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Hexavalent Chromium Reduction by Free and Immobilized Cell-free Extract of *Arthrobacter rhombi-RE*

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Abstract In the present study, hexavalent chromium (Cr(VI)) reduction potential of chromium reductase associated with the cell-free extracts (CFE) of *Arthrobacter rhombi-RE* species was evaluated. *Arthrobacter rhombi-RE*, an efficient Cr(VI) reducing bacterium, was enriched and isolated from a chromium-contaminated site. Chromium reductase activity of *Arthrobacter rhombi-RE* strain was associated with the cell-free extract and the contribution of extracellular enzymes to Cr(VI) reduction was negligible. NADH enhanced the chromium reductase activity. The enzyme activity was optimal at a pH of 5.5 and a temperature of 30 °C. Among the ten electron donors screened, sodium pyruvate was the most effective one followed by NADH and propionic acid. Michaelis–Menten constant, K_m, and maximum reaction rate, V_{max}, obtained from the Lineweaver–Burk plot were 48 μM and 4.09 nM/mg protein/min, respectively, in presence of NADH as electron donor and 170.5 μM and 4.29 nM/mg protein/min, respectively, in presence of sodium pyruvate as electron donor. Ca^{2+} enhanced the enzyme activity while Hg^{2+}, Cd^{2+}, Ba^{2+}, and Zn^{2+} inhibited the enzyme activity. Among the various immobilization matrices screened, calcium alginate beads seemed to be the most effective one. Though immobilized enzyme system was able to reduce Cr(VI), the performance was not very encouraging in continuous mode of operation.

Keywords *Arthrobacter rhombi-RE* · Chromium reductase · Cr(VI) bioremediation · Immobilized enzyme

Introduction

Hexavalent chromium is released into the environment through a large number of industrial operations which include manufacturing of alloys, dyes and pigments, electroplating, metal

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finishing, petroleum refining, leather tanning, and wood preservation. It is also used as corrosion inhibitor in conventional and nuclear power plants [1]. Hexavalent chromium is highly toxic, carcinogenic, and mutagenic in living organisms. Hence, treatment to reduce/remove this pollutant before discharging the effluent into the environment is necessary.

Conventional method for the removal of Cr(VI) involves reduction of toxic Cr(VI) to a less toxic Cr(III) using reducing agents, followed by precipitation. However, these methods require large quantities of chemicals and produce large amounts of sludge that pose serious problems to the environment [2].

Many reports are available on the biotransformation of Cr(VI) under aerobic and anaerobic conditions [3–5]. The reduction of Cr(VI) to Cr(III) was possible by pure and enriched mixed cultures of microorganisms [3, 4, 6]. Most of the biological systems for the treatment of Cr (VI)-containing wastewater are operated in batch mode [7–9], probably due to eventual loss of active biomass as a result of metal toxicity. Several researchers have reported that it is almost impossible to continuously remove Cr(VI) on a long-term basis without intermittently reseeding the biological system [10]. However, in recent years, continuous-flow and fixed-film bioreactors have been employed for biological reduction of Cr(VI) [11–14]. In these reactors, carbon sources as electron donors were supplied externally to the wastewater depending upon the requirement. Use of highly active Cr(VI) reducing enzymes may be a suitable option for the effective biotransformation of Cr(VI) [15–19].

It is reported that partially purified chromate reductase from *Pseudomonas ambigua* and *Pseudomonas putida* were able to reduce Cr(VI) using NADH and NAD(P)H as electron donors [15]. The presence of additional electron donors like NADH and NAD(P)H accelerated Cr(VI) reductase activity of CFE of *Bacillus sphaericus AND 303* [20]. Fractionation and assay of CFE of *Bacillus ES 29* have revealed that a soluble NADH-dependent enzyme was responsible for the catalytic reduction of Cr(VI) [16]. Most of the chromium reductases reported in aerobic conditions are normally associated with a soluble protein fraction utilizing NADH or NAD(P)H as electron donor and the occurrence of reduction process is either internal or external to the plasma membrane [14, 21]. Under anaerobic conditions, Cr(VI) is used as a terminal electron acceptor and is reduced in the membrane during anaerobic respiration [22]. Though many partially purified and crude enzymes are reported to have significant Cr(VI) reduction potential, their application in Cr (VI)-contaminated wastewater treatment is limited due to the expensive and tedious separation of free enzymes from the waste streams.

