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Isolation and Characterization of Chromium-Tolerant Bacteria from Chromium-Containing Waste Water

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ABSTRACT

Serious chromium (Cr^{6+}) contamination of both surface water and ground water has been reported in the Palar River Basin of Vellore District (Tamil Nadu), India due to the discharge of improperly treated effluents from tanneries and other industries in and around Ranipet. The primary objective of this study was to isolate and identify a potent chromate-reducing bacterial strain. Treated and untreated tannery effluent samples were collected from Ranipet at three different locations for Cr^{6+} analysis and the bacterial population was enumerated. The effluents characteristically contained high levels of Cr. Hence in the present study attempts were made to isolate and characterize chromate-resistant and -reducing bacterial strains. The majority of the chromate-resistant bacterial isolates from the tannery effluents showed a minimum inhibitory concentration (MIC) of Cr^{6+} ranging from 100 to 1500 mg/l. About 32.14% of the total 56 isolates of bacterial strains were able to grow at 200 mg/l Cr^{6+}. The potent Cr^{6+}-resistant strains of *Proteus* sp. V16 and *Pseudomonas mendocina* V42 showed a very high tolerance level to 1500 mg/l and were able to show 100% Cr^{6+} reduction up to 400 mg/l within 80 and 75 h, respectively. The present study conclusively demonstrates the ability of native microbial population present in tannery effluent to reduce Cr^{6+} compounds and is the first report on chromate-reducing bacterial strains from Ranipet tannery effluents showing tolerance up to 1500 mg/l. *Proteus* sp. V16 and *Pseudomonas mendocina* V42 have great potential for bioremediation of Cr^{6+}-containing wastes. This approach permits the selection of bacterial strains which could be used for specific environmental clean up operations.

Keywords: chromate reduction, *Pseudomonas mendocina*, tannery effluent, 16S rRNA
Abbreviations: **AAS**, atomic absorption spectrophotometer; **AAM**, acetate minimal medium; **AR**, analytical reagent; **CFU**, colony forming unit; **CETP**, common effluent treatment plant; **Cr^{6+}**, hexavalent chromium; **DPC**, S-diphenyl carbazide; **MIC**, minimum inhibitory concentration; **OD**, optical density; **PCR**, polymerase chain reaction

INTRODUCTION

Geographically Ranipet is a suburban and industrial area, a part of Vellore District in Tamil Nadu, India, located in 79°19'-79°22' E longitude and 12°53'-12°57' N latitude and is a chronically polluted area. It is one of the biggest exporting centers of tanned leather. The total number of industries located in and around Ranipet town includes 240 tanneries as well as ceramic, refractory, boiler auxiliaries plants, and chromium chemical manufacturing industries (Gowd and Govil 2008). A single tannery can cause the pollution of groundwater around the radius of 7 to 8 km (Ansari *et al.* 1999). During the process of leather manufacturing, several chemicals like $Cr(SO_4)_3$, NaCl, $Ca(OH)_2$, H_2SO_4, etc. are extensively used. Therefore, the resultant effluent is enriched with chromium and sodium salts. Many small-scale tanneries are processing leather in the study area and discharging their effluents onto open land and into surrounding water bodies (Govil *et al.* 2004). The Vellore district possesses vast groundwater potential along and near the Palar river course and its lands are fertile. Serious contamination of both surface water and groundwater has been reported in this basin as a result of uncontrolled discharge of untreated effluents by the tanning industries for the last three decades. About an 11,000 ha area of fertile land has lost its fertility (Thangarajan 1999).

Cr is a transition metal located in group VI-B of the periodic table. Although it is able to exist in several oxidation states, the most stable and common forms are the trivalent Cr^{3+} and the hexavalent Cr^{6+} species, which display quite different chemical properties. Cr^{6+} is considered as the most toxic form of chromium and is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions. Among the effects of increasing industrialization the discharge of heavy metals in effluents that grossly contaminates the environment, chromium compounds are some of the best documented mutagens and carcinogens (Montesano and Hall 2001; Mugica *et al.* 2002; Siva *et al.* 2006). Water containing more than 0.05 mg/l of Cr^{6+} is considered to be toxic (Miklos *et al.* 2000). Cr^{3+} is considered as an essential trace element for the maintenance of effective glucose, lipid and protein metabolism in mammals. On the other hand, Cr^{6+} is toxic for biological systems. Chromium is water soluble and extremely irritating and toxic to human body tissue owing to its oxidizing potential and permeability of biological membranes (Cervantes *et al.* 2001).

