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Aerobic chromate reduction by chromium-resistant bacteria isolated from serpentine soil

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Summary

A group of 34 chromium-resistant bacteria were isolated from naturally occurring chromium percolated serpentine soil of Andaman (India). These isolates displayed different degrees of chromate reduction under aerobic conditions. One of the 34 isolates identified as *Bacillus sphaericus* was tolerant to 800 mg l^{-1} Cr(VI) and reduced >80% Cr(VI) during growth. In Vogel Bonner broth, *B. sphaericus* cells (10^{10} cells ml^{-1}) reduced 62% of 20 mg l^{-1} of Cr(VI) in 48 h with concomitant discoloring of yellow medium to white one. Reduction of chromate was pronounced by the addition of glucose and yeast extract as electron donors. In the presence of 4.0 g l^{-1} of glucose, 20 mg l^{-1} of Cr(VI) was reduced to 2.45 mg l^{-1} after 96 h of incubation. Optimum pH and temperature for reduction were 6.0 and 25 °C, respectively. Increase in cell density and initial Cr(VI) concentration increased chromate reduction but was inhibited by metal ions like, Ni^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+}. Experiments with cell-free extracts indicated that the soluble fraction of the cell was responsible for aerobic reduction of Cr(VI) by this organism.

Introduction

Chromium, the transition metal has been designated as a priority pollutant in United States and many other countries due to its carcinogenicity in animals (Enterline, 1974) and mutagenicity in a number of bacterial systems (Petrilli and Flora, 1977). A large quantity of chromium is discharged into the environment mainly from industrial operations including metal finishing industry, petroleum refinery, leather tanning, iron and steel industries and causes a serious threat to human health (Beszedits, 1988). In the industrial wastes it is primarily present in the hexavalent form as

Abbreviations: MIC, minimum inhibitory concentration

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divalent oxyanions, chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). Hexavalent chromium, [Cr(VI)] is highly soluble in water and toxic for most organisms due to their strong oxidizing nature (Yassi and Nieboer, 1988), while trivalent chromium [Cr(III)] is less toxic and forms insoluble oxides and hydroxides above pH 5.0 and is impermeable to biological membranes (Rai et al., 1987).

The conventional methods to detoxify and remove Cr(VI) from the environment involve chemical reduction followed by precipitation, ion exchange and absorption on coal, activated carbon, alum, kaolinite, and flyash (Ohtake and Silver, 1994). During recent years, many bacteria have been reported to reduce Cr(VI) to Cr(III) under aerobic (Garbisu et al., 1998; Ishibashi et al., 1990), anaerobic (Komori et al., 1989; Pattanapipitpaisal et al., 2001) or both (Mclean and Beveridge, 2001; Srinath et al., 2001) conditions.

Chromate-reducing bacteria have been isolated and characterized mostly from chromium-contaminated soil (Mclean and Beveridge, 2001; Viti et al., 2003), wastewater and industrial effluents (Ganguli and Tripathi, 2001; Pattanapipitpaisal et al., 2001; Srinath et al., 2001). However, reports on the occurrence of Cr(VI)-reducing strains from naturally occurring chromium-percolated ecosystem such as serpentine soil are not common. Serpentine outcrops are metalliferrous soil developed over rocks rich in ferromagnesium minerals and are characteristically enriched with heavy metals like Cr and Ni (Brooks, 1987). Heavy metal-resistant microorganisms from serpentine areas of New Caledonia (Amir and Pineau, 1998; Stoppel and Schlegel, 1995) and Central Italy (Mengoni et al., 2001) were found to differ completely from strains occurring in anthropogenically polluted sites (Stoppel and Schlegel, 1995). Cr-resistant (7 mM) bacteria have been isolated from serpentine outcrops of Italy, but their mechanism of Cr-resistance have not been worked out (Mengoni et al., 2001). Serpentine soils of North and South Andaman Islands (India), particularly in Saddle Hills, Chidyatapu and Rutland are reported to be rich in Cr and Ni (Halder, 1984; Jafri et al., 2003; Roy et al., 1988; Vohra et al., 1989) but no microbiological studies have so far been made on these soils. In this paper, we report for the first time the isolation of Cr-resistant microorganisms from such naturally chromium-percolated serpentine soil of Andaman and evaluate their Cr(VI) reducing potential. *Bacillus sphaericus* AND303, a new Cr-resistant strain was isolated from this soil and its chromium reduction ability was examined. Further, Cr(VI) reduction by the new isolate was studied by using cell-free extracts.