Immobilized enzymes have many advantages such as (1) cost reduction of process because they can be reused many times, (2) easy separation from reaction mixtures, and (3) the possibility of using higher enzyme activity per unit volume in the reactor [23]. Several natural and synthetic support materials such as agar, alginate, carrageenan, cellulose, and its derivatives like collagen, gelatin, polyacrylamide, polyester, polystyrene, and polyurethane have been used for cell immobilization [24]. With the advancement in enzyme immobilization technology, it is speculated that the application of immobilized Cr(VI) reductase could be a promising approach for bioremediation of Cr(VI)-contaminated wastewater over a wide range of environmental conditions. Though many reports are available on the use of whole cell reactors for the treatment of Cr(VI)-contaminated wastewater, data on the application of enzyme-immobilized reactors for the same is scanty [25].

In the present study, Cr(VI) reduction potential of chromium reductase associated with the CFE of *Arthrobacter rhombi-RE* was evaluated. The optimal conditions for Cr(VI) reduction and the effect of various metals on chromium reductase activity were also evaluated. An attempt was made to employ immobilized chromium reductase in a continuous system for Cr(VI)-bearing wastewater treatment.

Materials and Methods

Enzyme Preparation

Arthrobacter rhombi-RE, a potential Cr(VI)-reducing bacterium, grown for 18 h in nutrient medium, was harvested by centrifugation, washed, and re-suspended in 50 mM potassium phosphate buffer of pH 6 (1 g (wet weight) cell in 5 mL buffer). The cells were lysed by sonication (five cycles of 40 s on and 40 s off at 175 W), centrifuged (10,000 ×*g* and 4 °C) and the CFE was collected. The crude CFE is referred as intracellular enzyme. Supernatants from the culture media, after separating the cells by centrifuging at 1,000 ×*g* for 10 min, were employed for extracellular enzyme activity studies. Total protein was estimated by Lowry's method [26]. All assays were carried out in triplicate. All the batch experiments were carried out in triplicate at 30 °C and the error bars represent the standard deviation from the mean values.

Chromium Reductase Assay

Chromium reductase activity was assayed using NADH as an electron donor. The reaction mixture consisted of 0.1 mL (0.39 mg protein) crude enzyme, 0.1 mM NADH (1 mM), and 38.46 μM of Cr(VI) in 50 mM potassium phosphate buffer (pH 6) in a total volume of 1 mL. Assay mixtures comprising similar composition as given above except enzyme or NADH were used as respective controls. The assay mixtures were incubated at 30 °C for 60 min. The amount of residual Cr(VI) in the reaction mixture was quantified.

Effect of pH and Temperature

Chromium reductase activity was measured at 30 °C and at different solution pH. pH of the mixtures were adjusted using various buffers, namely 50 mM sodium acetate, for pH between 4 and 5.5; 50 mM sodium phosphate, for pH between 5.5 and 8; and 50 mM sodium carbonate, for pH between 8 and 10. Enzyme samples were incubated in different buffers (pH 4.5–9.0) at 30 °C for 60 min and the residual enzyme activity was measured in terms of Cr(VI) reduction. To study the effect of temperature on enzyme activity, enzyme was incubated at temperatures ranging from 10 to 70 °C and at a pH of 6 for 60 min, cooled in an ice bath, and the residual enzyme activity measured in terms of Cr(VI) reduction.

Effect of Metal Ions

Metal ions employed in the present study were Zn^{2+}, Na^{+}, Hg^{2+}, Mn^{2+}, Ag^{2+}, K^{+}, Ni^{2+}, Cd^{2+}, Pb^{2+}, Li^{2+}, Ba^{2+}, Co^{2+}, Al^{3+}, and Ca^{2+}. The reaction mixture consisted of 0.1 mL (0.4 mg protein) of CFE, 0.1 mL of metal ions (10 mM), 0.1 mL of NADH (1 mM), and 38.46 μM of Cr(VI) in 50 mM potassium phosphate buffer (pH 6) in a total volume of 1 mL. The reaction mixture was incubated at 30 °C for 60 min and the residual activity was assayed as described earlier.

