Bull Environ Contam Toxicol (2008) 81:25–29
DOI 10.1007/s00128-008-9442-5

Chromium Tolerance and Reduction Potential of a *Bacillus* sp.ev3 Isolated from Metal Contaminated Wastewater

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Received: 9 September 2007 / Accepted: 15 April 2008 / Published online: 23 May 2008

Abstract This study was aimed at assessing the ability of *Bacillus* sp.ev3 to reduce hexavalent chromium into its trivalent form. *Bacillus* sp.ev3 could tolerate Cr^{6+} (4800 μg/mL), Pb^{2+} (800 μg/mL), Cu^{2+} (200 μg/mL), Cd^{2+} (50 μg/mL), Zn^{2+} (400 μg/mL), Ni^{2+} (4000 μg/mL) and Hg^{2+} (50 μg/mL). *Bacillus* sp.ev3 showed optimum growth at 37°C and pH at 7. *Bacillus* sp.ev3 could reduce 91% of chromium from the medium after 96 h and was also capable to reduce 84% chromium from the industrial effluents after 144 h. Cell free extracts of *Bacillus* sp.ev3 grown in the presence of Cr showed reduction of 70%, 45.6% and 27.4% at concentrations of 10 μg Cr^{6+}/mL, 50 μg Cr^{6+}/mL and 100 μg Cr^{6+}/mL, respectively.

Keywords Cr^{6+} reducing bacterium · Bioremediation · Cr^{6+} reduction · *Bacillus* sp.ev3

Heavy metals contamination in soil and water is a major environmental problem. Heavy metals have many industrial applications due to their technological importance. Wastewaters from these industries have permanent toxic effects to human and the environment. Chromium is a toxic heavy metal that is widely used in electroplating, leather tanning, textile dyeing, and metal processing industries. In metal cleaning, plating and metal processing industries, chromium concentration can approach 20,000–75,000, 15,000–52,000 and 100,000–270,000 μg/mL, respectively (Sag and Kutsal 1989).

Chromium exists in several oxidation states from chromium (II) to chromium (VI). In nature, chromium can be found either as chromium (VI) or as chromium (III). Chromium (III) is rather benign and easily adsorbed in soils and waters, whereas chromium (VI), which is the most toxic form, is not readily adsorbed and most of its salts are soluble (Kotas and Stasicka 2000). Industrial wastewaters contain both chromium and salts ions which have toxic affects on the microbial consortia of wastewater treatment systems (Stasinakis et al. 2003).

Conventional methods for removing metals from industrial effluents include chemical precipitation, chemical oxidation or reduction, ion exchange, filtration, electrochemical treatment, reverse osmosis, membrane technologies and evaporation recovery (Ahluwalia and Goyal 2007). These processes may be ineffective or extremely expensive especially when the metals in solution are in the range of 1000–100 μg/mL (Nourbakhsh et al. 1994). Therefore, it is important to develop an innovative, low cost and eco-friendly method for removal of toxic heavy metal ions from wastewater.

Several microorganisms have the exceptional ability to adapt to and colonize the noxious metal polluted environments, which are uninhabitable by higher organisms. These microorganisms have developed the capabilities to protect themselves from heavy metal toxicity by various mechanisms such as adsorption, uptake, methylation, oxidation and reduction. Many microorganisms have been reported to reduce the highly soluble and toxic chromium (VI) to the less soluble and less toxic chromium (III), e.g., *Acinetobacter* and *Ochrobactrum* (Francisco et al. 2002), *Arthrobacter* (Megharaj et al. 2003), *Pseudomonas* sp. (Rajkumar et al.

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2005), *Serratia marcescens* (Campos et al. 2005), *Ochrobactrum* sp. (Thacker and Madamwar 2005), *Bacillus* sp. (Elangovan et al. 2006), *Desulfovibrio vulgaris* (Goulhen et al. 2006), *Cellulomonas* spp. (Viamajala et al. 2007).

The present study deals with the isolation of chromium resistant bacterium from contaminated environment, its molecular characterization, the ability of the bacterium to reduce hexavalent chromium and optimization of temperature and pH for maximum chromium reduction.

Materials and Methods

Wastewater samples were collected in screw capped sterilized bottles from different industrial areas of Sialkot (Pakistan). Some physicochemical parameters of wastewater viz., temperature (°C), pH, dissolved oxygen and chromium (μg/mL) were measured (APHA 1989).

