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Biosorption of Hexavalent Chromium by Bacteria Isolated from Salt Rich Tannery Wastes

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IN THIS STUDY, two isolates capable of biosorption of Cr(VI) were isolated from chromium rich effluent treatment plant located at Magra Al-Oyoon, Old Cairo, Egypt. The abundance of bacteria residing in tannery waste water with high metal content was studied. Seventy-two bacterial isolates were recovered by direct plating; two of them were resistant to high chromium concentration (up to 30 mmol chromate). According to the morphological, biochemical and the 16S rRNA sequence analysis they were identified as *Bacillus tequilensis* and *Planococcus citreus* .

The atomic adsorption spectroscopy showed that reduction of chromate content in the cell free supernatant was 59.8% and 45.8% for *Bacillus tequilensis* and *Planococcus citreus*, respectively, after 24 hr of incubation at 30°C. Moreover, both scanning electron micrographs and EDX analysis showed accumulation of chromate on both isolates. *Bacillus tequilensis* and *Planococcus citreus* had high potential for hexavalent chromium removal and can be used for detoxification of waste containing hexavalent chromium along with other heavy metals in media with high salt concentration.

Keywords: Chromium, Heavy metals, Biosorption , SEM-EDX.

Chromium is widely used in a variety of industrial processes such as leather tanning, electroplating, dye and pigment manufacturing. It is mainly used as chromate and dichromate (Baldi *et al.*, 1990). Chromium is a transition element with electronic configuration of (Ar $3d^5 4s^1$). Although chromium exists in multiple valence states, hexavalant Cr(VI) and trivalent Cr(III) chromium are ecologically important because they are the most stable oxidation states in the natural environments (Megharaj *et al.*, 2003). Industrial effluents containing chromium compounds are released directly or indirectly into the soil-water systems resulting in the contamination of the environments (Thacker *et al.*, 2006). Cr (VI) is toxic to most organisms (Ganguli and Tripathi, 2002), it is listed as priority metal pollutant and is classified as a class A carcinogen by the US Environmental Protection Agency USEPA (Costa and Klein, 2006). Cr (III) is less mobile, less toxic and is mainly bound to organic matter in soil and aquatic environments (Becquer *et al*., 2003).

Due to the ubiquity, toxicity and occupational exposure of workers to chromium there is an interest in innovative, low cost methods for the remediation

of Cr(VI) from contaminated environment (De Flora 2000). Reduction of Cr(VI) to chromium (III) may alter the toxicity and environment mobility of the metal. The chemical methods currently used to treat chromium involve the addition of reductants, and subsequent pH adjustment to neutral ranges to precipitate the less soluble Cr (III). These processes requires large amount of chemicals and results in a metal-containing chemical sludge that further imposes threat to the environment (Shakoori *et al.,* 2000).

Biotechnological applications to remove chromate offer an advantage over the chemical procedure (Aravindhan *et al.,* 2007). The major processes being investigated are the adsorption onto biological materials and dissimilarly reduction of metal ions from higher to lower valance state through enzymatic reaction (Lovely and Philips 1994).

Biotransformation of Cr (VI) to less toxic Cr (III) by chromate resistant and reducing bacteria has offered an ecological and economical option for chromate detoxification and bioremediation (Lira-Silva *et al*., 2011). Chromate resistance has been demonstrated by various bacterial species including *Bacillus sp*. (Camargo *et al*., 2004 and Liu *et al*., 2006), *Pseudomonas sp*. (Ganguli and Tripathi 2002 and Park *et al.,* 2000), *Desulfovibrio sp*. (Mabbett and Macaskie 2001), *Mycobacterium sp*. (Pattanapipitpasal *et al*., 2001), *Shewanells sp.* (Myers *et al.,* 2000 and Vaimajala *et al.,* 2002), *Arthrobacter* sp. (Megharaj *et al*., 2003 and Asatiani *et al.,* 2004).

Chromium discharge in the area of study is due to the practice of leather tanning where animal skins are treated with high salt and chromate concentrations to make them more durable and less susceptible to decomposition. The present study aims to isolate endogenous bacteria that are tolerant to high concentrations of salt and chromium to investigate their potential application in the bioremediation technology.