Materials and methods

Isolation of chromium-resistant bacteria

Chromium-resistant bacteria were isolated from soil samples collected in sterile glass containers from serpentine areas of Saddle Hills, Chidyatapu and Rutland Island of Andaman, India. These soils are slightly acidic in nature and contain 1656.4–3495.4 mg Chromium, 3523.8–5115.5 mg Nickel and 255.2–433.4 mg Cobalt per kg of dry soil, whereas, extractable metals (in 0.05 N EDTA) per kg dry soil ranged from 11.96 to 16.84 mg Chromium, 447.7 to 591.9 mg Nickel and 44.9 to 244.7 mg Cobalt. Soil samples were serially diluted and plated on Peptone-Yeast Extract-Glucose (PYG) agar plates amended with 200 mg l^{-1} Cr(VI) (as K_2CrO_4) and incubated at 30 °C for 2–4 days. Chromium-resistant strains representing different colony morphologies were purified on the same agar medium, maintained on Cr(VI) amended PYG slants and stored at −20 °C.

Evaluation of chromium-resistance

The minimum inhibitory concentration (MIC) of all the Cr(VI)-resistant isolates were determined by broth dilution method (Calomoris et al., 1984) in PYG medium with Cr(VI) concentrations ranging from 200 to 800 mg l^{-1}. The minimum concentration of metal in the medium inhibiting complete growth was taken as the minimal inhibitory concentration (MIC).

Chromium reduction experiments

In culture

Reduction of chromium was determined by inoculating the isolates in PYG broth supplemented with 50 mg l^{-1} of Cr(VI) and incubated at 30 °C under continuous shaking (120 rpm). Reduction was estimated by measuring the decrease in hexavalent chromium in the culture filtrate at regular time intervals following 1, 5-diphenylcarbazide method (Snell and Snell, 1959). Total chromium was measured using a Varian Atomic Absorption Spectrometer (SpectrAA-20Plus). Chromium content of the biomass was determined following digestion in aqua regia at 80 °C for 2 h.

By suspended cells

Cells from overnight grown culture were harvested by centrifugation at 10,000 × *g* for 10 min at 4 °C, washed and suspended in sterile phosphate buffer (0.2 M; pH 7.0). Reduction of chromate by

suspended cells was determined following the method of Wang and Xiao (1995). Reduction was carried out in sterile medium (20 ml/100 ml flask) containing 20 mg l^{-1} Cr(VI) and a cell density of 10^{10} cells ml^{-1}. The flasks were incubated at 30 °C under continuous shaking (120 rpm) and hexavalent chromium was estimated following usual method.

With cell-free extracts

Cell-free extracts were prepared following the procedure as described by Wang and Xiao (1995). Washed cells from overnight grown culture were suspended in phosphate buffer (pH 7.0) at 5% the original culture volume and disrupted in an ice-bath using an ultrasonic probe. The sonicate was centrifuged at 12,000 × g for 10 min at 4 °C to obtain supernatant (S_{12}) and pellet (P_{12}). A second supernatant (S_{32}) and pellet (P_{32}) were prepared by centrifuging S_{12} fraction at 32,000 × g at 4 °C for 20 min. Fraction S_{32} was further centrifuged at 150,000 × g (Hitachi Automatic Preparative Ultracentrifuge 70P-72) at 4 °C for 40 min to yield the S_{150} supernatant and pellet (P_{150}). The pellets (P_{12}, P_{32} and P_{150}) were re-suspended in phosphate buffer at 5% the original culture volume. Cell-free extracts or pellet suspensions (10 ml), dispensed in 50 ml Erlenmeyer flasks containing 5 mg l^{-1} of Cr(VI) were incubated at 30 °C under continuous shaking (120 rpm) and residual Cr(VI) was measured at regular time interval.

