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IDENTIFICATION OF BACTERIA RESISTANT TO HEAVY METALS IN THE SOILS OF ISFAHAN PROVINCE*

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Abstract – During this research, bacteria resistant to metals were evaluated in the soil of Isfahan Province. According to the sampling locations the soils are classified as agricultural soils, non-agricultural soils and sediments of the Zayanderood Riverbank. The bacteria resistant to lead (2 mM), copper (5 mM), cadmium (0.5 mM) and arsenic (5 mM) were identified, and then during later stages, the minimum inhibitory concentration (MIC) for the growth of the bacteria resistant to metals were determined. The dry cellular weight and the metal uptake rate were also defined for the bacteria resistant to metals.

Keywords – Resistant, heavy metals, soils, bacteria, biomass

1. INTRODUCTION

In recent decades, the discharge of industrial wastewater from different resources containing heavy metals has resulted in a population increase of the resistant bacteria. This problem is considerably more common in industrial areas in which the discharge of wastewater containing heavy metals into rivers has caused the heavy metals to be sorbed by soil particles (especially clay) and transfer far distances from the discharging point. The results of some studies show that, in the Zayanderood river, the concentration of heavy metals in less settlable particles such as clay, which can travel far distances down the river, is significantly higher in lower parts of the river than the upper parts. This condition shows that, there is a direct relation between the discharge of wastewater in this river and the pollution of river sediments [1, 2]. In addition, the findings of some other researchers in other parts of Iran also indicate that the rate of metal exhibition concentration (As, Ni, Cu,…) exceeds the quality guidelines for the concentration of heavy metals in sediments [3]. Since soil is one of the most important environments for microbes and is easily exposed to many pollutants, evaluating the effects of pollutants on the microbial population is very valuable. Since the resistance of bacteria depends not only on their type and environment, but also on physical factors such as the type and concentration of the substances, the microorganisms with significant resistance can reveal the condition of their environment [4, 5].

2. MATERIALS AND METHODS

2.1. Metals

The nitrates of lead, copper and cadmium and arsenic acid were used for the preparation of a stock solution with 100 mM concentration [6].

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2.2. Sampling

The soil samples were collected from 42 points in Isfahan Province. All the soil samples were from the surface (0-15 cm depth). Determination of soil grain size was achieved by sieving and pH of saturated soil was conducted by pH-meter [7].

2.3. Soil analysis

Determination of heavy metals in soils was achieved by sieving and digestion of 2 mM dry samples in 20 ml aqua regia on a hot plate and dilution with distilled water to 100 ml final volume; after filtration with wahtman 42 it was analyzed with an atomic absorption spectrophotometer (Philips PU 9100). Soil texture was determined by sieving and the hydrometry method. The soil pH of the saturated soil was determined by pH-meter (ES-14) and electrical conductance of soil was determined by a conductivity meter (ES-14) [7, 8].

2.4. Bacteriological examination

At first, the total number of bacteria was determined in the soil samples using the SPC (Standard Plate Count) method [8].

2.4.1. Culture medium

For Standard Plate Count, (SPC), nutrient agar media was used. Then the population of the bacteria resistant to metals was defined using the PHG II media with metals. The PHGII media used contained pepton 4g, glucose 2g, yeast extract1g, and agar 15g which were dissolved in1000 ml water and adjusted at pH=7 until it was put in the autoclave [8]. In this study the concentration of 2 mM lead, 5 mM copper, 0.5 mM cadmium and 5 mM arsenic were used in order to compare with other performed researches [6]. The culture was used according to the standard method of the plate count in PHGII and then the plates were kept in an incubator for 2-4 days at $30^{\circ} \pm 2$ C [6].

2.4.2. Bacterial enumeration

The population of the resistant bacteria was counted and the percentage of the bacteria resistant to either compound was defined. In the later stage the purification of the resistant bacteria was performed in slant tubes containing metals [8, 9].

2.4.3. Identification of bacteria

Identification of various resistant bacteria was performed with key biochemical tests and different staining such as acid fast and gram staining. The resistance of the bacteria resistant to heavy metals has been defined with the agar dilution method. For evaluating the resistance of the bacteria, different media with different concentrations from each metal were prepared and then the bacteria cultured radially in the plate. The plates were then kept for 2 days in an incubator at 30˚C. The absence of bacterial growth indicated its sensitivity, while the presence of bacterial growth in this concentration indicated that bacteria were resistant.

