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# Bioremediation of Hexavalent Chromium Widely Discharged in Leather Tanning Effluents

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THE hexavalent chromium salts are widely used in many industries worldwide including leather tanning industry. The residues of these salts are discharged into the environment causing serious health hazardous to human, animals and plants. The chemical remediation of the Cr VI residues is costly and adds more pollutants to the environment. Therefore, the bioremediation of toxic hexavalent chromium residues is the aim of this study. For this purpose, the soil and wastewater samples from the heavily contaminated sites near tanneries were used for the isolation of Cr VI resistant bacteria. A total of 33 bacterial isolates was obtained from samples grown on LB medium amended with 50 $mgL^{-1}$ potassium di chromate (Cr VI). These isolates were screened for their growth in the medium amended with Cr VI concentrations ranging between 100 and 200 $mgL^{-1}$. Seven isolates showed tolerance to the highest concentration. These isolates were subjected to analysis of 16S rDNA genes followed by RFLP of the PCR product. The most promising isolate (No.3) that withstood the highest Cr VI concentration was further subjected to 16S rDNA gene nucleotide sequence. This isolate turned to be *Microbacterim spp.* with 98% similarity to the standard strain in the gene bank. The sequence was deposited in NCBI data bank under accession number mk878392. The efficiency of this indigenous strain of bacteria in removal of Cr VI from aquas solution showed that it was capable to remove 30% of Cr VI within first 20 hours then exponential increase took place after additional 20 hours. The total removal of Cr VI reached 97.2% after 96 hours of incubation. The immobilization of the strain on either alginate or chitosan accelerated the removal of Cr VI that reached 90% removal in 18 hours. This strain seems very promising as potential bioremediation agent for hexavalent chromium residues.

**Keywords:** Hexavalent chromium, *Microbacterium* spp., Bioremediation, Immobilization.

## Introduction

The discharge of the toxic wastes into or near the agricultural lands is reported to have adverse on affects the native beneficial microbes contributing to soil fertility [1–3]. The bioremediation of such wastes is essential to protect the soil biological activity. The bioremediation of toxic azo dye residues using fungal strain was affected to remove the toxic wastes from wastewater dischargers [4,5].

Chromium is one of the toxic heavy metals discharged into the environment through anthropogenic activities which contaminate soils and sediments as well as ground, and surface waters. This heavy metal is extremely toxic to biological and ecological systems [6]. Chromium (Cr) is a transition metal contaminant that exists in nature primarily in two forms; one is the soluble highly toxic Cr (VI) anion and the other is less soluble, less toxic Cr (III) [7,8]. Chromium (Cr) exists in several oxidation states,

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but the most stable ones are trivalent Cr (III) and hexavalent Cr (VI) states, which have different chemical properties and biological impacts on the environment [9-11]. Chromium metals released into the environment due to discharge of various effluents generated by large numbers of industries such as electroplating, animal skin tanning, paints, pigment production, pulp processing, wood preservation, metal corrosion inhibition and steel manufacture. All of these industries lead to discharge of huge amounts of wastes containing chromium into the environment [12,13]. The permissible concentration limit for hexavalent chromium Cr (VI) in drinking water is 0.05 mg $l^{-1}$ according to [14,15]. The United States Environmental Protection Agency (EPA) has formulated the maximum permissible levels of Cr (VI) into water bodies at 50 $\mu gdm^{-3}$ and in drinking water as 3 μgdm-3 and that of Cr (III) as 100 $\mu gdm^{-3}$ [16].

Tanneries as the major sources of chromium pollution are reported to release Cr (VI) in the range of 40 – 25,000 $mgL^{-1}$ in the industrial effluent [17,18].

The overexposure to Cr (VI) induces liver and kidney damage as well as skin lesions or rashes [19]. In addition the contamination by high doses of Cr VI causes as renal tubular necrosis and increases risks of respiratory-tract cancer as well as cytotoxic and genotoxic effects [20,21]. Chromium toxicity in plants results in alterations in the seed germination process, reduced growth of roots, stems and leaves which negatively affects the crops yields [22-26].

Several methods are used to detoxify Cr (VI) from the industrial effluents containing high concentration of chromium residues. Among those, reverse osmosis, precipitation, chemical reduction followed by precipitation, ion exchange and absorption on coal, activated carbon, alum or kaolinite, are employed [27.28]. The major disadvantages of these techniques apart from being economically expensive is the incomplete metal removal, high reagent and energy requirements, and generation of toxic sludge or other waste products which require disposal [29-31].

The bioremediation of chromium residues attracted the interest of scientists as a reliable approach that does not harm the environment [32,33]. Bioremediation technology is employed to transform toxic heavy metals such as chromium (VI) into a less harmful state using microbes [34-37]. The mechanism of microbial bioremediation involves removal and/ or recovery of toxic metals, by bio-accumulation, bio-sorption and / or enzymatic reduction. Bioremediation using soil bacteria is regarded as the most suitable technique for treatment of toxic metals including hexavalent chromium [38-41].

The aim of this study is to identify bacterial strains capable to remediate tannery industrial effluents containing toxic chromium (VI) residues, and enhancing Cr (VI) reduction capacity by these bacteria when immobilized on two immobilized media to remove Cr (VI) more efficiently.