Material and Methods

Sampling and bacterial isolation

Ten samples were collected from a tannery plant in Magra Al-Oyoon area in Cairo, Egypt, They were divided in two-five sample groups; group A obtained from waste water after washing of leather with $K_2Cr_2O_7$ pooled together, and group B from the skin shaves pooled. Both groups of samples were analyzed by the atomic absorption spectrophotometer to determine the total content of Cr, Cd, Zn, Pb, Cu, Fe. All samples were serially diluted and plated on Luria–Bertani (LB) agar. A control sample from sterile field soil was run aside. The plates were incubated for 24 hr at 30℃, and bacterial count was recorded.

Selection of most tolerant isolates

LB agar medium supplemented with K2Cr2O7 (as Cr/VI) to a final concentrations ranging from 5 mmol to 15 mmol were prepared. The Cr (VI)

stock solutions were filter- sterilized with 0.22µm membrane filter papers (Millipore Corp., Bedford, MA). Groups A and B were serially diluted and inoculated on surface of plates containing LB as control plates and LB were amended with $K_2Cr_2O_7$. The plates were incubated at 30℃ in the dark and examined after 24 hr. Bacterial colonies were purified on LB agar containing 5 mmol Cr(VI) and incubated for 24 hr at 30℃. Bacterial isolates with distinctive morphology were recovered from 15 mmol Cr amended plates. These isolates were grown on LB broth containing Cr(IV) in concentrations ranging from 5 to 30 mmol.

Determination of Chromium uptake

Each bacterial culture was grown in an a flask containing 200 ml LB medium supplemented with 15 mmol $K_2Cr_2O_7$ before incubation (in triplicates) at 30℃ in a shaking incubator at 120 rpm. The control sample was prepared aside without the addition of $K_2Cr_2O_7$. Single cultures were removed after 6, 12, 18, 24 and 30 hr of incbation, centrifuged and the cell free supernatants were analyzed for determination of total chromium using atomic absorption spectrophotometer at Ain Shams University.

Identification of bacterial isolates

Morphological examination, biochemical tests, and 16S RNA sequence analysis were carried out to identify the chromium tolerant isolates. Universal bacterial primers corresponding to *E. coli* positions 27F and 519R were used for polymerase chain reaction (PCR) amplification of the 16S rRNA gene (Edwards *et al.,* 1989). PCR Dream Taq master mix (Fermentas) was used according the manufacturer's instructions. The PCR product was analyzed on 1% agarose, purified with Quiagen gel extraction kit and sequenced using ABI sequencer. The nucleotide sequences were submitted in ncbi.nlm.nih.gov database.

Scanning Electron Microscopy-Electron Dispersion X-ray (EM-EDX) analysis

The bacterial isolates were cultured on agar plates amended with 5 mmol $K_2Cr_2O_7$ while plates without amendment were used as controls. A block from the agar plate was cut and fixed in 2% glutaraldehyde vapor at room temperature for 3 hours. Then dehydrated through a series of ethanol solutions (50, 60, 70, 80 and 95%), 15 min each; twice with 100% ethanol, 30 min/time. Then the ethanol was substituted with the acetone. Samples were dried using CO_2 critical point drier (Tousimis Audosamdri-815) and then sputters were coated with gold using a Gold Sputter (SPI-Module). Finally, samples were observed by scanning electron microscope (JSM -5500 LV) coupled with an analyzer EDX ZAF quantification (Gandhia *et al*., 2010). The size of the cells were measured by calculating the average of of twenty sizes (n=20). For confirming the biosorption of chromium on the bacterial cell surface, biosorption was detected in EDX at 20 kV where n= 20.

Results

When concentrations of heavy metals (Cr, Cd, Zn, Pb, Cu, Fe) in the waste water and skin shaves were measured, results showed that, in general, they were five times higher in the waste water samples compared to those in the skin shaves, except for chromium which was about 175 times higher than that in skin shaves (Table 1).

TABLE 1. Concentrations of heavy metals measured by atomic absorption spectrophotometer in waste water and skin shaves samples.

