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Hexavalent chromium removal from a tannery effluent by a bacterial consortium

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Abstract

The objective of this work concerns to exploit the power of bacteria for hexavalent chromium removal the tannery effluents. To reach this purpose, fifty bacterial isolates were isolated from abandoned lead mines soil in Morocco. Resistance of these bacteria to certain heavy metals (Cr(VI), Pb, Cd, Hg, Co, Ni, Cu, Zn and Mn) was tested in vitro under various concentrations (5 - 3500 mg/L). Four bacterial isolates have shown hexavalent chromium resistance up to 800 mg/L. In addition, we have observed a multi-metal resistant and no antagonistic activity between them. DNA sequencing of the 16S ribosomal RNA gene have allowed identifying these four bacteria: Bacillus subtilis, Kocuria rosea, Staphylococcus lentus and Bacillus subtilis subsp. spizizenii. These four bacteria multi-metal resistant were used at consortium for hexavalent chromium removal of tannery effluent. This elimination, is evaluated by reaction with 1,5-diphenylcarbazide (DPC). After five days of incubation at 30°C, the results show that rate of removal are 76%, 25%, 12.5% and 3.34% for the concentrations 50 mg/L, 100 mg/L, 200 mg/L and 300 mg/L respectively in the effluent.

Keywords: Heavy metals; Hexavalent chromium; Tannery effluent; Bioremediation.

1. Introduction

The growth of industrial sectors has become the root cause of environmental deterioration and has raised the concerns for efficient wastewater management and reuse. Due to its vital values, water is required in a cleaner form where plants, animals and humans cannot survive if it is loaded with high metal concentration, pathogenic microorganisms and/or hazard chemicals. Chromium hexavalent (Cr(VI)) has been designated as the priority pollutant by United States Environment Protection Act [1]. Tanneries are a major source of chromium pollution and release the Cr(VI). The general limit values for discharge into surface or underground waters of total chromium has been fixed at 2 mg/L and of Cr(VI) at 0.2 mg/L as per Moroccan standards [2]. Multiple technologies have been applied to eliminate Cr(VI) from aqueous solutions including precipitation, reverse osmosis, ion exchange, filtration, sand filtration, chemical reduction/oxidation, electrochemical precipitation, membrane filtration, solvent extraction and electrochemical deposition [3]. Nevertheless, all these techniques show various limitations including incomplete metal removal, high reagent consumption and energy utilization, low selectivity and generation of secondary wastes that are difficult to dispose of [3,4]. Currently, bio-removal of heavy metals is increasingly attracting scientific attention due to their significant role in purification of types different of wastewaters. Indeed, biological approaches may reduce Cr(VI) to Cr(III) intracellularly or by making the extra cellular environment more reducing or lowering pH to favor Cr(VI) reduction. In addition, the utilization of microorganisms may offer a highly selective removal of toxic metals coupled with considerable operational flexibility. Hence, they could be both *in-situ* and *ex-situ* in a range of bioreactor configurations [5]. Heavy metal resistant microorganisms play an important role in the bioremediation of heavy metal contaminated effluents [6]. The bacteria isolated from abandoned mines have developed the physiological mechanisms to grow in the presence of high concentrations of heavy metals and can be used to treat industrial effluents contaminated by heavy metals. In this context, this study describes the use of multiple-metal resistance bacteria, in consortium, to remove hexavalent chromium from a tannery effluent.

2. Materials and Methods

2.1. Study area and sampling

The study area is abandoned lead mines located in the high Moulouya of the region of Midelt-Morocco. This region is characterized by a cold arid climate with mountainous tendency [7]. The rainfall regime is extremely variable and erratic. Sometimes, the region receives snowfall. From each site, five samples were taken with the help of sterilized spatula from the 10 cm upper layer of soil. For the collection of each sample 5 x 5 m quadrate was selected [8].