## Materials and Methods

*Isolation of chromium (VI)-resistant bacteria*

Samples were collected from the tannery industrial areas at Magra EL-eyon, South Cairo, and Borg Al-Arab tanneries at Alexandria. Egypt. Samples were collected from soils, adjacent to tanneries where massive drain pipes are pouring the industrial effluents into dumping sites. Water samples were taken from the main drain containing mixture of both house hold sewage and tanneries effluents. Samples were collected in plastic bags and transported to the laboratory on ice, then cultivated on Luria–Bertani (LB) medium for the isolation. Samples were serially diluted and plated on LB agar medium containing ($gl^{-1}$); 10 tryptone, 5 yeast extract, 10 NaCl, 0.1 glucose, adjusted to pH 7. The liquid medium was supplemented with Cr (VI) as $K_2Cr_2O_7$ to reach the final concentration of 50 mg (VI) per liter using sterile filtered potassium di-chromate stock solutions. Plates were incubated at 30°C in dark and the growth was measured as OD at wavelength 600 nm after 2 days. Representative bacterial colonies were isolated and preserved on LB medium.

Tolerance of bacterial isolates to the three concentrations of hexavalent chromium

All isolates were grown on LB media containing 100, 150, 200 $mgL^{-1}$ of Cr (VI) and the growth was measured as OD at 600 nm after 3 days.

*Molecular identification of chromium removing bacteria*

Amplification of 16S rDNA gene using universal eubacterial PCR primers was carried out as previously described by Abd-El-Haleem et al. [42]. Genomic DNAs and PCRs were performed using EZ-10Spin Column DNA purification kit

according to the manufacturer's instructions (BIO BASIC INC). Sequencing was performed using ABI PRISM dye terminator cycle sequencing kit with Ampli Taq DNA polymerase and an Applied Bio systems 373 DNA sequencer (Perkin-Elmer, Foster City, Calif). The obtained PCR products were sequenced and the aligned sequences were subjected to homology search by using online tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast).

PCR conditions: initial denaturation 94ºC (5 min), 35 cycles of denaturation 94ºC (90 s), annealing at 56ºC (90 s), extension at 72ºC (90 s), and final extension step at 72ºC (7 min).

*PCR-RFLP of amplified 16S rDNA gene*

RFLP of amplified 16S rDNA gene product of four isolates was performed. The PCR amplified products were digested with 2 unit of restriction enzymes HaeIII and HincII (GIBCO B.R.L) according to the recommendations of the manufacturers and electrophoresed in 2% agarose gel in the subsistent of ethidium bromide. Gels were run in 1X TBE buffer and then visualized and photographed in the Multi Image light cabinet (Alpha Innotech Corporation, USA). Molecular weight markers 100 bp (Sigma, St. Louis, MO) was used.

*Enhancement of hexavalent chromium removal using immobilized bacteria*

Hexavalent chromium reduction was determined from the difference between total chromium (VI) and Cr (III) concentrations. Chromium Cr (VI) reduction was determined in the supernatant of growth culture using 1, 5-diphenylcarbazide method according toaccording to APHA [43] and Sarin and Pant [44]. The immobilization of bacterial cells was done according to the methods described by by Pal and Paul [45] and Srinath et al. [46]. The batch adsorption experiments were carried out to determine the reduction of Cr (VI) by the immobilized *Microbacterium spp.* on chitosan and alginate as well as using free cells of this bacterium as a control treatment. The experiment was conducted in 250 ml Erlenmeyer flask containing 50 ml of LB broth (pH 7.0) containing Cr (VI) at concentration of 300 mg $l^{-1}$,and 2 mg cells dry weight. The incubation was done at 30°C on orbital shaker 150 rpm and samples were taken from each flask every after 6, 12, 18 and 24 hours. Bacterial cell densities of the liquid cultures were determined by measuring optical density at 600 nm. The residual hexavalent chromium was determined using 1, 5- diphenylcarbazide [47].

Chitosan (2 w/v %) was dissolved in an aqueous solution of acetic acid (2 w/v %). The solution was added drop wise through a capillary into a gently stirred coagulation liquid (1 N sodium hydroxide and 26 v/v % ethanol). The obtained macrospheres were filtered and washed with distilled water until neutrality [48].

Calcium alginate beads were prepared by emulsion method. The polymer was dissolved in water with stirring at 100 rpm. Bacterial cells were added to polymer solution. The homogenized mixture was extruded into 5% calcium chloride solution with gentle agitation 37°C ± 0.5°C at ambient temperature. The formed beads were allowed to stand for 5 min in the solution, decanted, filtered, and finally dried at ambient temperature [49].