2.4.4. MIC determination

The MIC was defined as the minimum concentration of the metal, which inhibits the bacterial growth in the plate [7, 8, 10].

2.5. Determination of dry cellular biomass and metal uptake

In order to measure the amount of metal uptake by the isolated resistant bacteria, the following measures were conducted. At first, the dried biomass weight were determined and then inoculated in PHC II media and incubated for 24 hours in 30˚ C.

After incubation, the media were centrifuged at 200 rpm and the sediments washed by distilled water. The biomasses were placed on preweighted sterile aluminium foil and put in an oven for 3 hours at 120˚C [6]. The biomass and aluminium foil as the container were then placed in a disiccator containing P_2O_5 and, after cooling, weighted and the dried cellular biomasses determined. The difference between the first weight (aluminium foil and cellular mass) and the second weight (aluminium foil) was considered as cellular biomass. In the second stage, in order to determine the bacterial metal uptake, the broth was added to the PHGII medium and then it was centrifuged at the rate of 200 rpm for 24 hours at 30˚C. Next, the suspension was centrifuged at a rate of 7000 rpm for 10 minutes. After that, the samples were washed with 0.6 ml concentrated nitric acid and 0.25 ml sulphuric acid (75%).

In the next stage, 5ml of the metal solution with a specific concentration was added to the cellular mass and kept for 20 minutes in the incubator at 30˚C. Then the solution was centrifuged for 10 minutes at a rate of 10000 rpm, and the floating part added to plastic dishes containing 1% nitric acid [3, 5, 8, 11]. The concentrations of metals uptake were determined with an atomic absorption spectrophotometer (Phillips PU 9100). The metal uptake was determined based on the difference between the primary and secondary concentrations [6, 8, 12-14].

3. RESULTS

3.1. Soil analysis

Study of soil samples indicated that, the agricultural soils had a loamy texture, but the textures of non-agricultural soils were clayey, silt and loam. Since the increase of the clay fraction and organic carbon would increase the ion exchange capacity of the soil, a higher ion exchange capacity would also increase the uptake of metal in soils [7].

3.2. Bacterial resistance

The results from metal resistant microbes indicated that, the highest percentage of resistance was in agricultural soils (Table 1).The presence of lead has been confirmed in the analysis of these soils.

Table 1. Average percentage of the bacteria resistant to metals

Metal	First group Agricultural soil	Second group Non agricultural soil	Third group Soil and sediment of Zayanderood river
Lead	47%	22%	31%
Copper	15%	5%	9%
Cadmium	56%	31%	53%
Arsenic	45%	20%	55%

3.3. Bacterial identification

The results of the identification of the resistant bacteria are shown in Table 2.

The results indicated that, the most abundant type of bacteria resistant to lead was *Bacillus* and bacillus group B was more than other bacilli.

This condition was also true for the bacteria resistant to copper and arsenic

Table 2. Different types of bacteria resistant to metals

Heavy metals	Total number of bacterial isolated	Resistant bacteria to heavy metals %	Type of resistant bacteria isolated	Number of genuces resistant to heavy metal	Strain resistant to heavy metal %
			Bacillus set A	2	5.2
			Bacillus set B	14	36.8
Lead	38	10.4	Bacillus set C	7	18.4
			Bacillus set D	1	2.6
			Bacillus firmus	13	34.2
			Corynebacterium	1	2.6
			Bacillus set A	6	20.6
			Bacillus set B	10	34.4
			Bacillus firmus	6	20.6
Copper	29	8	Corynebacterium	4	13.7
			Kurthia	1	3.4
			Sporolactobacillus	1	3.4
			Pseudomonas	1	3.4
			Bacillus set B	4	9.3
			Bacillus set C	3	6.9
			Bacillus firmus	2	4.6
			Corynebacterium	4	9.3
Cadmium	43	11.8	Kurthia	1	2.3
			Sporolactobacillus	2	4.6
			Nocardia	1	2.3
			Lactobacillus	19	44.1
			Arcanobacterium	7	16.2
			Bacillus set A	3	6
			Bacillus set B	14	28
			Bacillus set C	5	10
Arsenic	50	13.8	Bacillus set D	4	8
			Bacillus firmus	8	16
			Corynebacterium	8	16
			Sporolactobacillus	3	6
			Staphylococcus	4	8
			Rhodococcus	1	2