The presence of high levels of chromate in the environment also has an inhibitory effect on most microorganisms. However, microorganisms have evolved resistance mechanisms that lead to the selection of resistant variants that can tolerate metal toxicity (Verma *et al.* 2001). Since the discovery of the first microbe capable of reducing Cr^{6+} in the 1970s, the search for Cr^{6+}-reducing microorganisms (both aerobic and anaerobic) has been enthusiastically pursued with numerous strains being isolated. Up to now many bacterial strains such as *Bacillus*, *Shewanella*, *Desulfovibrio*, *Microbacterium*, etc., have been reported to reduce the toxic Cr^{6+} to the less toxic Cr^{3+} which indicates an important bio-remedial step in detoxification of Cr^{6+}-contaminated wastes (He *et al.* 2009). However, the availability of

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an effective Cr^{6+}-removing bacterial strain with significant resistance to Cr^{6+} is an essential pre-requisite for developing a bioremediation process aimed at the detoxification of Cr^{6+}-contaminated waste waters. The objective of this study was to characterize the Cr^{6+} resistance and reduction potential of strains isolated from chromium-contaminated sites in Ranipet.

MATERIALS AND METHODS

Sampling and sample analysis

Effluent samples were collected from three selected location Ranipet, namely, five samples from location A – treated tannery effluent from Common Effluent Treatment Plant (CETP), five samples from location B – Untreated effluent from local tanneries and five sample from location C – discharged treated tannery effluent into the Palar river basin and surrounding fields. The temperature and pH of these samples were recorded at the time of collection. The effluent samples were collected in sterile glass bottles, transported to the laboratory and processed within 6 h of collection. Chromium in the samples was analysed with a Perkin-Elmer 5000 atomic absorption spectrophotometer (AAS) after digestion of samples with a mixture of concentrated nitric (six parts) and perchloric (one part) acid as per standard methods (APHA 1995).

Bacterial population

10 ml of effluent was diluted in 90 ml of $Na_4P_2O_7$ (1 g/l, pH 7.0). Standard serial dilutions followed and 0.1 ml aliquots of 10^{-5} dilution were spread on Nutrient agar (Himedia) plates. Total culturable aerobic bacteria were enumerated by the spread plate counting method. The bacterial populations were enumerated as colony-forming units (CFUs) from a serial dilution of the effluent suspensions. The colonies were counted after incubation for 3 days at 30°C (Hong *et al.* 2008).

Isolation of Cr^{6+}-resistant bacteria

Isolation of the bacterial isolates was done using an enrichment culture technique. Acetate minimal medium (AMM) was used which contained (in 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 $K_2Cr_2O_7$, 0.1. About 10% of each effluent sample was inoculated in a sterile 250 ml conical flask containing 100 ml AMM medium and incubated at 30°C for 24 h on an orbital shaker at 150 rpm. After incubation, the enriched bacterial strains were isolated by plating on AMM with 1.5% agar (Zahoor and Rehman 2008). The isolates were characterized further for chromate reduction.

Determination of MIC and relative growth

MIC of chromium was determined by the broth dilution method. AMM amended with various initial concentrations of chromium ranging from 100 to 2000 mg/l was inoculated with an overnight culture of chromate-resistant bacteria with an absorbance of 0.05 and was incubated at 30°C under shaking condition (150 rpm) for 48 h. The minimum concentration of chromate at which no turbidity was observed in the tube was considered as the MIC of that isolate (Verma *et al.* 2001). Relative growth of the isolates was measured by determining the dry weight of biomass. The biomass was harvested by centrifugation at 10,000 rpm. Washed thoroughly with sterile double distilled water, the pellet was transferred to pre-weighed aluminium cups and dried to constant weight at 80°C. Relative growth was calculated as a percentage of that obtained in the untreated control, which was considered as 100% (Pal *et al.* 2004).

