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# Hexavalent Chromium Reduction by *Bacillus* sp. Strain FM1 Isolated from Heavy-Metal Contaminated Soil

**Farhana Masood · Abdul Malik**

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**Abstract** A Cr(VI) reducing bacterial strain FM1 was isolated from heavy metal contaminated agricultural soil irrigated with tannery effluents of Jajmau, Kanpur (India), and was identified as *Bacillus* sp. on the basis of biochemical methods and 16S rDNA gene sequence analysis. FM1 strain was found to be resistant to some toxic heavy metals (Cr(VI), Cr(III), $Cu^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Ni^{2+}$ and $Zn^{2+}$) up to several fold concentrations to the normal levels occurring in highly polluted region. FM1 was resistant to very high concentration of Cr(VI) (1,000 mg/L) and completely reduced 100 mg/L Cr(VI) within 48 h. Factors (pH, temperature, initial Cr(VI) concentration) affecting Cr(VI) reduction under culture condition were also evaluated. Reduction was optimum at 37°C and pH 8. Cr(VI) reduction was enhanced by addition of glucose. The presence of heavy metal cations, such as $Cu^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Ni^{2+}$ and $Zn^{2+}$ showed differential effect on reduction. Since strain FM1 could grow in the presence of significant concentrations of metals and due to high Cr(VI) reduction ability, this bacterium may be potentially applicable in Cr(VI) detoxification.

**Keywords** Cr(VI) reduction · *Bacillus* sp · Bioremediation

F. Masood · A. Malik (✉)
Department of Agricultural Microbiology,
Faculty of Agricultural Sciences,
Aligarh Muslim University, Aligarh 202002, India
e-mail: ab_malik30@yahoo.com

*Present Address:*
A. Malik
Department of Infectious Diseases, Universität Klinikum
Freiburg, 79106 Freiburg im Breisgau, Germany

Chromium is one of the most widely used metals in industry, such as electroplating, leather tanning, wood processing and alloy preparation. Nevertheless, its release from improper storage and inappropriate waste-disposal practices caused serious environmental problems. Chromium exists in several oxidation states from Cr(II) to Cr(VI) (Avudainayagam et al. 2003). Hexavalent chromium Cr(VI) and trivalent chromium Cr(III) are the most dominant oxidation states of chromium that exist in the environment. Cr(VI) is highly mobile and water soluble as compared to Cr(III), whereas Cr(III) is relatively inert, chemically more stable than Cr(VI) and less bioavailable due to its negligible permeability to biomembranes (Pal et al. 2005). Therefore, reduction of Cr(VI) to innocuous Cr(III) rapidly and economically has become the key point of remediation strategies of Cr(VI) contaminated waters and soils. Conventional methods for treatment of toxic chromate include chemical reduction followed by precipitation, ion exchange, and adsorption on activated coal, alum, kaolinite and ash which require large amounts of chemicals and energy and therefore are unsuitable (Camargo et al. 2003). Biological approach has been considered as an alternative remediation for Cr(VI) contamination due to the lower costs and the significant smaller quantities of the produced sludge (Liu et al. 2006).

Several microorganisms have the exceptional ability to adapt and colonize the noxious metal polluted environments by developing mechanisms to evade metal toxicity like metal efflux channels, metal resistance plasmids, adsorption uptake, DNA methylation and metal biotransformation either directly by specific enzymes or indirectly by cellular metabolites. Biotransformation of Cr(VI) to Cr(III) using bacteria is the most pragmatic approach with a well-established feasibility in bioremediation. Reduction of Cr(VI) has been demonstrated in various bacterial

species including *Bacillus* sp. (Elangovan et al. 2006; Liu et al. 2006), *Pseudomonas* sp. (Ganguli and Tripathi 2002), *Escherichia coli* (Bae et al. 2005), *Exiguobacterium* sp. (Alam and Malik 2008), *Desulfovibrio* sp. (Mabbett and Macaskie 2001), *Microbacterium* sp. (Pattanapipitpaisal et al. 2001), *Shewanella* sp. (Vaimajala et al. 2002) and *Arthrobacter* sp. (Asatiani et al. 2004).

