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# Chromate Reduction Capability of a Gram Positive Bacterium Isolated from Effluent of Dying Industry

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Chromium is a priority pollutant in many countries and is well known for its toxic, mutagenic, carcinogenic and teratogenic effects on human beings and other living organisms (Ye and Shi 2001). Extensive use of chromium in diverse industries such as leather tanning, stainless-steel production, electroplating, pigment production, textiles, etc., has resulted in large-scale contamination of land and water (Wang and Xiao 1995; Turick et al. 1996; Kamaludeen et al. 2003) which poses a serious threat to human health.

Chromium is present in industrial wastes primarily in the hexavalent form as chromate or dichromate. The Cr(VI) compounds (chromates) are comparatively more toxic than Cr(III) compounds due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids (Basu et al. 1997; Cervantes et al. 2001; Kamaludeen et al. 2003). Chromates are also considered to be mutagenic and carcinogenic in biological systems (Cervantes et al. 2001). Conventionally chromates containing industrial effluents are treated by chemical means, which require high inputs of energy and chemicals, and may also be a source of potential metal pollution from the resultant metal containing chemical sludge (Srinath et al. 2001). Therefore, more practical and economical methods are being explored.

Since trivalent Cr is much less soluble and toxic than hexavalent Cr (chromates) (DeLeo and Ehrlich, 1994), reduction of chromates to Cr(III) represents a potentially useful detoxification process. Several reports have indicated biological reduction of chromate by microorganisms, both aerobes and anaerobes (Komori et al. 1989; DeLeo and Ehrlich 1994; Garbisu et al. 1998; Michel et al. 2001; Viti et al. 2003; Pal and Paul 2004). Biological reduction generates an insignificant quantity of chemical sludge as well as offers potential cost effective remediation strategy. The objective of present investigation was to isolate chromate resistant bacteria and assessment of their chromate detoxification potential for their possible application in environmental clean up.

## MATERIALS AND METHODS

The effluent sample was collected from a dying unit (Al-Amin Dying and

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Finishing Industries) located in Lahore, Pakistan in a sterilized glass bottle. A 50 µL inoculum was spread plated on a nutrient agar plates amended with 100 µg $mL^{-1}$ of chromate salt ($K_2CrO_4$) and incubated at 37°C. The chromate resistant bacterial isolates were purified and successively grown on increasingly higher levels of chromate. One isolate that was able to grow in the presence of >40mg $mL^{-1}$ of chromate was selected for further studies. The bacterial strain was subjected to morphological and biochemical characterization (Moir 1981; Gerhardt et al. 1994).

The effect of chromate on bacterial growth was checked in liquid medium containing a range of chromate concentrations. Culture flasks (250 mL) containing 50 mL nutrient broth supplemented with chromate (0-10 mg $mL^{-1}$) were inoculated with 100 µL from overnight bacterial culture and incubated at 37°C with 150 rpm shaking. Growth was measured at definite time intervals in terms of optical density at 600 nm. The resistance level of chromate resistant bacterial strain was also checked in M9 minimal medium with glucose as carbon source (Gerhardt et al. 1994) (0-6 mg $mL^{-1}$ of chromate). M9 medium (50 mL) adjusted at pH 7 and amended with desirable concentration of chromate in triplicate was inoculated and incubated as stated above. The growth was monitored after 24 hours in terms of optical density at 600 nm.

The resistance of the bacterial strain was also checked against salts (100 µg $mL^{-1}$) of other metals ($BaCl_2$, $CdCl_2$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $HgCl_2$, $MnSO_4$, $NiCl_2$, $Pb(NO_3)_2$, $ZnSO_4$) as well as antibiotics (ampicillin, 300 µg $mL^{-1}$; cefradine, 100; cefadroxil, 100; ciprofloxacin, 100; chloramphenicol, 5; doxycilline, 100; kanamycin, 50; streptomycin, 500; tetracycline, 20) (Hasnain and Sabri 1992; Srinath et al. 2001). The bacterial strain was streaked on nutrient agar plates containing desirable concentration of metallic salts or antibiotics and incubated at 37°C for 24 hours for visible growth.

