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Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater

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Abstract

The present study was aimed to assess the ability of *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* to reduce hexavalent chromium into its trivalent form. *Bacillus* sp. JDM-2-1 could tolerate Cr(VI) (4800 μg/mL) and *S. capitis* could tolerate Cr(VI) (2800 μg/mL). Both organisms were able to resist Cd^{2+} (50 μg/mL), Cu^{2+} (200 μg/mL), Pb^{2+} (800 μg/mL), Hg^{2+} (50 μg/mL) and Ni^{2+} (4000 μg/mL). *S. capitis* resisted Zn^{2+} at 700 μg/mL while *Bacillus* sp. JDM-2-1 only showed resistance up to 50 μg/mL. *Bacillus* sp. JDM-2-1 and *S. capitis* showed optimum growth at pH 6 and 7, respectively, while both bacteria showed optimum growth at 37°C. *Bacillus* sp. JDM-2-1 and *S. capitis* could reduce 85% and 81% of hexavalent chromium from the medium after 96 h and were also capable of reducing hexavalent chromium 86% and 89%, respectively, from the industrial effluents after 144 h. Cell free extracts of *Bacillus* sp. JDM-2-1 and *S. capitis* showed reduction of 83% and 70% at concentration of 10 μg Cr(VI)/mL, respectively. The presence of an induced protein having molecular weight around 25 kDa in the presence of chromium points out a possible role of this protein in chromium reduction. The bacterial isolates can be exploited for the bioremediation of hexavalent chromium containing wastes, since they seem to have a potential to reduce the toxic hexavalent form to its nontoxic trivalent form.

Key words: Cr(VI) reducing bacteria; bioremediation; *Bacillus* sp. JDM-2-1; *Staphylococcus capitis*

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Introduction

Heavy metal pollution represents an important environmental problem. Heavy metals have many industrial applications due to their technological importance. Wastewaters from these industries have permanent toxic effects to humans and environment. Heavy metal contamination and the problems posed to the biota have been well documented (Raskin and Ensley, 2000). Accumulation of toxic metals, e.g., Cd, Cr, Cu, Hg, and Zn, in humans has several consequences such as growth and developmental abnormalities, carcinogenesis, neuromuscular control defects, mental retardation, renal malfunction and a wide range of other illnesses (Thiele, 1995).

Chromium is a toxic heavy metal that is widely used in electroplating, leather tanning, textile dyeing, and metal processing industries. Chromium exists in several oxidation states from Cr(II) to Cr(VI). In nature, chromium can be found either as Cr(VI) or as Cr(III). Chromium(III) is rather benign and easily adsorbed in soils and waters, whereas Cr(VI), which is the toxic form, is not readily adsorbed and is soluble (Kotas and Stasicka, 2000). Industrial wastewaters contain both chromium and salt ions which have toxic effects on the microbial consortia of wastewater treatment systems (Stasinakis *et al*., 2003). Removal of Cr(VI) either by reduction (Kamaludeen *et al*., 2003) or by biosorption can significantly reduce the risks to human health.

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 1–100 mg/L (Nourbakhsh *et al*., 1994). Therefore, it is important to develop an innovative, low cost, and eco-friendly method for of toxic heavy metal removal from the wastewater.

A wide variety of microorganisms such as bacteria, yeast, algae, protozoa, and fungi are found in waters receiving industrial effluents. 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 Cr(VI) to the less soluble and less toxic Cr(III), e.g., *Acinetobacter* and *Ochrobactrum*

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(Francisco *et al*., 2002), *Arthrobacter* (Megharaj *et al*., 2003), *Pseudomonas* sp. (Rajkumar *et al*., 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 bacteria from a contaminated environment, their molecular characterization, the ability of the bacteria to reduce hexavalent chromium and optimization of temperature and pH for maximum chromium reduction.

1 Materials and methods

1.1 Sample collection

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

1.2 Isolation of Cr resistant bacteria

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(VI)/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 to 7–7.2 and then 1.5 g agar was added in the 250 mL flasks. The medium was autoclaved at 121°C and 15 lb/inch^2 pressure for 15 min. The growth of the bacterial colonies was observed after 24 h of incubation at 37°C. Effect of Cr(VI) on the growth of bacterial isolates was determined in acetate minimal medium which contained (g/L): NH_4Cl, 1.0; $CaCl_2 \cdot H_2O$, 0.001; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.001; sodium acetate, 5; yeast extract, 0.5; K_2HPO_4, 0.5 (pH 7) supplemented with $K_2Cr_2O_7$ (Pattanapipitpaisal *et al*., 2001). It was again incubated at 37°C for 24 h. This process was repeated with successively higher concentrations of Cr(VI) until the minimum inhibitory concentration (MIC) of bacterial isolate was obtained.

