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Chromate Reduction by Cell-Free Extract of *Bacillus firmus* KUCr1

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Abstract

Microbial enzymatic reduction of a toxic form of chromium [Cr(VI)] has been considered as an effective method for bioremediation of this metal. This study reports on the *in vitro* reduction of Cr(VI) using cell-free extracts from a Cr(VI) reducing *Bacillus firmus* KUCr1 strain. Chromium reductase was found to be constitutive and its activity was observed both in soluble cell fractions (S_{12} and S_{150}) and membrane cell fraction (P_{150}). The reductase activity of S_{12} fraction was found to be optimal at 40 μM Cr(VI) with enzyme concentration equivalent to 0.493 mg protein/ml. Enzyme activity was dependent on NADH or NADPH as electron donor; optimal temperature and pH for better enzyme activity were 70°C and 5.6, respectively. The K_m value of the reductase was 58.33 μM chromate having a V_{max} of 11.42 μM/min/mg protein. The metabolic inhibitor like sodium azide inhibited reductase activity of membrane fraction of the cell-free extract. Metal ions like Cu^{2+}, Co^{2+}, Ni^{2+} and As^{3+} stimulated the enzyme but others, such as Ag^{+}, Hg^{2+}, Zn^{2+}, Mn^{2+}, Cd^{2+} and Pb^{2+}, inhibited Cr(VI) reductase activity.

Key words: *Bacillus firmus*, cell-free extract, chromium reductase

Introduction

Application of Cr-resistant bacteria to detoxify Cr(VI) has been considered as an effective bioremediation method (Ohtake and Silver, 1994; Shakoori *et al*., 1999; Ganguli and Tripathi, 2002; Cheung and Gu, 2007; Sau *et al*., 2008). Bioremediation of Cr(VI) can occur by enzymatic reduction of Cr(VI) to Cr(III) *via* Cr(V) and Cr(IV) intermediates (Camargo *et al*., 2003; Xu *et al*., 2004; Xu *et al*., 2005; Pal *et al*., 2005; Cheung *et al*., 2006) or through immobilization (Losi *et al*., 1994). In the course of aerobic reduction, the cytosolic fractions of most chromium resistant bacteria exhibit Cr(VI) reductase activity (Campos *et al*., 1995; Cervantes *et al*., 2001), while under anaerobic conditions, CrO_4^{2-} is used as a terminal electron acceptor and is reduced in the cell membrane (McLean and Beveridge, 2001).

Requirement of electron supply is obvious during the reaction process, considering the process a redox reaction. The enzyme responsible for Cr(VI) reduction has not yet been well characterized, though there are reports on reduction kinetics of Cr(VI) reduction by cell-free extracts (CFE) with varied results (Ishibashi *et al*., 1990; Suzuki *et al*., 1992; McLean and Beveridge, 2001; Park *et al*., 2000; Camargo *et al*., 2003; Pal *et al*., 2005). Studies using partially purified Cr(VI) reductase from *Pseudomonas ambigua* G-1 (Suzuki *et al*., 1992) and *P. putida* MK1 (Park *et al*., 2000) have indicated Cr(VI) reduction using NADH or NADPH as a source of electrons. In *Bacillus* sp. ES29, Cr(VI) reductase was found in the soluble fraction of CFE, which utilizes NADH as electron donor (Camargo *et al*., 2003).

Cr(VI) reducing *Bacillus firmus* KUCr1 has been reported and its potential to reduce Cr(VI) using whole cells under culture condition was documented earlier (Sau *et al*., 2008). This study reports on the reduction of Cr(VI) by CFE of this strain, effects of different electron donors, metal ions, inhibitors, pH and temperature on its Cr(VI) reducing activity, and cellular localization of Cr(VI) reductase.

Experimental

Materials and Methods

Microorganism and growth condition. A Cr(VI) resistant *Bacillus firmus* KUCr1 (NCBI GenBank 16S rDNA sequence Accession No EU784699) was earlier reported (Sau *et al*., 2008) and used in this study.

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Cells were grown aerobically in PYG medium (peptone 10 g/l; yeast extract 5 g/l; glucose 3 g/l; pH 7.2) supplemented with or without (control) 0.1 mM Cr(VI) as K_2CrO_4 under continuous shaking on a rotary shaker at 35°C for 24 h.