2.2. Physicochemical analyses

The analyzed physico-chemical parameters were pH, Electrical Conductivity (EC), Salinity and heavy metal concentrations. pH and EC were measured by machine of type CONSORT-Model C535 (µS/cm, pH)-G. BOYER-S/N 71658. For pH, 10 g of soil sample was mixed with 50 mL of distilled water. After agitating for 15 min, the suspension was allowed to settle for 2 hours [9]. For EC, 5 g of the soil was agitated with 25 mL of distilled water, out at laboratory temperature at a constant speed of 120 rpm for 30 min. The suspension was allowed to settle for 15 min and centrifuged at 3000 rpm for 10 min [10]. All the experiments were done in triplicates. The EC was determined by using equations.

$$EC\ 25°C\ (dS/m) = ECt \times Ft$$

ECt: Conductivity at temperature of extract & **Ft**: Temperature coefficient.

Heavy metals concentrations were determined by Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES), HORIBA JOBIN YVON-Type Activa, S/N: OVOU/1048, in Innovation City of Sidi Mohamed Ben Abdellah University, Fez-Morocco. Soil samples were pre-treated according to protocol described by Alsac in 2007 before analysis at the ICP-AES: 5 g of soil samples were dried in oven at 40°C for 16 hours, milled and sifted it at 2 ~ 250 µm. 0.5 g of soil sample was mixed with 6 mL of hydrochloric acid and 2 mL of nitric acid. After heating at 95°C for 75 min, the volume of the obtained mineralized was adjusted to 50 mL prior to ICP-AES analysis [11].

2.3. Isolation of total bacteria

For each site, 5 g (1 g of each sample) was mixed, suspended in 50 mL sterile physiological solution (0.85% NaCl in distilled water) for 15 min at 30°C. From this suspension, a series of dilutions (10^{-1} - 10^{-8}) was prepared. Dilutions were then plated on Luria Bertani (LB) agar medium (Tryptone 10 g/L, Yeast Extract 5 g/L, NaCl 10 g/L, Agar 18 g/L) and incubated at 30°C for 48 hours. Bacterial isolates were purified by successive streaking on LB agar medium.

2.4. Screening of bacterial heavy metals resistant

The isolated bacterial were screened for their resistance to heavy metals using the agar diffusion method [12]. Plates containing LB agar medium were supplemented with heavy metals different Cr(VI), Pb(II), Cd(II), Hg(II), Co(II), Ni(II), Cu(II), Zn(II) and Mn(II) separately and at different concentrations (5 - 3500 mg/L). The inoculated plates were incubated at 30ºC for 48 hours. All the experiments were done in triplicates.

2.5. In vitro antagonistic test

Ten resistant bacteria isolates were tested for their antimicrobial activities, against each other, using disk diffusion method [13]. It consists in depositing disks impregnated with bacterial isolates in broth (optical density (OD) at 600 nm = 0.8) on the surface of petri dishes previously inoculated with the target isolate. After incubation at 30°C for 24 hours, the inhibition is manifested by the presence of clear zones around the disks. It is considered as positive when diameter of clear zone is greater than 1 mm [14].

2.6. Molecular identification

Four isolates showing high resistance to Cr(VI) and no antagonistic activity, have been subject to PCR amplification targeting the 16S rRNA with universal primers fD1 and rP2. For each bacterial isolate, after an overnight culture at 30°C, a volume of 1 mL was centrifuged at 8000 rpm for 5 min. Resuspend the pellet in 100 µL of distilled water. The suspension was subjected to thermal shock: 3 min at -20°C followed by 3 min at 95°C. After centrifugation at 12 000 rpm for 5 min, 2 µL of supernatant containing the DNA was used for PCR reaction. The universal primers, fD1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and rP2 (5′-TACGGCTACCTTGTTACGACTT-3′) were used to amplify 16S rDNA [15]. The amplification reaction was performed in a final volume of 20 µL containing 0.5 µM of each primer, 200 µM of each dNTP, 0.2 units Taq DNA polymerase, 1.5 mM $MgCl_2$ and 2 µL DNA sample in 1x Taq polymerase buffer. The amplification was performed on a thermal cycler (Techne Genius, Cambridge, UK). The mixture was first denatured at 94°C for 5 min. Then, 35 cycles of PCR were performed by denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 30 s. At the end of the last cycle, the mixture was incubated at 72°C for 10 min. The sequencing was carried in Innovation City of Sidi Mohamed Ben Abdellah University, Fez-Morocco, using an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). For identification of the isolated bacteria, the partial 16S rRNA gene sequence was compared with the full sequence available in the GenBank database using a BLAST search (NCBI: National Center for Biotechnology Information). To ascertain the taxonomic position