The objective of this study was to characterize the Cr(VI) resistance and reduction potential of strain FM1 isolated from heavy metal contaminated soil where the level of hexavalent chromium is very high.

## Materials and Methods

In India, Kanpur (UP) lies in Indo-Gangatic plains between the parallels of 26° 28N and 80° 24E. About 310 tanneries are located at Jajmau (Kanpur), which is one of the major centers for the processing of raw hides. The treated effluent is being used for the irrigation of crops and vegetables growing in an area of 2,100 acre. Composite soil samples were vertically collected with the depth of 5–10 cm from contaminated site. Due to long-term irrigation, the area is highly contaminated and is selected for the present study. These soils contain 1,126.25 mg Chromium, 14.7 mg Cadmium, 95.6 mg Copper, 41.16 mg Nickel and 84 mg Zinc per kg of dry soil as estimated by atomic absorption spectrophotometer (GBC 932 plus, Australia). Soil samples were serially diluted and plated onto Luria–Bertani (LB) agar plates amended with 100 mg/L Cr(VI) as $K_2Cr_2O_7$ and incubated at 37°C for 48 h. Colonies of different morphologies were then selected on LB agar containing 100 mg/L of Cr(VI). Chromium tolerance was checked by transferring morphologically different colonies on Luria agar plate amended with 50–400 mg/L of hexavalent chromium. One culture (FM1), which could grow on plate containing 300 mg/L chromium was identified by biochemical methods and 16SrRNA analysis. All further studies were done using FM1.

The isolate was identified based on standard biochemical tests according to Holt et al. (1994). Further identification was done by 16S rDNA analysis using the primers 27 f (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1,492 r (5′ GGTTACCTTGTTACGACTT 3′). Sequence was initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov) using BLAST (n) program and corresponding sequences were downloaded. The phylogenetic tree was constructed by the neighbor-joining method using the MEGA package version (Tamura et al. 2007). The sequence was deposited at GenBank with accession No. GQ334453.

The minimum inhibitory concentration (MIC) was determined in LB agar amended with heavy metal salts such as $CdCl_2$, $CuSO_4$, $CoCl_2$, $CrCl_3.6H_2O$, $K_2Cr_2O_7$, $NiCl_2$ and $ZnCl_2$ in varying concentrations ranging from 3.12 to 1,600 mg/L (Aleem et al. 2003; Ansari et al. 2008). The minimum concentration of the metal inhibiting complete growth was taken as the MIC.

Growth curves of bacterial isolate was determined in LB medium with (100 mg Cr(VI)/L) and without chromium (control). LB inoculated with 1% exponential growth phase bacterial culture and incubated at 37°C with shaking (120 rpm). An aliquot of culture was taken out in sterilized tube at regular intervals and absorbance was measured at 600 nm.

The isolate was inoculated in 100 mL of medium in flasks supplemented with 100 mg/L of Cr(VI) as $K_2Cr_2O_7$ and incubated at 37°C with shaking. Samples were drawn at regular time intervals and centrifuged at 10,000 rpm for 15 min. Cr(VI) concentration in the supernatant was determined colorimetrically using diphenylcarbazide reagent in acid solution (Clesceri et al. 1998). The absorbance was measured at 540 nm by UV 5704 SS model spectrophotometer.

To characterize the Cr(VI)-reduction efficiency of strain FM1, the effects of temperature (27, 37, 47°C), initial pH (4, 5, 6, 7, 8, 9, 10, 11) and initial Cr(VI) concentration (50–200 mg/L) were investigated. Cr(VI)-reduction was studied in aerobic batch cultures. Autoclaved nutrient medium (100 mL) in 250 mL culture flasks was supplemented with Cr(VI), inoculated from log phase bacterial culture and incubated at the appropriate temperature with shaking (120 rpm). Samples were drawn at regular time intervals and analyzed for disappearance of Cr(VI) as described above. In order to monitor any abiotic Cr(VI)-reduction, cell-free controls were also used for each Cr(VI)-reduction assay.