Effect of Electron Donors

Effect of different electron donors on the performance of CFE was also evaluated. Electron donors employed for the present study were propionic acid, sodium lactate, formic acid, sodium pyruvate, NADH, dextrose, lactose, and maltose. The reaction mixture (total volume of 1 mL) consisted of 0.4 mg of CFE (measured as protein), 100 mM of electron donor (except NADH

(1 mM)), and 38.46 μM of Cr(VI) in 50 mM potassium phosphate buffer (pH 6). The residual Cr(VI) concentration was measured after 60 min of incubation at 30 °C.

Kinetic of Cr(VI) Reduction

Kinetics of Cr(VI) reduction by CFE was evaluated using different initial Cr(VI) concentrations varying from 10 to 860 μM. Reaction mixtures consisted of 0.1 mL CFE (0.47 mg protein), 0.1 mL of NADH (1 mM), and different concentrations of Cr(VI) in 50 mM sodium acetate buffer (pH 5.5) in a total volume of 1 mL, and they were incubated for 30 min at 30 °C. The amount of residual Cr(VI) in the reaction mixture was quantified. Michaelis–Menten equation was used to determine the Michaelis constant (K_m) and maximum velocity of the enzyme (V_{max}). K_m and V_{max} were estimated by linear regression analysis of the values obtained in the assay.

Immobilization Matrices

Five different matrices, namely polyvinyl alcohol (PVA)–boric acid beads, alginate beads, polyacrylamide beads, gelatin gel, and zeolite resin, were employed as enzyme immobilization matrices. The following procedures were employed for the immobilization.

Immobilization of reductase enzyme in PVA–boric acid beads Twenty milliliters of PVA aqueous solution (20%, *w*/*v*) was mixed thoroughly with 5 mL of CFE (21 mg protein). Drops of resulted mixture were allowed to fall into saturated boric acid solution to form spherical beads. To complete the polymerization, the beads were kept in saturated boric acid solution for 20 h. They were then taken out and washed with distilled water before use.

Immobilization of reductase enzyme in polyacrylamide beads Twenty milliliters of aqueous solution containing 5 g of acrylamide monomer (ACAM), 0.75 g of *N*,*N*′-methylene bisacrylamide, and 0.05 g of sodium alginate was mixed with 5 mL of CFE (21 mg protein). Drops of the resulted mixture were allowed to fall into a solution containing 0.1 g of potassium persulfate, 1 mL of 25% *N*,*N*,*N*′,*N*′ tetramethylene diamines, and 1.0 g of calcium chloride. The beads were immersed in the solution for 30 min at room temperature and then washed with distilled water before further use.

Immobilization of reductase enzyme in gelatin gel Two grams of gelatin was dissolved in 20 mL of water to prepare a 10% (wt) aqueous solution. The solution was gently heated to facilitate the dissolution process and the temperature was adjusted to 35–40 °C. Temperature was kept relatively high so that the gelatin solution was not too viscous to cause enzyme denaturation. Five milliliters (21 mg protein) of CFE and 2 mL of the hardening solution (formaldehyde 20% (*v*/*v*); ethanol 50% (*v*/*v*); water 30% (*v*/*v*)) were added to 20 mL of this solution. Then the mixture was poured into a small beaker and frozen at −28 °C for 4 h to facilitate the gel formation. The gel thus formed was cut into small cubes of approximately 3 mm size and was gently washed with distilled water before use.

Immobilization of reductase enzyme in alginate beads Five milliliters (21 mg protein) of CFE was mixed with 20 mL of 3% (*w*/*v*) sodium alginate solution. The beads were allowed to form by dripping the polymer solution from a height of approximately 20 cm into a beaker containing 100 mL of stirred 0.2 M $CaCl_2$ solution, using a syringe and a needle at room temperature. A typical hypodermic needle produced beads of 0.5–2 mm in diameter.

The beads were left in the calcium solution to cure for 0.5–3 h. Beads thus formed were then washed with distilled water. Effects of alginate concentration on CFE immobilization was studied by varying the alginate concentration from 1% to 4% (*w*/*v*).

Immobilization of reductase enzyme in Zerolite resin (cation exchange resin) Extrudate-shaped Zerolite resin (20 g) was added to the flask containing 50 mL of deionized water. The flasks were shaken at 200 rpm for 3 h in order to remove the weak binder. After the treatment was complete, the Zerolite particles were washed with deionized water and the washed wet Zerolite particles were then dried at 100 °C for 6 h. Ten grams of dried Zerolite resin and 50 mL of CFE (0.8 mg protein) in the relevant buffer (pH 6) were added to 125-mL flasks. Each flask was sealed to prevent evaporation and placed in a shaking water bath at 200 rpm and 25 °C for 30 min. After that, the resin was gently washed with distilled water.