Group	Heavy metal (mg/l)					
	Cr	Cd	Zn	Pb	Cu	Fe
A	1008.0	0.071	1.072	0.25	0.487	0.515
B	5.73	0.035	0.679	0.16	0.138	0.139

(A): Waste water group (B): Skin shaves group

Isolation and purification of chromate tolerant bacteria

Total bacterial numbers (as CFU/g) of raw tannery effluents for samples of both groups A and B were recorded; the CFU ranged between $7.2x10^4$ and $2.4\ x10^5$, respectively. The CFU from tannery effluent was lower than that found in the sterile soil ($7.8\ x10^7$) Cultivation on chromium amended plates containing (5, 10 and15) mmol resulted in reducing the number of colonies as the chromium concentration increases. Four percentages of the CFU(of group A and 1% of group B samples were resistant to 15 mmol of chromium (Fig.1).

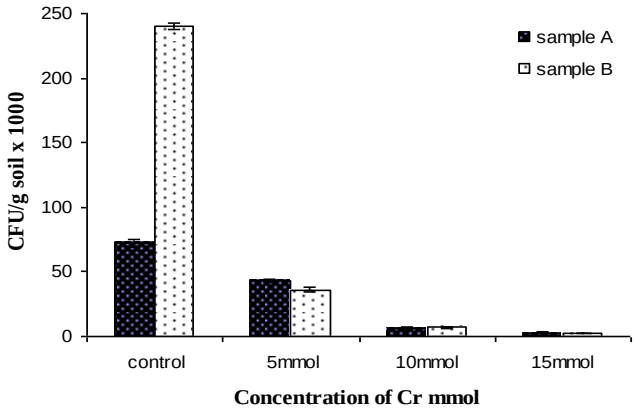

Fig. 1. Total colony forming units (CFU/ g) of samples A and B cultivated on LB medium amended with Cr IV concentrations ranging from 5 to 15 mmol.

Only five morphotypes (CrB1, CrB4, CrB7, CrB10 and Cr05) were able to grow on plates amended with 15 mmol chromium. They were selected and grown on LB broth medium containing Cr averaging a range from 5 - 30 mmol (Fig. 2). The two most resistant isolates, designated Cr B7 and Cr O5, were selected for further studies.

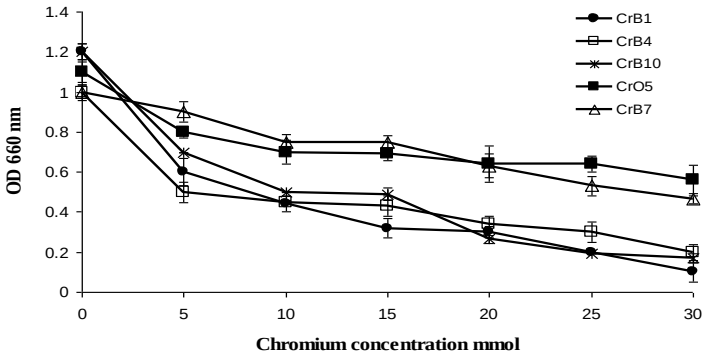

Fig. 2. Effect of increasing Cr concentration on growth of the five chromium tolerant isolates.

Chromium uptake by the most tolerant isolates

The selected isolates CrB7 and CrO5 were grown on LB broth containing an initial concentration of 15 mmol (4412 mg/l) $K_2Cr_2O_7$ before incubation for 30 hr. The uptakes of chromate were measured at time intervals. Results declared that the maximum uptake was observed after 24hr of incubation; the amount of chromium found in culturw supernatant was reduced by 59.8% and 45.8% using Cr B7 and Cr O5 respectively.

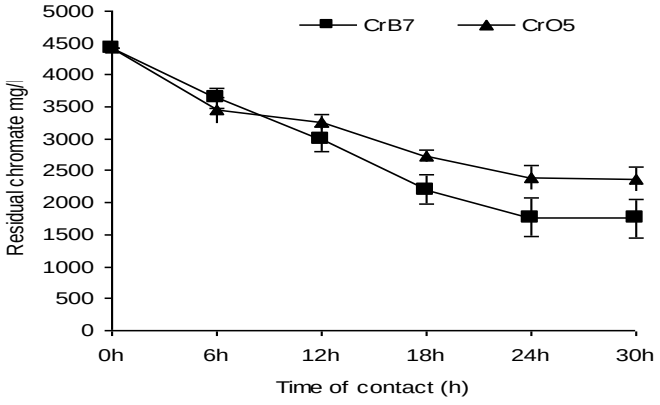

Fig. 3. Chromium uptake by tolerant isolates CrB7 and CrO5 of what?