The effects of glucose (supplemented 1% glucose (w/v) as the electron donor) and heavy metals on Cr(VI)-reduction by strain FM1 were also investigated. LB broth having 100 mg/L Cr(VI) with 0.1 mM of different test metals ($Cd^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Ni^{2+}$, $Zn^{2+}$) separately incubated at 37°C with shaking. The experiments were performed as described above and the mean values were reported.

All experiments were done in triplicates and mean value for the replicates was used for comparison. Standard deviation and one way ANOVA at $p \leq 0.05$ were done using MINITAB Software, USA.

## Results and Discussion

Agricultural soil irrigated with tannery effluents had accumulated multi-fold elevated level of chromium and other heavy metals. Others workers have also reported high concentration of chromium in the same area (Singh et al.

2004). Heavy metal contamination is known to cause shifts in microbial communities with emergence of bacterial species with elevated metal tolerance (Stepanauskas et al. 2005; Ansari et al. 2008). In search for chromium-resistant microorganisms, a total of 50 Cr-resistant bacteria were isolated on plates amended with 50–300 mg/L of $K_2Cr_2O_7$. Out of which only one isolate (FM1) could tolerate up to 300 mg/L of $K_2Cr_2O_7$ and hence, all further studies were done using FM1 as it showed maximum tolerance. Chromium contamination exerts a selective pressure on microbial flora of tannery soils (Viti et al. 2003). Chromium-resistant bacteria capable of chromate reduction have been reported from chromium polluted environments (Srinath et al. 2001). The strain belongs to genus *Bacillus* sp. (97% similarity with *Bacillus thuringiensis* strain INRS10) on the basis of 16S rRNA gene sequence analysis and biochemical tests. Figure 1 showed the phylogenetic tree drawn using neighbor-joining program (Saitou and Nei 1987) in MEGA 4.0 software between different members of genus *Bacillus* (sequences retrieved from NCBI database) and present bacterial isolate FM1 (GQ 334453).

The bacterial isolate showed resistance to various other heavy metals. MICs of various heavy metal salts viz $K_2Cr_2O_7$, $CdCl_2$, $CuSO_4$, $CrCl_3.6H_2O$, $CoCl_2$, $NiCl_2$, $ZnCl_2$ were found to be 1,000, 200, 800, 1,600, 800, 800 and 1,000 mg/L, respectively. Cr(VI) bearing wastewater from industrial process generally consists of a wide variety of other heavy metal cations, therefore the resistance of the isolate to various metallic salts was also determined. It was found that bacterial strain was resistant to multiple metal ions and the degree of inhibition caused by tested metal cations was $Cd^{2+} > Cu^{2+} = Ni^{2+} > Cr^{6+} > Cr^{3+} = Zn^{2+}$. Viti et al. (2004) have compared the MIC of the chromium resistant isolates to various heavy metals and reported that different isolates exhibited different level of metal tolerance. Tolerance to other metals has an added advantage of withstanding the presence of different metallic ions while performing the desired activity.

Growth curves of the bacterial isolate with or without 100 mg/L Cr(VI) were plotted (Fig. 2). The cells were grown well in the medium with 100 mg/L Cr(VI), although the growth curve of FM1 in the medium containing Cr(VI) did not follow the same growth pattern as the control and indicates the toxic effect of Cr(VI) on the cells during the incubation time of 24 h. Cr(VI)-reduction potential of *Bacillus* sp. FM1 was assessed by adding Cr(VI) at 100 mg/L in the culture medium. Strain FM1 efficiently reduced 33%, 54%, 72% and 100% from the medium after 12, 24, 36 and 48 h, respectively. Pal et al. (2005) reported almost 72% Cr(VI) removal by *Bacillus sphaericus* AND303 within 24 h.