Chromate reduction ability of the chromate resistant bacterial strain was estimated in the medium used by DeLeo and Ehrlich (1994) (contained g/L: tryptone, 10.0; yeast extract, 5.0; NaCl, 5.0; citric acid, 1.0; $Na_2HPO_4$, 6.9). The effect of temperature, pH, initial cell concentration and chromate concentration on chromate reduction was investigated following Wang and Xiao (1995). Chromate reduction was studied in aerobic batch cultures in 250 mL conical flasks containing 50 mL medium. The autoclaved medium in flasks was amended with appropriate amount of chromate and was inoculated with overnight bacterial culture (containing desirable number of cells $mL^{-1}$) and incubated at desirable temperature with shaking (150 rpm). Samples were removed at definite time intervals, centrifuged (6000 rpm for 10 minutes) and chromate reduction was monitored over time by measuring the disappearance of chromate in the supernatant fluid by using diphenylcarbazide method (APHA 1989). For each chromate reduction experiment cell free controls were also employed to monitor any abiotic chromate reduction. The effect of other metals ($Co^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Pb^{2+}$) on chromate reduction was also checked as described above. All experiments were performed in triplicate.

Results were statistically analyzed for standard error of the means and Duncan's New Multiple Range Test following Steel and Torrie (1981).

## RESULTS AND DISCUSSION

The ever-increasing concern about the toxicity of chromates in industrial effluents coerce the isolation of chromate resistant bacteria and their use for detoxification of toxic chromate contaminated industrial wastes. One chromate resistant bacterial strain SDCr-4 was isolated from the effluents of dyeing industries that could tolerate more than 40 mg $mL^{-1}$ of chromate in the agar medium. Isolation of bacteria resistant to varying degrees of Cr(VI) salts have been reported. A pseudomonad strain CRB5 was tolerant to 500 μg $mL^{-1}$ of chromate (McLean et al. 2000) while *Desulfomicrobium* sp. was able to endure chromate up to 97 μg $mL^{-1}$ (Michel et al. 2001). A gram positive bacterium from tannery wastes tolerated 2.91 mg $mL^{-1}$ $CrO_4^{-2}$ in L-broth (Pattanapipitpaisal et al. 2001). Chromate tolerant bacteria isolated from tannery effluents by Verma and co-workers (Verma et al. 2001) were able to tolerate chromate up to 200 μg $mL^{-1}$. *Bacillus sphaericus* isolated from serpentine soil was tolerant to 800 μg $mL^{-1}$ Cr(VI) (Pal and Paul 2004). Although comparing metal resistance levels with values reported in the literature is not possible due to different types of media and growth conditions employed, however, what this study as well as the cited studies show is that the resistance level of this strain is quite high.

The bacterial strain grew into yellow, convex and circular colonies with entire margin varying in size from 0.5 – 1.0 mm. The cells were gram-positive, non-motile, aerobic cocci. It had cytochrome oxidase and catalase enzyme, showed weak positive result for ONPG test and could hydrolyze gelatin. It gave negative results for methyl red, nitrate reduction, denitrification, starch hydrolysis, sodium citrate, sodium malonate, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, $H_2S$ production, urea hydrolysis, tryptophan deaminase, indole, acetoin and fluorescent pigment tests. The bacterial strain also failed to produce acid from glucose, maltose, sucrose, mannitol, arabinose, sorbitol and inositol but produced acid from rhamnose. It was also able to grow on Simon's citrate agar but not on MacConkey agar, EMB agar and brilliant green agar. On the basis of these characteristics it was tentatively identified as *Micrococcus* sp. (Holt et al. 1994). Previously gram-positive bacteria have been reported to be resistant to hexavalent Cr (Basu et al. 1997; Garbisu et al. 1998; Srinath et al. 2001; Viti et al. 2003; Pal and Paul 2004).