Phenotypic and genotypic characteristics of the isolates

The bacterial colonies of CrB7 were circular, smooth and creamy. The strain was Gram positive, rod-shaped with (1.4x 0.63mm). Spores are located centrally. While the bacterial colonies of CrO5 were smooth, glistening, circular and orange in color. The isolate was Gram positive non motile none sporulating with (0.640 mm) in diameter. Physiological characteristics for both strains are presented in (Table 2). Interestingly, both isolates were found to tolerate 20% NaCl as NaCl is used in leather treatment process. Partial sequence of 16S rRNA gene of CrB7 indicated that its blast identity was 99% similar to *Bacillus tequilineis*. The sequence was given accession number (KC631633) and the phylogenetic tree indicated that it grouped with *B. tequilineis* with boot strap value 58% (Fig4). Strain CrO5 was given accession number (KC631632) and the sequence was 99% identical to *Planococcus citreus.* The Neighbor joining phylogenetic tree revealed its grouping with *P. citreus* with 76% bootstrap value (Fig. 4).

TABLE 2. Physiological characteristics of isolates CrB7 and CrO5.

Isolate	oxidase	Catalase	NO_3 reduction	H_2S	Tolerance to 20% NaCl	citrate	Starch hydrolysis
CrB7	+	+	+	-	+	+	-
CrO5	_	+	+	-	+	-	-

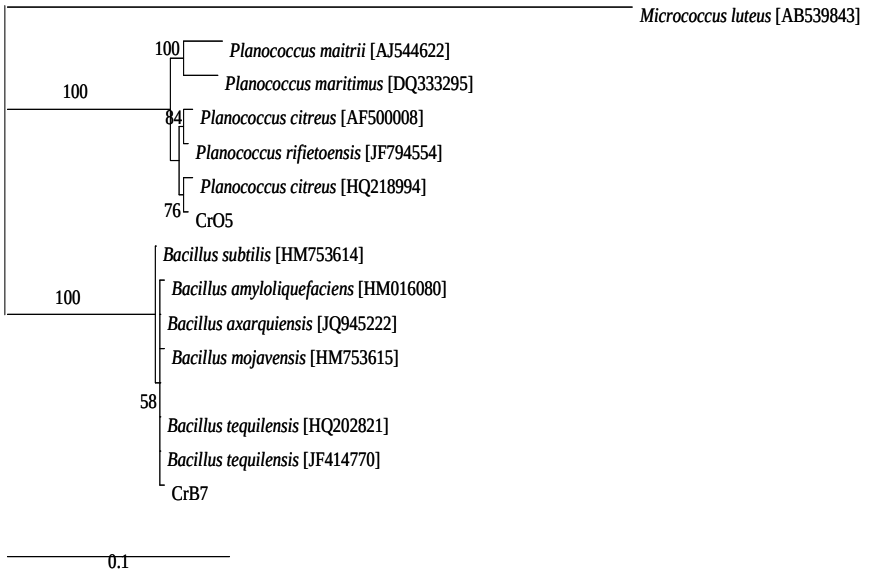

Fig. 4. Neighbor joining phylogenetic tree of partial 16S rRNA sequence of chromium resistant isolates CrB7 and CrO5. The scale bar represents 10% nucleotide substitutions. Percentages of bootstrap values recovered from 100 trees are presented on the nodes.

SEM-EDX findings

SEM photographs of CrB7 and CrO5 isolates taken before and after Cr (VI) exposure are presented in (Fig. 5). SEM results revealed that the cells in chromium free medium appeared to have smooth surfaces in a loosely-bound form. When chromium was added to the growth medium, insoluble particles were deposited in the form of amorphous substances which aggregated all over cell surface. Analysis of the energy dispersion X-ray showed that these particles were rich in chromium (Fig. 6).