3.4. MIC for bacterial resistant

The percentage of the isolates susceptible to various concentrations of the four heavy metal ions are shown in Table 3

Table 3. MIC concentration for metals resistant bacteria

Metal	Metal concentration 3	4	6	8	9	10	11	12	Number of strain
Lead	*5 (14)	13 (35)	6 (16)	4 (11)	-	2 (5)	7 (19)	-	37

Table 3. (Continued)

Copper	-	- (31)	9 (34)	10 (10)	3 (10)	3 (3)	1 (10)	3	29
Metal	Metal concentration 1.5	2.5	3.5	4.5	5.5	6.5	7.5		Number of strain
Cadmium	2 (5)	11 (25)	1 (2)	10 (23)	7 (16)	7 (16)	5 (12)		43
Metal	Metal concentration 20	30	40	50	60	70	80	90	Number of strain
Arsenic	1 (2)	1 (2)	6 (12)	6 (12)	13 (27)	-	9 (18)	13 (28)	49

*The numbers present the rate of MIC concentration for metal. The number in parenthesis show the percentage of isolated bacteria with the same MIC concentration.

3.5. Bacterial biomass

The effect of bacterial biomass in different soil samples was affected by the type and the concentration of the metal and the type of microorganism [15]. In this research the results were obtained based on r=0.05. The percentage of resistance for the 362 strain at standard levels of the four heavy metals tested are presented in Table 4.

Table 4. Comparison of numbers and percentages of the bacteria resistant

Metal ion	Number of bacteria resistant	Resistant (%)
Lead	180	49.7
Copper	157	43.3
Cadmium	193	53.3
Arsenic	231	63.8

3.6. MMR pattern for resistant bacteria

Multi metal resistance (MMR) and individual resistance patterns are shown in Table 5.

Table 5. Phenotype pattern with resistance to several metals in the isolated bacteria

Type of multiple resistance	Isolates No	%	Isolates with different multiple resistance No	%	%R	Resistance pattern Pb	Cu	Cd	As
Tetra-R	47	12.98	-	-	-	+	+	+	+
Tri-R	91	25.13	13	14.28	3.59	+	+	+	-
			19	20.87	5.24	+	+	-	+
			30	32.96	8.28	+	-	+	+
			29	31.86	8.01	-	+	+	+

Table 5. (Continued)

Double-R	111	30.66	14	12.61	3.86	+	+	-	-
			15	13.51	4.14	+	-	+	-
			25	22.52	6.90	+	-	-	+
			10	9	2.76	-	+	+	-
			14	12.61	3.86	-	+	-	+
			33	29.72	9.11	-	-	+	+
Mono -R	78	21.54	17	21.79	4.69	+	-	-	-
			11	14.1	3.03	-	+	-	-
			16	20.51	4.14	-	-	+	-
			34	43.58	9.39	-	-	-	+

4. DISCUSSION

4.1. Physicochemical indexes of soil

Physical and chemical properties of soil have a partial effect on the resistance of the microorganisms. Since the resistance of the microorganisms would be affected by the variation of pH and the pH of these soil samples were about neutral, there would be little effect on bacterial resistance. Salt concentration, electrical conductance and resistivity of soil also had no significant effect on bacterial resistance [6, 7, 16]. Some researches showed the concentration of metals in the lower parts was greater than that of upper parts due to the capabilities of clay particles to uptake metals and travel by river water in suspension [3].

