

F. A. O. Camargo · B. C. Okeke · F. M. Bento ·
W. T. Frankenberger

In vitro reduction of hexavalent chromium by a cell-free extract of *Bacillus* sp. ES 29 stimulated by Cu^{2+}

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Abstract Chromium-resistant bacteria (CRB) isolated from soils can be used to reduce toxic Cr(VI) from contaminated environments. This study assessed in vitro reduction of hexavalent Cr using a cell-free extract (CFE) of CRB isolated from soil contaminated with dichromate. One isolate, ES 29, that substantially reduced Cr(VI) was identified as a *Bacillus* species by 16S rRNA gene-sequence homology. The isolate reduced Cr(VI) under aerobic conditions, using NADH as an electron donor and produced a soluble Cr(VI)-reducing enzyme stimulated by copper (Cu^{2+}). The CFE of the bacterial isolate reduced 50% of Cr(VI) in 6 h. The Cr(VI)-reduction activity of the CFE had a K_m of $7.09 \mu\text{M}$ and a V_{\max} of $0.171 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein. Mercury inhibited the enzyme, but not competitively, with a V_{\max} of $0.143 \mu\text{mol min}^{-1} \text{mg}^{-1}$ protein, a K_m of $7.07 \mu\text{M}$ and a K_i of $1.58 \mu\text{M}$. This study characterizes the enzymatic reduction of Cr(VI) by *Bacillus* sp. ES 29 which can be used for the bioremediation of chromate.

Introduction

High concentrations and toxic levels of hexavalent chromium in the environment are of concern worldwide (Losi et al. 1994). Soluble Cr(VI) is extremely toxic and exhibits mutagenic and carcinogenic effects on biological systems due to its strong oxidizing nature (McLean and Beveridge 2000, 2001). Conventional physical and chemical methods for cleanup of Cr(VI) are not cost-effective and not recommended for full-scale remediation. However, application of Cr-resistant bacteria (CRB) for detoxification of Cr(VI) has been considered as an

economical, effective and safe procedure (Ganguli and Tripathi 2002).

Bioreduction of Cr(VI) can occur directly as a result of microbial metabolism (enzymatic), or indirectly through a bacterial metabolite such as H_2S (Losi et al. 1994). Two enzymatic mechanisms are reported to reduce Cr(VI), both through metabolic activities of the CRB. In aerobic conditions, most of the chromate reductase reported is soluble in the cytosol and reduces Cr(VI) to Cr(III) inside or outside the plasma membrane (Campos et al. 1995; Cervantes et al. 2001). Under anaerobic conditions, CrO_4^{2-} is used as a terminal electron acceptor and is reduced in the membrane during anaerobic respiration (McLean and Beveridge 2001).

In previous work, we showed that the Cr(VI)-reducing activity of intact cells of five CRB isolated from soils contaminated with dichromate may be useful for Cr(VI) detoxification in polluted environments (Camargo et al. 2003). To assess the potential of these CRB for bioreduction of Cr(VI) we studied the nature of the reduction and the involvement of enzymatic reactions. The objectives of this study were to assess in vitro reduction of Cr(VI) by cell-free extracts (CFE) of five bacterial isolates, elucidate the cellular location of Cr(VI) reductase, determine the effect of electron donors, metal ions, and inhibitors on Cr(VI)-reducing activity and obtain the kinetic parameters of Cr(VI) reduction.

Materials and methods

Microorganisms and culture conditions

Five CRB isolated from soils contaminated with potassium dichromate (Camargo et al. 2003) were used in this study. Cells of each bacterium were grown overnight in 500 ml of Luria-Bertani (LB) medium at 30°C with orbital shaking (200 rpm). Thereafter, cells were harvested by centrifugation (4,800 g), and washed twice with phosphate buffer (0.1 M; pH 7.0). Cells were then re-suspended in 10 ml of the same buffer and chilled in an ice bath.

F. A. O. Camargo · B. C. Okeke · F. M. Bento ·
W. T. Frankenberger (✉)
Department of Environmental Science,
University of California,
Riverside, CA, 92521, USA
e-mail: william.frankenberger@ucr.edu

Preparation of CFE

Cells in an ice bath were disrupted with an ultrasonic probe (550 Sonic Dismembrator, Fischer Scientific, Pittsburgh, Pa.). Power was applied five times in 1-min pulses at 50 W each. The sonicate was centrifuged at 6,000 g (7,200 rpm) at 4°C for 15 min and filtered (0.22 µm) to produce a CFE.