of the resistant bacteria of Cr(VI) isolated from the soil samples, we amplified and sequenced the 16S rDNA. As reported in the literature, PCR amplification of the 16S rRNA gene with the fD1 and rP2 primers amplifies a DNA fragment of approximately 1.5 kbp [15]. These sequences were compared with database sequences. The program used is BLAST NR 2.8.1 through the NCBI. The molecular definition of gender states requires that the homology of 16S rDNA should be greater than or equal to 97%. 99% homology allows the identification of species, while a score below 97% of homology does not allow it identification [16].

2.7. Hexavalent chromium removal from tannery effluent by the bacterial consortium

An industrial effluent sample was collected from lower part of the discharge point of chromium-laden tannery in Ain Nokbi industrial zone, Fez city, located at 4°56'W and 34°3'N at altitude of 248 m. The sample was stored at 4°C to inhibite any biological activity and was filtered using Whatman N°.1 filter paper. A volume of 50 mL of the effluent was inoculated by the bacterial consortium formed by 100 µL of each bacterial isolate (10^{+6} – 10^{+7} CFU/mL). After incubation at 30°C with stirring 125 rpm, the growth of cells was examined by measuring of optical density (OD) at 600 nm using Spectrophotometer. Then, the decreasing Cr(VI) concentration was measured by the diphenyl carbazide essay (DPC 0.25% w/v) prepared in acetone. The reaction mixture was set up in 10 mL volumetric flasks as follows: after centrifugation at 6 000 rpm for 10 min, 200 µL of sample was added at 330 µL of H_2SO_4 (6M) and 4 mL of DPC. Final volume was made to 10 mL using distilled water. Spectrophotometric measurements were made immediately at 540 nm (JENWAY 6100 spectrophotometer) [17].

3. Results and Discussion

Microorganisms have a big potential to control environmental pollution, particularly industrial sources of water pollution. Chromium, one of the hazardous pollutants discharged from tanneries. In the environment, chromium is present in two stable oxidation states such as Cr(VI) as an anion and Cr(III) as a cation. Cr(VI) is water soluble in nature and is highly toxic, whereas Cr(III) is completely insoluble and known as an important micronutrient for lives, which plays role in the metabolic activities [18]. Because of the high mobility and acute toxicity of Cr(VI), it affects humans by causing different diseases in body parts such as skin, liver, kidney and respiratory organs.

Effective treatment of tannery effluent is a dire need of the era as a part of environmental management. Among all the wastewater treatment technologies, bioremediation is the most effective and environment-friendly tool to manage the water pollution.

3.1. Physical-chemical characterization of soils samples

The first step of our study was to evaluate the physio-chemical characteristics of soil samples collected from the abandoned mines of Midelt and of Zaïda, Morocco (Table 1). The measured pH value ranges between 8.70 and 8.85. Based on previous studies [19], this alkalinity can be attributed to the presence of carbonates. In addition, soils have low salinity because the electrical conductivity values ranged from 0.37 to 1.08 dS/m. In the other hand, the results ICP-AES analysis showed high concentrations and variety of heavy metals in the collected soil samples (Table 2). The abandoned mines of Midelt and Zaïda in the Upper Moulouya, Morocco, were among the main deposits exploited between 1972 and 1985. Mining activities represents a source of potential environmental impacts for all natural resources. Contamination by heavy metal is one of the most prominent environmental risks at these sites. At these two sites, several studies have highlighted the presence of high levels of heavy metal in the environment around the site including soils [7].

Table 1. Physico-chemical characteristics of the soil samples.

	pH	EC (dS/m at 25°C)	Salinity
Site N°1: Midelt mine	8.70±0.10	1.08±0.10	Salty
Site N°2: Zaïda mine	8.85±0.04	0.37±0.02	Unsalty

Table 2. Heavy metals concentrations in the soil samples.