## Results and Discussion

A total of 33 isolates was obtained from the soil and wastewater samples collected from three locations namely: Magra el-Eyon tannery, Borg El-Arab tannery and Elagamy wastewater treatment station, the isolation was done using LB medium amended with 50 mg $L^{-1}$ potassium dichromate Cr (VI). The isolated bacteria were grown on LB media containing three concentrations of Cr (VI) (100, 150 and 200 ppm). Results are presented in Tables (1, 2 and 3). The isolates from Magra El-Eyon location (Table 1) showed resistance to 100 ppm Cr (VI). Isolates 2, 3 and 4 were more resistant than the others grown on LB containing 100 ppm Cr (VI)/L. On 150 ppm hexavalent chromium in the LB medium, the growth of Magra el-Eyon isolates was Affected in comparison with the growth on 100 ppm Cr (VI). Isolates 2, 3 and 6 were resistant to Cr VI concentration of (150 ppm) compared with other isolates. Regarding the growth of the isolates on LB medium amended with 200 ppm Cr VI, the isolate No. 3 observed the highest significant growth compared with other isolates, which indicates that this isolate is tolerant to the highest tested Cr VI at all concentration (100, 150 and 200 ppm).

The isolates from Borg EL- Arab tannery location (12 isolates), showed comparatively higher resistance to Cr VI concentrations in the growth media compared with isolates from Magra el-Eyon tannery location (Table 1). The increase of hexavalent chromium concentration in the growth media was accompanied by decrease in bacterial growth (Table 2). The most resistant bacterial isolates to 200 ppm Cr VI concentration

**TABLE 1. Effect of Cr VI concentrations on the growth of bacterial isolates from Magra EL-eyon tannery locations (growth as measured OD at 600 nm).**

| Isolate No. | 100 ppm | 150 ppm | 200 ppm |
|---|---|---|---|
| **1** | 0.773 b | 0.445 b | 0.147 b |
| **2** | 0.565 abc | 0.578 ab | 0.085 cd |
| **3** | 0.868 a | 0.591 a | 0.380 a |
| **4** | 0.613 ab | 0.371 c | 0.129 bc |
| **5** | 0.466 c | 0.495 b | 0.108 bc |
| **6** | 0.733 b | 0.614 a | 0.099 c |

Duncan's test: Values followed by different letters are significantly different.

**TABLE 2. Effect of Cr VI concentrations on growth of bacterial isolates from Borg Al-Arab tanneries location (growth as measured OD at 600 nm).**

| Isolates | 100 ppm | 150 ppm | 200 ppm |
|---|---|---|---|
| **7** | 0.278 fg | 0.139 gh | 0.068 f |
| **8** | 0.822 a | 0.666 a | 0.189 cd |
| **9** | 0.475 e | 0.410 e | 0.105 e |
| **10** | 0.699 c | 0.436 de | 0.080 f |
| **11** | 0.769 ab | 0.623 ab | 0.342 bc |
| **12** | 0.737 b | 0.651 a | 0.136 d |
| **13** | 0.712 bc | 0.534 bc | 0.387 b |
| **14** | 0.258 g | 0.397 f | 0.277 c |
| **15** | 0.518 de | 0.622 ab | 0.364 b |
| **16** | 0.798 a | 0.675 a | 0.561 a |
| **17** | 0.681 cd | 0.553 b | 0.130 d |
| **18** | 0.415 ef | 0.172 g | 0.062 f |

Duncan's test: Values followed by different letters are significantly different.

**TABLE 3. Effect of Cr VI concentration on the bacterial isolates from El-agamy wastewater treatment station (growth as measured OD at 600 nm).**

| Isolates | 100 ppm | 150 ppm | 200 ppm |
|---|---|---|---|
| **19** | 0.865 b | 0.571 b | 0.327 a |
| **20** | 0.266 de | 0.302 bcd | 0.160 bcde |
| **21** | 0.706 ab | 0.394 abc | 0.114 c |
| **22** | 0.625 bc | 0.571 b | 0.202 b |
| **23** | 0.672 abc | 0.761 a | 0.081def |
| **24** | 0.345 d | 0.083 f | 0.104 c |
| **25** | 0.495 c | 0.295 cde | 0.159 b |
| **26** | 0.253 e | 0.063 g | 0.082 bcd |
| **27** | 0.356 d | 0.307 bcd | 0.380 a |
| **28** | 0.922 a | 0.384 bc | 0.118 cde |
| **29** | 0.111 e | 0.113 de | 0.058 abc |
| **30** | 0.177 ef | 0.101 e | 0.043 e |
| **31** | 0.392 cd | 0.501 ab | 0.101 def |
| **32** | 0.170 ef | 0.494 ab | 0.092 abc |
| **33** | 0.299 de | 0.091 f | 0.056 de |

Duncan's test: Values followed by different letters are significantly different.

were isolate No. 16 followed by isolates nos.15 and 20.

The isolates from wastewater treatment station at Ela gamy (15 isolates) showed growth retardation in LB medium amended with 100,150 and 200 ppm of Cr VI with the growth at 200 ppm being significantly lower (Table 3). One isolate (29) showed constant growth pattern on the three concentrations of Cr VI being 0.356, 0.307 and 0.380 OD at 100, 150 and 200 ppm chromium hexavalent in LB medium respectively. Isolate No. 21 gave significantly higher growth on the highest concentration of Cr VI. Both isolates 21 and 29 showed resistance to the concentrations of hexavalent chromium up to 200 ppm in LB growth medium.

Based on previous assessments, seven isolates were selected, for further studies, as hexavalent resistant bacterial isolates, there Nos, 3, 11, 15, 16, 20, 21 and 29.