Preparation of cell-free extract (CFE) and cellular fractions. Cell-free extract (CFE) and cellular fractions were prepared following McLean and Beveridge (2001) with little modification. Cells grown in PYG medium were harvested at the exponential phase, washed three times in 0.2 M phosphate buffer (pH 7.2) by centrifugation (4000 × g at 4°C), resuspended in the same buffer to have a 10 ml suspension and kept in an ice bath. Cells were disrupted with an ultrasonicator (Hielscher Ultrasound Technology, UP50H). Power was applied five times in 1min pulses with 100% amplitude. After sonication the suspension was centrifuged at 12 000 × g for 10 min at 4°C to obtain a soluble fraction (S_{12}). Five ml of S_{12} fraction was centrifuged at 150 000 × g for 90 min at 4°C to obtain S_{150} fraction. The pellet fraction was washed twice with 0.2 M phosphate buffer (pH 7.2) to remove soluble chromium reductase, if any and was resuspended in same buffer (5 ml) to obtain membrane fraction (P_{150}). Equivalent volume of S_{12}, S_{150} and P_{150} fraction were assayed for studying the cellular localization of Cr(VI) reductase enzyme.

Cr(VI) reductase assay. Cr(VI) reductase activity of CFE was assayed following the procedure of Park *et al*. (2000). The reaction mixture (1 ml) for the enzyme assay contained 0.2 mM K_2CrO_4, 0.2 mM NADH and 400 µl of CFE in 0.2 M phosphate buffer (pH 7.2). The reaction was stopped by adding 0.5 ml of 20% trichloroacetic acid (Horitsu *et al*., 1987). Reduction of Cr(VI) was measured by estimating the decrease in Cr(VI) in the reaction mixture after 30 min of incubation at 35°C and quantified colorimetrically using 1,5-diphenylcarbazide as the complexing reagent (Urone, 1955). To eliminate the abiotic reduction of Cr(VI), if any, a control set was prepared through out the course of the study without putting any biotic component including bacterial cell. Whenever required, abiotic reduction of Cr(VI) has been subtracted from the total reduction to get the exact influence on chromium reduction by the biotic factors. Amount of protein in the CFE was estimated by the folin-phenol method (Lowry *et al*., 1951) using bovine serum albumin as the standard. One unit of Cr(VI) reductase activity was defined as the amount of enzyme which decreased l.0 µM Cr(VI) per min at 35°C. The effect of pH and temperature on Cr(VI) reductase were measured at different pH values (4 to 10.6) of the reaction mixture at 35°C and at different reaction temperature (20 to 100°C) at pH 7.2 respectively.

Results and Discussions

The reductase activities of the soluble fraction (S_{12}) from both the induced and uninduced cells of *B. firmus* KUCr1 were almost similar with regard to time course (Fig. 1). This study revealed that the chromate reductase in this strain is constitutive, which supports earlier reports on the enzymatic reduction of Cr(VI) under aerobic conditions (Bopp and Ehrlich, 1988; Campos *et al*., 1995; Wang and Xiao, 1995; McLean and Beveridge, 2001; Pal *et al*., 2005), though an inducible reductase in the soluble fraction of CFE of *Ochrobactrum* sp. was reported (Thacker and Datta, 2005). As the reductase activity was found to be constitutive, thus for further experiments CFE (S_{12}) was prepared from cells grown in Cr(VI)-free medium for characterization of chromate reductase activity.

The effect of initial concentration of Cr(VI) on reductase activity of S_{12} fraction was determined at a concentration range of 0 to 80 µM of Cr(VI). Specific activity increased with increasing initial concentration of chromate up to 40 µM, after that it slowed down but reduction continued (Fig. 2a) with enzyme equivalent to 0.493 mg protein/ml. The saturation kinetics of Cr(VI) reduction of S_{12} fraction fit with the linearized Lineweaver-Burk plot, and the apparent Michaelis-Menten constant (K_m) was found at 58.33 µM chromate and V_{max} was 11.42 µM per min/mg protein (Fig. 2b). The K_m and V_{max} values differed from the enzyme activity of the CFE of *Bacillus* sp. ES 29 (Camargo *et al*., 2003), *B. subtilis* (Garbisu *et al*., 1998), *B. Sphaericus* AND 303 (Pal *et al*., 2005), *P. putida* (Ishibashi *et al*., 1990; McLean and Beveridge