4.2. Metal resistance in bacteria

According to statistical analysis (the t-test and P=0.05), for the resistance percentage of microbes to lead, there was a great difference between the first group (agricultural soils) and the second group (non agricultural soils), while there was little difference between the first and third group. Table 1 shows the average percent of resistance bacteria for each group. The average population of resistant bacteria in 2 mM lead in the soil of the first group was 47%, and in the second group 22%. The average in the third group was 31%. The high percentage of resistance in the first group was not only due to the presence of lead in some soils but was also due to the drainage of the Zayanderood river water which affected the increase in the resistant bacteria population. The effect of the river water on the soils of the Zayanderood river bank is completely clear.

Concerning microbial resistance to copper, there was a meaningful difference between the first and the second group, while no significant difference between the second and the third group, and also between the first and the third group was found. The average bacteria resistant to cadmium showed a significant difference between the first and the second group, and this problem was also observed between the second and the third group, however no difference was observed between the first and the third group.

Several agents such as fertilizers, pesticides and discharge of wastewater from different industrial sources into the Zayanderood river and its surrounding area was one of the most important factors in the cadmium concentration increase in the area and in bacterial resistance.

Regarding resistance to arsenic, there was a difference between the first and the second group, but there was no difference between the first and the third group. However, significant differences were seen between the first and the second group. In addition, the percentage of resistant bacteria in the first and the third group was more than that in the soil of the second group. Further, the highest percentage of resistant bacteria population for the four metals was related to cadmium and then arsenic, and this problem was not

only due to the environmental condition, but it could also be due to the role of the genetic structure of the microorganism. Different factors (plasmids, stress proteins, membrane pumps…) were effective in these conditions [12, 16].

4.3. Identification of resistant bacteria

The abundance of bacillus was possibly due to the spore structure in the bacilli group, which increases its power of uptake and resistance against the metals. However, among the bacteria resistant to cadmium, the abundance of *Lactobacillus* was significant and this problem was to some extent due to membrane structure and membrane pumps [17]. According to Table 2, the diversity of resistant types among the bacteria resistant to cadmium and arsenic was more than that of lead and copper. In the bacteria resistant to arsenic, a relative equivalence was observed in the resistance percentage among the different types.

4.4. Results of MIC of the metals in three soil groups

According to statistical data, the MIC of the bacteria resistant to lead, between the first and second groups and the first and the third groups revealed a meaningful difference, while there was little difference between the second and the third group. The average MIC in the bacteria resistant to lead in the first group was 7 mM and 5.3 mM in the second group and 5 mM in the third group. The high level of MIC in the first group was possibly due to the existence of lead in some of the soils of the first group which has caused not only the increase in population of resistant bacteria, but also increased their resistance. According to Table 3, in 60% of the bacteria resistant to lead, the MIC was between 4-8 mM, while according to Sabry et al., [8] research on the estuaries in the Alexandria region in Egypt, the highest level of lead resistance was 2.5-5 mM and 98% of the bacteria were sensitive to this concentration range. In the research conducted by Choundhury and Kumar [10] made on water samples, MIC was 10 mM. According to Roane and Kellogg [7] the level of resistance to lead in the soil samples was between 2.5 and 5.6 mM. The MIC of the bacteria resistant to copper revealed little difference between the three soil groups.

The average MIC in the first group was 8.3 mM, in the second group 7 mM, and in the third group 7.8 mM. According to Table 3, 60% of the resistant bacteria had their MIC in the concentration range of 6-8 mM. According to Sabry et al., [8] the level of copper resistance was reported to be 2.5 mM. In another study it has been reported that copper MIC for *Escherichia coli* was 10 mM, for *Vibrio cholera* 5 mM and for *Pseudomonas* 7 mM [7]. Thus, according to the obtained results it could be concluded that, the resistance power in the isolated bacteria was more than in previous studies. This problem may be due to environmental conditions or the type of bacteria. The level of MIC for cadmium revealed little difference between the three groups.

The average MIC in the first group was 4.5 mM, in the second group 4.9 mM and in the third group 3.8 mM. Table 3 shows that the concentration range of 4.5-6.5 mM includes more than half of the cases. According to Mullen et al., [5] research at this level was in the range of 2-2.5 mM and according to another study it was 10 mM for *E.coli*, 10 mM for *V.cholera*, and 3 mM for *Pseudomonas* [10]. According to Javadi and Kargaran's research [4], the level of cadmium MIC was 0.5-1.2 mM. From the results of this study it is clear that the isolated bacteria have a higher tolerance with respect to the bacteria isolated by other researches. The average MIC of the arsenic revealed little difference between the three soil groups. The average arsenic tolerance in the first group was 66 mM, in the second group 68mM, and in the third group 65 mM. About 70 % of MIC has been in the range of 60-90 mM, while in the research it was 20mM [8]. Thus, the high level of MIC was probably due to the genetic structure of the bacteria or the environmental factors.