Growth kinetics

Growth of Cr^{6+}-resistance potential bacterial isolates V16 and V42 were studied in 1000 ml flasks containing 500 ml of AMM supplemented with different potassium dichromate concentrations: 200, 500, 1000 and 1500 mg/l. Flasks were inoculated with 0.2 ml of freshly prepared inoculum and incubated at 30°C with 150 rpm shaking for 48 h. Samples were drawn at regular 1-h time intervals. The changes in optical density of the culture during growth were recorded at 540 nm using a SHIMADZU spectrophotometer UV-1601 model (Pal *et al.* 2004).

Identification of potent isolated strain

A standard microbial identification procedure (Cappuccino and Sherman 1999) was used to characterise the isolated bacterial strains and the results were compared with Bergey's Manual of Systematic Bacteriology (Holt *et al.* 1994).

Reduction of hexavalent chromium by bacteria

AMM amended with various initial concentrations of chromium ranging from 100 to 400 mg/l was inoculated with selected bacterial isolates V16 and V42 culture so as to get an OD of 0.05 from overnight grown culture; it was then incubated at 30°C in a shaker at 150 rpm. 10 ml aliquots were withdrawn at regular 5-h intervals and analyzed for chromium reduction, cell viability and other bacterial contamination (Thacker *et al.* 2006).

Cr^{6+} analysis

Samples (10 ml) were withdrawn at regular time intervals and centrifuged at 10,000 rpm for 20 min at 30°C. The supernatant was used to measure chromium concentration. Chromium reducing activity was estimated as the decrease in chromium concentration in supernatant with time using hexavalent chromium-specific colorimetric reagent S-diphenyl carbazide (DPC) at 0.25% (w/v) prepared in acetone (AR) to minimize deterioration (Monteiro *et al.* 2002). The reaction mixture was set up in 10 ml volumetric flask containing 200 or 400 µl sample and the same volume of standard $K_2Cr_2O_7$ (10 mg/l) volume was made up to 1 ml using glass distilled water. A further 330 µl of 6 M H_2SO_4 and 400 µl of DPC were added and the final volume was made up to 10 ml using glass distilled water. Optical density was measured immediately at 540 nm using a SHIMADZU spectrophotometer UV-1601 model (Thacker *et al.* 2006).

Molecular identification of strain V42 by 16S rRNA studies

Since the relative growth of strain V42 was higher than strain V16 in medium containing 1500 mg/l Cr^{+} and since it took strain V42 less time to reduce 400 mg/l Cr^{+} than strain V16, strain V42 was further identified up to the species level using 16S rRNA molecular identification studies. The 16S rRNA sequence of bacterial strain V42 was determined by sequencing the polymerase chain reaction (PCR)-amplified 16S rRNA. Total bacterial DNA was extracted from the isolate and purified. The genes encoding 16S rRNA were amplified using 0.1 µg of the total DNA as the template. The primers used for PCR amplification F27 (5′-AGA GTTTGATCATGGCTCAG-3′) and R1492 (5′-GGCTACCTTGTT ACGACTT-3′) (Heuer *et al.* 1997) were synthesized at Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India. Genes were amplified by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 7 min. The PCR product was sequenced by Bioserve Biotechnologies. The 16S rRNA sequence was examined using BLASTN (NCBI, USA) for similarities and a phylogenetic tree was constructed (Jiang *et al.* 2009).

RESULTS AND DISCUSSION

Cr^{6+} concentration in tannery effluents

Effluent samples were collected from Ranipet at three locations (A, B and C) for Cr^{6+} analysis. The total Cr^{6+} concentrations in the effluents of the study site were generally high while the temperature and pH of effluents in these three locations varied (**Table 1**). The Cr^{6+} concentration ranged from 4.29 to 5.41 mg/l (mean = 4.72 mg/l) at location A. The total Cr^{6+} concentration is in the range of 18.17 to

Table 1 Properties of tannery effluents collected from different locations in Ranipet.