The growth response of *Micrococcus* sp. SDCr-4 towards different concentrations of chromate in liquid culture is shown in Fig. 1A. Growth of SDCr-4 occurred at all concentrations used. The lag phase of the isolate extended to 6 hours both in the absence and presence of chromate. The log phase of SDCr-4 ended after 24 hours in the absence of chromate but it progressed to 72 hours (at 1 and 5 mg $mL^{-1}$ chromate) and 96 hours (at 10 mg $mL^{-1}$ chromate) in the presence of chromate. Although chromate resistant bacterium was able to tolerate more than 40 mg $mL^{-1}$ of chromate in the nutrient agar medium but in M9 minimal medium,

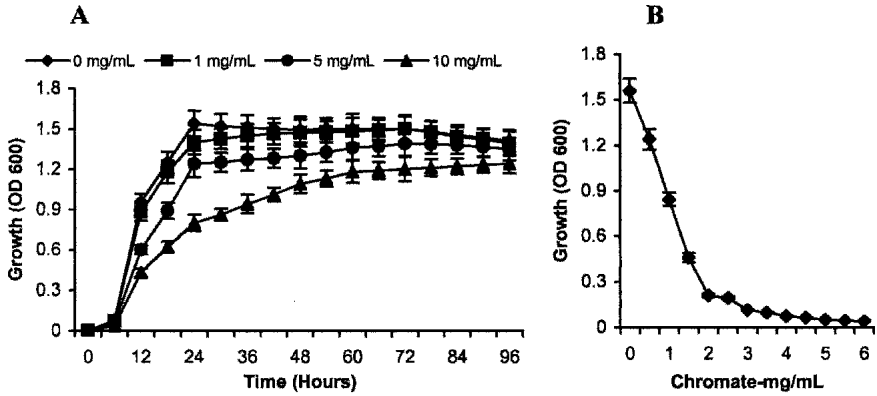


**Figure 1.** Growth response of chromate resistant *Micrococcus* sp. SDCr-4 at varying concentrations of chromate in nutrient broth (a) and M9 medium (b).

it was able to tolerate chromate up to 3.0 mg $mL^{-1}$ (Fig. 1B). *P. fluorescens* LB300 was capable of growth in minimal medium containing 1.5 mg $mL^{-1}$ of chromate (Bopp et al. 1983) while chromate resistant bacterial strains described by Viti and co-workers (Viti et al. 2003) showed chromate MICs of 1.746-4.268 mg $mL^{-1}$. This is because metals are relatively more freely available in liquid than in solid medium thereby increasing metal toxicity in liquid cultures (Yilmaz 2003). Moreover the toxicity of metal ions depends upon the chemical composition of the medium. The various components of rich media such as peptones, yeast extract etc., complex metals thus making them less available and consequently leading to increased resistance to metals in rich media than in minimal media (Hughes and Poole 1991; Nair and Krishnamurthi 1991).

Contaminated habitats are generally characterized by the co-existence of a large number of toxic cations and, therefore, it is necessary to study the multiple metal resistances of microorganisms. Tolerances to other metals have an added advantage of withstanding the presence of different metallic ions while performing the desired activity. The chromate resistant isolate exhibited tolerance against salts of $Ba^{2+}$, $Cd^{2+}(W^{+})$, $Cu^{2+}$, $Fe^{3+}$, $Mn^{2+}$, $Ni^{2+}$ and $Pb^{2+}$ but showed sensitivity to $Co^{2+}$, $Hg^{2+}$ and $Zn^{2+}$. Multiple metal ion resistances in Cr(VI) resistant bacteria have been reported (McLean and Beveridge 2001; Srinath et al. 2001; Viti et al. 2003). The chromate resistant strain also exhibited resistance against ampicillin, cefadroxil, chloramphenicol, kanamycin and tetracycline and was sensitive to other antibiotics tested. Chromate resistant bacteria isolated by Verma et al. (2001) and Viti et al. (2003) also exhibited resistance against various antibiotics.

