putida* S83 due to high concentration of metal salts . Magnification powers 60000X.

Bioremediation of heavy metal solution at different influent concentration and time.

In this application used pilot plant was designed to examine efficiency of P. putida S83 removal Fe^{+2} and Mn^{+2} from polluted water at different concentration and time shown in fig. (4).

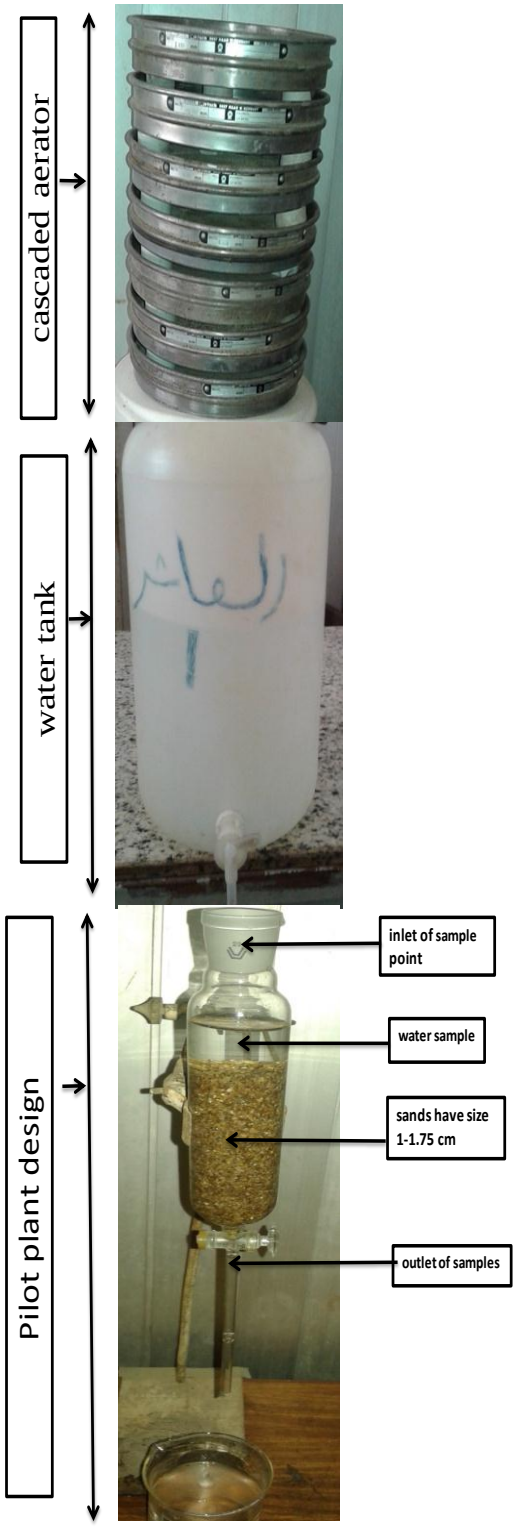

Fig. (4): *The components of the pilot plant design for biological removal of Fe^{+2} and Mn^{+2}.*

Removal of iron by P. putida S83.

Bioremediation of different concentration iron (II) at different times was carried out by P. putida S83, the results in tables (3). Revealed that P. putida S83 was accumulated (94.5%) at concentration 2 mg/l of Fe^{+2} and 96 hours. Meanwhile the maximum percentage of total removal of iron (II) at concentration (3, 4, 5, 6, 7 and 8 mg/l) of Fe^{+2} were (92, 91.3, 90.4, 89.2, 88.2 and 87.8%) respectively were noticed at 96 hours. And the maximum percentage of total removal of iron (II) (93.5, 87.5 and 81%) at concentration 2 mg/l of Fe^{+2} were noticed at time (72, 48 and 24 hours) respectively for P. putida S83.