Parameters	Effluent samples		
	Location A	Location B	Location C
Temperature	30.6 ± 0.2	27.4 ± 0.2	29.0 ± 0.2
pH	7.66 ± 0.2	8.20 ± 0.2	7.36 ± 0.1
Cr^{6+} concentration (mg/l)	4.72 ± 0.4	19.30 ± 0.1	7.08 ± 0.2
Bacterial count CFU/ml × 10^{-4}	109.6 ± 0.5	68.2 ± 0.3	78.2 ± 0.5

Average ± standard deviation (n=5)

19.69 mg/l, with a mean value of 19.30 mg/l at location B. The mean concentration of total Cr^{6+} as 7.08 mg/l in the effluents at location C (6.77–7.39 mg/l). These results clearly show that the Cr^{6+} concentration is higher in untreated effluent and is reduced during effluent treatment and the concentration again builds up at the discharge site due to pileup. Thus, there is an urgent need to remove chromium from these locations otherwise there will be an environmental impact on plants and animals as studied previously (Cervantes *et al.* 2001; Ramírez *et al.* 2008). In addition, the entry of these heavy metals into the food chain through contaminated edibles may cause health hazards (Filali *et al.* 2000). Also, the air-borne respirable dust generated from various industrial sites causes a number of health problems (Panigrahi *et al.* 2006). A number of diseases due to metal pollution, especially chromium, are very common among tannery workers in India (Gianello *et al.* 1999; Padma and Bajpai 2008) as they are in constant touch with tannery wastes, the cause of most microbial infections, particularly pathogens present in the effluents and wastes.

Aerobic bacterial counts

The bacterial numbers as CFUs of effluents were measured and their counts are given in **Table 1**. The population of aerobic bacteria in effluents from locations A, B and C show mean values of 109.6 × 10^{-4}, 68.2 × 10^{-4} and 78.2 × 10^{-4} CFU/ml, respectively. This suggests that the very high chromium concentrations in the effluents have been primarily responsible for the decrease in the microbial number whereas, during effluent treatment at location A (CETP), more congenial environmental conditions are maintained, which favors maximum microbial growth.

Isolation of Cr^{6+}-resistant bacteria

Bacterial cultures isolated from three locations of Ranipet were screened for their Cr^{6+} resistance. In total, 56 bacterial isolates representing morphologically different bacterial colonies were able to grow on 100 mg/l chromium-containing plates. Out of 56 bacterial isolates 18 were able to grow at 200 mg/l, while 5 isolates could tolerate chromium up to 400 mg/l, 4 up to 500 mg/l and 2 up to 1500 mg/l. The growth of selected chromate-resistant isolates in Cr^{6+}-containing media (**Table 2**) revealed that the relative growth of 11 isolates (200–1500 mg/l) decreased with increasing concentration of Cr^{6+}. Bacterial isolates V16 and V42 showed a high degree of resistance (1500 mg/l) with relative growth, 26.8 ± 0.1 and 38.2 ± 0.1% higher than the control, respectively. Chromate-resistant bacterial species appear to be abundant in all three locations, although they differ noticeably in their degree of resistance. He *et al.* (2009) reported a bacterial strain from a chromium landfill, chromate factory in Changsha, China which was resistant to 200 to 800 mg Cr^{6+}/l. The strains isolated from Cr^{6+}-contaminated soil could grow up to 100 mg/l in minimal medium. *Ochrobactrum intermedium* CrT-1 and *Brevibacterium* sp. CrT-13 tolerated 1000 mg/l chromate in AMM (Megharaj and Avudainayagam 2003). Based on these results two isolates V16 and V42 were selected for further studies.

Effect of Cr^{6+} on bacterial growth

The growth responses of the two strains towards different Cr^{6+} concentrations in AMM are shown in **Fig. 1**. The two bacterial strains were able to grow even at 200-1500 mg/l Cr^{6+}. There was no remarkable difference in growth patterns for each strain among the tested concentrations, indicating that strains V16 and V42 were tolerant to Cr^{6+} concentrations as high as 1500 mg/l. Nevertheless, the growth of strain V16 was slightly inhibited after 35 h in media containing 500–1500 mg/l Cr^{6+}. Both isolates showed a more or less similar pattern of growth in Cr^{6+}-containing media and a decrease in biomass yield was noted with increasing concentration of Cr^{6+}. Irrespective of the isolate the lag phase of growth was prolonged with increasing chromium concentration and it was maximum with isolate V42. The extended lag period could be due to acclimatization of bacterial cells with a high concentration of Cr^{6+} in the medium.