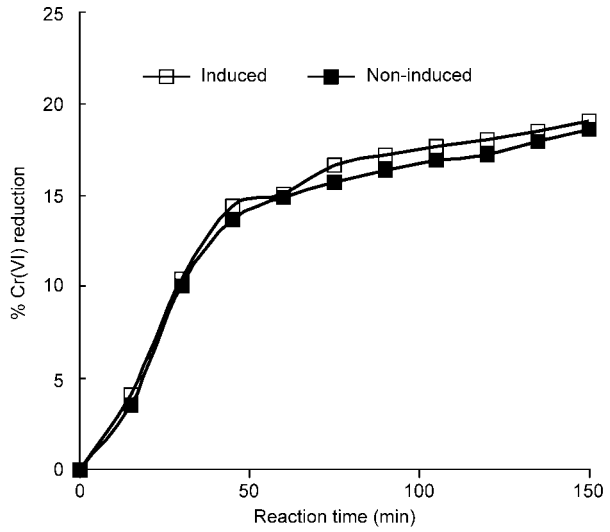

Fig. 1. Cr(VI) reduction by the extracts (S_{12}) from both induced [0.1 mM Cr(VI) in the medium] and non-induced cells of *B. firmus* KUCr1. The reaction mixture contained 0.2 mM Cr(VI) and 0.2 mM NADH as electron donor in 0.2 M phosphate buffer (pH 7.2) and the reaction temperature was 35°C.

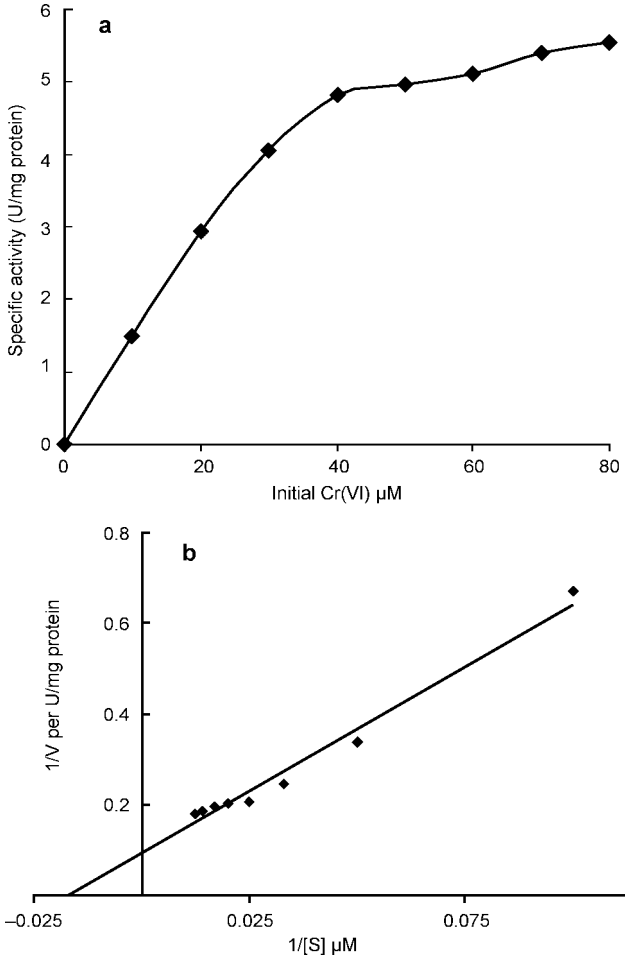

Fig. 2. Kinetics of Cr(VI) reduction of cell-free extract (S_{12}) of *B. firmus* KUCr1 at different Cr(VI) concentrations. Reaction time was 30 min at 35°C (a). Linearized Lineweaver-Burk plot for Cr(VI) reduction of cell-free extract (S_{12}) (b).