Table (3): *Iron removal efficiency at different influent concentrations and time.*

Removal system of Fe^{+2}											
Influent concentration of Fe^{+2} (mg/L)	After cascade aeration (mg/L)	Removal% after aeration	After biofilteration (mg/L)	Removal% through the biofilter	Total %Removal	Influent concentration of Fe^{+2} (mg/L)	After cascade aeration (mg/L)	Removal% after aeration	After biofilteration (mg/L)	Removal% through the biofilter	Total %Removal
At 24 hours.						At 48 hours.					
2	1.32	34	0.38	71.2	81	2	1.25	37	0.25	80	87.5
3	1.88	37	0.75	60.1	75	3	2.02	32	0.41	79.7	86.3
4	2.24	44	1.02	54.5	74.5	4	2.15	46	0.57	73.5	85.8
5	3.56	28	1.28	64	74.4	5	3.36	32	0.73	78.3	85.4
6	4.44	26	1.65	62.8	72.5	6	3.17	47	0.91	71.3	84.8
7	5.36	23	1.88	64.9	73.1	7	5.96	14	1.15	80.7	83.6
8	6.39	20	2.18	66.3	72.7	8	6.95	13	1.27	81.7	84.1
At 72 hours.						At 96 hours.					
2	1.15	42	0.13	88.7	93.5	2	1.24	38	0.11	91.1	94.5
3	1.85	38	0.26	85.9	91	3	2.11	29	0.24	88.6	92
4	2.19	45	0.37	83.1	90.7	4	2.68	33	0.35	86.9	91.3
5	3.33	33	0.52	84.4	89.6	5	3.96	20	0.48	87.9	90.4
6	4.15	30	0.67	83.8	88.8	6	4.68	22	0.65	86.1	89.2
7	4.63	33	0.88	81	87.4	7	5.74	18	0.82	85.7	88.2
8	6.87	14	1.12	83.7	86	8	6.89	13	0.98	85.7	87.8

Removal of manganese by P. putida S83.

Bioremediation of different concentration manganese (II) at different times was carried out by P. putida S83 the results in tables (4). Revealed that P. putida S83 was accumulated (94%) at concentration 2 mg/l of Mn^{+2} and 96 hours. Meanwhile the maximum percentage of total removal of manganese (II) at concentration (3, 4, 5, 6, 7 and 8 mg/l) of Mn^{+2} were (93.7, 93.5, 93, 92.5, 92.4 and 91.1%) respectively were noticed at 96 hours. And the maximum percentage of total removal of manganese (II) (88.5, 73.5 and 47%) at concentration 2 mg/l of Mn^{+2} were noticed at time (72, 48 and 24 hours) respectively for P. putida S83.

Table (4): *Manganese removal efficiency at different influent concentrations.*

Removal system of Mn^{+2}												
Influent concentration of Mn^{+2} (mg/L)	After cascade aeration (mg/L)	Removal% after aeration	After biofilteration (mg/L)	Removal% through the biofilter	Total %Removal	Influent concentration of Mn^{+2} (mg/L)	After cascade aeration (mg/L)	Removal% after aeration	After biofilteration (mg/L)	Removal% through the biofilter	Total %Removal
At 24 hours.						At 48 hours.					
2	1.32	34	1.06	19.7	47	2	0.69	65	0.53	23.2	73.5
3	2.02	32	1.66	17.8	44.7	3	2.45	18	0.94	61.6	68.7
4	2.35	41	2.25	4.2	43.7	4	3.33	16	1.12	66.4	62.7
5	3.62	27	2.89	20.2	42.2	5	4.12	17	1.88	54.4	62.4
6	4.74	21	3.52	25.7	41.3	6	5.45	9	2.28	58.2	62
7	6.33	9	4.18	34	40.3	7	5.17	26	2.84	45	59.4
8	7.48	44	4.95	33.8	38.1	8	6.41	19	3.32	48.2	58.5
At 72 hours.						At 96 hours.					
2	1.62	19	0.23	85.8	88.5	2	1.19	40	0.12	89.9	94
3	1.96	34	0.36	81.6	88	3	2.34	23	0.19	91.9	93.7
4	3.49	12	0.53	84.8	86.7	4	3.66	8	0.26	92.9	93.5
5	3.65	27	0.68	81.4	86.4	5	4.42	11	0.35	92	93
6	5.28	12	0.82	84.5	86.3	6	4.19	30	0.45	89.3	92.5
7	6.45	7	1.05	83.7	85	7	5.68	18	0.53	90.7	92.4
8	6.95	13	1.22	82.4	84.7	8	6.56	18	0.71	89.2	91.1