Table 1 shows the effect of varying temperatures on chromate reduction. Maximum chromate reduction was observed at 37°C. Chromate reduction was comparatively more at 42°C than at 28°C. Optimum temperature for chromate reduction in *Enterobacter cloacae* HO1 was also around 37°C (Ohtake and Silver

1994) while in *Pseudomonas fluorescens* LB300 (Bopp and Ehrlich 1988) and *Bacillus* sp. (Wang and Xiao 1995) it was 30°C. The optimum pH for chromate reduction in chromate resistant bacterial strain was 7 (Table 1). Optimum pH for chromate reduction was also 7 in *P.fluorescens* LB300 (Bopp and Ehrlich 1988), *E. cloacae* HO1 (Komori et al. 1989) and *Bacillus* sp. (Wang and Xiao 1995).

**Table 1.** Effect of varying temperature and pH on % chromate reduction by SDCr-4 after 24 hours of incubation at initial chromate concentration of 100 μg $mL^{-1}$ and initial cell concentration of $10^8$ cells $mL^{-1}$.

| | Temperature °C | | | pH | | |
|---|---|---|---|---|---|---|
| | **28** | **37** | **42** | **6** | **7** | **8** |
| % chromate reduction | 32.90 ±1.15 | 82.03 ±2.54 | 62.70 ±2.38 | 54.23 ±1.84 | 82.03 ±2.54 | 64.88 ±2.33 |

To optimize the inoculum size for chromate reduction, four initial cell concentrations $8\times10^5$, $4\times10^6$, $2\times10^7$ and $1\times10^8$ cells $mL^{-1}$ were used. The trend of chromate reduction by this strain is shown in Fig. 2A. Chromate reduction increased with increase in initial cell concentration. Complete chromate reduction was observed within 32 hours of incubation with higher initial cell concentration of $10^8$ cells $mL^{-1}$ while with other initial cell concentrations the complete reduction was observed within 48 hours of incubation. Similar pattern in chromate reduction was observed in *P. fluorescens* LB300 and *Bacillus* sp. (Wang and Xiao 1995) and *B. sphaericus* AND303 (Pal and Paul 2004). The chromate reduction potential of this strain was also evaluated over initial chromate concentration of about 100 – 1000 μg $mL^{-1}$ (100-1000 mg $L^{-1}$). The chromate resistant bacterium completely reduced 115.5 μg $mL^{-1}$ (115.5 mg $L^{-1}$) chromate within 32 hours whereas complete reduction of 232 μg $mL^{-1}$ chromate (232 mg $L^{-1}$) was achieved within 72 hours (Fig. 2B). The higher chromate concentrations of 532.5 μg $mL^{-1}$ (532.5 mg $L^{-1}$) and 1030 μg $mL^{-1}$ (1030 mg $L^{-1}$) were, however, not completely reduced within 96 hours but the extent of chromate reduction at these concentrations was quite high i.e., 97% at 532.5 μg $mL^{-1}$ and 60.92% at 1030 μg $mL^{-1}$. High concentrations of chromate require longer time for complete reduction. (Ohtake and Silver 1994; Megharaj et al. 2003). Negligible chromate reduction was observed in cell free controls during all chromate reduction experiments. *P. fluorescens* LB300 over a period of 289 hours reduced Cr(VI) by 99.7% with initial chromate concentration of 112.5 mg $L^{-1}$ (112.5 μg $mL^{-1}$), 69% with initial chromate concentration of 200 mg $L^{-1}$ (200 μg $mL^{-1}$) and 61% with initial chromate concentration of 314 mg $L^{-1}$ (314 μg $mL^{-1}$) (DeLeo and Ehrlich 1994). *Bacillus* sp. strain QC1-2 completely reduced 64 μg $mL^{-1}$ chromate in 22 hours (Campos et al. 1995). The pseudomonad strain CRB5 showed complete reduction of 20 μg $mL^{-1}$ chromate after 120 hours (McLean and Beveridge 2001). The chromate resistant strains studied by Viti and co-workers (Viti et al. 2003) showed 56% to 69% chromate reduction after 96 hours of growth with 38.8 μg $mL^{-1}$ chromate. The presence of other metallic ions such as $Co^{2+}$, $Cu^{2+}$, $Mn^{2+}$and $Ni^{2+}$ significantly increased chromate reduction by SDCr-4 (Table 2). $Pb^{2+}$ did not