As shown in Table 1, the optimum pH for Cr(VI) reduction by the strain FM1 was pH 8.0. Nonetheless, FM1 was also capable of reducing Cr(VI) at the pH range of 7–9 with an appreciable efficiency on either side of the optimum pH. Isolate FM1 reduced 3.3, 2.6, 2.1, 1.1, 1.2, 1.7 and 2.0 times more chromate at pH 8 as compared to pH 4, 5, 6, 7, 9, 10 and 11 respectively after 36 h and found to be significant ($p \leq 0.05$). Camargo et al. (2003) reported that optimal pH for Cr(VI) reduction by bacterial isolates was 7–8. Wang and Xiao (1995) have reported that reduction of chromium in *Bacillus* and *Pseudomonas fluorescens* occurred at pH 7, but it was restricted at pH 6. The change in optimal pH indicates that pH modification is important for different cultures to achieve the maximum Cr(VI) reduction in Cr(VI) detoxification.

Temperature is an important factor that has an effect on microbial Cr(VI)-reduction. Cr(VI)-reduction by the strain

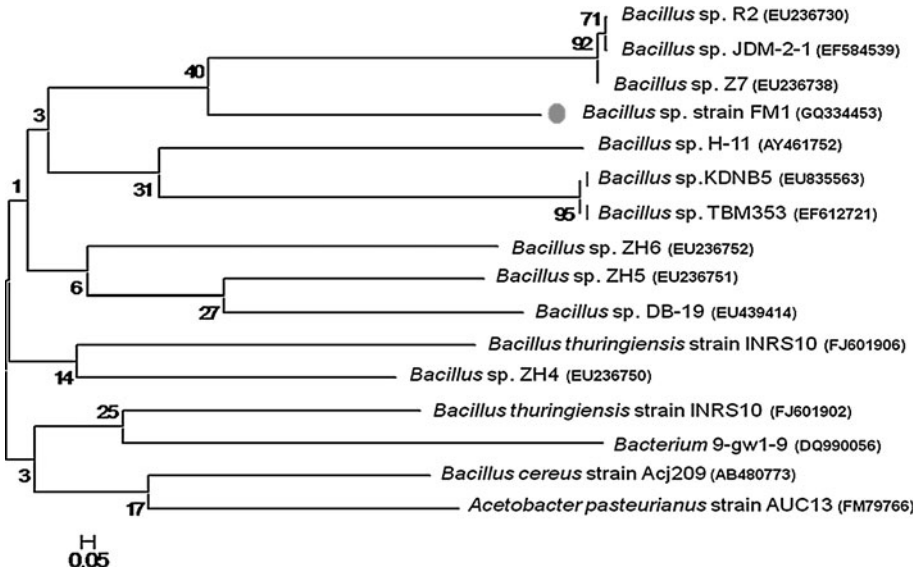


**Fig. 1** Phylogenetic tree derived from 16S rRNA gene sequence of *Bacillus* sp. FM1 (GQ 334453) and sequence of closest phylogenetic neighbors obtained by NCBI BLAST (n) analysis. The *bar* represents distance values calculated in MEGA and values at nod represent percentage of 500 bootstrap replicates. The *scale bar* corresponds to 0.05 substitution per nucleotide position. *Numbers in bracket* represents GenBank accession numbers

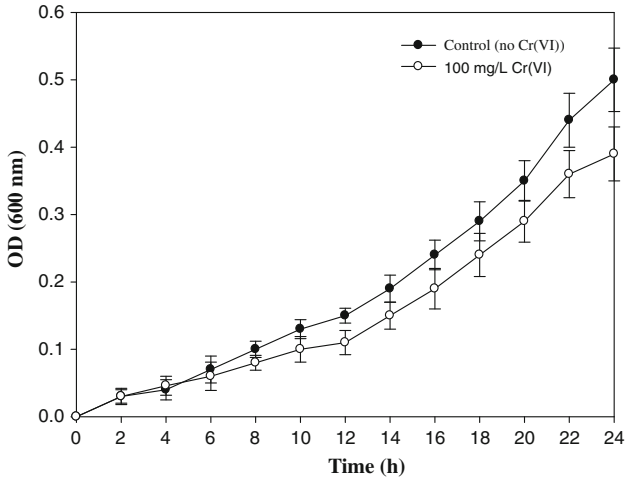


**Fig. 2** Growth curves of *Bacillus* sp. FM1 in absence and in presence of 100 mg/L Cr(VI). *Error bars* represent standard deviations

