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Reduction of Hexavalent Chromium by Cell-Free Extract of *Bacillus sphaericus* AND 303 Isolated from Serpentine Soil

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Abstract. Cell-free extracts (CFEs) of chromium-resistant bacterium *Bacillus sphaericus* AND 303 isolated from serpentine soil of Andaman, India reduced Cr(VI) in *in vitro* condition, and the reductase activity was solely localized in the soluble cell-fractions (S_{12}, S_{32}, and S_{150}). The enzyme was constitutive as the CFEs from cells grown in Cr(VI)-free and Cr(VI)-containing media reduced a more or less equal amount of Cr(VI). Optimum Cr(VI) reductase activity was obtained at an enzyme (S_{150}) concentration equivalent to 4.56 mg protein/mL, 300 μM Cr(VI) and pH 6.0 after 30 min incubation at 30°C. The enzyme was heat labile; 80% of its activity was lost when exposed at 70°C for 15 min. Kinetics of Cr(VI) reductase activity fit well with the linearized Lineweaver-Burk plot and showed a V_{max} of 1.432 μmol Cr(VI)/mg protein/min and K_m of 158.12 μM Cr(VI). The presence of additional electron donors accelerated Cr(VI) reductase activity of CFE, and an increase of 28% activity over control was recorded with 1.0 μM NADH. Heavy metal ions such as Ni(II), Cu(II), and Cd(II) were strong inhibitors of Cr(VI) reductase unlike that of 100 μM Co(II), which retained 93% activity over control.

Hexavalent chromium [Cr(VI)], a widespread pollutant, is released into the environment from industrial processes and exhibits mutagenic and carcinogenic effects on biological systems [8, 10]. Biotransformation of Cr(VI) to relatively nontoxic trivalent chromium [Cr(III)] by chromium-resistant bacteria (CRB) [1, 5, 7, 12] has offered an economical as well as eco-friendly option for chromate detoxification and bioremediation [11]. Microbial reduction of Cr(VI) occurs either directly (enzymatically) or indirectly through production of hydrogen sulfide that precipitates Cr(VI) as chromium sulfide (Cr_2S_3) [8]. Enzymatic reduction of chromate by CRB proceeds aerobically, anaerobically, or both. In aerobic organisms, chromate reductase activity is mostly localized in the cytosolic fraction of the cell [4] while under anaerobic reduction; chromate is reduced in the membrane [18]. Reduction of hexavalent chromate is a redox reaction and requires supply of electrons. The bacterial enzymes responsible for direct reduction of chromate have not been well characterized to date. Studies using partially purified chromate reductase from *Pseudomonas ambigua* G-1 [15] and *P. putida* MK1 [13] have indicated Cr(VI) reduction using NADH or NAD(P)H as a source of electrons. In *Bacillus* sp. ES 29, chromate reducing activity was localized in the soluble fraction of cell-free extract (CFE), which utilizes NADH as the sole electron donor [1].

Isolation of chromium-resistant bacteria from naturally occurring chromium percolated serpentine outcrops of Andaman, India has been reported, and their potential to reduce chromate during growth was assessed. Whole cell suspension of *Bacillus sphaericus* AND 303, the most potent strain, reduced chromate using glucose as an effective electron donor [12]. The present study was envisaged to elucidate the *in vitro* reduction of Cr(VI) by CFEs of *B. sphaericus* AND 303.

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Materials and Methods

Microorganism and culture condition. *Bacillus sphaericus* AND 303 (MTCC 6512), a chromate-resistant and reducing bacterium [12] isolated in this laboratory from serpentine soil of Andaman, India, was

Table 1. Localization of chromate reductase activity in cell fractions of *Bacillus sphaericus* AND 303

Cell fractions	Growth condition			
	Medium − Cr(VI)		Medium +1mM Cr(VI)	
	Cr(VI) reductase activity,[a] U/mg protein	Cr(VI) reduction,[b] %	Cr(VI) reductase activity,[a] U/mg protein	Cr(VI) reduction,[b] %
Soluble Fraction				
S_{12}	0.752 ± 0.005	45.2 ± 0.3	0.731 ± 0.01	44.4 ± 0.02
S_{32}	0.805 ± 0.037	55.0 ± 2.6	0.811 ± 0.06	56.3 ± 0.09
S_{150}	0.822 ± 0.004	56.2 ± 0.2	0.825 ± 0.05	57.0 ± 0.50
Pellet Fraction				
P_{150}	0.190 ± 0.010	3.0 ± 0.18	0.220 ± 0.01	3.68 ± 0.01