The resistance of the selected seven isolates to the high concentration of Cr VI 200 ppm reflects the tolerance of these isolates that may be due to the presence of strong reduction enzymatic system within the bacterial cells. These results are in line with those obtained by Baldiris et al. [50], Joutey et al. [51], and Pradhan et al. [52].

The selected seven bacterial isolates were subjected to analysis of 16S rDNA gene followed by RFLP of the PCR product. The 16S rDNA analysis showed identical bands with molecular weight at 1500 as presented in (Fig. 2). The RFLP of the 16S rDNA PCR product from the seven isolates gave four specific groups as presented in (Fig. 3). The first includes isolate 3, the second includes isolates 11, 15 and 16, whereas the third includes isolate 20 and the fourth includes isolate 29 (Fig. 3). Four isolates were selected; each one represents one of the aforementioned groups. Those four isolates are nos.: 3, 11, 21 and 29. Among these isolates three were tolerant to 200 ppm Cr VI concentration and one isolate (21) was less tolerant to the same concentration.

The correlation between the bacterial growth and Cr VI illustrate the percentage of Cr VI reduction to Cr III by different bacterial isolates is presented in Fig. (1). the growth of bacterial isolates was correlated with their capacities to reduce Cr VI. Isolates 3 and 29 gave the highest growth and the highest Cr VI reduction. However, the other two isolates (11 and 21) gave less growth and less Cr VI reduction. Isolate 3 was selected as the most efficient biomass accumulator and the highest Cr VI reducing isolate. This isolate was identified to the species level by sequencing the 16S rDNA gene. It was identical as *Microbacterium spp*. with 96% similarity to the standard strain in the gene bank. The nucleotide sequence analysis was deposited in NCBI data bank under the accession number mk878392

Chromium (VI) reduction efficiency by *Microbacterium i*solate from Magra el-Eyon tannery.

The chromium (VI) reduction efficiency by *Microbacterium spp*. decreased by increasing the Cr (VI) concentration in the medium and reached to 97.2% at 300 $mgl^{-1}$ Cr.

In this experiment, the concentration of Cr VI in LB medium was elevated to 300 ppm to assess the efficiency of Cr VI reduction by this tolerant bacterial strain. Results in Fig. (4) show that 30% of Cr VI was reduced in the first 20 hours, then exponential increase in Cr VI reduction took place within the next 20 hours, where the reduction reached 85% of the total Cr VI in the growth medium. The reduction of the Cr VI reached 96% after 84 hours and 97.2% at 96 hours incubation period.

Several authors studied the Cr VI remediation by several bacterial strains [53]. This is agreement with the results obtained in this study, where the selected bacteria tolerated the Cr VI at the concentration of 300 ppm and efficiently reduced it to Cr III. The contrary failed to reach complete reduction of Cr VI at the concentration of 20 ppm of Cr (VI). Sikander [54] showed that *Ochrobactrum* grew in the presence of Cr (VI) concentrations up to 1500 $\mu gml^{-1}$. This indicates the capability of this bacterium to reduce the Cr VI.

*Chromium reduction using free and immobilized bacterial cells*

The ability of the chromate-resistant bacterium *Microbacterium spp*. to reduce Cr (VI) at 300 $mgl^{-1}$concentration was performed using free and immobilized cells. Sodium alginate and chitosan were used as immobilization media Fig. (5) Showed that the reduction of hexavalent chromium increased as the time of incubation increased. However, immobilization of *Microbacterium spp*. on either chitosan or

alginate markedly increased the reduction as compared with the free cells. In addition, the incubation for 12 hours resulted in reduction of Cr (VI) by 88, 26 and 88% using immobilized cells on alginate and chitosan respectively whereas, with the free cells maximum reduction was at 18, hour of incubation and did not exceed 49.56%. This clearly shows that immobilization of *Microbacterium spp*. particularly on chitosan was the best for Cr (VI) reduction, where it reached 84% and almost 92% after 12 and 18 hours of incubation respectively.

*Enhancing bioremediation capacity of Microbacterium spp. strain using immobilization technique.*

The strain of *Microbacterium spp.* showed potential for use as bioremediation agent to remove hexavalent chromium. However, this strain requires long time (96 hours) to perform complete removal of Cr VI. A trial was made to speed the removal through immobilization of *Microbacterium spp.* cells on two immobilization media. Results in Figs. (6 and 7) showed that the immobilization on alginate induced 90% removal of hexavalent chromium in 18 hours, when the initial Cr VI concentration in the medium was 300 ppm. Similar results were obtained with using chitosan as immobilization medium Fig. (7). both alginate and chitosan immobilization media without cells reduced the residual Cr VI in the liquid phase of the media by 50% in 18 hours. This may be due to absorption of Cr VI by binding to immobilization media. Similar results were reported by by Bayramoğlu and Arica [55]. These results clearly show that the bacterial cell immobilization supports the bioremediation of Cr VI compared with free cells Fig. (6 and 7).