Results

In search for chromium-resistant microorganisms, a total of 34 Cr-resistant bacteria were isolated from serpentine soil following dilution and plating on media amended with 200 mg l^{-1} Cr(VI). The majority (about 62%) of these isolates showed an MIC value of >600 mg l^{-1} Cr(VI), but only about 9% of isolates tolerated 800 mg l^{-1} Cr(VI). The isolates were capable of reducing chromate in different degrees without any accumulation of Cr(VI) in the biomass. About 38.2% of the isolates were able to reduce >40% of Cr(VI) in the medium, while only one strain (AND303) showed >80% chromate reduction after 48 h of growth at 30 °C and was tolerant to 800 mg l^{-1} of Cr(VI) (Table 1).

Isolate AND303, the most potent one, was selected to determine the effect of various environmental factors on chromate reduction. The bacterium was Gram-positive, peritrichously flagellated, rod-shaped, aerobic, and formed terminal endospores. Physiological and biochemical characteristics of the isolate are described in Table 2. Based on comparison of characters as described in Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1989) the isolate was tentatively identified as *Bacillus sphaericus*.

Chromate reduction by washed cells of AND303 was studied in Vogel Bonner (VB) and Minimal salts (MS) broth supplemented with 20 mg l^{-1} of Cr(VI). Significant discoloration of the medium along with 62% reduction of Cr(VI) was recorded in VB broth, while it was comparatively much lower in MS broth (Fig. 1). Chromate reduction in either of the media was without any significant growth. Screening of electron donors in VB broth has indicated pronounced Cr(VI) reduction with glucose and yeast extract. However, cells of *B. sphaericus* were able to utilize a variety of other organic compounds like amino acids, organic acids and complex nitrogenous substances as electron donors during Cr(VI) reduction, but comparatively to a lesser extent. Identical experiments with autoclaved cells failed to detect Cr(VI) reduction (data not shown). The rate of reduction, irrespective of electron donors tested, was faster during the initial stages (24 h) of incubation, which slowed down gradually with time. Glucose (1.0 g l^{-1}) alone reduced 20 mg l^{-1} Cr(VI) to 8.9 mg l^{-1} in 24 h at the rate of 0.46 mg

Table 1. Screening of chromium resistant bacteria from serpentine soil for chromate reduction

MIC[a] (mg l^{-1})	No. of isolates	Percent reduction of Cr(VI)[b]			
		<40	40–60	60–80	>80
>800	3	–	1	1	1
601–800	18	13	3	2	–
400–600	9	5	4	–	–
< 400	4	3	–	1	–
Total	34	21	8	4	1

[a]MIC of isolates was determined by broth dilution method in PYG medium.
[b]Chromate reduction was measured by 1,5-diphenylcarbazide method.

Table 2. Morphological, physiological and biochemical characteristics of bacterial isolate AND303

Characters	Response
Morphological characteristics	
Colony morphology	White, round with smooth margin
Diffusible pigments	None
Gram reaction	+
Micromorphology	Cells rod shaped, single, 0.6 × 1.9 μm
Endospore	Terminal, round, 0.8–1.0 μm diameter
Motility	+
Physiological characteristicss	
UV fluorescence	−
Growth under aerobic condition	+
Growth under anaerobic condition	−
Growth on Mac Conkey agar	−
Growth in presence of lysozyme	+
Growth pH	5.7–11.0, optimum 7.2
Growth temperature	15–42 °C, optimum 30 °C
NaCl tolerance	<7%
Biochemical characteristics	
Production of catalase	+
Production of oxidase	+
Indole production	+
Methyl red test	−
Voges Proskauer test	−
Hydrolysis of starch	−
Hydrolysis of urea	−
Hydrolysis of casein	−
Gelatin liquefaction	+
Oxidation/fermentation (O/F)	−
Nitrate reduction	−
Nitrite reduction	−
H_2S production	−
Acid production from: Melibiose, trehalose salicin inositol sorbitol	+
Arabinose, xylose dextrose, fructose, galactose, mannose, rhamnose, lactose, maltose, sucrose, cellobiose, raffinose, inulin, adonitol, mannitol, dulcitol	−