For isolation of chromium resistant bacteria, 100 μL of the wastewater sample was spread on Luria-Bertani (LB) agar plates containing 100 μg of Cr^{6+}/mL of the medium. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100 mL distilled water, pH adjusted at 7.2–7.5 and then 1.5 g agar was added in the 250 mL flasks. The medium was autoclaved at 121°C and 15 Lb pressure for 15 min. The growth of the bacterial colonies was observed after 24 h of incubation at 37°C. Isolated colonies were picked up with sterilized wire loop and streaked on LB agar medium plate containing 100 μg Cr^{6+}/mL. It was again incubated at 37°C for 24 h. This process was repeated with successively higher concentrations of Cr^{6+} until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained. The MIC is defined as the lowest concentration of Cr^{6+} at which a single colony-derived streak could not grow.

For further identification, genomic DNA was isolated and the 16S rRNA gene was amplified by PCR using two general bacterial 16S rRNA primers (RS-1; 5′-AAACTCAAATGAATTGACGG-3′, RS-3; 5′-ACGGGCGGTGTGTAC-3′). The PCR product of 0.5 kb was removed from the gel and cloned in pTZ57R/T vector. The amplified 16S rRNA gene was purified with a Fermentas purification kit and the amplified products were electrophoresed on 1% agarose gel. Sequencing was carried out by Genetic analysis system model CEQ-800 (Beckman) Coulter Inc. Fullerton, CA, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

For optimum growth of the bacterial isolate, two parameters, i.e., temperature and pH were considered. For determination of optimum temperature, 5 mL LB broth was added in 4 sets, each of three test tubes, autoclaved and inoculated with 20 μL of freshly prepared culture of the isolate. The four sets of tubes were incubated at 25, 30, 37 and 42°C. After an incubation of 12 h, their absorbance was taken at 600 nm.

For determination of optimum pH, test tubes having 5 mL LB broth were prepared in 9 sets, each containing 3 test tubes and their pH was adjusted at 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 then autoclaved. These tubes were inoculated with 20 μL freshly prepared culture of the isolate. After incubation period of 12 h, their absorbance was taken at 600 nm.

Growth curves of bacterial isolate were determined with (100 μg Cr^{6+}/mL) and without chromium (Control). For bacterial isolate 50 mL LB broth was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 20 μL of the freshly prepared inoculum. These cultures were incubated at 37°C in a shaker at 80–100 rpm. An aliquot of culture was taken out in an oven sterilized tube, at regular intervals of 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h. Absorbance was taken at 600 nm wavelength. Growth was plotted graphically.

The cross heavy metal resistance of bacterial isolate was determined by using stock solutions of 10,000 μg/mL of different metal salts such as, lead nitrate, cadmium chloride, copper sulphate, potassium dichromate, zinc sulphate and nickel chloride. The cross metal resistance was checked by increasing the concentration of respective metal in a stepwise manner with 50 μg/mL of metal increased every time. Streaked plates containing metal ions, incubated at 37°C for 24 h and growth was observed for four days.

In order to determine the ability of bacterial isolate to reduce Cr^{6+} to Cr^{3+}, diphenylcarbazide method was used (Fulladosa et al. 2006). Samples (1 mL) from culture were taken after 24, 48, 72 and 96 h, spun down at 14,000 rpm for 5 min and supernatant was used for estimation of Cr^{6+} left in the medium. Supernatant (100 μL) was added to 10 mL of glass-distilled water in a test tube, followed by addition of 1 mL of diphenylcarbazide solution (prepared by dissolving 0.25 g diphenylcarbazide in 100 mL acetone) and 1 drop of H_3PO_4. The mixture was kept at room temperature for 10 min to allow for color development and then O.D. was taken at 540 nm.

To check the efficacy of the bacterium to reduce Cr^{6+} in natural environment a large-scale experiment was set up. Three plastic containers were used. In first container 10 L of tap water was taken along with 1.5 L of bacterial isolate grown to log phase. In second container 10 L of industrial effluent was taken along with 1.5 L of log phase grown bacterial culture. In the third container only 10 L of industrial effluent was taken and 100 μg/mL of Cr stress was maintained in each container. Cr^{6+} reduction in these containers was determined by using diphenylcarbazide method after 48, 96 and 144 h of incubation at room temperature.

To prepare the crude cell free extract, the bacterial culture was grown in 200 mL Luria-Bertans broth for 24 h at 37°C with chromium (100 μg/mL) and without chromium. Cells were harvested by centrifugation at 9,000 rpm for 15 min. Pellets were washed twice with 10 mM Tris HCl buffer (pH 7.2) and were suspended in 3 mL of the same buffer. Cells were disrupted by sonication for 5 min (Sonics VC 500 USA) in cold condition. The resultant homogenate was centrifuged at 8,000 rpm for 30 min at 4°C; the supernatant was used as a crude extract. The crude extracts were then subjected to three different concentration of chromium, i.e., 10, 50 and 100 μg/mL for 12 h and crude extracts that were heated at 100°C for 30 min act as control. Chromium reduction was assessed using diphenylcarbazide method.