	Metallic trace elements concentrations (mg / 1kg soil)											
	As	**Cd**	**Cu**	**Cr**	**Zn**	**Pb**	**Ba**	**P**	**K**	**Ca**	**Mn**	**Na**
Site N°1: Midelt mine	< 1	< 1	10.4	43	136	367.45	276.33	766.8	16 067	92 196	474.11	1520
Site N°2: Zaïda mine	< 1	< 1	55	36	127.36	4535.30	1916.30	350.65	8582	$> 10^{+5}$	2972.6	4110.9

3.2. Screening of bacterial isolates resistant to different heavy metals

A total of 50 isolates were purified on LB agar medium from different soils samples. The purified isolates were tested for their resistance to heavy metal using the agar diffusion method on LB agar medium. The degree of heavy metals resistance to the highest concentration in the LB agar medium was evaluated based on the ability of the isolated bacteria to grow on the higher concentrations. Among these 50 isolates, 10 were resistant to various metals at high concentrations. The results obtained are shown in Table 3.

-: Negative ; +: Positive.

Most of bacterial isolates have been able to grow in the presence of low concentrations of the heavy metals, while no bacterial growth was observed at higher concentrations. These differences in toxicity toward the bacterial isolates may be explained by the conditions of bacterial isolation and the nature and physiological characteristics of each bacterial isolate [12]. Of these ten isolates multiple-metal resistance, four (X1, X2, X3 and X7) showed no antagonist activity between them.

3.3. Molecular identification

The results obtained are given as a percentage of identity (Table 4). Isolate X1 showed 99% identity with *Bacillus subtilis strain GA CAS11*, isolate X2 showed 99% identity with *Kocuria rosea strain RR75*, isolate X3 showed 99% identity with *Staphylococcus lentus strain FC3104* and isolate X4 showed 99% identity with *Bacillus subtilis subsp. spizizenii strain MV20*. The 16S rRNA sequences of these four isolates was deposited in GenBank under accession number MK571421, MK571422, MK571435 and MK571447 (Table 4). According to the literatures, *Bacillus sp* is characterized by its tolerance to heavy metals (Fe, Cd and Cr) [20]. *Bacillus subtilis* is known for its ability to reduce chromium [21,22,23]. Also, *Kocuria sp* is proven to be resistant to heavy metals (Cu, Zn, Pb, Ni and Cd) [24]. *Kocuria sp*. is marked by its ability to remove Ni^{+2} and Cr^{+3} from an aqueous solution [25]. Some species of *Staphylococcus* (*S. epidermidis, S. aureus, S. saprophyticus and S. arlettae*) are resistant to chromium [26]. Also, *Staphylococcus sciuri* is known for its Multiple metal resistance and for its ability to reduce Cr(VI) [27]. On the other hand, *Staphylococcus lentus* has never been reported as chromium-resistant bacterium.

Table 3. Resistance of bacterial isolates to different heavy metals and concentrations.