The reuse of the immobilized *Mycobacterium* spp, showed that using the same immobilized

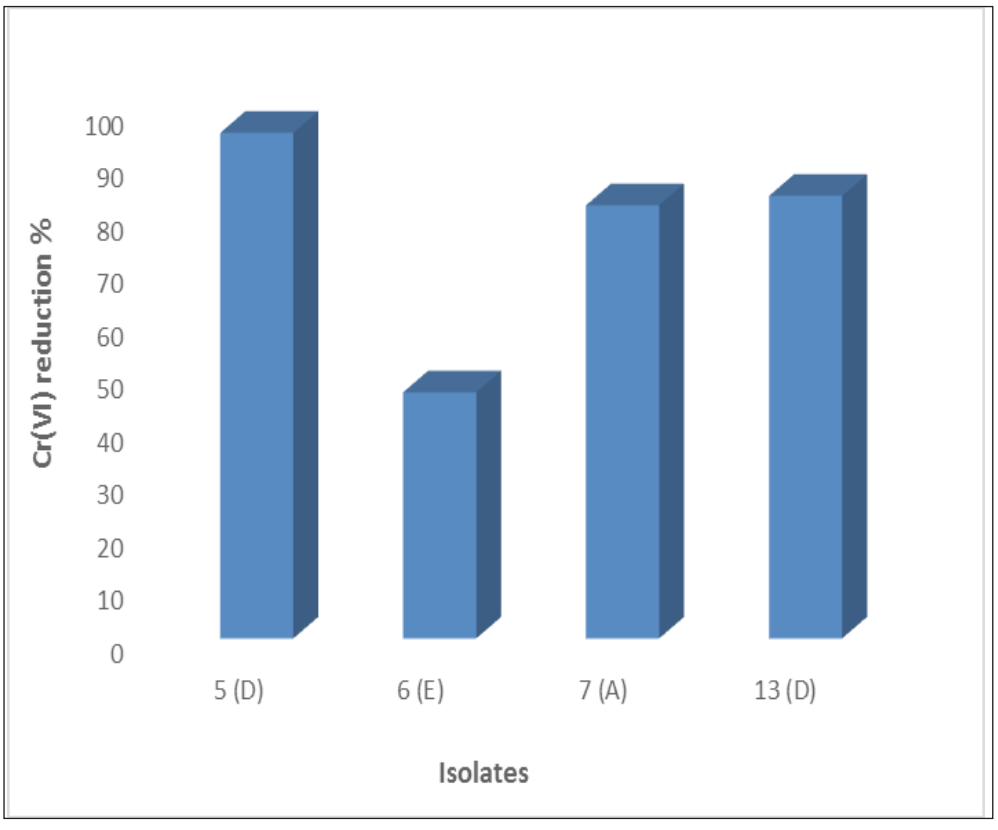


**Fig. 1. Percentage of Cr VI reduction by representative of bacterial isolates in LB amended with 200 ppm Cr VI.**

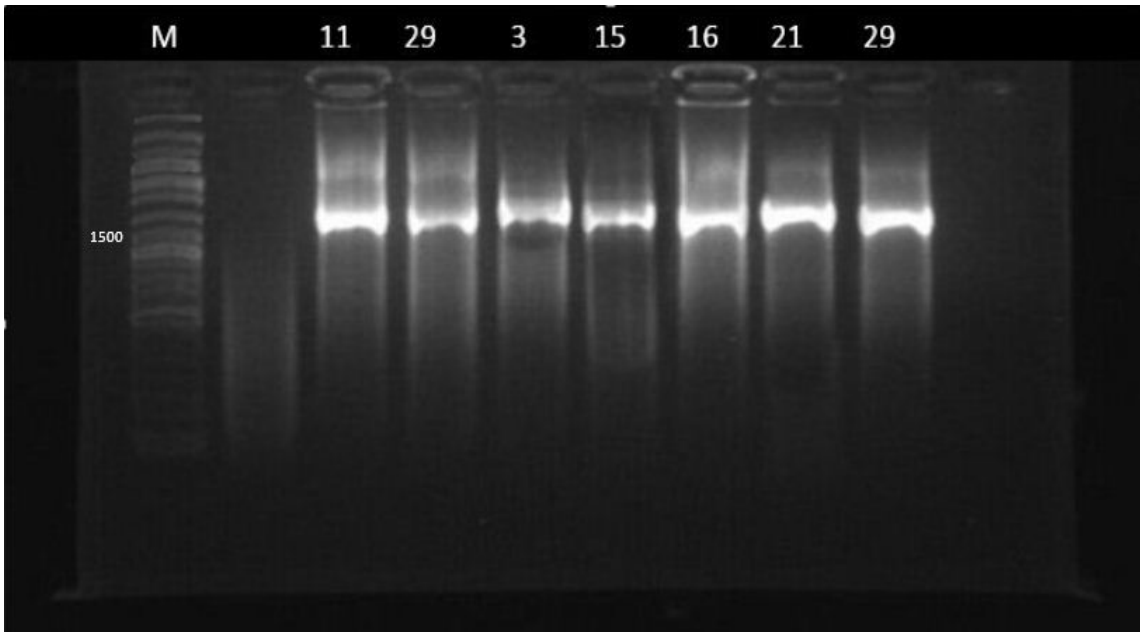