1.3 Identification of the bacterial isolates

For molecular identification, genomic DNA was isolated as described by Carozzi *et al*. (1991) and the 16S rRNA gene was amplified by PCR using two general bacterial 16S rRNA primers (RS-1; 5'-AAACTC-AAATGAATTGACGG-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 (#K0513, Fermentas International Inc., Canada) 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, USA. The 16S rRNA gene sequences were compared with known sequences in the GenBank database to identify the most similar sequence alignment.

1.4 Determination of optimum growth conditions

For optimum growth of the bacterial isolates, two parameters, i.e., temperature and pH were considered. For the determination of optimum temperature, 5 mL LB broth was added into 4 sets, each set consisted of three test tubes. The tubes were autoclaved and inoculated with 20 μL of freshly prepared culture of each bacterial isolate growth overnight at 37°C. The four sets of tubes were incubated at 25, 30, 37, and 42°C, respectively. After an incubation period of 12 h, their absorbance was measured at 600 nm using a LAMBDA 650 UV/Vis Spectrophotometer (PerkinElmer, USA). To determine the optimum pH, test tubes having 5 mL LB broth were prepared in 9 sets, each containing 3 test tubes and their pH was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0, and then autoclaved. These tubes were inoculated with 20 μL freshly prepared culture of each bacterial isolate. After an incubation period of 12 h, their absorbance was measured at 600 nm.

1.5 Effect of Cr on bacterial growth

Growth curves of bacterial isolates were determined in acetate minimal medium with (100 μg Cr(VI)/mL) and without chromium (control). For each bacterial isolate 50 mL medium was taken in one set consisting of 3 flasks, autoclaved and then inoculated with 20 μL of the freshly prepared inoculum. The cultures were incubated at 37°C in a shaker at 80–100 r/min. 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 measured at 600 nm. Growth was plotted graphically.

1.6 Resistance to heavy metal ions

The cross heavy metal resistance of bacterial isolates was determined by using stock solutions of 10 mg/mL of different metal salts (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 checked resistance in acetate minimal medium. Culture flasks containing 100 mL medium and metal ions were inoculated with 20 μL overnight bacterial cultures and incubated at 37°C for 24 h. Growth was measured as optical density at 600 nm.

1.7 Reduction of hexavalent chromium by bacteria

In order to determine the ability of bacterial isolates to reduce Cr(VI) to Cr(III), the diphenylcarbazide method was used (Fulladosa *et al*., 2006). Samples (1 mL) from cultures were taken after 24, 48, 72, and 96 h, spun down at 14000 r/min (6500 ×*g*) for 5 min and supernatants were used to estimate Cr(VI) remained in the medium. Supernatant (100 μL) was added to 10 mL of glass-distilled water in a test tube, followed by the 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 for color development and then optical density was

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measured at 540 nm.

To check the efficacy of the bacteria to reduce Cr(VI) in the natural environment a large-scale experiment was set up. Three plastic containers were used. In the first container 10 L of tap water was taken along with 1.5 L of bacterial isolate grown to log phase. In the 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(VI) reduction in these containers was determined according to the diphenylcarbazide method after 48, 96, and 144 h of incubation at room temperature.

1.8 Crude cell extract

To prepare the crude cell free extract, the bacterial cultures were grown in 200 mL acetate minimal medium for 24 h at 37°C with chromium (100 μg/mL) and without chromium. Cells were harvested by centrifugation at 9000 (3800 $\times g$) for 15 min. Pellets were washed twice with 10 mmol/L Tris HCl buffer (pH 7.2) and were suspended in 3 mL of the same buffer. Cells were disrupted by sonication for 5 min (Heilscher Ultrasonic Processors UP 400, S) in cold condition. The resultant homogenate was centrifuged at 8000 r/min (3300 $\times g$) for 30 min at 4°C; the supernatant was used as a crude extract. The crude extracts were then subjected to three different concentrations 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 acted as control. Chromium reduction was assessed according to the diphenylcarbazide method.