Identification of isolates

The isolates were characterized morphologically and biochemically (**Table 3**) and identified as *Proteus* sp. V16 and *Pseudomonas* sp. V42 strains as described in Bergey's Manual of Systematic Bacteriology (Holt *et al.* 1994).

Cr^{6+} reducing capacity of bacterial isolates

The chromate-reducing abilities of V16 and V42 bacterial isolates were periodically monitored up to 96 h at different initial concentrations of Cr^{6+} ranging from 100 to 400 mg/l under aerobic condition at pH 7 and 30°C. As shown in **Fig. 2**, V16 *Proteus* sp. could reduce 100, 200, 300 and 400 mg/l of chromate maximally to zero at 30, 55, 60 and 80 h, respectively. *Pseudomonas* sp. V42 was able to reduce 100, 200, 300 and 400 mg/l of chromate to zero at 20, 45, 50 and 75 h, respectively. A high concentration of Cr^{6+} (400 mg/l) was almost completely reduced within 75-80 h. The results indicated that V16 and V42 strains can significantly reduce Cr^{6+}.

In the literature, *Bacillus* sp., isolated from chromate-contaminated soil, reduced Cr^{6+} from 80 to 40 mg/l after 42

Table 2 Relative growths of selected chromate-resistant isolates in Cr^{6+}-supplemented media and minimum inhibitory concentration of Cr^{6+}.

Isolate No.	% of Relative growth in Cr^{6+}, mg/l						MIC[a] of Cr^{6+}
	200	300	400	500	1000	1500	mg/l
V11	42.3 ± 0.1	36.7± 0.2	23.1 ± 0.1	-	-	-	200 - 500
V12	39.6 ± 0.1	27.6 ± 0.1	13.5 ± 0.1	-	-	-	200 - 500
V16	57.6 ± 0.1	52.6 ± 0.2	49.8 ± 0.1	46.2 ± 0.1	43.1 ± 0.2	26.8 ± 0.1	>1500
V23	36.2 ± 0.1	25.4 ± 0.2	14.2 ± 0.05	-	-	-	200 - 500
V32	42.6 ± 0.2	40.2 ± 0.1	36.7 ± 0.2	22.1 ± 0.1	-	-	200 - 1000
V35	43.3 ± 0.1	38.2 ± 0.1	29.3 ± 0.1	19.1 ± 0.1	-	-	200 - 1000
V36	28.3 ± 0.2	23.2 ± 0.1	18.1 ± 0.2	-	-	-	200 - 500
V42	93.7 ± 0.3	89.2 ± 0.2	75.5 ± 0.2	67.7 ± 0.2	50.2 ± 0.1	38.2 ± 0.1	>1500
V43	36.5 ± 0.2	29.8 ± 0.1	24.3 ± 0.1	-	-	-	200 - 500
V44	44.3 ± 0.1	41.5 ± 0.2	36.0 ± 0.1	26.5 ± 0.2	-	-	200 - 1000
V50	40.2 ± 0.05	35.6 ± 0.1	29.3 ± 0.2	18.5 ± 0.1	-	-	200 - 1000

- No growth, Percent relative growth of isolates were measured by determining dry weight of the biomass in metal-amended broth as compared to control set without metal that was taken as 100%, [a]Minimal Inhibitory Concentration, Average ± standard deviation (n=3)