**Fig. 2. Gel electrophoresis of PCR products of 16 rDNA. Lanes 1–7are isolates Lane M 1kb DNA ladder.**

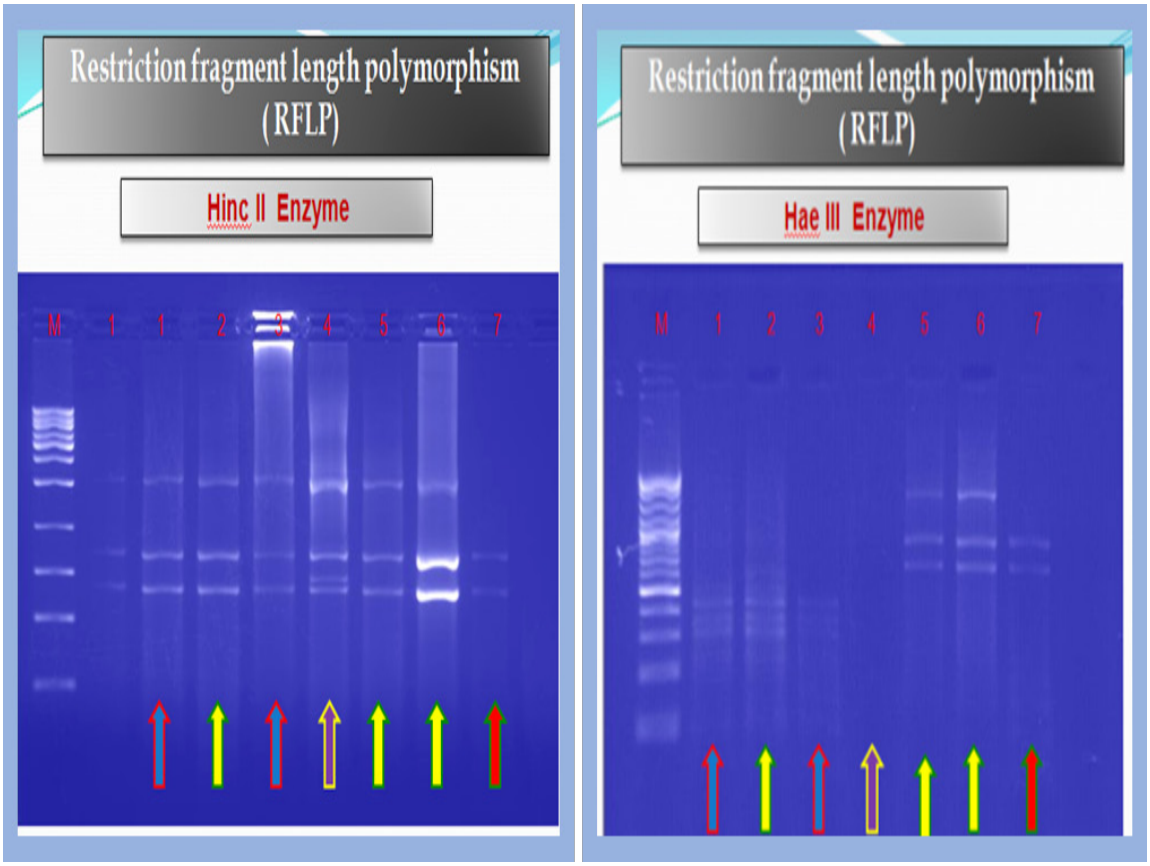


**Fig. 3. RFLP pattern of 16S rDNA products from seven potential isolates treated with HincII (A) and HaeIII (B) restriction enzymes.**