**Table 1** The effects of factors on Cr(VI) reduction, including pH, temperature and initial Cr(VI) concentration

| Parameters | Residual Cr(VI) (mg/L) | | | |
|---|---|---|---|---|
| | Incubation (h) | | | |
| | 12 | 24 | 36 | 48 |
| pH | | | | |
| 4 | 95 ± 1 | 89.3 ± 1.5 | 75 ± 2 | 70 ± 3 |
| 5 | 93 ± 2 | 88 ± 1.5 | 68 ± 3 | 63 ± 3.6 |
| 6 | 90 ± 2.6 | 80 ± 1.8 | 61 ± 5 | 53 ± 4.3 |
| 7 | 63 ± 3 | 45.3 ± 2.1 | 28 ± 4 | 0 |
| 8 | 55 ± 5.3 | 40 ± 3.6 | 18 ± 3 | 0 |
| 9 | 67 ± 3 | 50 ± 4 | 30 ± 3 | 0 |
| 10 | 70 ± 4 | 61 ± 4.6 | 53 ± 4.3 | 40 ± 3 |
| 11 | 76 ± 5.3 | 65 ± 3 | 60 ± 4 | 50 ± 5 |
| LSD ($p \leq 0.05$) | 6.53 | 6.05 | 6.19 | 5.64 |
| Temperature (°C) | | | | |
| 27 | 65 ± 1.8 | 50 ± 1.5 | 45 ± 2 | 30 ± 2.6 |
| 37 | 55 ± 3 | 37 ± 2 | 26 ± 4 | 0 |
| 47 | 69 ± 3 | 58 ± 2.7 | 49 ± 3.6 | 36 ± 4 |
| LSD ($p \leq 0.05$) | 4.64 | 5.82 | 4.13 | 7.51 |
| Initial Cr(VI) concentration (mg/L) | | | | |
| 50 | 28 ± 3 | 0 | 0 | 0 |
| 100 | 53 ± 4.3 | 20 ± 4.5 | 10 ± 4.4 | 0 |
| 200 | 168 ± 4 | 100 ± 4 | 70 ± 5 | 54 ± 3.6 |
| LSD ($p \leq 0.05$) | 3.48 | 9.69 | 6.41 | 4.7 |

Results are mean ± standard deviations (SD) of 3 replicates (n = 3).
*LSD* Least significance difference

FM1 was evaluated within a temperature range of 27–47°C. The strain FM1 significantly ($p \leq 0.05$) reduced Cr(VI) (70–64%) from 27 to 47°C with an optimum (100%) at 37°C (Table 1). Optimal temperature for Cr(VI) reduction by *Ochrobactrium intermedium* SDCr-5 was reported at 37°C (Sultan and Hasnain 2007).

Chromate reduction was monitored at different initial chromium concentration ranging from 50 to 200 mg/L (Table 1). Cr(VI)-reduction occurred even at the highest concentration of 200 mg/L, but complete reduction was not observed at this initial concentration in 96 h. Complete Cr(VI)-reduction was observed for 50 and 100 mg/L at 12 h and 48 h, respectively. It seems higher concentration posed a selection pressure due to Cr toxicity and thus lengthened the growth phase, and for having substantial cell mass it took longer period for complete reduction. DeLeo and Ehrlich (1994) reported 99.7% reduction of Cr(VI) by *Pseudomonas fluorescens* LB300 at an initial concentration of 112.5 mg/L within a period of 289 h, while, Pattanapipitpaisal et al. (2001) reported that *Microbacterium* sp. completely reduced 20 mg/L of Cr(VI) within 72 h.