+, Positive response, −, negative response.

Cr(VI) $l^{-1} h^{-1}$ while the same for yeast extract was 0.42 mg Cr(VI) $l^{-1} h^{-1}$ (Table 3). The efficiency of reduction increased with increasing concentration of glucose and about 20 mg l^{-1} of Cr(VI) was reduced to 2.45 mg l^{-1} in the presence of 4.0 g l^{-1} glucose (Fig. 2). This was accompanied by a sharp decline in glucose level, which was completely exhausted after 60 h of incubation.

The optimum conditions for Cr(VI) reduction by the isolate AND303 were observed at pH 6.0 and 25 °C showing 57.5% and 53.4% reduction after 24 h, respectively (data not shown). Chromate reduction by AND303 increased with increasing cell density and significant reduction was recorded at an initial cell concentration of 10^{10} cells ml^{-1} (Fig. 3). The effect of initial Cr(VI) concentration on reduction was investigated over a range of 10–100 mg l^{-1} Cr(VI). The rate of reduction increased with increase in initial Cr(VI) concentration up to 100 mg l^{-1} and it was highest [1.42 mg Cr(VI) $l^{-1} h^{-1}$] over the initial 24 h of incubation (Fig. 4). The rates, however, declined with time irrespective of the initial metal concentration. But complete reduction was not achieved even at the lowest concentration [10 mg l^{-1} Cr(VI)] used. The presence of additional heavy metals such as Ni^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} were in general inhibitory to chromate reduction. Results as summarized in Table 4 showed that Ni^{2+} at the lowest concentration (20 mg l^{-1}) was most toxic. At higher concentration, Co^{2+} also showed a significant decrease in chromate reduction while there was no considerable variation with respect to Cd^{2+} and Pb^{2+}.

Chromate reduction occurred with cell-free extracts of AND303 under aerobic condition. The soluble fractions (S_{12}, S_{32} and S_{150}) were almost

equally effective in reducing chromate (Fig. 5) but no significant reduction was detected with the insoluble fractions (P_{12}, P_{32} and P_{150}). The active soluble fractions after autoclaving at 15 p.s.i for 15 min, however, failed to show any chromate reduction.

Discussion

Chromium-resistant bacteria capable of reducing chromate have been reported from chromium-polluted environments (Ganguli and Tripathi, 2001; Mclean and Beveridge, 2001; Srinath et al., 2001). However, bacteria resistant to Cr (7 mM) are not uncommon in serpentine outcrops of Italy (Mengoni et al., 2001). But so far none from serpentine soils have been reported to reduce chromate. The present study has clearly indicated the prevalence of chromate-resistant and reducing bacteria in serpentine soils of Andaman.