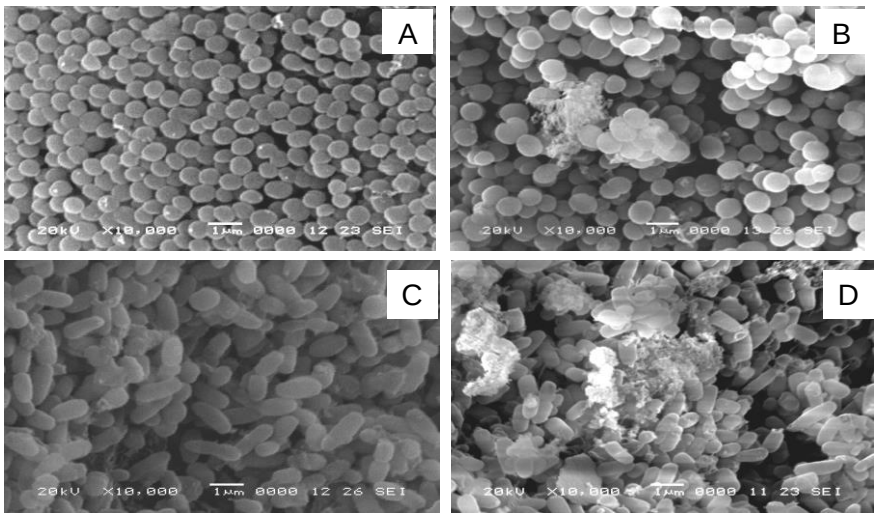

Fig. 5. SEM photograph; (A) CrO5 control, (B) CrO5 treated with CrVI, (C) CrB7 control and (D) CrB7 treated with Cr(VI).

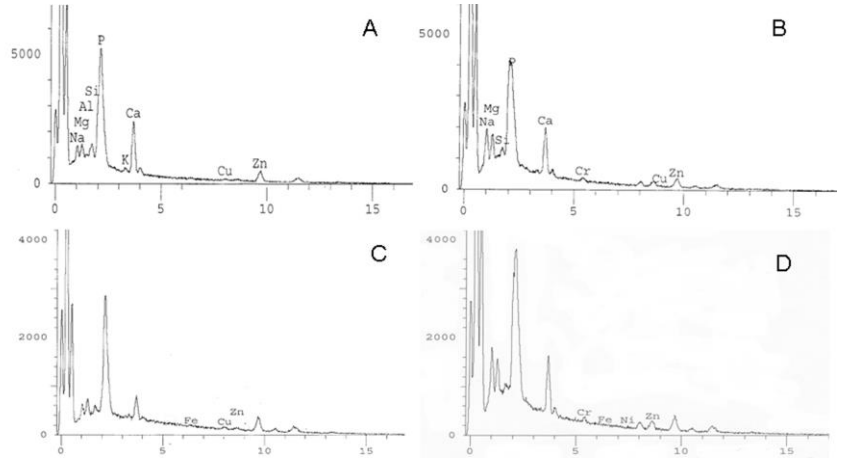

Fig. 6. EDX spectra of CrB7 and CrO5 isolates, (A) control CrB7, (B) treated CrB7, (C) control CrO5 (D) treated CrO5.

Discussion

Continuous discharge of chromium containing wastes used in tannery industries in the area of Magraa Al- Oyoun, Cairo, Egypt resulted in prolonging the exposure of to high concentration of chromium. Concentration of chromium in the collected samples was 5.7 and 1008 mg/l in groups A, B, respectively. According to Saboor and Aly (2000), soils of industrial areas in Egypt show significant accumulation of metals and several orders of magnitude increase in the concentration above the permissible levels. They recorded that the permissible level of chromium ranges between (75-100) ppm. Rich chromium soil provided an enrichment environment for the selection of potent chromium resistant bacteria. Moreover, high concentration of chromium represents a stress factor that reduces microbial diversity as a result of selective pressure. Few resistant bacteria were dominated in such contaminated areas. In this study, comparison between the sterile soil sample and the chromate contaminated samples revealed lower diversity in chromate contaminated samples which led to domination of only five distinctive morphotypes. Microscopic examination indicated that shape of 4 isolates (B1, B5 B7 and B10) were bacilli and only one isolate (Cro5) was coccid. Similar results were reported by Kamala-Kannan *et al.,* (2007) and Rehman *et al*., (2008) when they found that *Bacillus* spp. were dominant in the chromium contaminated sites.

The two isolates selected in this study were found to tolerate high concentrations of what? reaching 30 mmol when grown in the LB medium. Mergeary (1995) reported that this medium could complexes itself with the chromate and / or reduces it; this could indicate that the isolated bacteria could be resistant to even higher concentrations of chromate in its natural environment.

Resistance and tolerance to chromate is common among Gram positive and Gram negative bacteria (Mishra *et al.,* 2012; Minyan *et al.,* 2010; Pei *et al.,* 2009; Desai *et al.,* 2008).

