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Assessment of Chromium Resistant Bacteria Isolated from Tannery Waste Contaminated Soil in the Region of Fez (Morocco): A Statistical Approach

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The environmental risk of chromium pollution is pronounced in soils adjacent to tannery effluents. The influence of contamination by chromium on the diversity of soil microorganisms was investigated in Sebou-Fez system. Soil samples obtained from five sites of the same area were analyzed for chromium contamination. A group of 35 chromium-resistant bacteria were isolated. A negative correlation between soil microbial population and chromium content was observed. These isolates displayed different degrees of resistance to chromium, approximately 77.14% of these isolates showed an MIC value of 400- 600 mg/L Cr(VI). A large number of the cultures reduced >20% Cr(VI) during growth (57.1%). Three of the culture showed >80% chromate reduction (about of 8.57% of the total). These strains were tested for their ability to tolerate zinc, mercury, lead, cobalt, copper, and nickel in their growth medium. A large number of the cultures tolered 1500, 2000, and 2000 mg/L Zn, Ni, and Pb, respectively. Some strains can be exploited for bioremediation of hexavalent chromium containing wastes, since it seems to have the potential to reduce the toxic hexavalent form of chromium to its non toxic trivalent form.

Key words: Bioremediation, Microbial population, Chromium, Heavy metal resistance, Cr(VI) reduction.

Chromium (Cr) is an essential trace metal for living organisms, but above critical level it is toxic[1], mutagenic[2-3] and carcinogenic[4].The wide use of chromium (Cr) in industries such as leather tanning, electroplating, textile, production of paint pigments and dyes has resulted in large quantities of chromium containing industrial effluent. In Morocco, tannery wastes constitute a major anthropogenic source of chromium in the environnement. Chromium exists in the environment in several diverse forms such as hexavalent Cr(VI) and trivalent Cr(III). Cr(VI) is considered the most toxic form of Cr, which usually occurs associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanions while Cr(III) is less mobile, less toxic and is mainly found bound to organic matter in soil and aquatic environments[5-6]. Therefore reduction of Cr(VI) to Cr(III) is an effective way for remediation of Cr (VI) in the contaminated soils.The conventional

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methods to detoxify and remove Cr(VI) from the environment include chemical reduction followed by precipitation, absorption on alum or kaolinite and ion exchange etc. However, most of these technologies need high energy or large input of chemical reagents that may cause secondary environmental pollutions[7]. Alternatively, The search for new and innovative technology for the remediation of Cr(VI) pollution has attracted to use bioremediation approaches through selective microorganisms have the potential to accumulate chromium and reduce Cr(VI) to Cr(III)[8]. In recent years, reduction of Cr(VI) has been reported in several bacteria under aerobic or anaerobic conditions, including *Pseudomonas* sp.[9], *Intrasporangium* sp.[10], *Bacillus* sp.[11], *Thermoanaerobacter*[12], and *Escherichia coli*[13] etc. The present investigation aims with the isolation of chromate resistant bacteria from tannery waste contaminated soil of Fez (Morocco) and the study of their heavy metal resistance and chromate reduction.

MATERIALS AND METHODS

Study sites

The study area (Fez) is located in the North of Morocco (North Africa) between 33°55'44''Lat. north and 4°58'46"Long. west. Several rivers that cross the region currently serve as foul water conduits for both domestic and industrial waste waters (Figure 1). To characterize the soils of these areas polluted, five sites were selected: Site R1 (control site), in the River Sebou, upstream from the confluence of the Sebou River with the Fez River. Site R2 located in Stream Mehraz. Site R3 in the Fez River out of the medina. Site R3'located on the same site as R3, but directly in the effluent of a tannery. Site R4 in the River Sebou, a few meters downstream from confluence of the Sebou River with the Fez River. The sites (R2, R3, R3', R4) are sites polluted by effluent from tannery while the site R1 is the control site.

Soil sampling, characteristics

The soil samples were collected from the upper 20 cm of the five selected sites located in Fez, Morocco. The samples were placed in sealed plastic bags and transported to the laboratory. The soil samples were air- dried over three days and passed through a 2 mm sieve. The physicochemical properties such as pH, total chromium of the soil samples obtained from these were digested with nitric acid then the mixture was heated coolant over a sand bath at 120 °C for 4 hours, ûltered through a Whatman 540 ûlter paper made up to volume and then analysed on the atomic absorption spectrophotometer[14].