Batch Experiments with Immobilized Enzymes

Activities of immobilized enzymes on various matrices were determined using batch experiments. A reaction mixture consisting of 10 mM of electron donor (pyruvate) and 38.46 μM of Cr(VI) in 50 mM buffer (pH 5.5) medium (40 mL) was taken in a 125-mL conical flask. Five grams (wet weight) of enzyme-immobilized beads was added to the reaction mixture and placed on a rotary shaker at 150 rpm and at 30 °C. The residual Cr(VI) concentration with respect to time was monitored.

Continuous Reactor Studies with Immobilized Enzyme

Experiments were conducted in continuous packed-bed reactors containing enzyme-immobilized alginate beads. Reactors were made of Pyrex glass with an internal diameter of 1.2 cm and a height of 20 cm. Ninety percent of the reactor volume was occupied by enzyme-immobilized alginate beads. Influent solution (38.46 μM of Cr(VI) and 1 mM of sodium pyruvate in 50 mM acetate buffer with a pH of 5.5) was pumped upwards through the columns at a flow rate of 10 mL/h. The reactor was operated at ambient temperature with a hydraulic retention time (HRT) of 2 h corresponding to a Cr(VI) loading rate of 24 g of Cr(VI)/m^3/day. Samples were withdrawn at 1-h intervals and analyzed for residual Cr (VI) concentration. Control column packed with alginate beads without enzyme was also operated in the same manner.

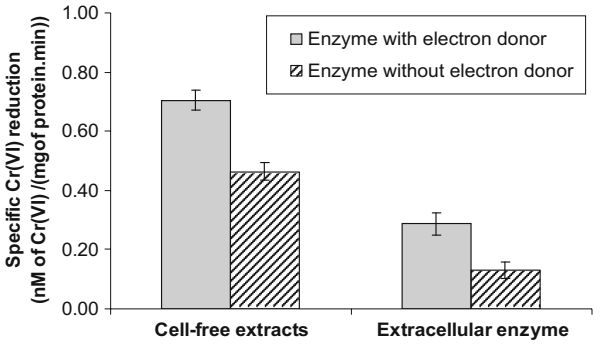


Fig. 1 Cr(VI) reduction by extracellular and cell-free extracts (CFE). The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0), 1 mM of NADH, 0.1 mL of enzyme, incubation 30 °C for 60 min

Analytical Procedures

Cr(VI) concentration was determined by diphenyl carbazide method (reference number 3500-Cr B colorimetric method) by measuring the absorbance at 540 nM using a UV–vis spectrophotometer (Techcom, UK) [27]. pH was measured using a Cyber scan 510 meter (Eutech Instrument, UK). Total protein was estimated by Lowry's method [26].

Results and Discussion

Kinetics of Cr(VI) Reduction by CFE and Extracellular Enzymes of *Arthrobacter rhombi-RE*

Cr(VI) reduction experiments were conducted using CFE and extracellular enzymes of *Arthrobacter rhombi-RE* to understand the component responsible for biotransformation of Cr(VI) and the results are presented in Fig. 1. The specific Cr(VI) reduction activity obtained for CFE at 30 °C and a pH of 6.0 was 0.46 nM Cr(VI) reduction/mg protein/min. Addition of NADH enhanced the chromate reductase activity (0.77 nM Cr(VI) reduced/mg protein/min). Even when there was no external electron donor supply, a lower but significant level of Cr(VI) reduction was observed in presence of CFE. Residual organic matter present in the CFE might have acted as the electron donor for Cr(VI) reduction [4]. The supernatant obtained after harvesting the cells (extracellular enzymes) showed less chromium reductase activity (0.13 nM Cr(VI) reduction/mg protein/min) compared to CFE. This clearly showed that the Cr(VI) reduction was associated with the soluble fraction of the cells and not with extracellular enzymes, which corroborates the earlier studies with *Escherichia coli* [2], *Desulfovibrio vulgaris* [28], *P. putida* [29], and *Bacillus* QC1-2 [30]. Bopp and Ehrlich reported that necessary enzymes for the transfer of electrons from NADH to chromate are membrane bound in a strain of *Pseudomonas fluorescens* grown aerobically [31]. Garbisu et al. [21] have shown that reduction of chromium by *Bacillus subtilis* was affected by a constitutive system associated with the soluble protein fraction and not with the membrane fraction. However, Wang et al. [32] found that the chromate reductase