[a]One unit of Cr(VI) reductase activity was defined as the amount of enzyme that convert 1.0 μM Cr(VI) per min at 30°C.
[b]Cr(VI) reduction was measured after 30 min of incubation.
Results represent mean ± standard error of three experiments.
Pellet fractions P_{12} and P_{32} as well as autoclaved supernatant fractions S_{12}, S_{32}, and S_{150} were without any reductase activity and are not shown in the table.

used throughout this study. The bacterium was grown in Peptone Yeast Extract Glucose medium amended with 1.0 mM Cr(VI) (as K_2CrO_4) at 30°C for 24 h under continuous shaking (120 rpm).

Preparation of cell-free extracts. CFEs of *B. sphaericus* AND 303 were prepared following the modified procedure of Wang and Xiao [17]. Cells from overnight-grown culture were harvested, washed, and sonicated in phosphate buffer (pH 7.0) at 5% of the original culture volume using an ultrasonic probe (150 kHz) for 20 min. The sonicate was centrifuged (6000 *g*, 4°C, 10 min) and the supernatant was filtered (0.22 μm) to produce the crude CFE. The CFE was subsequently centrifuged (4°C) at 12,000 *g* (10 min), 32,000 *g* (20 min), and 1,50,000 *g* (40 min) to yield the supernatants S_{12}, S_{32}, and S_{150} and the corresponding pellets P_{12}, P_{32}, and P_{150}, respectively. Chromate reduction experiments were performed using freshly prepared CFEs.

Chromate reductase assay. Chromate reductase activity of CFE was assayed following the procedure of Camargo et al. [1]. The reaction mixture (1.0 mL) contained 200 μM K_2CrO_4 in 0.6 mL of 0.2 M phosphate buffer (pH 7.0). An aliquot of 0.4 mL of CFE was added as the enzyme to initiate the reaction. Reduction of Cr(VI) was measured by estimating the decrease in Cr(VI) in the reaction mixture after 30 min of incubation at 30°C. One unit of Cr(VI) reductase activity was defined as the amount of enzyme that converts 1.0 μM Cr(VI) per min at 30°C. Cr(VI) was quantified colorimetrically using 1, 5-diphenylcarbazide as the complexing reagent [13, 16]. Protein was estimated by the folin-phenol method of Lowry et al. [9] using bovine serum albumin as the standard.

Results

The cell-free extracts (CFEs) of *B. sphaericus* AND 303 fractionated into soluble (S_{12}, S_{32}, and S_{150}) and insoluble (P_{12}, P_{32}, and P_{150}) fractions were tested for Cr(VI) reduction. All soluble fractions reduced Cr(VI) readily under aerobic condition without the presence of any additional electron donors. However, reductase activity was negligible to almost nil in suspended pellet fractions. The chromate reduction efficiency of CFEs obtained from cells pre-grown in the absence or presence of 1.0 mM Cr(VI) was almost equal irrespective of their growth conditions (Table 1). The soluble S_{150} fractions from both sources were most active and reduced 56.2% and 57% of Cr(VI), respectively, in 30 min. However, fraction S_{32} was not inferior to S_{150}. The active soluble fractions, when treated at 15 p.s.i. for 15 min, failed to show any chromate reduction. A soluble component of the cell and not the membrane fraction of *B. sphaericus* AND 303, therefore, appeared to be responsible for Cr(VI) reduction under aerobic condition. Characterization of chromate reductase of CFE was based on soluble fraction S_{150} only, prepared from cells pre-grown in Cr(VI)-free medium.

Time course of Cr(VI) reduction by the soluble fraction S_{150} at an initial Cr(VI) concentration of 200 μM shows that the extent of reduction was rapid during the first 10 min of incubation and continued till 30 min when more than 55% of Cr(VI) was reduced (Fig. 1). In subsequent experiments Cr(VI) reduction was, therefore, measured after 30 min of incubation. A gradual increase in Cr(VI) reduction was obtained with increasing concentration of S_{150} and the optimum concentration was found to be 0.4 mL of the fraction, which was equivalent to 4.56 mg protein/mL.