Enzyme assay

Hexavalent chromium was determined colorimetrically (λ_{540}) (Spectronic 1001, Milton Roy, Rochester, N.Y.) using the *s*-diphenylcarbazide method (Bartlett and James 1996) with a detection limit of 5 µg l⁻¹. Protein was determined using Coomassie blue (Pierce) at 595 nm, with bovine serum albumin as the standard. The reaction mixture for the enzyme assay contained 1 mg l⁻¹ Cr(VI) as K₂Cr₂O₇ in 0.8 ml of 100 mM phosphate buffer, pH 7.0. After 5 min of pre-incubation at 30°C, the reaction was initiated by the addition of 0.2 ml of the enzyme (as a CFE) and Cr(VI) reduction was measured after 30 min. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 µmol Cr(VI) min⁻¹ at 30°C.

Identification of selected CRB isolate ES 29

The bacterial isolate was identified by 16S rRNA sequencing as follows: ES 29 was grown in LB agar containing 500 mg Cr(VI) l⁻¹ for 2 days at 30°C. Bacterial colonies were suspended in nuclease-free water. DNA was extracted from the suspension according to the method described by Asubel et al. (1997). Colonies of the isolate, suspended in a mixture of TE buffer, SDS (10% w/v) and proteinase K, were incubated for 1 h at 37°C. NaCl (5 M) and CTAB/NaCl solution [4.1 g NaCl and 10 g CTAB (*N*-cetyl-*N,N,N*,-trimethylammoniumbromide) in pre-warmed 100 ml distilled water] were added and incubated for 10 min at 65°C. The solution was extracted by adding 780 µl of chloroform-isoamyl alcohol (24:1), and centrifuged for 5 min to separate aqueous and organic phases. The aqueous phase containing the DNA was further extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) to remove impurities. DNA present in the aqueous phase was precipitated with 0.6 vol. of isopropanol and the DNA precipitate was washed with 70% (v/v) ethanol. The DNA pellet was dried using a lyophilizer and re-suspended in nuclease-free water. Universal bacterial primers corresponding to *Escherichia coli* positions 27F and 1492R were used for PCR amplification of 16S rRNA gene. PCR master mix (Catalogue no. M7502, Promega, Madison, Wis.) was used according to the manufacturer's instructions. Genomic DNA (1 µl) was the template. DNA was amplified using a 35-cycle PCR (initial denaturation, 95°C for 3 min; subsequent denaturation, 95°C for 1 min; annealing temperature, 48°C for 1 min; extension temperature was 72°C for 1 min and final extension was 72°C for 5 min). The PCR product was analyzed on 2% agarose gel and purified using a Qiaex II gel kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, the gel slice was suspended in buffer QxI and the QiaexII suspension was added and mixed by vortexing. The suspension was incubated at 50°C for 10 min, centrifuged for 30 s and the pellet was washed twice with buffer QxI and one time with buffer PE. After air-drying for 15 min, the DNA was eluted with nuclease-free water. DNA cycle sequencing was done with the ABI Prism BigDye terminator kit (Perkin Elmer Applied Biosystems, Foster City, Calif.) and an Applied Biosystems ABI 3100 genetic analyzer. MEGABLAST (Altschul et al. 1997) was used for homology searching and the isolate was deposited with the American Type Culture Collection (ATCC BAA-696).

Effects of electron donors, metal ions and respiratory inhibitors on Cr(VI) reduction

The CFE of ES 29 was characterized to determine the effects of different electron donors, inhibitors and metal ions. The electron donors tested were glucose (0.5 mM), fumarate (0.1 mM), glycerol (1.0%; v/v), ascorbate (0.1 mM), acetate (0.5 mM) and NADH (0.4 mM). The respiratory inhibitors used were NaCN and NaN₃ at 1.0 mM. The metal ions tested were Na₂SO₄, ZnSO₄, MnSO₄, CuCl₂, HgCl₂, AgCl, FeCl₃, MgCl₂ and CoCl₂ at a concentration of 1.0 mM for each metal ion. The reaction mixture for the enzyme assay contained 1 mg l⁻¹ of Cr(VI) as K₂Cr₂O₇, and the inhibitor, electron donor or metal ion to be tested in 0.8 ml of 100 mM phosphate buffer, pH 7.0. After 5 min of pre-incubation at 30°C, the reaction was initiated by the addition of 0.2 ml of enzyme (as CFE). Cr(VI) was measured after 30 min at 30°C, as described above.