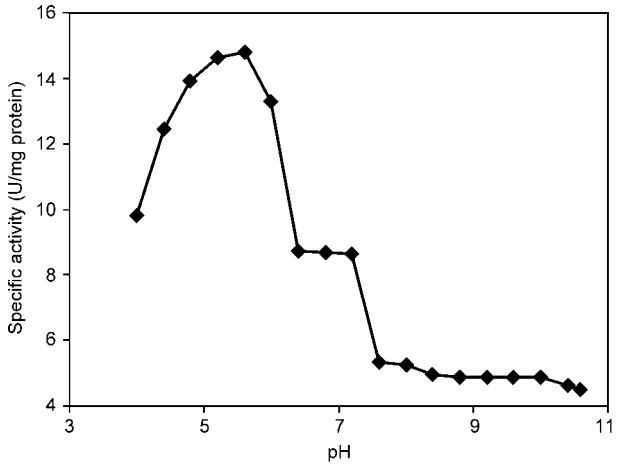

Fig. 3. Effect of pH on chromate reductase activity (S_{12}) of *B. firmus* KUCr1 at 35°C for 30 min of incubation.

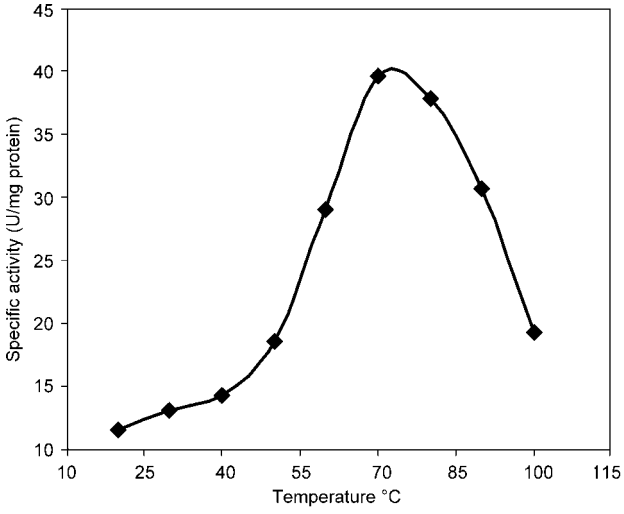

Fig. 4. Effect of temperature on chromate reductase activity (S_{12}) of *B. firmus* KUCr1. The assay was conducted in 0.2 M phosphate buffer (pH 7.2) for 30 min.

2001; Park *et al*., 2000) and in *P. ambigua* G-1 (Suzuki *et al*., 1992). Lower K_m value of Cr(VI) reductase suggests higher affinity for the substrate, at least with what was found with the cell-free extract in this strain.

The effect of pH on reductase activity was assessed at a pH range of 4.0 to 10.6 using 0.2 M of citrate buffer (pH 4.0 to 5.6), 0.2 M of phosphate buffer (pH 6.0 to 7.6), 0.2 M of tris-HCl buffer (pH 8.4 to 8.8) and 0.2 M of glycine-NaOH buffer (pH 9.2 to 10.6) separately. The reductase activity achieved a maximum at pH 5.6 (Fig. 3). Similarly, the optimum temperature for highest Cr(VI) reduction was found at 70°C (Fig. 4). These results varied from other earlier reports with *B. sphaericus* AND 303, where they were 30°C and 6.0 respectively (Pal *et al*., 2005) and with *P. putida* MK1 (Park *et al*., 2000) or *P. ambigua* G-1 (Suzuki *et al*., 1992), the optimal temperature and pH being 80°C, 50°C, and pH 5.0, 8.6 respectively.

The effect of electron donors, inhibitors and metal ions on chromate reduction by CFE (S_{12}) of *B. firmus* KUCr1 was determined. 0.2 mM each of NADH, NADPH, glutathion, D-glucose, and D-fructose were used as electron donors. Among the electron donors used, only NADH showed a significant effect on chromate reductase activity (72% activity over control). NADPH gave 32% less activity than NADH (Table I).

Table I
Effect of electron donor on Cr(VI) reductase activity in cell-free extracts (S_{12}) of *B. firmus* KUCr1

Electron donor (0.2 mM)	Specific activity[a] (U/mg protein)	Relative specific activity[b] (%)
None (control)	2.40 (±0.01)	27.74
NADH	8.65 (±0.06)	100.00
NADPH	5.87 (±0.08)	67.86
Glutathione	2.53 (±0.02)	29.24
D-glucose	2.89 (±0.01)	33.41
D-fructose	2.70 (±0.07)	31.21

[a] Data are the mean of three replications plus standard error. The reaction mixture contained 0.2 mM Cr(VI) in 0.2 M phosphate buffer (pH 7.2) and was incubated for 30 min at 35°C.