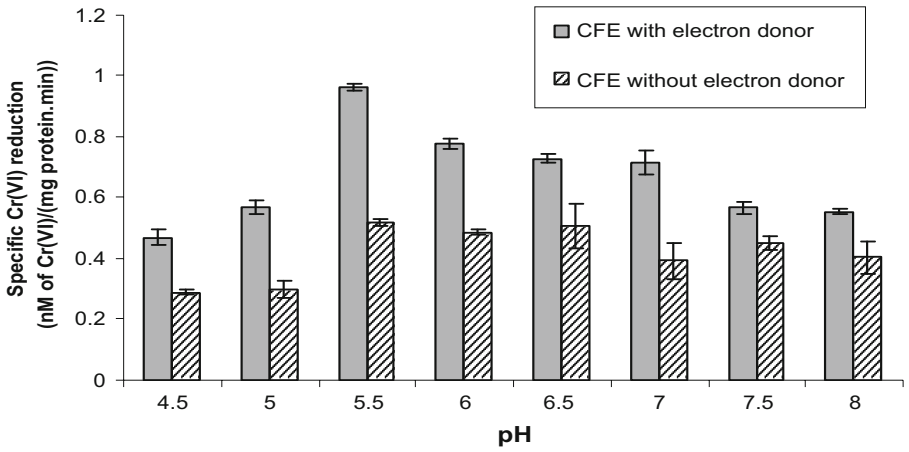


Fig. 2 Effect of pH on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr (VI), 1 mM of NADH, and 0.1 mL of CFE, incubated at 30 °C for 60 min

activity of the anaerobically grown *Enterobacter cloacae* HO1 was preferentially associated with the membrane fraction of the cells.

Effect of pH

The effect of pH on Cr(VI) reduction by crude CFE was assessed in a pH range of 4.5 to 8.0 using acetate buffer (4.5 to 5.5) and phosphate buffer (6.0 to 8.0). Significant chromium reductase activity was observed in a narrow pH range with an optimum at pH 5.5 (Fig. 2). More than 40% of the activity at optimum pH (at pH 5.5) was lost when the pH was changed to 4.5 or 8.0. As seen in Fig. 2, at pH less than 5.5, the enzyme stability (activity) reduced drastically. The decrease in enzyme activity was less in alkaline condition (with respect to optimum pH) compared to acidic condition. Wang et al. [32–33] have reported that *Enterobacter* strain was able to reduce Cr(VI) at a pH range of 6.5–8.5 and Cr(VI) reduction was strongly inhibited at pH 5 and 9. Chromate reduction in *Agrobacterium radiobacter* EPS-916 was observed in a range of 5 to 8 and optimum reduction was between pH 7 and 7.5 [34]. It may be noted that Cr(VI) reduction is enzyme mediated. Changes in pH affect the degree of ionization of the enzyme and change the protein's conformation. As a result, the enzyme activity gets affected [35].

Effect of Temperature

The optimal temperature of chromium reductase activity was determined by changing the incubation temperatures from 10 to 50 °C. Maximum chromium reductase activity was observed at 30 °C for a pH of 6 (Fig. 3). Activity at 20–25 °C was 85% of that at 30 °C and at 15 °C, the activity was only 75% of the optimum activity. Decrease in chromium reductase activity was observed for temperatures above 30 °C. Further increase of temperature resulted in rapid loss of enzyme activity. Losi et al. [36] reported an optimal temperature of 30 to 37 °C for Cr(VI) reduction by Cr(VI)-resistant bacteria. Wang et al. [32] reported that no chromate reduction was observed at 4 and 60 °C by *E. cloacae*. The optimum pH and temperature for chromate reductase extracted from *Bacillus sp. ES 29*, *Bacillus sphaericus*