Elements	Concentrations mg/L	Strains/Results									
		Site N°1					Site N°2				
		X1	X2	X3	X4	X5	X6	X7	X8	X9	X9
Mercury (Hg(II))	10	+	+	+	+	+	+	+	+	+	+
	25	-	+	-	+	+	+	+	+	-	+
	50	-	+	-	-	-	+	+	+	-	-
	75	-	+	-	-	-	-	-	+	-	-
	100	-	-	-	-	-	-	-	-	-	-
Cadmium (Cd(II))	10	+	+	+	+	+	+	+	-	-	+
	25	-	-	+	+	+	+	+	-	-	+
	50	-	-	+	+	+	+	+	-	-	-
	75	-	-	+	+	+	+	+	-	-	-
	100	-	-	+	-	+	-	+	-	-	-
	200	-	-	-	-	-	-	-	-	-	-
Chromium (Cr(VI))	100	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+
	400	+	+	+	+	+	+	+	+	+	+
	600	+	+	-	+	+	+	+	+	-	-
	800	-	-	-	-	-	-	+	+	-	-
	1000	-	-	-	-	-	-	-	-	-	-
Cobalt (Co(II))	100	+	+	+	+	+	+	+	+	+	+
	200	+	-	-	+	+	+	+	+	+	-
	400	-	-	-	+	-	-	-	+	-	-
	600	-	-	-	-	-	-	-	-	-	-
	800	-	-	-	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-	-	-	-
Nickel (Ni(II))	100	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+
	400	+	+	+	+	-	+	+	+	+	+
	600	-	-	-	-	-	-	-	-	+	+
	800	-	-	-	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-	-	-	-
Copper (Cu(II))	100	+	+	+	+	+	+	+	+	+	+
	200	-	+	+	+	+	+	-	+	+	-
	400	-	-	+	-	-	-	-	-	-	-
	600	-	-	-	-	-	-	-	-	-	-
	800	-	-	-	-	-	-	-	-	-	-
	1000	-	-	-	-	-	-	-	-	-	-
Zinc (Zn(II))	100	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+
	400	-	-	+	+	-	-	-	+	-	-
	600	-	-	+	-	-	-	-	-	-	-
	800	-	-	+	-	-	-	-	-	-	-
	1000	-	-	+	-	-	-	-	-	-	-
	1500	-	-	-	-	-	-	-	-	-	-
Lead (Pb(II))	100	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+
	400	+	+	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+	+	+
	800	+	+	+	+	+	+	+	+	+	+
	1000	+	+	+	+	+	+	+	+	+	+
	1500	-	-	+	+	-	-	+	+	+	-
	2000	-	-	+	+	-	-	-	+	-	-
	2500	-	-	-	-	-	-	-	-	-	-
Manganese (Mn(II))	100	+	+	+	+	+	+	+	+	+	+
	200	+	+	+	+	+	+	+	+	+	+
	400	+	+	+	+	+	+	+	+	+	-
	600	+	+	+	+	+	+	+	+	+	-
	800	+	+	+	+	+	+	-	+	-	-
	1000	+	-	+	+	+	+	-	+	-	-
	1500	-	-	+	+	+	-	-	-	-	-
	2000	-	-	+	+	+	-	-	-	-	-
	2500	-	-	+	+	+	-	-	-	-	-
	3000	-	-	+	+	+	-	-	-	-	-
	3500	-	-	-	-	-	-	-	-	-	-

Table 4. Identification of Cr(VI)-resistant bacteria by 16S rRNA gene sequence analysis.

Isolates	Organism	Accession no.	Identity (%)
X1	*Bacillus subtilis strain GA CAS11*	MK571421	99%
X2	*Kocuria rosea strain RR75*	MK571422	99%
X3	*Staphylococcus lentus strain FC3104*	MK571435	99%
X4	*Bacillus subtilis subsp. spizizenii strain MV20*	MK571447	99%

3.4. Hexavalent chromium removal

Figure 1 presents the results obtained for Cr(VI) removal and growth of the bacterial consortium in tannery effluent. After five days of contact of free bacteria in effluent under stirring, the abatement rate of Cr(VI) is 76%, 25%, 12.5% and 3.34%, respectively for the Cr(VI) concentrations 50 mg/L, 100 mg/L, 200 mg/L and 300 mg/L in the effluent (Figure 1(A)). For concentrations of Cr(VI) in the effluent of 50 mg/L and 100 mg/L, reduction of Cr(VI) was found to be growth associated of bacterial cells forming the consortium (Figure 1 (B)). Cr(VI) removal efficacy by the bacterial consortium can be improved by increasing the amount of inoculum. The rate of reduction of Cr(VI) by the bacterial consortium decreases with increasing of its concentration in the effluent. Also, the increase of Cr(VI) concentration in the effluent, adversely affects the growth of the bacterial consortium this strongly reduces their capacity to reduce Cr(VI).