Time course and kinetic parameters of Cr(VI) reduction

The time course of Cr(VI) reduction by CFE of isolate ES 29 was evaluated using Cr(VI) as K₂Cr₂O₇ at different Cr concentrations (5, 10, 20 and 40 µM) and incubated for 4 h at 30°C. Time course data were analyzed by application of an exponential decay equation ($Cr-red = Cr-red_{max}e^{-kt}$) to estimate the rate constant (*k*) of Cr reduction and by application of a hyperbolic equation $Cr-red = Cr-red_{max}t/(t_{1/2}+t)$; (where *t* = time in hours) to estimate half-life (*t*_{1/2}). To determine the kinetic parameters, Cr reduction by the CFE was tested at different Cr(VI) concentrations (0–25 µM). The assay mixture was incubated at 30°C for 1 h. The inhibition of 1 mM HgCl₂ on Cr(VI) reduction was evaluated in the same experiment. Kinetic parameters (*K*_m and *V*_{max}) were determined using the hyperbolic equation described for Michaelis-Menten $v_o = V_{max}[S]/[S]_{1/2} + [S]$; where $[S]_{1/2} = K_m$ (Farrel and Ranallo 2000), and *V*_{max} is the maximum nonspecific rate or maximum specific velocity. The kinetic parameters were obtained after fitting the results to the equations using the software, Sigma Plot 2000 (Jandel Scientific Software, San Rafael, Calif.).

Cell fractionation and localization of Cr(VI) reduction

Cells of bacterial isolate ES 29 were grown, harvested and disrupted as described above and the supernatant was centrifuged at 12,000 g at 4°C for 15 min to produce cell-extract supernatant (S_{12k}). A 1 ml volume of S_{12k} was set aside and diluted to 10 ml with water for further assay. An 8-ml volume of the S_{12k} supernatant was centrifuged at 150,000 g and 4°C for 60 min to produce a cell-extract supernatant S_{150k} (soluble fraction) and a membrane pellet (membrane fraction). The membrane pellet fraction was re-suspended in 10 ml of phosphate buffer (0.1 M; pH 7.0). All cellular fractions were analyzed for protein content using the Coomassie blue method. Cell extracts (supernatants S_{12k} and S_{150k}, and membrane fraction) were analyzed for Cr(VI) reduction activity as described above.

Results

The CFE of five CRB tested substantially reduced Cr(VI) (Table 1). Isolates ES 04 and ES 29 showed the most reduction: 81.2% and 79.5% respectively. Isolate ES 29 was selected for further studies based on specific chromate reductase activity and its previous high reduction of Cr(VI) (Camargo et al. 2003). GenBank Blast analysis of the 16S rRNA gene sequence revealed 98% similarity to several *Bacillus* spp., including *Bacillus* sp. LCSAOTU7 (EMBL accession no. AF506057) and *Bacillus* sp. SVM (EMBL accession no. AF503203).

Table 1 Chromate reductase activity in cell-free extracts (CFE) of Cr-resistant bacterial isolates (mean values)

Isolates	Initial protein concentration (mg ml ⁻¹)	Chromate reductase activity (units mg ⁻¹ protein)	Cr(VI) reduction (%)
ES 04	1.167	0.44 ^a	81.2 ^a
ES 23	0.844	0.41 ^{a,b}	54.3 ^b
ES 29	1.066	0.48 ^a	79.5 ^a
ES 32	0.980	0.38 ^{a,b}	57.0 ^b
ES 39	0.662	0.34 ^b	34.9 ^c

^{a,b,c} Means within each column followed by the same letter are not significantly different at the 0.05 level of probability, according to Tukey's test (mean of three replicates)