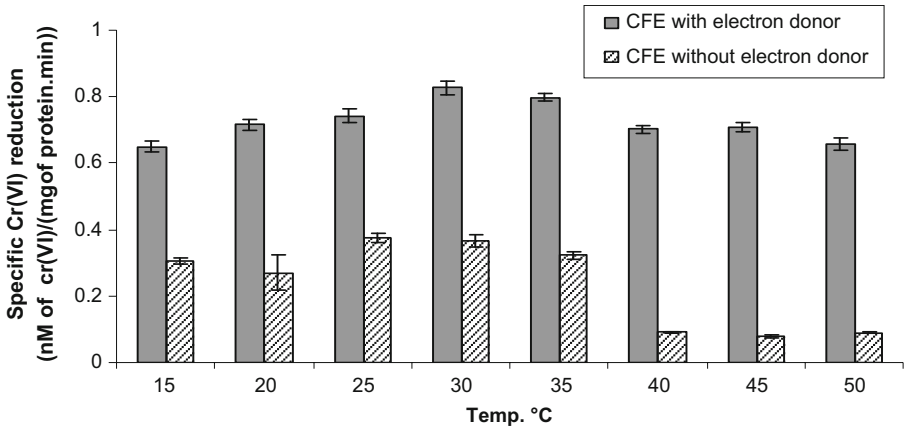


Fig. 3 Effect of temperature on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0), 1 mM of NADH, and 0.1 mL of CFE, incubated for 60 min