1.9 Chromium reductase induction in the presence of chromium

Protein profile of bacterial cells grown with 100 μg/mL chromium and without chromium was done to compare the inducibility of chromium reductase. The proteins of bacterial isolates were isolated according to Sayyed *et al.* (2000), estimated according to Lowry *et al.* (1951), and electrophoresed according to Laemmli (1970). Aliquots of 15–20 μL were loaded onto a 12% SDS polyacrylamide linear resolving gel overlaid with a 6% stacking gel. Gels were stained with Coomassie Brilliant Blue R-250.

1.10 Statistical analysis

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

2 Results

2.1 Physicochemical characteristics of wastewater

Some physicochemical characteristics of industrial wastewater were ascertained, from where chromium tolerant bacteria were isolated. The temperature of different samples ranged from 24 to 38°C, pH ranged from 6.5 to 8.7, dissolved oxygen ranged from 0.45 ± 0.01 to 1.30 ± 0.03 mg/L, and Cr(VI) ranged from 0.70 ± 0.03 to 1.84 ± 0.04 μg/mL.

2.2 Identification of the bacterial isolates

The partially amplified (500 bp) and sequenced 16S rRNA gene from local isolates (CBL-Cr2 and CBL-Cr3) was uploaded to the National Center for Biotechnology Information (NCBI) website to search for similarity to known DNA sequences and to confirm the species of this local isolate. The BLAST query revealed that this gene is 98% homologous to *Bacillus* sp. JDM-2-1 (CBL-Cr2) and 94% homologous to *Staphylococcus capitis* (CBL-Cr3). The nucleotide sequences coding for the 16S rRNA gene of *Bacillus* sp. JDM-2-1 and *S. capitis* have been submitted to the GenBank database under accession numbers EUO17506 and EUO17509, respectively.

2.3 Optimum growth conditions

The most suitable temperature for Cr-resistant bacterial isolates was found to be 37°C. *Bacillus* sp. JDM-2-1 showed maximum growth at pH 6 while *S. capitis* showed maximum growth at pH 7. The growth curve pattern was studied by growing the organism in the presence of Cr(VI) (100 μg/mL) and comparing with the control culture in which no metal ion was added. Although the growth pattern of the *Bacillus* sp. JDM-2-1 was not significantly different from those of control but the growth rate of *S. capitis* was lower in the presence of Cr(VI). The growth pattern is shown in Fig. 1.

2.4 Metal resistant bacteria

Bacillus sp. JDM-2-1 and *S. capitis* were found to be resistant to chromium up to a concentration of 4500 and 2800 μg/mL, respectively. The bacterial isolates were also checked for their resistance to various other heavy metals. *Bacillus* sp. JDM-2-1 was able to resist Cd^{2+} (50 μg/mL), Cu^{2+} (200 μg/mL), Pb^{2+} (800 μg/mL), Hg^{2+} (50 μg/mL), Ni^{2+} (4000 μg/mL), and Zn^{2+} (50 μg/mL). *S. capitis* was also resisted Cd^{2+} (50 μg/mL), Cu^{2+} (200 μg/mL), Pb^{2+} (800 μg/mL), Hg^{2+} (50 μg/mL), Ni^{2+} (4000 μg/mL), and Zn^{2+} (50 μg/mL).

2.5 Cr reduction ability of the bacterial isolates

Chromium reducing capability of the bacterial isolates was checked by adding Cr(VI) at 100 μg/mL in the culture medium. *Bacillus* sp. JDM-2-1 could reduce Cr(VI) (100 μg/mL) 40%, 66%, 77%, and 85% from the medium after 24, 48, 72, and 96 h, respectively. *S. capitis* was also able to reduce 29%, 53%, 65%, and 81% from the medium after 24, 48, 72, and 96 h, respectively (Fig. 2). As shown in Fig. 3, *Bacillus* sp. JDM-2-1 was able to reduce 18%, 72%, and 86% of Cr(VI) after 48, 96, and 144 h of incubation directly in industrial effluent. *Bacillus* sp. JDM-2-1 was also able to reduce 85% of Cr(VI) in distilled water after 144 h. In comparison, the microbial flora alone of the industrial effluent was able to reduce only 52% of Cr(VI) after 144 h. *S. capitis* was able to reduce 17%, 70%, and 89% of Cr(VI) after 48, 96, and 144 h, respectively. In distilled water with 100 μg Cr/mL, the isolate was able to