[b] {(Specific activity) / (specific activity)$_{NADH}$ } X 100

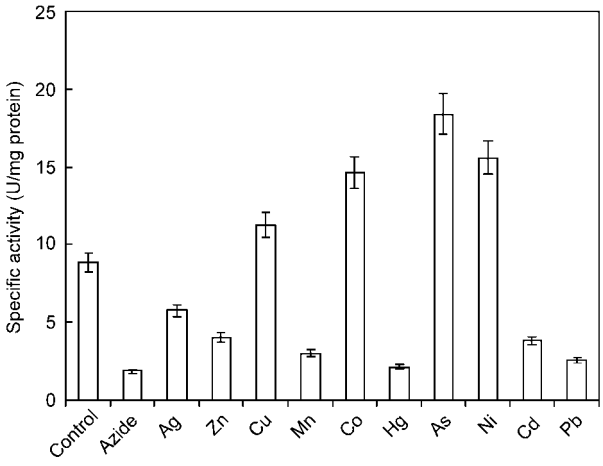

Fig. 5. Effect of azide (NaN_3, 0.2 mM)) and some selected metals (0.2 mM) on Cr(VI) reductase activity in the cell-free extracts (S_{12}) of *B. firmus* KUCr1. Data are the mean of three replications with error bars.

In the presence of glutathion, D-glucose, and D-fructose, the non-enzymatic reductants of Cr(VI), the activity of the reductase was almost equal to that of the control.

The cell-free enzyme of *B. firmus* KUCr1 required NADH or NADPH as an electron donor for better enzymatic Cr(VI) reduction. The reductase became sharply more active in the presence of NADH than NADPH, suggesting the requirement of a cofactor for catalytic activity. NADH dependent Cr(VI) reduction was also advocated by several researchers earlier in *Bacillus* (Garbisu *et al*., 1998; Camargo *et al*., 2003) and in *Pseudomonas* (Suzuki *et al*., 1992; Park *et al*., 2000). Our study also supports the earlier observations on the role of nonenzymatic reductants like glutathione, D-glucose and D-fructose on Cr(VI) reduction by CFE (Branca *et al*., 1990; Shi and Dalal, 1990; Suzuki *et al*., 1992).

Cr(VI) reduction by CFE was significantly inhibited in the presence of 0.2 mM sodium azide (Fig. 5). The metal cations, Ag^+, Zn^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+}, and Hg^{2+} inhibited reductase activity by more than 50% over the control in the reaction mixture (Fig. 5). However, $Co^{2+,}$ Ni^{2+}, As^{3+} and Cu^{2+} stimulated the activity of CFE. The order of stimulation by these metal cations in reductase activity was found to be $As^{3+} > Ni^{2+} > Co^{2+} > Cu^{2+}$.

In this study, Cr(VI) reductase activity was found to be inhibited by a respiratory inhibitor, sodium azide (0.2 mM). Though chromate reductase in CFE was reported to be unaffected by azide in *Escherichia coli* ATCC 33456 (Shen and Wang, 1993), *Bacillus* sp. ES29 (Camargo *et al*., 2003) and in *B. megaterium* TKW3 (Cheung *et al*., 2006) by 1.0 mM NaN_3. However, inhibition of Cr(VI) reduction due to NaN_3 in live cells of a *Bacillus subtilis* strain was reported (Garbisu *et al*., 1998). Inhibition of a cytoplasmic membrane associated Cr(VI) reductase by azide was also reported in *Shewanella putrefaciens* MR-1 (Myers *et al*., 2000). The interference of azide in Cr(VI) reduction in this study and views from earlier reports suggest the possible coexistence of Cr(VI) reductase in the cytosol and membrane as well. However, further investigations are required to elucidate this particular feature.