To the best of our knowledge, it is the first time to isolate halotolerant *Bacillus tequielnsis* and *Planococcus citreus* isolates that are simultaneously resistant to Cr VI (30mmol) and other heavy metals. Verma *et al.* (2009) reported that chromate resistant bacilli were found to be tolerant to multiple metals found in the developing biological treatment of waste water containing chromium and several other metals.

Results of scanning electron micrographs indicated accumulation of chromium on the chromium treated cells of *Bacillus tequielnsis* and *Planococcus citreus.* In a similar study, Cr(IV) reduction outside microbial cells generated the insoluble Cr(III) ions that unable to cross the cellular membranes (Cervantes *et al.,* 2001). Ehrlish (2002) suggested a possible mechanisms of chromate resistance by *Bacillus tequielnsis* and *Planococcus citreus* depending on their ability to reduce chromium (VI) to (III) which precipitates as hydroxides [$Cr(OH)_3$] or ($Cr_2O.H_2O$). Furthermore, exposure to the chromate resulted in the enlargement of bacterial

cells. Almost similar observation was recorded by Ackerley *et al.,* (2006) and Yang *et al.,* (2007). The later found that cells of *Enterobacter cloacae* cell were elongated after 24hr incubation in 400 mg/l chromate. From the EDX analysis, chromium peaks were observed on cells grown in presence of chromium. Similar results were demonstrated also by Pei *et al.,* (2009).

The chromate concentration used in this study (15 mM) (4400 mg/l) was relatively higher than that previously studied. Previous study mentioned that the rate of Cr(VI) reduction is dependent on the initial concentration of chromium used a well as on the total bacterial load. According to Ma *et al.,* (2007) and Opperman *et al.,* (2008), the time required to reduce chromate varies from 2-9 days among different bacterial species tested. It took the isolates selected in this study 24 hr to reduce the initial concentration of 15 mmol by 59.8 and 45.8% for *Bacillus tequielnsis* and *Planococcus citreus*, respectively, Therefore, both isolates provided potential tools for removal of toxic elements from water and characterized high salt and multi-metal loads.

Further investigation is required to elucidate the mechanism of chromate reduction by *Bacillus tequielnsis* and *Planococcus citreus*.

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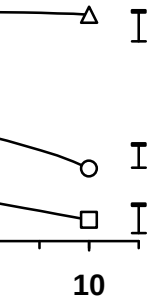

الإدمصاص الحيوي للكروم سداسي التكافؤ باستخدام بكتريا معزوله من مخلفات الدباغه الغنيه بالملح

ايناس حامد الشطورى و سحر طلبه محمد
قسم الميكروبيولوجى – كلية العلوم – جامعة عين شمس – القاهرة – مصر .

تم فى هذه الدراسة عزل سلالتين من البكتريا قادرة على إدمصاص الكروم سداسى التكافؤ من موقع معالجة جلود غنى بالكروم بمنطقة مجرى العيون بالقاهرة القديمة - مصر. تم دراسة المحتوى البكتيرى الموجود حيث امكن عزل سبعون عزلة منها و كـان من بينها عزلتان قادرتـان على تحمل تركيز مرتفع من الكـروم وصل الى ٣٠ مل مول. ومن خلال دراسة الشكل المظهرى و الخصائص البيوكيميائية و استخدام تقنية التتابع الجينى لجين 16S rRNA تم تشخيص العزلتين بأنهما باسيلس تيكويلينسس و بلانوكوكاس سترس. و باستخدام التحليل الطيفى للعناصر, وجد ان الكائنات محل الدراسة لها القدرة على خفض تركيز الكروم فى الوسط الغذائى خلال ٢٤ ساعة عند درجة حرارة ٣٠ م° بنسبة ٥٩,٨ و ٤٥,٨ على التوالى. بالإضافه الى ذلك فإن صور المسح بواسطة المجهر الإلكترونى و كذلك التحليل الطيفى للعناصر أظهرت تراكم الكروم على سطح كلا العزلتين .

و بذلك فان الكائنين محل الدراسة لديهما الكفاءة على ازالة الكروم فى وسط يحتوى على العديد من المعادن الثقيلة و فى وجود تركيز مرتفع من الاملاح.