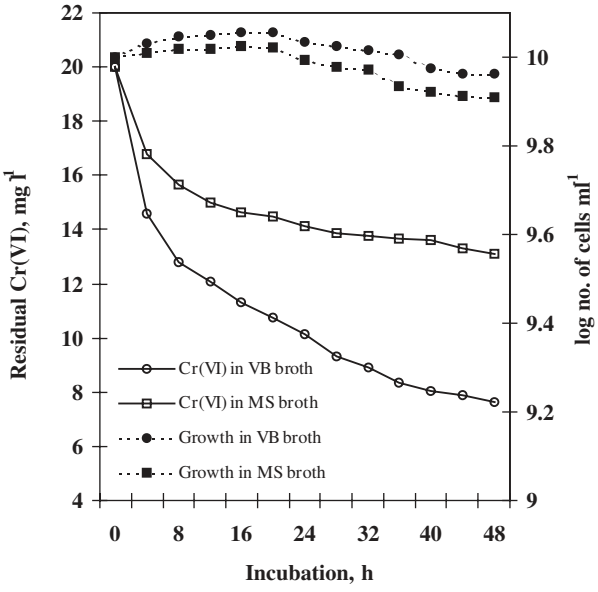

Figure 1. Changes in residual Cr(VI) and cell density during reduction of chromate by cells of *B. sphaericus* AND303 in Vogel Bonner Broth and Mineral Salts Broth.

The most potent strain (AND303) has been tentatively identified as *B. sphaericus* AND303 by morphological, physiological and biochemical analysis. This bacterium reduced chromate aerobically using glucose as one of the efficient electron donors and was accompanied by a change in yellow color of the medium. Similar changes in color were recorded by Mclean and Beveridge (2001) with *Pseudomonas* CRB5. Aerobic Cr(VI) reduction by *Agrobacterium radiobacter*, *B. cereus*, *Escherichia coli* ATCC33456, and *P. fluorescens* LB300 utilized glucose as the electron donor (Wang and Shen, 1995) and in *Bacillus* sp. 2.7 g l^{-1} of glucose was required to reduce 20 mg l^{-1} Cr(VI) to about 12 mg l^{-1} in 24 h (Wang and Xiao, 1995).

A high cell density has been recommended for significant chromate reduction to occur (Wang and Shen, 1995). As with *Microbacterium* MP30 (Pattanapipitpaisal et al., 2001), the rate of Cr(VI) reduction by the present isolate increased with increase in cell concentration ranging from 10^7 to 10^{10} cells ml^{-1}. Likewise an initial cell concentration of 10^{10} cells ml^{-1} was used for aerobic Cr(VI) reduction by *Bacillus* sp. and *P. fluorescens* LB300 (Wang and Xiao, 1995). The rate of chromate reduction is greatly influenced by the initial Cr(VI) concentration (Pattanapipitpaisal et al., 2001; Shen and Wang, 1994; Wang and Xiao, 1995); however, complete reduction is of rare occurrence even at the lowest concentration of metal. The

Table 3. Screening of electron donor for chromate reduction by suspended cells of *B. sphaericus* AND303

Electron donor, 1.0 g l^{-1}	Rate of chromate reduction[a] (mg Cr(VI) l^{-1} h^{-1})			
	Incubation (h)			
	24	48	72	96
Glucose	0.46	0.30	0.22	0.17
Glycine	0.33	0.21	0.16	0.125
Na-acetate	0.19	0.15	0.11	0.086
Na-propionate	0.26	0.17	0.13	0.105
Yeast extract	0.42	0.27	0.20	0.15
Beef extract	0.17	0.12	0.09	0.07
Peptone	0.18	0.13	0.102	0.08
Tryptone	0.14	0.106	0.08	0.06

Reduction was not detected when autoclaved cells were used under identical conditions.
[a]Chromate reduction was studied in VB broth with an initial Cr(VI) concentration of 20 mg l^{-1}.