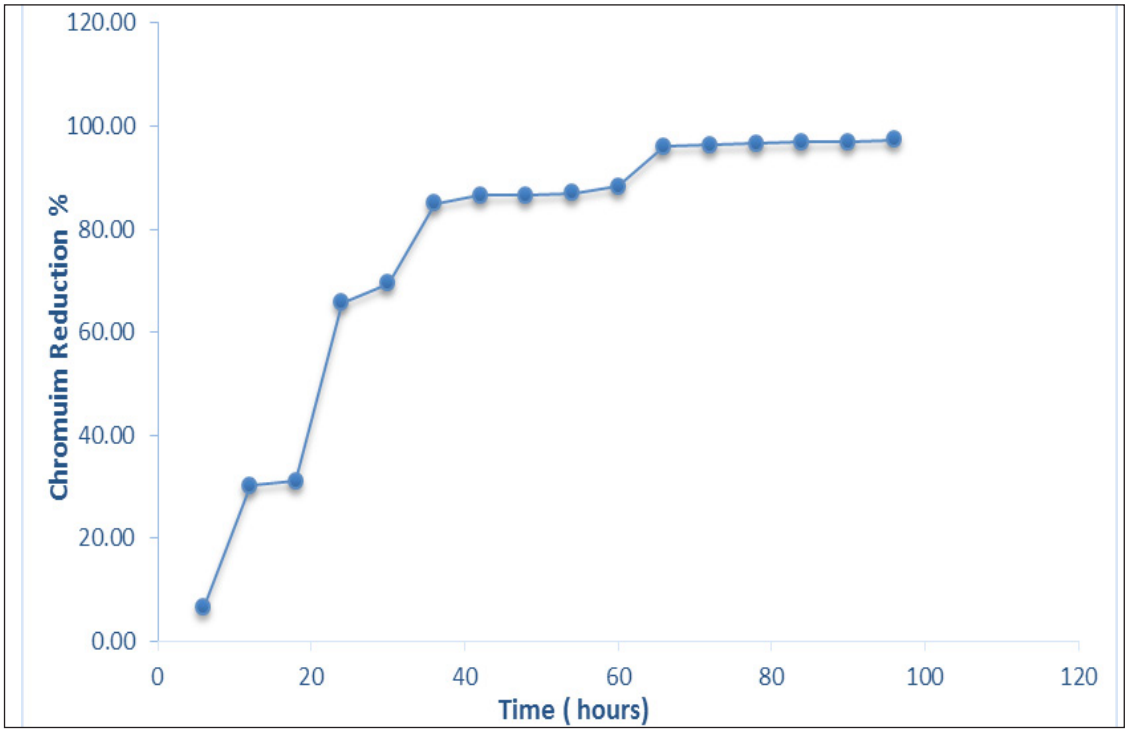


**Fig. 4. Reduction of Cr VI by *Microbacterium* spp. throughout 96 hours of incubation.**

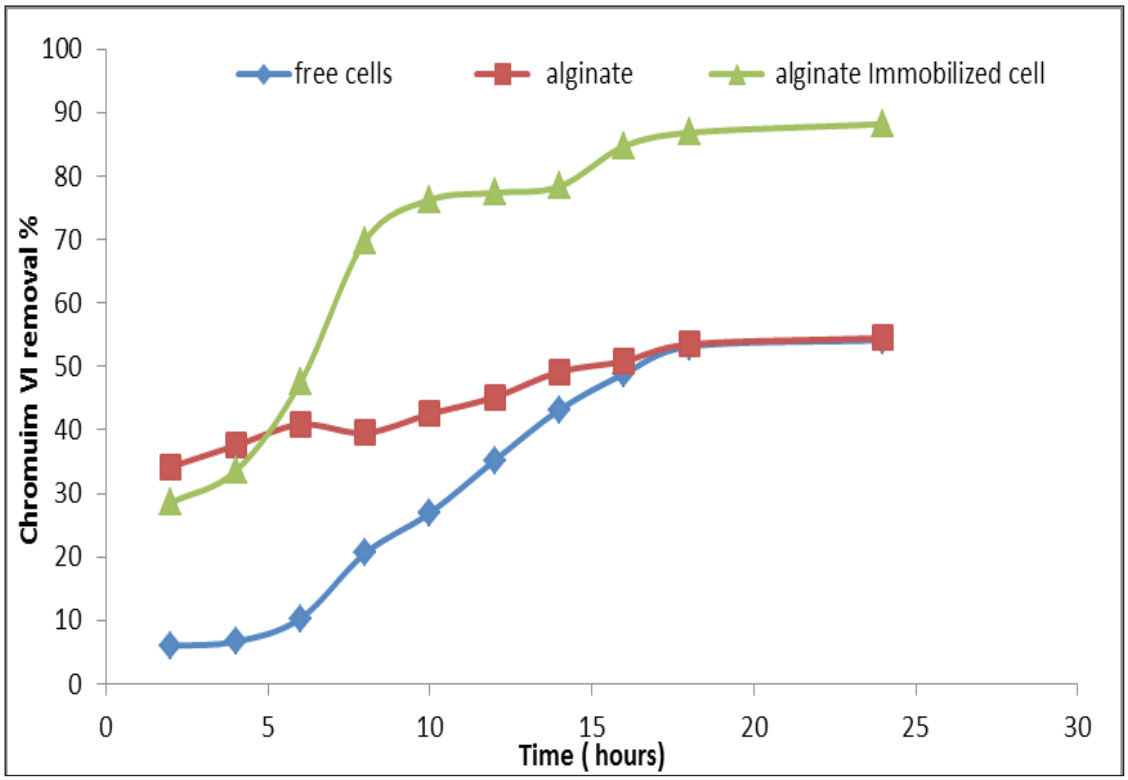


**Fig. 5. Enhancing bioremediation of Cr VI using *Mycobacterium* spp. immobilized on immobilization alginate media.**

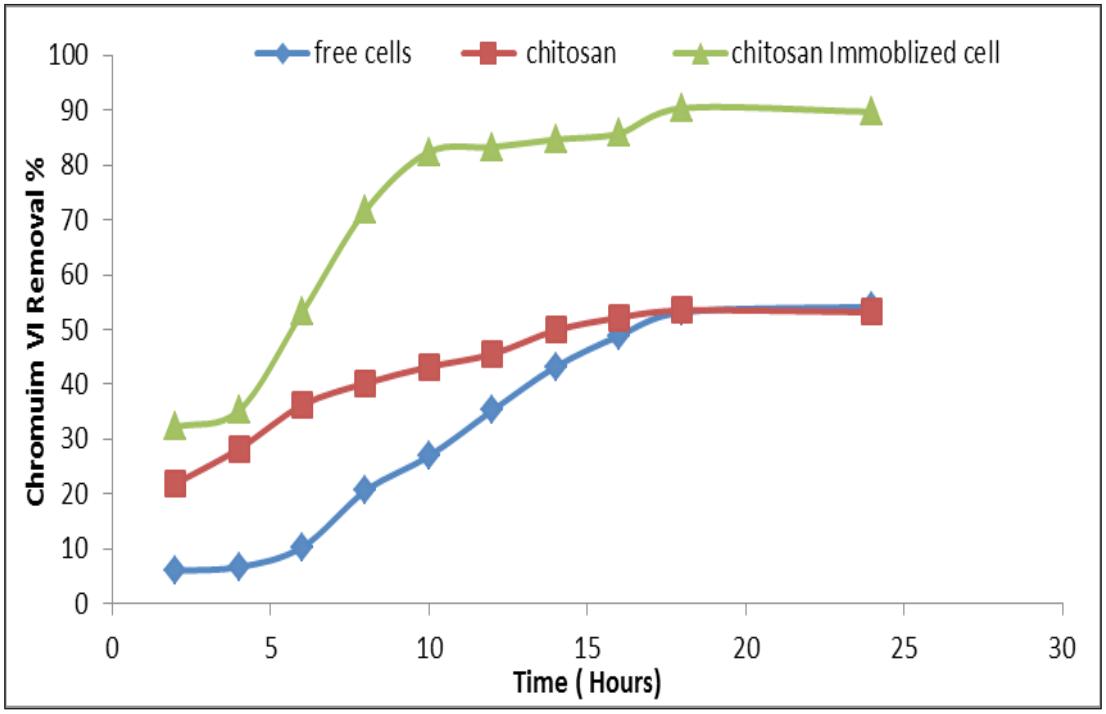


**Fig. 6. Enhancing bioremediation of Cr VI using Mycobacterium spp. immobilized on immobilization chitosan media.**

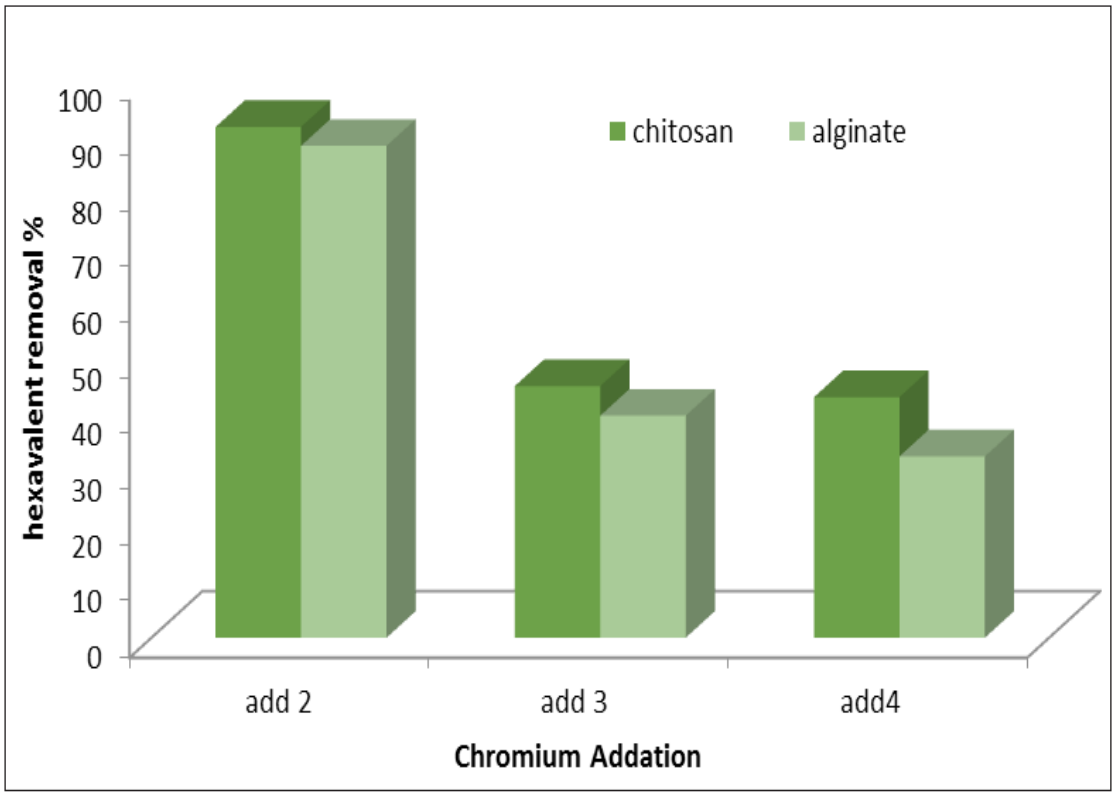


**Fig. 7. Reuse of immobilized Mycobacterium spp. for bioremediation of hexavalent chromium.**

## Conclusion

This study shows that bacterial bioremediation of Cr VI residues is a promising technique to remove the toxic hexavalent chromium. The immobilization of the potential bacterial stain (*Microbacterium* spp., No. 3) on alginate and chitosan has accelerated the reduction of the soluble hexavalent chromium to precipitated Cr III. The immobilizations reduced the time necessary to perform the bioremediation process.

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