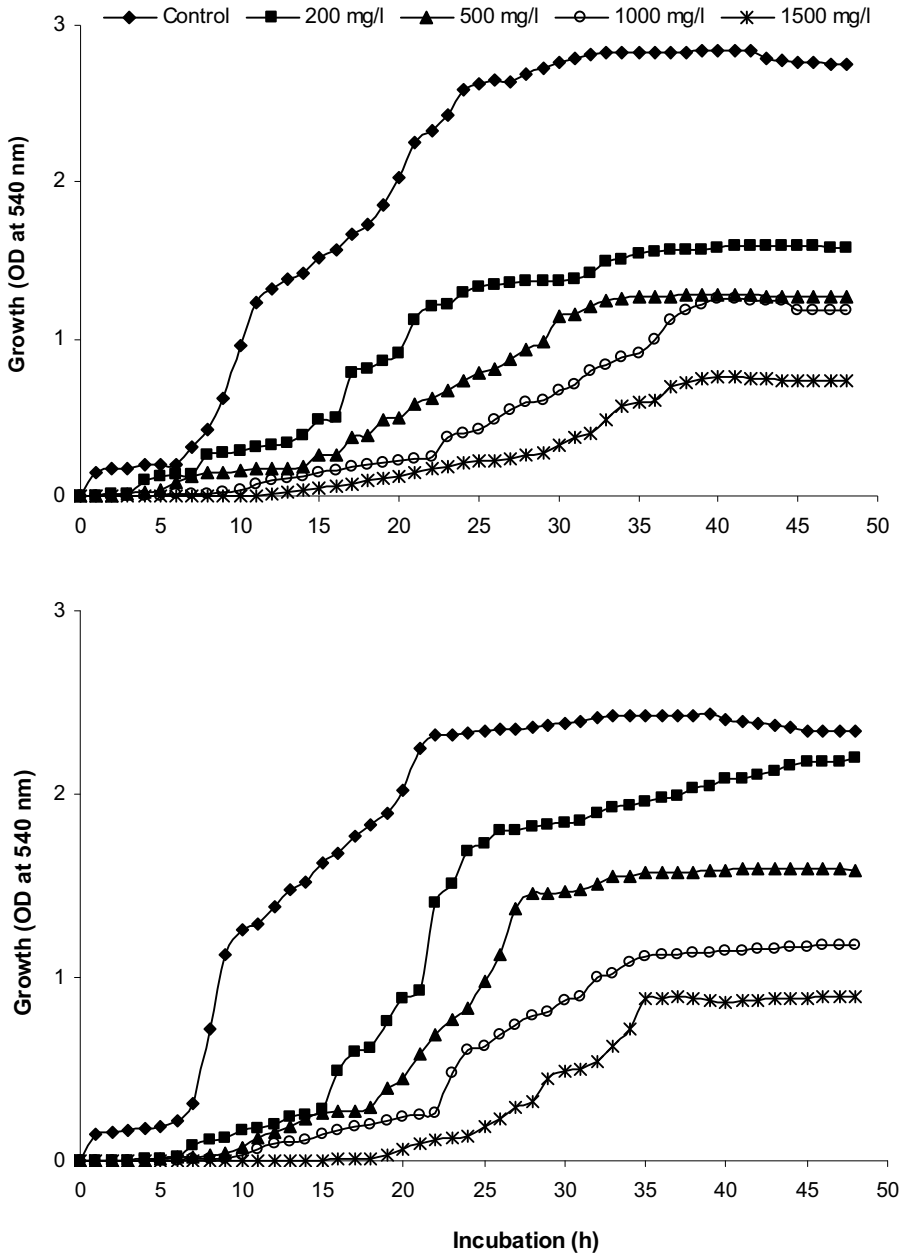

Fig. 1 Growth kinetics of selected chromium-resistant bacterial isolates (V16, top and V42, bottom) in potassium dichromate-containing media. The isolates were grown in acetate minimal medium (♦) supplemented with 200 mg Cr^{6+}/l (■), 500 mg Cr^{6+}/l (▲),1000 mg Cr^{6+}/l (○) and 1500 mg Cr^{6+}/l (*) under 150 rpm shake condition at 30°C.