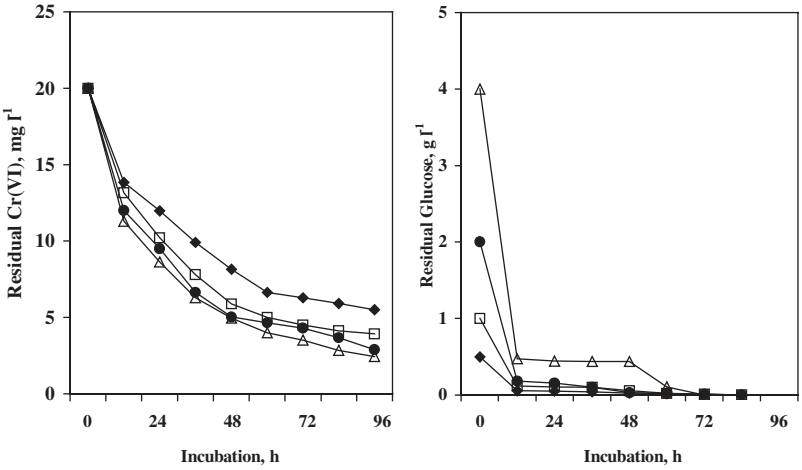

Figure 2. Changes in residual Cr(VI) A and glucose B during the course of chromate reduction by *B. sphaericus* AND303 cells. Reduction was conducted in Vogel Bonner Broth containing 0.5 (-◆-), 1.0 (-□-), 2.0 (-●-) and 4.0 (-△-) g l^{-1} of glucose, respectively.

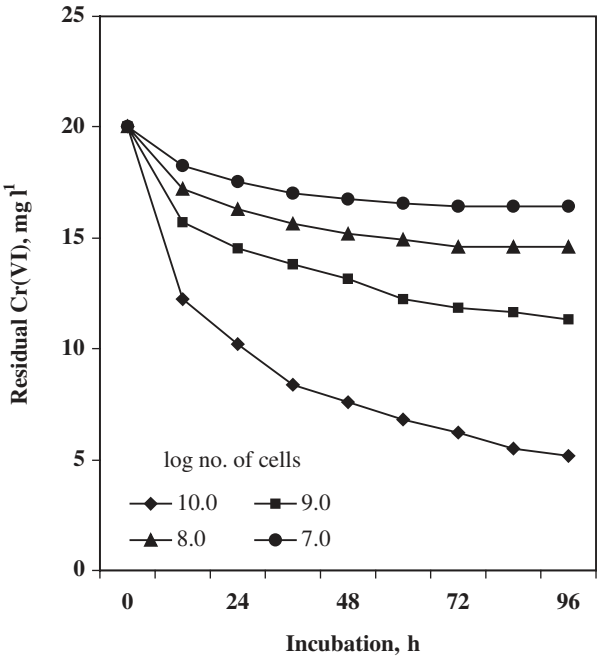

Figure 3. Chromate reduction by *B. sphaericus* AND303 as influenced by cell density.

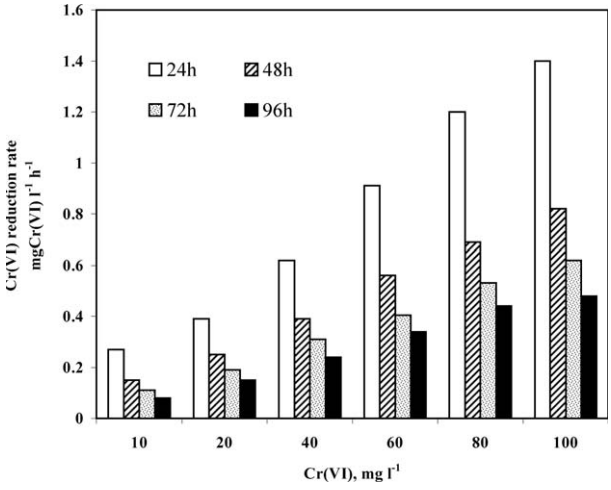

Figure 4. Influence of Cr(VI) concentration on the rate of chromate reduction by *B. sphaericus* AND303 cells in suspension. (Rates were determined based on chromate reduction within corresponding incubation period.)

present isolate AND303, likewise failed to cause complete reduction even at initial concentration of 10 mg l^{-1} Cr(VI). Presence of divalent metal ions such as Ni^{2+}, Co^{2+}, Cd^{2+} and Pb^{2+} were inhibitory to chromate reduction by the bacterium. Nickel, which is prevalent in serpentine soil, was most toxic. Chromate reduction by *E. coli* ATCC33456, however was unaffected by Cd^{2+} and Pb^{2+} at a concentration range of 1–20 mg l^{-1} (Shen and Wang, 1994).