The effect of initial concentration of chromate on reductase activity of CFE was determined at a concentration range of 0 – 500 μM of Cr(VI). The specific chromate reduction activity increased rapidly with increase in initial Cr(VI) level up to 300 μM, beyond which the activity slowed but continued until 500 μM Cr(VI) (Fig. 2A). The kinetics of Cr(VI) reductase activity fit well with the linearized Lineweaver-Burk plot (Fig. 2B), and the K_m and V_{max} values thus ob-

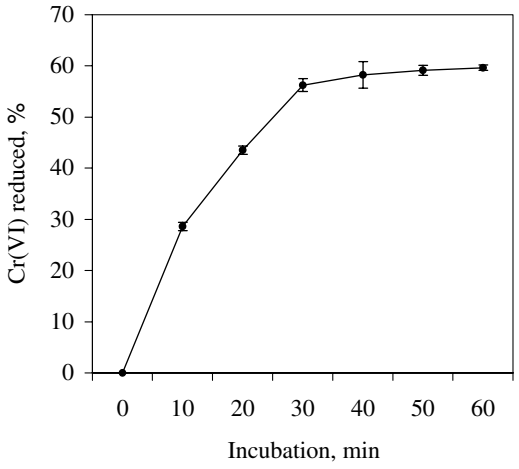

Fig. 1. Chromate reduction by cell-free extract (S_{150}) of *Bacillus sphaericus* AND 303 as a function of time. [The assay mixture (1.0 mL) contained 200 μM Cr(VI) in 0.2 M phosphate buffer (pH 7.0) and incubated at 30°C.]

tained from the Lineweaver-Burk plot were 158.12 μM Cr(VI) and 1.432 μmol Cr(VI)/mg protein/min, respectively.

The effect of pH on Cr(VI) reduction by crude CFE (S_{150}) was determined at a pH range of 4.0 – 8.0 using citrate phosphate (4.0 to 6.5), sodium phosphate (6.0 to 7.5), and Tris-HCl (7.0 to 8.0) buffers of overlapping pH. The Cr(VI) reductase activity was maximum at pH 6.0, and it declined sharply on either side of the pH scale. Similarly, the optimum temperature for Cr(VI) reduction by the soluble fraction S_{150} of *B. sphaericus* AND 303 was 30°C. However, Cr(VI) reductase activity of S_{150} was found to be heat labile, and 80% of the enzyme activity was lost when S_{150} was treated at 70°C for 15 min.

An increase in Cr(VI) reductase activity of CFE (S_{150}) was recorded with addition of NADH (0.1 μM), glucose, and yeast extract (1.0 g/L each) as electron donors, and the activity was further increased with increasing concentration of the respective electron donors. An increase of 28% activity over control was recorded when NADH concentration was raised by 10-fold. However, the presence of additional metal cations, Ni(II), Cu(II), and Cd(II) inhibited Cr(VI) reduction activity (Table 2). Cu(II) and Cd(II) in particular caused nearly 70% inhibition at 100 μM. Co(II) was apparently noninhibitory, causing about 7% loss of activity at 100 μM.

Discussion

Bacillus sphaericus AND 303 has been reported to reduce chromate under aerobic condition [12]. The present findings with soluble fractions (S_{12}, S_{32}, and S_{150}) of