h in a nutrient medium (Elangovan *et al.* 2006). An *Arthrobacter* sp. isolated from a long-term tannery waste contaminated soil was examined for reduction of hexavalent chromium and found to be able to reduce 30 mg/l of Cr^{6+} during 46 h incubation and did not show any Cr^{6+} reduction at 100 mg/l Cr^{6+} concentration during this incubation period (Megharaj and Avudainayagam 2003). A *Pseudomonas fluorescens* LB 300 strain was able to reduce a high concentration of Cr^{6+} in a batch culture; 61% of Cr^{6+} was reduced over 289 h of observation with an initial Cr^{6+} concentration of 314 mg/l, 69% reduction was observed in a flask with an initial concentration of 200 mg Cr^{6+}/l and 99.7% reduction was obtained with an initial concentration of 112.5 mg Cr^{6+}/l (Deleo and Ehrlich 1994). *Microbacterium* sp. completely reduced 20 mg/l of Cr^{6+} within 72 h (Pattanapipitpaisal *et al.* 2001) while a *Pseudomonad* strain CRB5, however, showed complete reduction of 20 mg/l of chromate after 120 h (Mclean *et al.* 2001). *B. sphaericus* AND303 failed to completely reduce 10 mg/l of Cr^{6+} (Pal and Paul 2004). The results of our study clearly demonstrate that V16 and V42 strains exhibited a higher ability for Cr^{6+} reduction than the bacterial strains previously reported.

Molecular identification of strain V42 by 16S rRNA studies

The bacterial strain V42 with a strong ability to reduce Cr^{6+} was selected for identification using the 16S rRNA gene sequence. An approximately 1.5 kb fragment of the 16S rRNA gene was amplified and purified. The first 779 bases were sequenced using the same PCR primers. This sequence showed 99% similarity with *Pseudomonas mendocina*. The phylogenetic analysis (**Fig. 3**) performed on partial 16S rRNA gene using the neighbor joining method showed that the nearest neighbors are *P. mendocina* strain Lyso H and *P. mendocina* strain PA553 with more than 99% similarity. Hence, strain V42 of present study was identified as *P. mendocina*. This sequence was submitted to GenBank and accepted with accession number GQ910744.

CONCLUSIONS

The present study is the first report on a *Pseudomonas mendocina* strain, identified by 16s rRNA gene studies, which can tolerate Cr^{6+} concentrations up to 1500 mg/l and

Table 3 Morphological and biochemical characteristic of potential chromate-resistant bacterial isolates.

Character	Bacterial isolates	
	V16	V42
Morphological		
Gram staining	Gram (-)	Gram (-)
Cell morphology	Short rods	Short rods
Motility	Swarming motile	Motile
Endospore staining	-	-
Florescence (UV)	-	+
Biochemical		
Catalase production	+	+
Oxidase production	-	+
Indole	-	+
Methyl red	+	+
Voges proskauer	-	-
Citrate utilization	+	+
Urease	+	-
Stretch hydrolysis	+	+
Fermentation of sugar		
Glucose	+	+
Fructose	+	+
Sucrose	-	+
Lactose	-	-
Maltose	+	+
Galactose	+	+
Arabinose	-	-
Raffinose	+	-
Xylose	+	-
Mannitol	-	+

+ Positive response, - Negative response.