Observations were made and all the experiments run in triplicate. At least three separate flasks were usually maintained for one treatment. Each time three readings were taken; their mean, and standard error of the mean were calculated.

Results and Discussion

Some physicochemical characteristics of industrial wastewater were ascertained, from where chromium tolerant bacterium was isolated. The temperature of different samples ranged between 23.5°C and 38.3°C, pH ranged between 7.0 and 8.4, dissolved oxygen between 0.48 ± 0.01 and 1.40 ± 0.03 mg/L and Cr^{6+} ranging between 0.80 ± 0.03 and 1.70 ± 0.04 μg/mL.

The partially amplified (500 bp) and sequenced 16S rRNA gene from local isolate (CBL-Cr) was blast to check the maximum homology of this gene to confirm the species of this local isolate. The blast query revealed that this gene is 97% homologous to already reported gene of *Bacillus* sp.ev3. The nucleotide sequences coding for 16S rRNA gene of *Bacillus* sp.ev3 have been submitted to the GenBank database under accession number EU017505.

The most suitable temperature for Cr-resistant bacterial isolate was found to be 37°C and *Bacillus* sp.ev3 showed maximum growth at pH 7. The growth curve pattern was studied by growing the organism in the presence of Cr^{6+} (100 μg/mL) and comparing with the control culture in which no metal ions were added. Although the growth pattern of the isolate was not significantly different from those of control but the growth of isolate was inhibited in the presence of Cr^{6+}. It is interesting to note that the lag phase of the isolate was extended from 4 to 12 h in *Bacillus* sp.ev3. The growth pattern has been shown in Fig. 1.

Bacillus sp.ev3 was found to be resistant to chromium at a concentration of 4800 μg/mL. The bacterial isolate was also checked for its resistance to various other heavy metals, viz., cadmium, copper, lead, zinc, mercury and nickel (Table 1). *Bacillus* sp.ev3 showed maximum resistance against Ni^{2+} at a concentration of 4000 μg/mL and the order of resistance regarding the metal concentration was $Ni^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+} > Cd^{2+} > Hg^{2+}$.

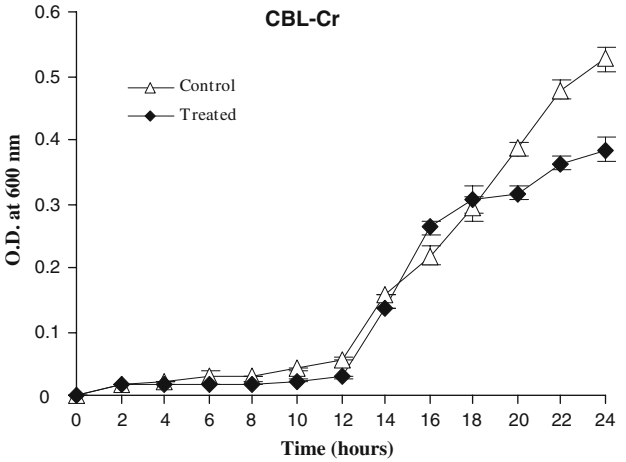

Fig. 1 Growth curves of Cr-resistant *Bacillus* sp.ev3 in LB medium containing chromium (100 μg/mL) and without chromium after incubation at 37°C

A variety of mechanisms exist for the removal of heavy metal from aqueous solution by bacteria, fungi, algae, mosses, macrophytes and higher plants (Holan and Volesky 1995; Pattanapipitpaisal et al. 2002; Rehman et al. 2007).

Cr (VI) is a common pollutant introduced into natural waters from a variety of industrial effluents and its removal either by a reductive process (Pattanapipitpaisal et al. 2002; Sultan and Hasnain 2007) or by biosorption (Rehman et al. 2007; Ziagova et al. 2007) significantly reduces the risks to human health.

Chromium reducing capability of the bacterial isolate was checked by adding Cr^{6+} at 100 μg/mL in the culture medium (Fig. 2). *Bacillus* sp.ev3 could reduce 91% of chromium from the medium after 96 h. The *Bacillus* sp.ev3 was also capable to reduce Cr^{6+} (100 μg/mL) 49.7%, 74.3% and 84.2% from the medium after 24, 48 and 72 h, respectively. One potential method is microbially catalyzed reduction of Cr (VI) to Cr III, which was first reported with *Pseudomonas* spp. (Romanenko and Koren'Ken 1977). Since then, significant progress has been made towards understanding the processes controlling enzymatic reduction of Cr (VI) in Gram-negative bacteria, especially those belonging to the genera *Pseudomonas*, *Desulfovibrio* and *Shewanella* (Chardin et al. 2003; Ackerley et al. 2004). Several Gram-positive bacteria are also known to reduce Cr (VI) including several members of the genus *Bacillus* (Campos et al. 1995; Camargo et al. 2003).