The chromate-reducing microorganisms may utilize a variety of organic compounds as electron donors for Cr(VI) reduction (Philip and Venkobachar 1998; Liu et al. 2004). The effect of glucose on Cr(VI) reduction was tested (Fig. 3), the concentration of Cr(VI) decreased to 28.0 ± 1.5 mg/L when glucose was applied, while the medium without glucose the Cr(VI) concentration dropped only to 37.2 ± 1.8 mg/L after incubation of 36 h. This implies that glucose significantly ($p \leq 0.05$) promoted the bacterial Cr(VI) reduction. No Cr(VI)-reduction was observed in the medium without any cells inoculated, which confirms the ability of bacteria (FM1) to reduce Cr(VI). Wang and Shen (1995) found that glucose promoted Cr(VI) reduction by *Agrobacterium radiobacter*, *Bacillus cereus, Escherichia coli* ATCC33456, and *Pseudomonas fluorescens* LB300.

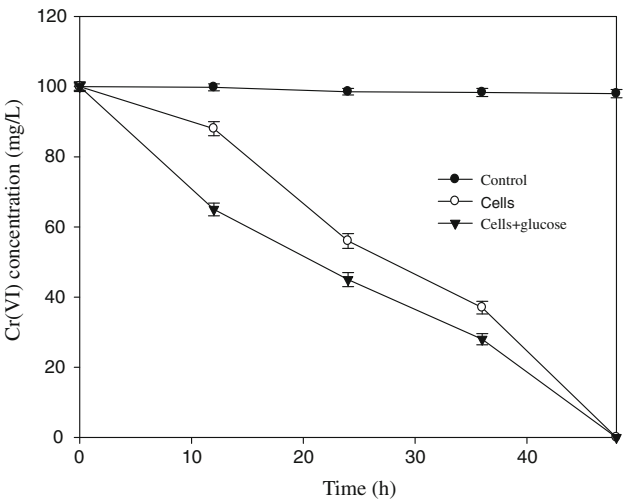


**Fig. 3** Effect of glucose on Cr(VI) reduction by *Bacillus* sp. FM1. *Error bars* represent standard deviations

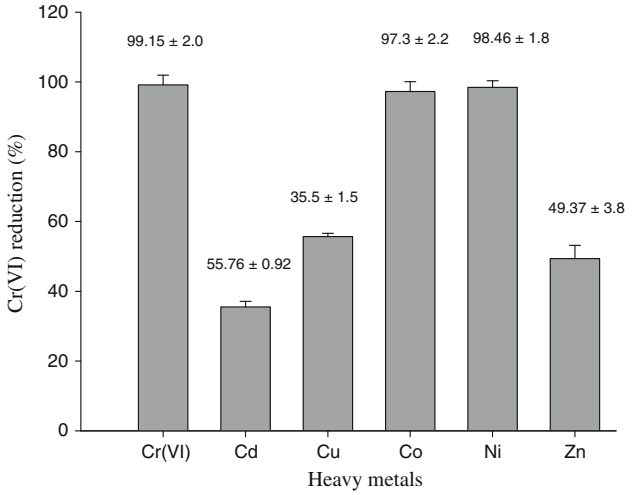


**Fig. 4** Effect of heavy metals on Cr(VI) reduction (%) by *Bacillus* sp. FM1. *Error bars* represent standard deviations

Cr(VI) reduction was variedly affected by the addition of different heavy metals ($Cd^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Ni^{2+}$ and $Zn^{2+}$) under culture condition (Fig. 4). Metals as $Cd^{2+}$, $Zn^{2+}$ and $Cu^{2+}$ significantly ($p \leq 0.05$) inhibited Cr(VI) reduction by 35%, 49.37% and 55.65% respectively. The inhibition of Cr(VI) reduction by the addition of $Cd^{2+}$ and $Zn^{2+}$ corroborated with the results obtained in *Bacillus* sp. G1DM22 (Desai et al. 2008). Park et al. (2000) also reported that $Cu^{2+}$ inhibits Cr(VI) reduction in *Pseudomonas putida*. $Co^{2+}$ and $Ni^{2+}$ had negligible effect on Cr(VI) reduction ($p \leq 0.05$), similar results also reported by Pal and Paul (2004).

The results obtained may provide useful information for the bioremediation of chromate under a wide range of environmental conditions. Further researches will be conducted on the mechanisms by which the bacteria reduce Cr(VI).

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