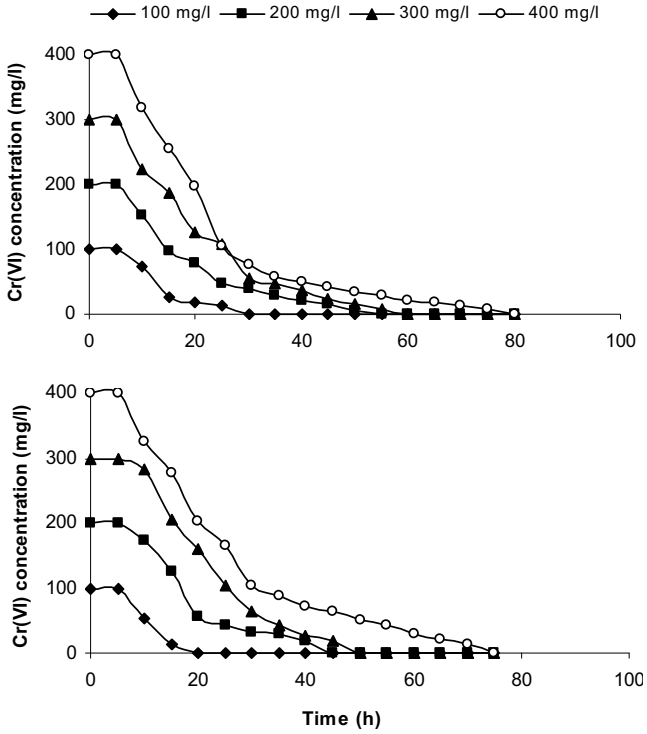

Fig. 2 Time course of Cr^{6+} reduction by *Proteus* sp. V16 (top) and *Pseudomonas mendocina* V42 (bottom) at different initial Cr^{6+} concentrations at pH 7 and 30°C over a period of 80 h.

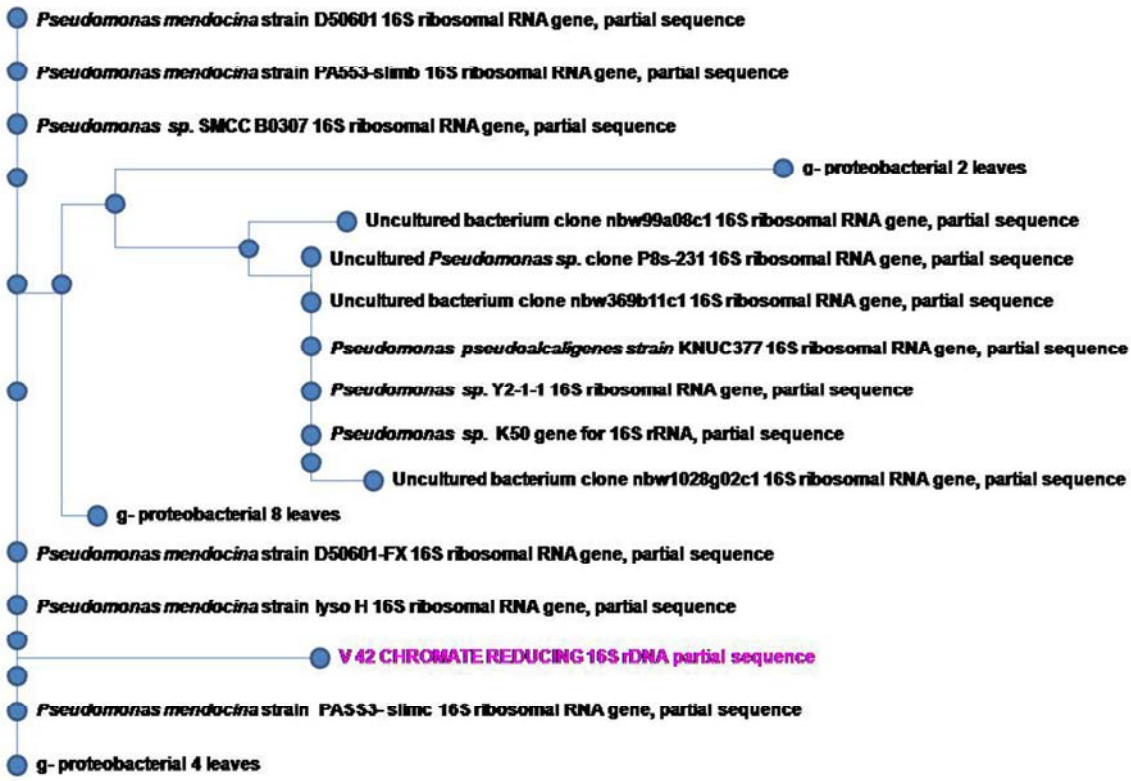

Fig. 3 Phylogenetic tree of partial 16S rRNA gene sequences by neighbor joining method.

capable of 100% reduction of 400 mg/l chromate within 75 h. This isolate may be utilized in biological treatment of tannery effluents, since chemical processing, though less expensive, is a major hazard by itself. Industrial workplaces provide novel enrichment environments for natural selection of potent strains of metal-resistant bacteria since there is little regulatory control over the industrial emissions and thus the environmental burden is severe.

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