DISCUSSION

In this study, the bacterial isolates were isolated from 34 collected ground water samples. It was isolated on nutrient agar supplemented with 1mg/l of Fe^{+2} and Mn^{+2}. Out of 34 bacterial isolates, only 4 bacterial isolates no. (S75, S84, S85 and S83) were resistant to heavy metals. Similar work was done by different workers as [9, 10, 11, 12 and 13]. The isolates were inoculated on broth media containing different concentrations of heavy metals (2, 3, 4, 5, 6, 7, 8 and 9 mg/L) of Fe^{+2} and Mn^{+2} showed that the 4 bacterial isolates able to resist the iron and manganese at concentration 4 mg/L. Out of 4 bacterial isolates, one bacterial isolates no. (S83) showed high resistance to iron and manganese at concentration 8 mg/L. These results were agreement with others [12, 13, 14 and 15]. The highly tolerant isolate for most metals was selected for identification and further study. Selected tolerated isolate no 83 was identified as Pseudomonas putida based on the morphological and biochemical characterization according to Bergey's Manual of Determinative Bacteriology [7]. These results were agreement with these reported [16 and 17]. Electron microscopic examination study showed P. putida no. S83 was chosen to study the mechanism of heavy metal removal using Transmission Electron Microscope (TEM). the results showed that there was a dramatic changes in cell shape and formation of amorphous irregular cellular structure as a result of Fe^{+2} and Mn^{+2} salts treatment of P. putida S83.Whereas Fe^{+2} and Mn^{+2} salts were accumulated inside the vacuoles of cells P. putida isolate no. S83 and the deposited metal salts were appeared as dark precipitates. The cells of P. putida no. S83 which accumulated Fe^{+2} and Mn^{+2} salts in vacuoles were degradation of the cells due to high salt concentration. These results were agreement with others [18, 19 and 20]. Bioremediation of different concentration Fe^{+2} and Mn^{+2} at different times was carried out by P. putida S83. P. putida S83 have higher removal efficiency of Fe^{+2} 94.5% at concentration 2 mg/l of Fe^{+2} and 96 hours. As well as the P. putida S83 have higher removal efficiency of Mn^{+2} 94% at concentration 2 mg/l of Mn^{+2} and 96 hours. The major part of removed Fe^{+2} could be due to the abiotic oxidation in the cascade aeration is probably due to physico-chemical oxidation [21 and 22]. Accordingly, [23] mentioned that biological Fe^{+2} removals are likely to be supplementary to conventional physico-chemical removal in the presence of oxygen. [24] reported that the biological removal of iron is very efficient over a long operational period and residual iron concentrations below 10 µg/L can be constantly achieved. Even when this procedure was cut off for one month, the efficient restart of this method and the effective removal of iron were re-achieved within only few days (2–3) after restarting the operation. Mn^{+2} oxidation in freshwater is thought to be from a combination of bacterial oxidation and chemical oxidation with oxygen [25]. Aeration does not oxidize organically bound Mn^{+2}, but in situ treatment is considered a good primary treatment for Mn^{+2}removal [26]. Mn^{+2} removals from drinking water sources, minimizing chemical oxidants that could form unwanted by-products [27]. Biological oxidation of Mn^{+2} is a biofiltration process that has not been fully explored, although it is believed that Mn (II) oxidation causes Mn (IV) oxide accumulation on bacterial surface, attached to the filter media. This accumulated Mn^{+2} are to be removed together with excess bacteria and biofilm during backwashing [28].

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