Microbial population

1 g of soil was diluted in 100 ml distilled water. Soil suspensions were shaken at 110 rpm for 1 hours and then rest for 10 min to allow settling of the soil. Standard serial dilutions followed and 100 µL aliquots of dilution were spread on plates. Enumeration of soil microorganisms was performed using the spread plate counting method. The microbial populations were enumerated as colony forming units (CFU) from a serial dilution of the soil suspensions. The colonies were counted after incubation for 48 h.

Isolation of chromium resistant bacteria

The sample suspension was prepared by adding about 1g of soil to 100 mL distilled water. 1 mL of the sample suspension was transferred to 9 mL distilled water. This process was repeated to obtain the serial 10- fold dilutions of sample suspension (10^{-1} to 10^{-8}). 100 µL aliquots were withdrawn from 10^{-1} to 10^{-8} sample suspension dilutions and then dropped respectively to Luria Broth (LB) agar amended with Cr(VI) as $K_2Cr_2O_7$ to final concentration (200 mg/L) with sterile Cr(VI) stock solution. Plates were incubated at 37°C for 24 h. Chromium-resistant strains representing different colony morphologies were purified on the same agar medium and then stored at 4°C.The LB agar medium consisted of peptone (10 g), sodium chloride (10 g), yeast extract (5 g), agar (15 g) in 1 L distilled water. The pH value of the medium was adjusted to 7 with 10% (w/v) NaOH and 10% (w/v) HCl.

Evaluation of chromium resistance

Maximum resistance of the strain against increasing concentrations of $K_2Cr_2O_7$ (200 to 800 mg/L) on LB agar plates was evaluated until the strain unable to give colonies on the agar plates. Based on the evaluation, minimum inhibitory concentration (MIC) was determined after 48 h of incubation at 37°C.

Evaluation of metal tolerance

Analytical grades of metal salts ($ZnSO_4 .7H_2O$; $CuSO_4 .5H_2O$; $NiCl_2$; $HgCl_2$; $CoCl_2 .6H_2O$

and $Pb(NO_3)_2$) were used to prepare 200 g/Lstock solutions. Each stock solution was filter sterilized and added separately in the agar medium to final concentrations of 5 to 2000 mg/L of each metal for determinations of the minimum inhibitory concentrations (MICs) of the metal ions for each isolate. Duplicate plates were prepared for each metal concentration and then they were incubated at 37°C.

Chromium reduction experiments

Reduction of chromium was determined by inoculating the isolates in LB medium supplemented with 100 mg/L of Cr(VI) concentration and incubated at 37°C under continuous shaking (120 rpm). Reduction was estimated by measuring the decrease in hexavalent chromium in the supernatants of culture at regular time intervals.

Analytical methods

Chromate reducing activity was determined as decrease of chromate over time using the Cr(VI) specific colorimetric reagent S-diphenyl carbazide (DPC) 0.25% (w/v) prepared in acetone (AR) to minimize deterioration. The reaction mixture was set up in 10 mL volumetric flask as follows: 200 µL or 400 µL sample or standard $K_2Cr_2O_7$ (10 mg/L) volume was made to 1 mL using glass distilled water followed by addition of 330 µL of 6 M H_2SO_4 and 4 mL of DPC and final volume was made to 10 mL using glass distilled water[15]. Spectrophotometric measurements were made immediately at 540 nm.

RESULTS AND DISCUSSION

Physicochemical parameters in the soil samples

Soil samples were collected from the upper 20 cm of the five locations (R1, R2, R3, R3' and R4) for physicochemical parameters. The pH of the soil samples were in the range of 7.6- 8.05 (Table 1). This indicates that waste contaminated soil is slightly alkaline in nature. The concentrations of total Cr concentration in the soil samples vary significantly (Table 1).The concentrations of total Cr in soil varies greatly according to the distance from tannery effluents. The lowest total Cr concentration (1.6 mg/L) is found in the soil sample far away from the main source of metal chromium such as tannery (R4). In comparison with local background values (Table 1) in the soil at the control site (R1), the value of total Cr in the contaminated soils (R3, R3' and R2) is much higher. The concentrations of total Cr in the soils at sites R2, R3 and R3' are 3, 3 and 10 times higher than the soil at the control site, respectively, but in the River Sebou, a few meters downstream from the confluence of the Sebou River with the Fez River (R4) is generally "clean". The results show that there are serious pollutions by chromium from tannery effluents. On the selected four sites, site R3' (directly in the effluent of a tannery) has the highest contaminant level, followed by Sites R3 (in the Fez River out of the medina), R2 (in Stream Mehraz) and R4 has the lowest contaminant level. Most researches focus on the comparison of the contamination degree among the soils around the contaminant sources and coincident results that total Cr concentration decrease with distance from the contaminating sources were reported in previous literatures[16-17].