The present findings suggest that a soluble component of the cell-free extract was responsible for chromate reduction by the isolate AND303, which corroborates the earlier studies with *E. coli* (Shen and Wang, 1994); *D. vulgaris* (Lovley and Phillips, 1994); *P. putida* (Ishibashi et al., 1990) and *Bacillus* QC1-2 (Campos et al., 1995). Garbisu et al. (1998) have shown that reduction of chromate by *B. subtilis* was effected by a constitutive system associated with the soluble protein fraction and not with the membrane fraction. Membrane associated Cr(VI) reductase activity, however, was detected in anaerobic bacteria *Enterobacter cloacae* (Wang et al., 1990).

Table 4. Effect of heavy metal supplementation on chromate reduction by *B. sphaericus* AND303 cells

Metal added	Initial concentration[a] ($mg\,l^{-1}$)	Percent Cr(VI) reduced[b]
Cr(VI)	20	47.2
Ni(II)+Cr(VI)	20 (20)	29.25
Ni(II)+Cr(VI)	100 (20)	27.5
Co(II)+Cr(VI)	20 (20)	41.75
Co(II)+Cr(VI)	100 (20)	26.0
Pb(II)+Cr(VI)	20 (20)	35.0
Pb(II)+Cr(VI)	100 (20)	35.0
Cd(II)+Cr(VI)	20 (20)	36.0
Cd(II)+Cr(VI)	100 (20)	35.0

[a]Values in parenthesis represent Cr(VI) concentration.
[b]Chromate reduction was measured by 1,5-diphenylcarbazide method after 24 h of incubation.

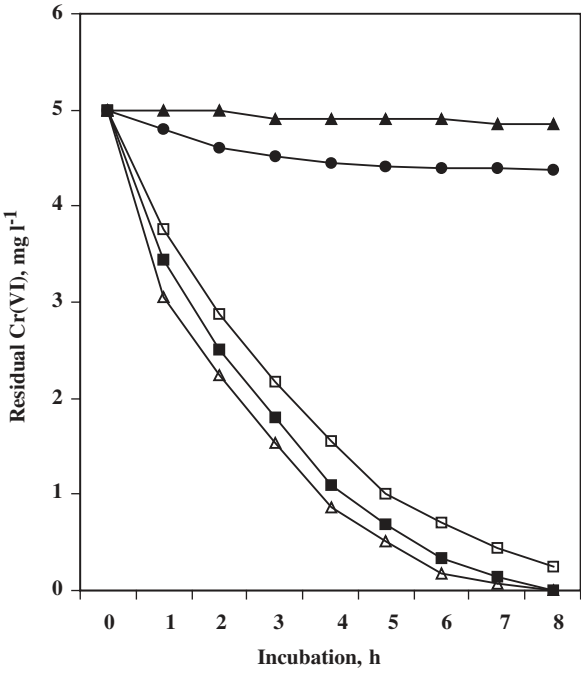

Figure 5. Chromate reduction using cell-free extracts [S_{12} (-□-), S_{32} (-■-), S_{150} (-△-) and autoclaved S_{150} (-▲-)] and pellet suspension P_{150} (-●-) of *B. sphaericus* AND303. (Autoclaved S_{12} & S_{32} and P_{12} & P_{32} were without any Cr(VI) reductase activity and are not shown in the figure.)

The results thus obtained have characterized and identified a new chromate-resistant and Cr-reducing strain, *B. sphaericus* AND303 from serpentine soil, which has established that chromium-resistant bacteria are prevalent in naturally chromate-percolated ecosystem and possess equal potential of reducing chromate under aerobic condition, a process of environmental and biotechnological significance. However, the role of this bacterium in reducing Cr(VI) in serpentine soil and leaching of metals from serpentine minerals is yet to establish.

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