4.5. Effect of biomass on microbial uptake of metals

The results indicated that the microbial biomass effects on lead uptake were in the form of negative dependence and were meaningful. Thus it could be concluded that, in the bacterial presence of lead, the level of receipt is decreased as the biomass level increases. This was also true for copper, while the microbial biomass concentration has had little effect on the level of cadmium and arsenic receipt. The above results were different from other studies conducted on chrome uptake [16], as in that study the level of chrome uptake increased with the increase in cellular mass, which was possibly due to an increase in cellular connection sites and more metal contact to the cell. However, the uptake of metals is in different forms. For example the cytoplasmic precipitation of silver. According to some studies, when the biomass weight increased more than 30-35mgl^{-1} the uptake level decreased [12]. This could be due to the viscosity of the cellular suspension increase that inhibits the uptake or mixing of the metal in the cell [13]. According to some researches the increase in biomass resulted in a decrease in the uptake level [15]. The effect of lead and copper on *Arthrobacter* was reviewed by Veglio et al., [15]. It was found that the highest level of uptake was with the lowest level of biomass and this problem could be due to interference between metal connection sites. According to another research, the increase in cellular biomass was effective in the cellular uptake and its effect was positive. Overall, not only the microbial biomass, but also the biomass age is important for the level of uptake [18].

4.6. Results of microbial uptake of metals based on bacteria types

For determining the most common bacteria types results show that, the resistant bacteria to lead, bacilli, were the main resistant types and their average metal uptake was more than 90%.

Thus, it could be concluded that the bacilli had a great capacity for lead uptake. Among the bacteria resistant to copper, the bacilli are also the main group, with an average uptake higher than 90%. Among the bacteria resistant to cadmium, the bacilli had also an uptake average higher than 90%, however the uptake rate in other types such as *Sporolactobacillus* was considerable. The uptake level of arsenic was lower than that of other metals and it has absorbed 34% of the metal. Also, in this group the bacilli were the dominant type, but other types such as *Staphylococcus* and *Corynebacterium* were also considerable. The results of this study indicated that the highest level of uptake was related to the microorganisms resistant to lead, while the lowest uptake level was related to arsenic retention in the microorganisms resistant to arsenic. The low level of uptake for arsenic was possibly due to the membrane pumps that cause metal to be desorbed from bacteria.

4.7. Evaluating the multi resistant bacteria

Among the 362 isolated bacteria, based on the number and percentage of metal resistances, the highest level of resistance was related to arsenic (63.8%) and the lowest to copper (43.3%).

This problem may be due to the microbe structure or concentration of the solutions. The number and the percentage of bacteria resistant to metals are shown in Table 4.

4.8. Evaluating the multi bacterial resistances to metals

According to the results obtained from the determination of bacterial resistance to other metals, it was found that among 362 resistant bacteria, 47 showed resistance to all of the four metals, which was 12.98% of the total resistant bacteria. 91 bacteria showed resistance to three metals, or 25.13% of the total resistant bacteria.

Resistant types are shown in Table 5. In three aspects of resistances, the highest resistance percentage was concurrently related to lead, cadmium and arsenic.

This pattern of resistance forms 8.28% of the total resistant bacteria, while 111 bacteria had, concurrently, two resistance metals which was 30.66% of the total isolated resistant bacteria. The highest pattern of resistance in this phenotype was related to the concurrent resistance to arsenic and cadmium, which included 9.11% of the total 362 isolated bacteria. In the phenotype of one metal resistance with 78 cases includes 21.54% of the total cases, with the resistance to arsenic being the highest case. Considering the resistant pattern, the resistance to arsenic is considerably greater than other metals which was due to the cytoplasmic pumps of the bacteria which discharge metals.

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