Table 1. Physiochemical properties of sampling sites in Sebou-Fez system

Sampling site	pH	Cr(VI) (mg/L)
R1(control site)	8,29	1.1
R2	7.9	3.7
R3	7.6	3.9
R3'	8.7	10.0
R4	8.05	1.6

Population of microorganisms

The population of microorganisms in soil from site R3' (directly in the effluent of a tannery) is very low, only 32×10^4 CFU/g (Figure 2). However, microbial number is great in soil from control site (R1) and (R4), with the value of 216×10^5 CFU/g and 198×10^5 CFU/g, respectively. The increase of distance from the soils around the contaminant sources, the number of soil microbes increases. Population of soil microorganisms shows the order of R1> R4> R2> R3>R3'. No significant difference in microbial population was found between site R4 and control site R1. The statistical analysis also shows that microbial number is negatively correlated with total chromium (data not shown). This suggests that the very high chromium concentration in the contaminated sites have been primarily responsible for the decrease in the microbial number.

Table 2. Screening of chromium resistant bacteria from tannery waste contaminated soil

Sites	Number of isolates	MIC			
		< 400	400-600	600-800	> 800
R1	6	2	4	-	-
R2	3	-	3	-	-
R3	3	-	3	-	-
R3’	3	1	2	-	-
R4	20	1	15	2	2
Total	35	4	27	2	2
% of isolates	100	17.14	77.14	5.71	5.71

Table 3. Tolerance of 35 soil bacterial strains to 6 metals

Metal	% of isolates susceptible to the following metal concentration (mg/L)							
	≤ 100	200	300	400	500	1000	1500	2000
Zn	-	-	-	2.85	31.42	25.71	25.71	14.28
Cu	5.71	5.71	37.14	42.85	5.71	2.85	-	-
Ni	2.85	-	17.14	11.42	65.71	2.85	-	-
Pb	-	-	-	-	-	2.85	94.28	2.85
Co	-	-	-	34.28	48.57	17.14	-	-
Hg	17.14	-	-	-	-	-	-	-

Table 4. Correlation (Pearson’s linear coefficient) between MICs of heavy metals

	MIC Cr	MIC Zn	MIC Cu	MIC Ni	MIC Pb	MIC Co
MIC Cr	1					
MIC Zn	0.829	1				
MIC Cu	0.116	0.216	1			
MIC Ni	0.285	0.098	0.825	1		
MIC Pb	0.069	0.523	-0.084	-0.178	1	
MIC Co	0.696	0,970	-0.349	0.281	0.057	1

Correlation is significant at the $p<0.01$ level

Table 5. Screening of chromium resistant bacteria from tannery waste contaminated soil for chromate reduction

MIC (mg/L)	Number of isolates	Percent reduction of Cr(VI) (%)			
		< 20	20-60	60-80	> 80
<400	1	-	-	1	-
400-600	21	9	8	2	2
600-800	10	3	1	5	1
>800	3	3	-	-	-
Total	35	15	9	8	3

Isolation of chromium resistant bacteria

A group of 35 chromium resistant bacteria were isolated from the five soils sites. Twenty isolates from site R4, six isolates from site R1 and three from each site (R2, R3 and R3') (Table 2). Approximately 77.14% of these isolates showed an MIC value of 400- 600 mg/L Cr(VI) and 5.71% isolates were able to tolerate more than 800 mg/L Cr(VI). Parameswari *et al.*,[18] showed that most Cr (VI) resistant microorganisms tolerate over a wide range (0 to 100 mg/L). However the Cr(VI) resistance above 1500mg/L has been reported by McLean[19].