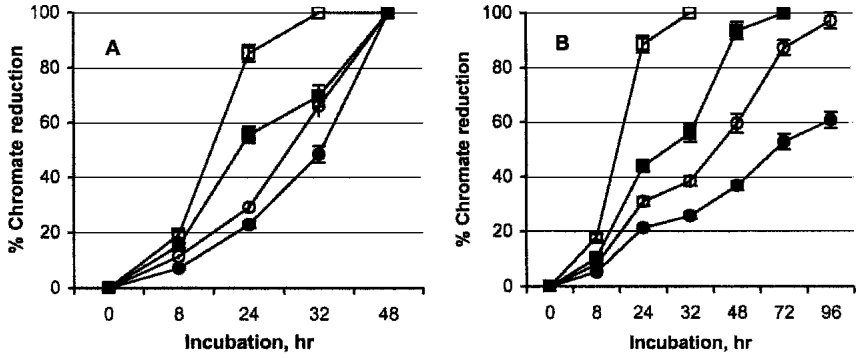


**Figure 2.** A. Effect of initial cell concentration (□, $1\times10^8$ cells $mL^{-1}$; ■, $2\times10^7$ cells $mL^{-1}$; ○, $4\times10^6$ cells $mL^{-1}$; ●, $8\times10^5$ cells $mL^{-1}$) on % chromate reduction by SDCr-4 at pH 7 and 37°C with about 100 μg $mL^{-1}$ of chromate. B. Effect of initial chromate concentration over a range of about 100-1000 μg $mL^{-1}$ (□, 115.5 μg $mL^{-1}$; ■, 232 μg $mL^{-1}$; ○, 532.5 μg $mL^{-1}$; ●, 1030 μg $mL^{-1}$) on chromate reduction by SDCr-4 at pH 7 and 37°C over a period of 96 hours.

affect it but presence of $Cd^{2+}$ was significantly inhibitory for chromate reduction. Presence of divalent metal ions such as $Ni^{2+}$, $Co^{2+}$, $Cd^{2+}$ and $Pb^{2+}$ was inhibitory to chromate reduction by *B. sphaericus* (Pal and Paul 2004). Hence the most prominent feature of the bacterial strain described in this study as compared with bacterial strains reported in cited studies is its high resistance to chromate as well as very high chromate detoxification potential that is also not affected by the presence of other metallic ions. Such strains have become extremely important for developing bioremediation process for detoxification of chromate-contaminated wastewaters.

**Table 2.** Effect of other metals on chromate reduction by SDCr-4 after 24 hours of incubation at 37°C with initial chromate concentration of 200 μg $ml^{-1}$.

| Metals added | Initial concentration μg $ml^{-1}$ | Chromate reduction (%) | Relative (%) chromate reduction |
|---|---|---|---|
| Cr (VI) | 200 | $52.00^{b} \pm 2.42$ | 100.00 |
| Co (II) + Cr (VI) | 20 + 200 | $61.08^{d} \pm 2.85$ | 117.40 |
| Cd (II) + Cr (VI) | 20 + 200 | $48.18^{a} \pm 2.35$ | 92.65 |
| Cu (II) + Cr (VI) | 100 + 200 | $65.56^{e} \pm 3.28$ | 126.07 |
| Mn (II) + Cr (VI) | 100 + 200 | $56.24^{c} \pm 2.74$ | 108.15 |
| Ni (II) + Cr (VI) | 100 + 200 | $65.96^{e} \pm 3.26$ | 126.84 |
| Pb (II) + Cr (VI) | 100 + 200 | $52.21^{b} \pm 2.08$ | 100.40 |

[a,b,c,d,e] Means followed by the same letter are not significantly different at the 0.05 level of significance according to Duncan's New Multiple Range Test.

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