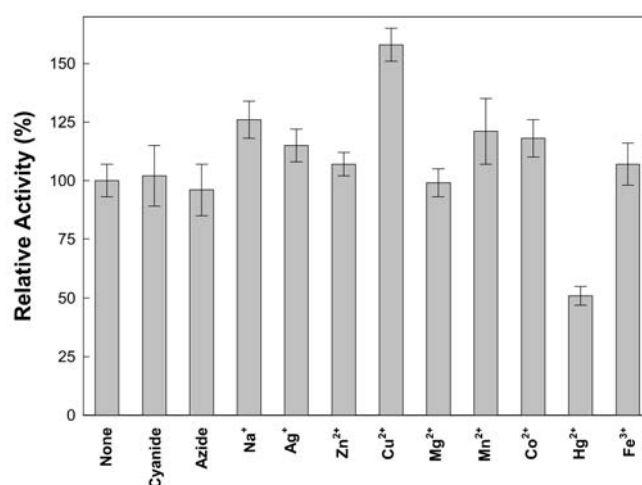
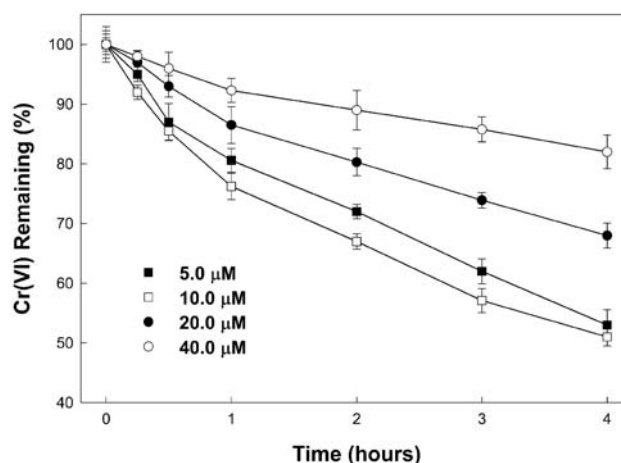
Table 2 Localization of chromate reductase activity in cell fractions of *Bacillus* sp. ES 29 using NADH (0.4 mM) as electron donor (mean values)

Cell fractions	Initial protein concentration (mg ml ⁻¹)	Chromate reductase activity (units mg ⁻¹ protein)	Cr(VI) reduction (%)
Cell-free extract (S _{12k})	0.826	0.467	53.8
Soluble fraction (S _{150k})	0.929	0.517	74.3
Membrane fraction	0.249	0.080	3.1

The effects of electron donors, inhibitors and metal ions on chromate reduction by *Bacillus* sp. ES 29 were also evaluated. Among the electron donors tested, only NADH had a significant effect on chromate reductase activity. A slight abiotic reduction of Cr(VI) by NADH was observed, but it was not substantial compared to Cr(VI) reduction with the cell-free enzyme plus NADH. The respiratory inhibitors tested at 1.0 mM (cyanide and azide) did not significantly affect the relative activity of the CFE of the *Bacillus* sp. ES 29 (Fig. 1). However, the addition of monovalent, divalent and trivalent cations did affect the relative activity of Cr(VI) reduction by the cell-free enzyme extract (Fig. 1). The monovalent cation Ag⁺ did not affect the activity but Na⁺ stimulated chromate reductase activity (26% increase in the relative activity). The trivalent cation Fe³⁺ showed no significant effect on the relative activity (Fig. 1) at 1.0 mM. Among the divalent cations, Zn²⁺ and Mg²⁺ neither stimulated nor inhibited chromate reductase activity. However, Mn²⁺ and Co²⁺ slightly stimulated the enzyme. Cu²⁺ substantially stimulated the activity of the CFE of *Bacillus* sp. ES 29. Mercury (Hg²⁺) strongly inhibited the enzyme with 49% reduction in relative activity.

To identify the cellular location of Cr(VI) reduction, the CFE of *Bacillus* sp. ES 29 was fractionated by ultracentrifugation (Table 2). Analysis of the enzyme activity revealed that most of the activity was in the soluble fraction (74.3% reduction). Very low chromate reductase activity was detected in the re-suspended membrane fraction. The addition of NADH as an electron donor in the reaction increased the Cr(VI) reduction 4.6 times after 30 min of incubation.