Response of bacterial isolates to heavy metals

Contaminated habitats are generally characterized by the co-existence of a large number of toxic cations and, therefore, it is necessary to study the multiple metal resistances of microorganisms. Tolerances to other metals have an added advantage of withstanding the presence of different metallic ions while performing the desired activity. The percentage of the isolates that were susceptible when challenged with various concentrations of the six heavy metals are shown in Table 3. From comparisons of the results across the metals, it was evident that 31.42%, 42.85%,

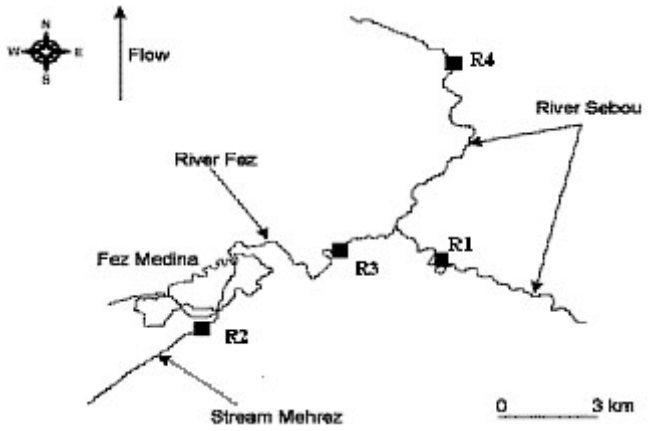

Fig. 1. Location of sampling sites in Sebou-Fez system

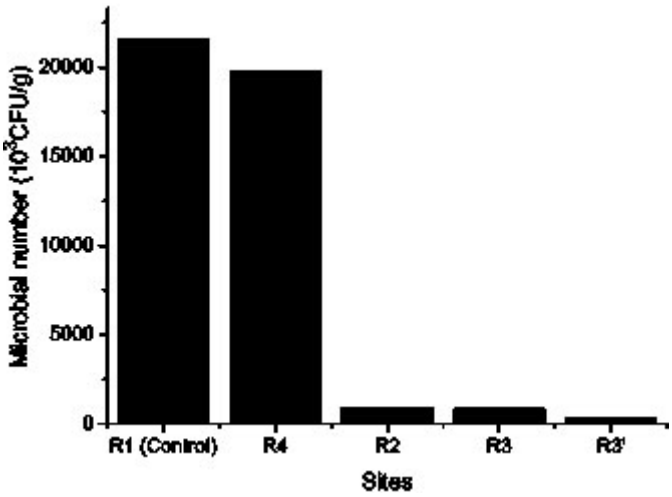

Fig. 2. Total microbial population at five sampling sites

65.71%, 94.28%, 48.57% and 17.14% of the isolates were tolerant to Zn, Cu, Ni, Pb, Co and Hg with MICs of 500, 400, 500, 1500, 500 and 5 mg/L, respectively. All the cultures showed some tolerance to heavy metals with a large proportion even tolerating 1500, 2000, and 2000 mg/L Ni, Zn and Pb, respectively. The high levels of resistance and the widespread tolerance that was found among the isolates is probably attributed to the high metal contents of the sites (Sebou River and Fez stream)[20]. Pearson's correlation coefficients among MICs of heavy metals were represented in table 4. A significant positive correlation (p<0.01) exists between Cr and Zn, Cu and Ni, Zn and Co.

Cr(VI) reduction

The isolates were capable of reducing chromate in different degrees. About 57.1% of the isolates were able to reduce >20% of Cr(VI) in the medium, while three strains showed >80% chromate reduction after 48 h of growth at 37°C and were tolerant 400 to 800 mg/L of Cr(VI) (Table 5). Pal and Paul[21] reported that about 34 isolates 38.2% reduced > 40% of 50 mg/ Lof Cr(VI) only one strain was able to reduce >80% of Cr(VI).

CONCLUSION

Bacterial isolates obtained from tannery waste contaminated soil were found to have an ability to adapt in hexavalent Cr(VI) polluted environment, approximately 77.14%(27) of total isolates showed an MIC value of 400- 600 mg/L Cr(VI) and 5.71% isolates were able to tolerate more than 800 mg/ L Cr(VI). The study revealed that the isolates can tolerate heavy metals at different concentrations over a wide range (5- 2000mg/ L). Soil contamination by heavy metals is often irreversible and may repress or even kill parts of the microbial community, and it is generally assumed that the exposure to heavy metals lead to the establishment of a tolerant microbial population. These isolates displayed different degrees of chromate reduction under aerobic conditions; about 57.1% of the cultures reduced >20% Cr(VI) during growth. The results obtained in this study may provide significant information for the bioremediation of chromate.

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