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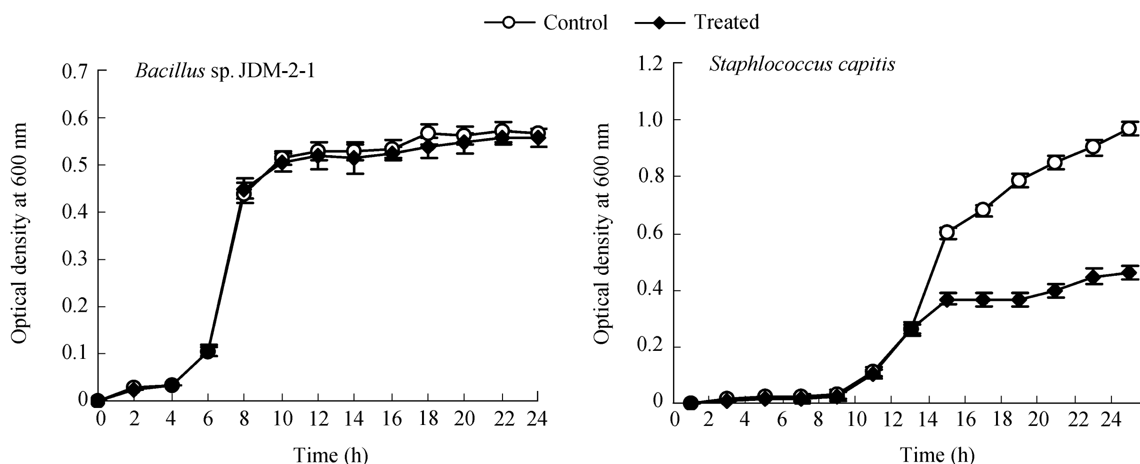


Fig. 1 Growth curves of Cr-resistant *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* in LB medium containing chromium (100 μg/mL) and without chromium after incubation at 37°C.

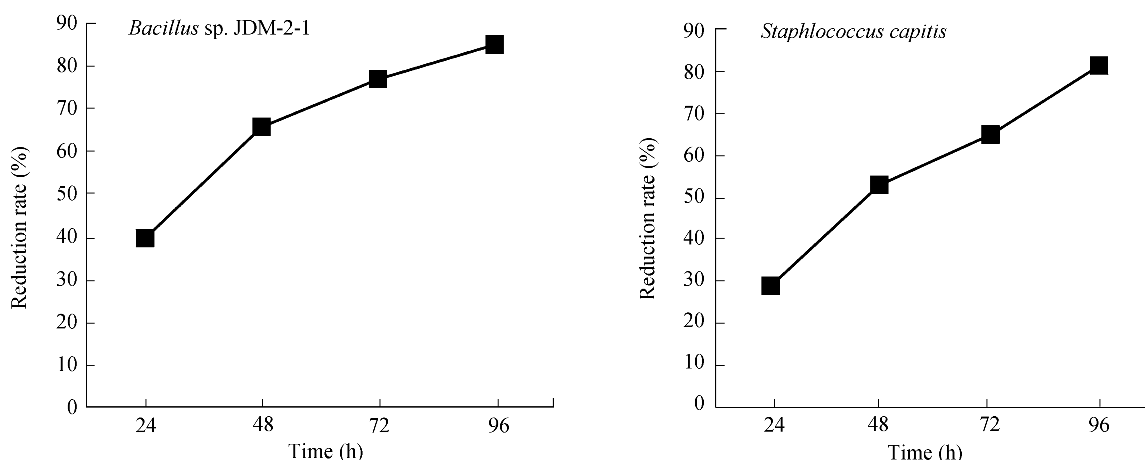


Fig. 2 Chromium reduction by *Bacillus* sp. JDM-2-1 and *Staphylococcus capitis* from the medium containing (100 μg Cr(VI)/mL). Estimations were done at different time periods.

reduce 87% of Cr(VI) after 144 h. The micro-flora of the effluent could reduce up to 62% of Cr(VI) after 144 h of incubation at room temperature.

2.6 Cell free assay

Cell free crude extracts from the *Bacillus* sp. JDM-2-1 reduced Cr(VI) to Cr(III) as shown in Table 1. The cell free extracts of *Bacillus* sp. JDM-2-1 and *S. capitis* were exposed to three different concentrations of Cr, i.e., 10, 50, and 100 μg Cr(VI)/mL and the reduction ability was assessed after 12 h of incubation at 37°C. *Bacillus* sp. JDM-2-1 showed Cr(VI) reduction of 83%, 58%, and 30%, respectively. Cell free extract of *S. capitis* illustrated reduction of 70%, 46%, and 28% at concentrations of 10, 50, and 100 μg Cr(VI)/mL, respectively (Table 1).