The time course of Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29 at different Cr(VI) concentrations is shown in Fig. 2. After 1 h, approximately 15% of the Cr(VI) was reduced in all Cr(VI) concentrations tested. The highest rate of Cr(VI) reduction was observed at 5–10 μM. After 4 h of incubation of the enzyme with 10 μM Cr(VI), approximately 50% reduction was observed. At

**Fig. 1** Effects of inhibitors and metallic cations on chromate reductase activity in cell-free extracts (CFE) of *Bacillus* sp. ES 29. Error bars indicate SD**Fig. 2** Time course of Cr(VI) reduction in CFE of *Bacillus* sp. ES 29 at different Cr(VI) concentrations

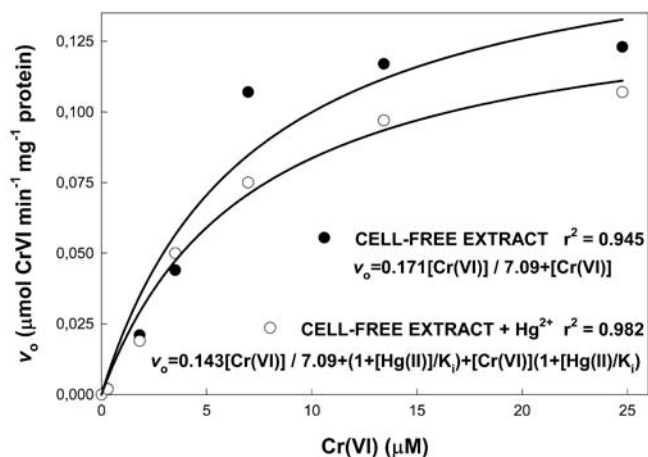


Fig. 3 Kinetics of Cr(VI) reduction in a CFE of *Bacillus* sp. ES 29 at different Cr(VI) concentrations in the absence or presence of HgCl_2 (1 mM)

10 μM , the rate of Cr(VI) reduction was 0.1715 h^{-1} and the $t_{1/2}$ was calculated to be 4.17 h. The least reduction of Cr(VI) was noted at the highest Cr(VI) concentration (40 μM). At 40 μM , the rate of Cr(VI) reduction was estimated to be 0.0481 h^{-1} with a $t_{1/2}$ of 18.83 h.

The saturation kinetics of Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29 fit the hyperbolic equation $v_o = V_{\max}[S]/[S]_{1/2} + [S]$ (Fig. 3). The nonspecific half-saturation or Michaelis-Menten constant, K_m , was $7.09 \mu\text{M}$ and V_{\max} , was $0.171 \mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$. Mercury (Hg^{2+}) significantly inhibited Cr(VI) reduction in the CFE of *Bacillus* sp. ES 29 (Fig. 1). The kinetic data of the inhibitor (Hg^{2+}) showed a reduction in the V_{\max} ($0.143 \mu\text{mol min}^{-1} \text{ mg}^{-1} \text{ protein}$) and did not affect the K_m ($7.07 \mu\text{M}$) of Cr(VI) reduction. The K_i was $1.58 \mu\text{M}$.

Discussion

Biologically mediated reduction of Cr(VI) to Cr(III) is a cost-effective approach in the bioremediation of Cr(VI). A number of studies have demonstrated Cr(VI) detoxification using whole cells (Cervantes et al. 2001); however, only a few studies have demonstrated in vitro reduction of Cr(VI) by microbial enzymes (Cervantes et al. 2001). The use of cell-free enzymes has advantages over using whole cells. Cell-free enzymes are not affected by growth inhibitors, toxins, predators of microbial growth or microbial competition in the environment. Moreover, cell-free enzymes do not require transport mechanisms that may impair microbial uptake of chromate. Furthermore, cell-free enzymes can be immobilized for pollutant removal in reactors. The enzyme can be re-used and there is no need for post-reaction treatment of biomass.

Isolate ES 29 has been described as a Gram-positive rod that forms long, branching chains and heat-stable spores (80°C for 15 min). ES 29 is catalase-positive, hydrolyzes casein gelatin and utilizes carbohydrates under

aerobic and facultative anaerobic conditions (Camargo et al. 2003). It was identified as a *Bacillus* sp. by analysis of its 16S rRNA gene-sequence homology. Probable *Bacillus* spp. identified by biochemical and physiological characteristics have been reported to reduce Cr(VI) (Campos et al. 1995; Shakoori et al. 2000).

Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29 was an NADH-dependent reaction. Our work supports other studies that have reported enzymatic reduction of Cr(VI) under aerobic conditions and which are dependent on NADH (Suzuki et al. 1992; Shen and Wang 1993; Campos et al. 1995; Alvarez et al. 1999; Park et al. 2000; McLean and Beveridge 2001). According to Suzuki et al. (1992), NADH donates an electron to Cr(VI) reducing it to an intermediate form, Cr(V), that accepts two electrons from two molecules of the same coenzyme to produce Cr(III).

Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29 was not inhibited by 1 mM cyanide (NaCN) and azide (NaN_3). Similarly, these respiratory-chain inhibitors did not affect Cr(VI) reduction as reported by Shen and Wang (1993). The inhibitory effect of Hg^{2+} was expected, because mercury binds to a variety of enzyme systems, with a particular affinity for ligands containing SH groups. The inhibitory effect of Hg^{2+} on Cr(VI) reduction in *Pseudomonas putida* was reported by Ishibashi et al. (1990). The other metallic cations supplied as sulfate salts (Na_2SO_4 , ZnSO_4 and MnSO_4) did not affect the relative activity of Cr(VI) reduction. Sulfate is a competitive inhibitor of chromate transport (Cervantes et al. 2001) and can inhibit the chromate reductase activity noncompetitively (Park et al. 2000).

We report for the first time that Cu^{2+} stimulates Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29. Most studies have reported an inhibitory influence of Cu^{2+} on Cr(VI) reduction. Ohtake et al. (1990) demonstrated that the addition of 0.5 mM of Cu^{2+} inhibited 32% of Cr(VI) reduction by a membrane-associated chromate reductase under anaerobic conditions. However, the addition of 5–25 mM Cu^{2+} did not affect Cr(VI) reduction of a soluble chromate reductase under aerobic conditions (Park et al. 2000; McLean and Beveridge 2001). McLean and Beveridge (2001) reported a small increase in Cr(VI) reduction by a pseudomonad when 0.4 mM of Cu^{2+} was added.

The mechanism of stimulation of chromate reductase activity by Cu^{2+} is not clear. However, Cu^{2+} is a transition metal that is a prosthetic group for many reductase enzymes. Its main function has been reported to be related to electron-transport protection or acting as a single-electron redox center and, in some cases, as a shuttle for electrons between protein subunits (Abe et al. 2001). In concentrations above 1 mM, Cu^{2+} may be toxic to microorganisms, but at 1 mM we observed an increase in the relative activity of Cr(VI) reduction. It is also possible that Cu^{2+} is indirectly involved in the protection of chromate reductase from O_2 , for oxygen-sensitive enzymes (Ettinger 1984).

Fractionation and assay of the CFE of *Bacillus* sp. ES 29 revealed that a soluble NADH-dependent enzyme is responsible for the catalytic reduction of Cr(VI). NADH is not a cost-effective electron donor for practical applications in large-scale bioremediation projects. However, addition NADH will be necessary for enzyme activity determination in purification and characterization studies.

The analysis of the kinetic data of Cr(VI) reduction by the CFE of *Bacillus* sp. ES 29 showed differences when compared to the results obtained with the intact cells (Camargo et al. 2003). At the same Cr(VI) concentrations, the intact cells of the isolate ES 29 were responsible for 62% of Cr(VI) reduction after 4 h of incubation (Camargo et al. 2003), while the CFE reduced only 50% of the Cr(VI) added after the same time of incubation. This difference could be attributed to limited availability of NADH and/or other biomolecules that promote Cr(VI) reduction in actively growing cells, or to a decrease in enzyme activity during extraction. The K_m obtained in the CFE was about 50% less than the K_m observed for the intact cells. Hg^{2+} was a noncompetitive inhibitor (probably reversible) of Cr(VI) reduction because only the V_{max} was affected. The K_m was not significantly altered by this divalent cation.

In summary, we assessed the in vitro reduction of Cr(VI), using a CFE of a CRB isolate identified as *Bacillus* sp. ES 29. Characterization of the chromate reductase present in this isolate may be an important step toward establishing a cleanup strategy for soil contaminated with hexavalent Cr.

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