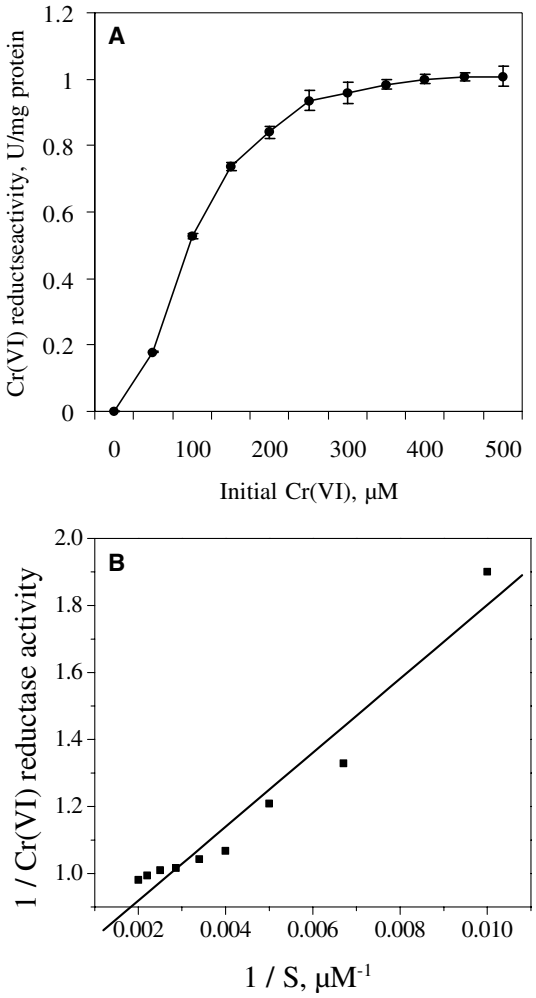

Fig. 2. Effect of initial Cr(VI) concentration on chromate reduction (A) and linearized Lineweaver-Burk plot for Cr(VI) reduction by cell-free extract (S_{150}) (B). [The assay mixture (1.0 mL) contained increasing concentration of Cr(VI) in 0.2 M phosphate buffer (pH 7.0), 0.4 mL of CFE; incubation 30°C for 30 min.]

CFE of *B. sphaericus* AND 303 have clearly indicated the localization of Cr(VI) reductase activity solely in the soluble component of the cell (Table 1). Furthermore, the reductase activity of *Bacillus* AND 303 has been found to be a constitutive one, which is in conformity with the earlier studies on aerobic chromate reduction by constitutive soluble reductases [2, 17] from chromium-resistant *Pseudomonas putida* MK1 [13] and *Bacillus* ES 29 [1].

The optimal reductase activity of the crude soluble fraction (S_{150}) was obtained at an enzyme concentration equivalent to 4.56 mg protein/mL and at 300 μM Cr(VI) (Figure 2A). On the other hand, temperature and pH optima of chromate reductase in *in vitro* condition coincide closely with those of optimal growth conditions

Table 2. Effect of metal cations on chromate reductase activity by CFE (S_{150}) of *Bacillus sphaericus* AND 303.

Metals[a]	Concentration (μM)	Cr(VI) reductase activity (U/mg protein)	Activity (%)
None (control)	—	0.816 ± 0.007	100.0
Ni(II)	10.0	0.746 ± 0.016	91.4
	50.0	0.671 ± 0.018	82.2
	100.0	0.597 ± 0.005	73.2
Co(II)	10.0	0.838 ± 0.009	102.7
	50.0	0.837 ± 0.024	102.6
	100.0	0.762 ± 0.032	93.4
Cu(II)	10.0	0.482 ± 0.015	59.1
	50.0	0.385 ± 0.009	47.2
	100.0	0.266 ± 0.013	32.6
Cd(II)	10.0	0.615 ± 0.021	75.4
	50.0	0.260 ± 0.003	31.9
	100.0	0.253 ± 0.002	31.0

[a]The Cr(VI) content of the assay medium was maintained at 200 μM. Results represent mean ± standard error of triplicate experiments.

of the organism [12]. However, differences in such parameters were recorded with *E. coli* [14], *P. putida MK1* and *P. putida* PRS2000 [13]. The crude enzyme in the present study appeared to be heat labile with a significant loss (80%) of activity at 70°C. CFE of *P. putida* MK1 was reported to retain reductase activity even at 80°C [13]. Comparison of enzyme kinetics revealed that the K_m value obtained with S_{150} fraction (Fig. 2B) of *Bacillus* AND 303 was nearly equal to that of *Bacillus subtilis* $168t^+$ [6], but the maximal velocity of Cr(VI) reduction by AND 303 was recorded to be highest among other chromate reducing Bacilli [1, 3].

The soluble fraction (S_{150}) of CFE of *Bacillus* AND 303 was capable of reducing chromate in the absence of any added electron donor, which supported the concept that endogenous electron reserves can act as electron donors during Cr(VI) reduction [14]. The enzyme activity of AND 303 was enhanced further in the presence of additional NADH, glucose, or yeast extract. Similar enhancement of Cr(VI) reduction by external NADH has also been demonstrated in *E. coli* ATCC 33456 [14]. However, Cr(VI) reductase activity of *Pseudomonas aeruginosa* and *P. putida* was NADH or NAD(P)H-dependent [5, 13]. Inhibitory effects of metal ions on Cr(VI) reduction by intact cells [12] and CFEs [13] have been reported. Similarly, Cr(VI) reduction by CFE (S_{150}) of *B. sphaericus* AND 303 was inhibited by 100 μM Ni(II), Cd(II), and Cu(II), whereas Co(II) at a similar concentration failed to show any significant inhibition (Table 2). On the contrary, chromate reductase activity of CFE of *Bacillus* ES 29 was stimulated by 1.0 mM Cu(II) [1].

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