The cations Hg^{2+} and Ag^+ (0.2 mM) inhibited reductase activity. The noncompetitive inhibitory effect of Hg^{2+} and Ag^+ on Cr(VI) reduction in *P. putida* was reported earlier (Ishibashi *et al*., 1990). The inhibitory effect of Hg^{2+} is expected, because of its affinity for ligands containing thiol (-SH) group of a variety of enzymatic proteins. Enzymatic reduction of Cr(VI) by CFE of *B. firmus* KUCr1 was stimulated by Cu^{2+}, As^{3+}, Ni^{2+} and Co^{2+} at 0.2 mM concentration separately, though Pal *et al.* (2005) reported the inhibition of Cr(VI) reductase activity of *B. sphaericus* AND 303 by Ni^{2+} and Co^{2+} at 100 µM concentration. The reductase activity was found to be unaffected by As^{3+} in *P. putida* MK1 (Park *et al*., 2000). Stimulation of enzyme activity by Cu^{2+} might be due to its nature as a prosthetic group of many reductase enzymes and also indirectly involved in the protection of chromate reductase from O_2, for oxygen-sensitive enzyme (Ettinger 1984; Camargo *et al*., 2003). Abe *et al*. (2001) reported that Cu^{2+} acting as electron-transport protection or acting as a single electron redox center and as a shuttle for electron between protein subunits. Zn^{2+}, Cd^{2+}, Pb^{2+} and Mn^{2+} inhibited the reductase activity at 0.2 mM. Inhibitory effects by Cd^{2+} and Zn^{2+} support the earlier views (Park *et al*., 2000; Pal *et al*., 2005; Desai *et al*., 2008). On the contrary, Camargo *et al.* (2003) showed slight stimulatory effect of Mn^{2+} on Cr(VI) reductase in *Bacillus* sp. ES 29. These variations seem to be due to the different functional nature of the Cr(VI) reductase in *B. firmus* KUCr1, which warrants further investigation.

Cr(VI) reductase activities by different cellular fractions are presented in Table II. In the presence of electron donor (0.2 mM NADH) S_{150} fraction showed higher activity than fractions S_{12} and P_{150}.

Table II
Localization of Cr(VI) reductase in cell fractions of *B. firmus* KUCr1 and its catalytic activity

Cellular fraction[a]	% Cr(VI) reduction[b]	Specific activity[c] (U/mg protein)
S_{12}	14.21	9.12 (±0.03)
S_{150}	15.66	14.68 (±0.04)
P_{150}	6.98	7.34 (±0.04)

[a] S stands for soluble cellular fractions and P stands for membrane fractions (see Materials and Methods).
[b] Reaction mixture contained 0.2 mM Cr(VI) in phosphate buffer (pH 7.2) and was incubated for 30 min at 35°C.
[c] Data are the mean of three repetitions plus standard error.

The percentage of Cr(VI) reduction by fraction P_{150} is significantly less compared to other fractions, but the specific activity signifies its catalytic function, suggesting the occurrence of some membrane-bound protein responsible for Cr(VI) reduction. In *Bacillus* QC1-2 (Campos *et al*., 1995), *B. sphaericus* AND 303 (Pal *et al*., 2005) and *P. putida* (Ishibashi *et al*., 1990; Park *et al*., 2000) the chromium reductase activities were reported to be associated with the cytosolic and soluble fractions. In *Enterobactor cloacae* (Wang *et al*., 1990), *S. putrefaciens* MR-1 (Myers *et al*., 2000), *B. megaterium* TKW3 (Cheung *et al*., 2006) and *P. fluorescences* (Bopp and Ehrlich, 1988) Cr(VI) reductase appears to be membrane associated. Lovley and Phillips (1994) reported that both the soluble and membrane fractions reduced chromate in *Desulfovibrio vulgaris* but soluble protein fraction reduced Cr(VI) faster than the membrane fraction did. In *Bacillus cereus* S-6 (Iftikhar *et al*., 2007) Cr(VI) reduction occur both in cytosolic and membrane fraction of this strain but percentage of reduction in cytosolic fraction is higher than membrane fraction. It seems that KUCr1 harbors Cr(VI) reductase constitutively both in membrane and cytosol. Thus further investigations to visualize the exact pathway of Cr(VI) reduction in this particular strain are required.

Enzyme inhibition by respiratory inhibitors like NaN_3 and Cr(VI) reductase activity by the P_{150} fraction suggest the co-existence of a membrane-bound, respiratory-chain-linked reductase activity in this strain; study with purified proteins of both the cytosolic and membrane fractions would provide more knowledge about the exact pathway of Cr(VI) in this particular strain. Moreover, the feature of higher temperature optima of Cr(VI) reductase of the cytosolic soluble fraction in KUCr1 suggests the possibility to clone the gene into a thermophilic bacteria, so that it could offer a decisive advantage in chromium bioremediation under conditionally elevated temperature or in a bioreactor system using immobilized enzyme.

Acknowledgement

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