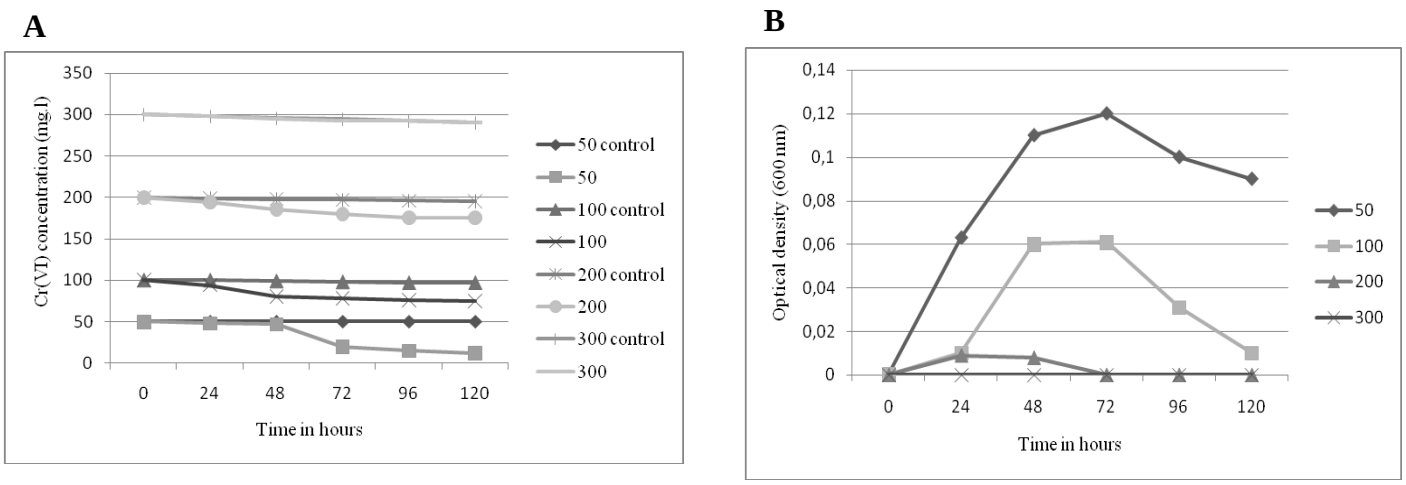

Figure 1: Cr(VI) removal (**A**) and growth of the bacterial consortium (**B**) in the effluent enriched with Cr(VI) (50 mg/L - 300 mg/L).

We have seen that the effluent enriched with 50 mg/L of Cr(VI) turned to a colorless white after 72 hours of incubation in the presence of our bacterial consortium. Also, there is formation of a white precipitate which has occurred in effluent enriched with 200 mg/L and 300 mg/L of Cr(VI). Chromate tolerance mechanisms in bacteria have been reported to include reduction, methylation, precipitation at the cell surface, blocking cellular uptake by altering the uptake pathway and removal from the cytoplasm by efflux pumps [28]. The Cr(VI) reduction may be due to chromate reductase activity [29]. In the strain *Bacillus subtilis strain BYCr-1* the reduction of Cr(VI) is due to the

nfrA gene [30]. In the present study the mechanism of chromium tolerance was not investigated. However, our results provide a basis for assessing the potential of using Cr(VI)-reducing novel bacteria for bioremediation application. So, our four bacteria can be good candidates for removal of Cr(VI) in industrial effluents. Application of chromium resistant bacteria for detoxification of Cr(VI) has been considered as an economical, effective and safe procedure over conventional physical and chemical methods [31].

4. Conclusion

The soils exposed to heavy metals leading to the establishment of a tolerant microbial population. The exploitation of these extreme biotopes to isolate new bacteria resistant to heavy metals and used them for removal hexavalent chromium of tannery effluents is important in protection and preservation of environment. The benefits of biological techniques over physico-chemical techniques can be a clean and efficient alternative for the removal of chromium.

This study establishes the role of *Bacillus subtilis*, *Kocuria rosea*, *Staphylococcus lentus subsp*. and *Bacillus subtilis subsp. spizizenii*, isolated from the soil of abandoned lead mines in the Midelt-Morocco, in consortium for hexavalent chromium removal in tannery effluents.

This is the first study, which shows the role of *Staphylococcus lentus* in the reduction of hexavalent chromium. This study is the basis for implementation of advanced technologies like bioreactor technology for rapid and effective removal of chromium from polluted water.

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