Table 1 Hexavalent chromium recovery after incubation with crude cell extracts at different Cr(VI) concentrations

Sample	Cr(VI) (μg /mL)					
	0 h	12 h	0 h	12 h	0 h	12 h
Control	10	10	50	50	100	100
Bacillus sp. JDM-2-1	10	1.7	50	21	100	70
Staphylococcus capitis		10	3.0	50	27	100

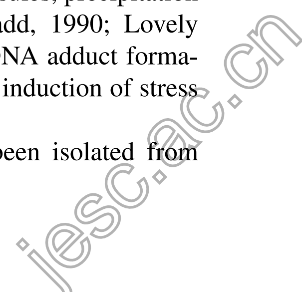
2.7 Induction of chromium reductase

Figure 4 shows the SDS profile of proteins of bacterial isolates grown with 100 μg/mL of chromium and without chromium. Comparison of the profiles shows that a protein of molecular weight around 25 kDa is induced on SDS-PAGE gel in the presence of chromium in both *Bacillus* sp. JDM-2-1 and *S. capitis*.

3 Discussion

A variety of mechanisms exist for the removal of heavy metals from aqueous solution by bacteria, fungi, ciliates, algae, mosses, macrophytes and higher plants (Holan and Volesky, 1995; Pattanapipitpaisal *et al*., 2002; Rehman *et al*., 2007, 2008). The cellular response to the presence of metals includes various processes such as biosorption by cell biomass, active cell transport, binding by cytosolic molecules, entrapment into cellular capsules, precipitation and oxidation-reduction reactions (Gadd, 1990; Lovely and Coates, 1997) as well as protein-DNA adduct formation (Zhitkovitch and Costa, 1992) and induction of stress proteins (Ballatori, 1994).

Chromium resistant bacteria have been isolated from



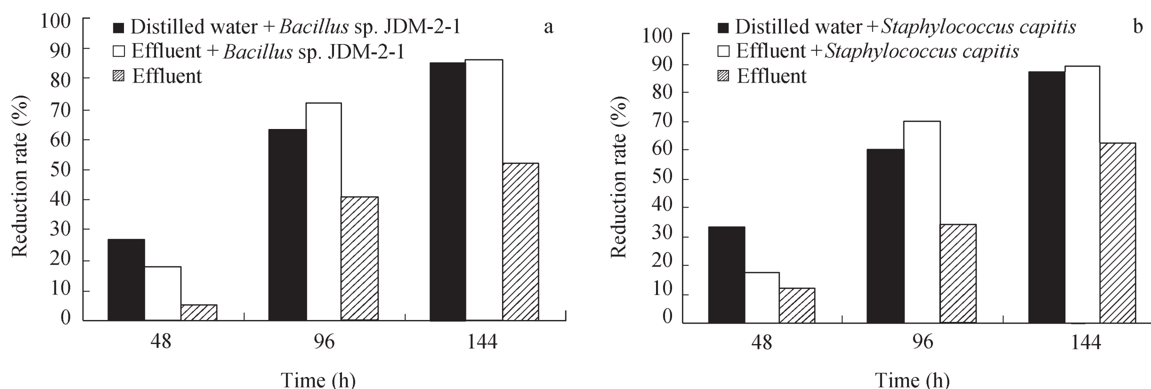


Fig. 3 Percentage reduction of Cr(VI) by *Bacillus* sp. JDM-2-1 (a) and *Staphylococcus capitis* (b) from the solutions (distilled water and industrial effluent) with initial concentration of 100 μg/mL of Cr(VI) after 48, 96, and 144 h of incubation at room temperature.

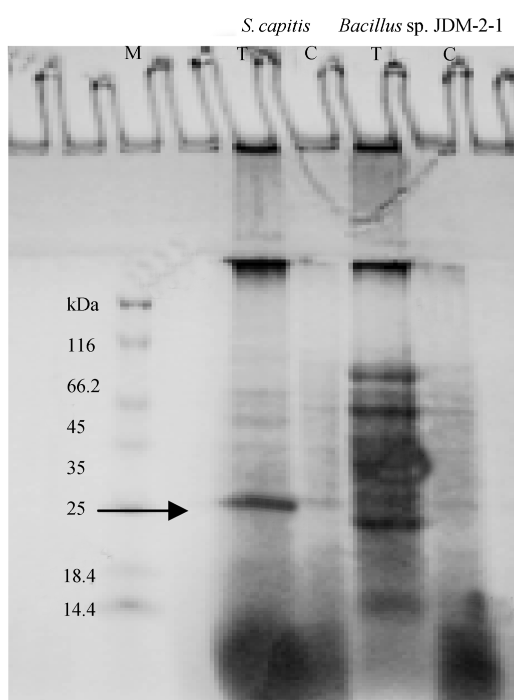


Fig. 4 SDS-PAGE pattern of total proteins of Cr resistant bacterial isolates. M: protein marker; C: control (without Cr); T: Cr treated. The gel is 12% stained with Coomassie Blue.