Bacillus sp.ev3 was able to reduce 84% of Cr^{6+} after 144 h of incubation directly in industrial effluent. It could also reduce 13.3% and 18% of Cr^{6+} after 48 and 96 h,

Table 1 Cross metal resistance of Cr-resistant bacterial isolate from industrial wastewater against other heavy metals

Bacterial isolate	Cr^{6+} (μg/mL)	Cd^{2+} (μg/mL)	Cu^{2+} (μg/mL)	Pb^{2+} (μg/mL)	Zn^{2+} (μg/mL)	Ni^{2+} (μg/mL)	Hg^{2+} (μg/mL)
Bacillus sp.ev3	4800	50	200	800	400	4000	50

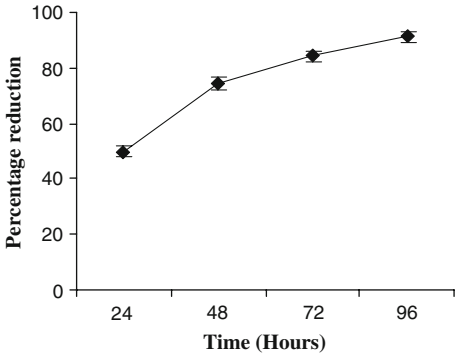

Fig. 2 Chromium reduction by *Bacillus* sp.ev3 from the medium containing (100 μg Cr^{6+}/mL). Estimations were done at different time periods

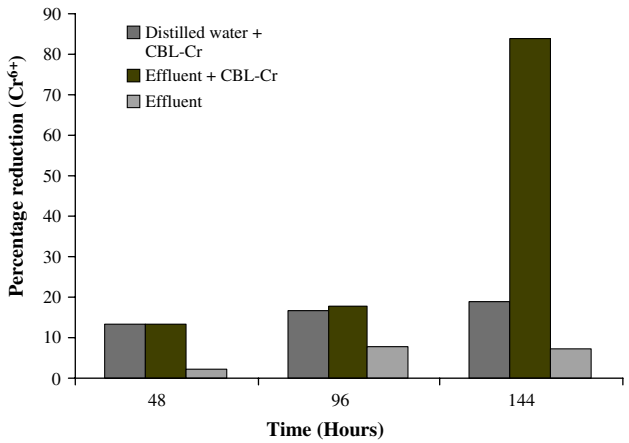

Fig. 3 Percentage reduction of Cr^{6+} by *Bacillus* sp.ev3 from 10 L aqueous solutions (distilled water and industrial effluent) with initial concentration of 100 μg/mL of Cr^{6+} after 48, 96 and 144 h of incubation at room temperature

respectively (Fig. 3). CBL-Cr was also able to reduce 19% of Cr^{6+} in distilled water after 144 h. The nutrient stress conditions have a retarding effect on the Cr^{6+} reducing ability of CBL-Cr. On the contrary the microbial flora alone of the industrial effluent was able to reduce only 7.4% of Cr^{6+} after 96 h. Hence CBL-Cr not only exhibited the ability to survive synergistically in contaminated wastewater but also demonstrated a marked increase in remediation of toxic Cr^{6+} in its presence.

Cell free crude extract from the *Bacillus* sp.ev3 reduced Cr^{6+} to Cr^{3+} as shown in Table 2. The cell free extracts of *Bacillus* sp.ev3 were exposed to three different concentrations of Cr, i.e., 10, 50 and 100 μg Cr^{6+}/mL and the reduction ability was assessed after 12 h of incubation at

Table 2 Hexavalent chromium recovered (%) after incubation with crude cell extract at different Cr^{6+} concentrations

	At 10 (μg/mL)		At 50 (μg/mL)		At 100 (μg/mL)	
Time (h)	0	12	0	12	0	12
Control	10	10	50	50	100	100
Experimental	10	3.0	50	22.8	100	72.6

37°C. Cell free extracts of CBL-Cr grown in the presence of Cr illustrated reduction of 70%, 45.6% and 27.4% at concentrations of 10, 50 and 100 μg Cr^{6+}/mL, respectively. Ganguli and Tripathi (2002) reported that *Pseudomonas aeruginosa* cells reduced chromate 10 μg/mL completely within 2 h. Hexavalent chromium was reduced to undetectable level from 10 μg/mL.

During the present investigation *Bacillus* sp.ev3 showed excellent ability to reduce hexavalent chromium to non-toxic trivalent chromium, i.e., 91%. The chromate resistant *Bacillus* sp.ev3 showed high level of chromium tolerance (4800 μg/mL) and reduced substantial amount of Cr^{6+} from the medium and therefore may be employed for the treatment of Cr^{6+} contaminated wastewater.

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