tannery effluents by several groups (Basu *et al*., 1997; Sultan and Hasnain, 2007). During the present investigation *Bacillus* sp. JDM-2-1 and *S. capitis* both were found to be highly resistant to chromium at a concentration of 4500 and 2800 μg/mL, respectively. *Bacillus* sp. JDM-2-1 showed maximum resistance against Ni^{2+} at 4000 μg/mL and the order of resistance regarding the metal concentration was $Ni^{2+} > Pb^{2+} > Cu^{2+} > Zn^{2+} > Cd^{2+} > Hg^{2+}$. *S. capitis* also showed maximum resistance against Ni^{2+} at 4000 μg/mL and the order of resistance regarding the metal concentration was $Ni^{2+} > Pb^{2+} > Zn^{2+} > Cu^{2+} > Cd^{2+} > Hg^{2+}$.

Chromium(VI) is a common pollutant introduced into natural waters from a variety of industrial effluents and its removal by reduction has been well documented (Pattanapipitpaisal *et al*., 2002; Sultan and Hasnain, 2007). In the present study both *Bacillus* sp. JDM-2-1 and *S. capitis* could reduce Cr(VI) (100 μg/mL) 85% and 81% from the medium after 96 h, respectively. *Bacillus* sp. JDM-2-1 and *S. capitis* were also able to reduce 86% and 89% of Cr(VI) after 144 h of incubation directly from industrial effluent. The nutrient stress conditions have no retarding effect on the Cr(VI) reducing ability of *Bacillus* sp. JDM-2-1 and *S. capitis*. Hence both bacteria not only exhibited the ability to survive in contaminated wastewater but also demonstrated a marked increase in remediation of toxic Cr(VI) in their presence. Several researchers have also reported the direct reduction of Cr(VI) in contaminated effluents of the metal finishing industry (Hardoyo and Ohtake, 1991; Ganguli and Tripathi, 2002).

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).

In the present study the cell free extracts of *Bacillus* sp. JDM-2-1 and *S. capitis* resulted in reduction of 83% and 70% at concentration of 10 μg Cr(VI)/mL, respectively. Ganguli and Tripathi (2002) reported that *Pseudomonas aeruginosa* cells reduced 10 μg/mL chromate completely within 2 h. Hexavalent chromium was reduced from 10 μg/mL to undetectable levels. Experiments with cell free extracts of *Bacillus* sp. indicated that the soluble type of enzymes were responsible for Cr(VI) reduction (Wang and Xiao, 1995).

During the present investigation a protein that has a molecular weight around 25 kDa is induced in the presence of chromium (100 g/mL). Similar types of protein induction studies have been done and chromate reductase has been purified from *P. putida* MK1 which has a molecular weight of 20 kDa on SDS-PAGE (Park *et al*., 2000). In *P. ambigua* chromate reductase was shown to have a molecular weight of 25 kDa on SDS-PAGE but the native protein was 65 kDa. It has been reported in *P. aeruginosa* that a

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30-kDa protein was induced in the presence of chromium (Ganguli and Tripathi, 2001). Thacker and Madamwar (2005) reported that a protein with a molecular weight around 30 kDa was induced in *Ochrobactrum* sp. in the presence of chromium.

4 Conclusions

The possible mechanism of hexavalent chromium reduction by bacteria, isolated from industrial wastewater has been evaluated. *Bacillus* sp. JDM-2-1 and *S. capitis* showed excellent ability to reduce hexavalent chromium to nontoxic trivalent chromium, i.e., 85% and 81%. The induced protein having molecular weight around 25 kDa in the presence of chromium points out a possible role of this protein in chromium reduction. The bacterial isolates can be exploited for the bioremediation of hexavalent chromium containing wastes, since it seems to have a potential to reduce the toxic hexavalent form to its nontoxic trivalent form.

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