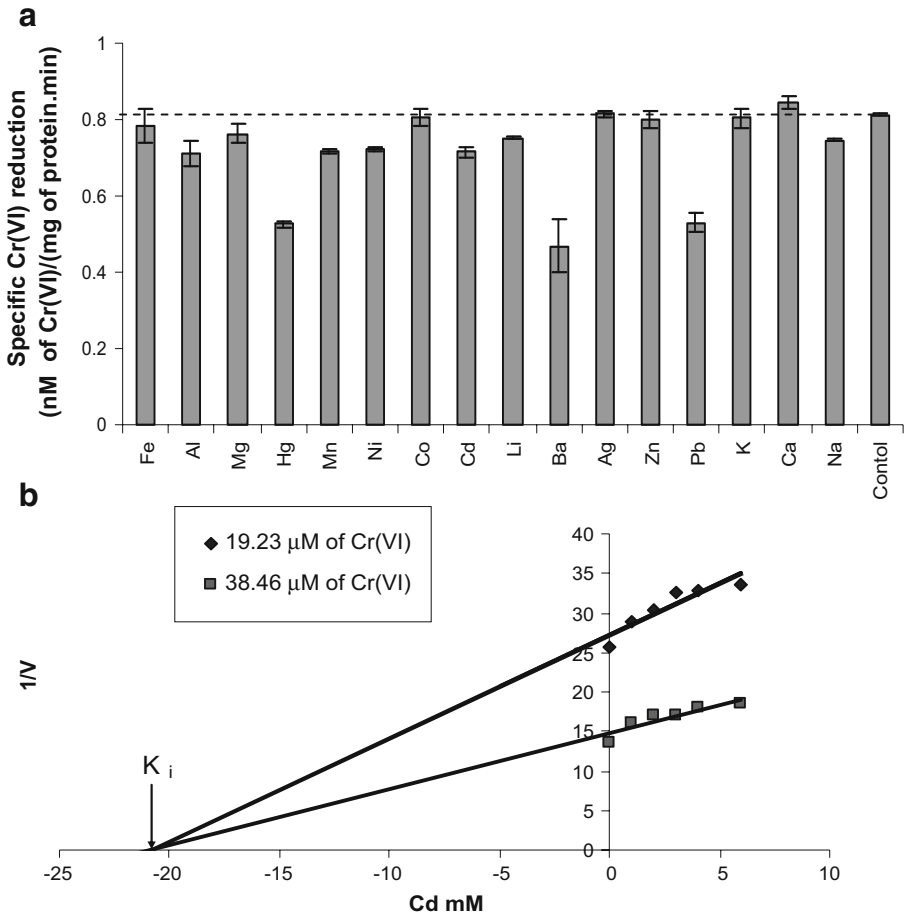


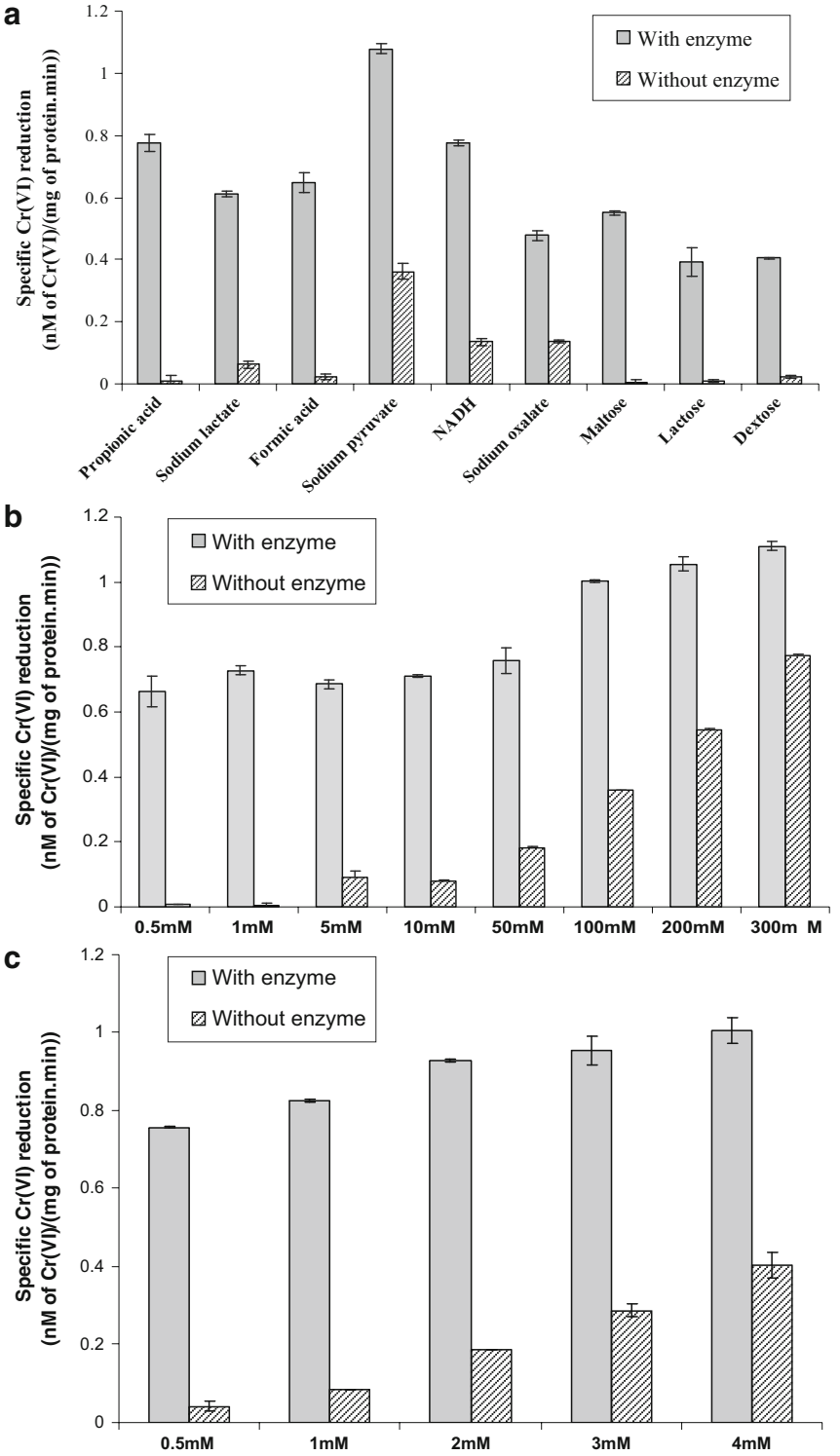
Fig. 4 **a** Effect of metal ions on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0), 1 mM of NADH, 0.1 mL of CFE, and 10 mM of metal ions, incubated at 30 °C for 60 min. **b** Dixon plot of chromium reductase at different concentrations of Cd^{2+}

AND 303, *E-coli ATCC 33456*, *Actinomycete*, and *Arthrobacter crystallopoietes* ES 32 were reported to be between pH 5 to 9 and 30 °C, respectively [16, 18, 37].

Effect of Metal Ions and Metabolism Inhibitors on Chromium Reductase

The effect of metal ions on Cr(VI) reduction by crude CFE was studied at a pH of 5.5 and a temperature of 30 °C, using NADH as electron donor. Among the various metal ions

Fig. 5 **a** Effect of various electron donors on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0), 0.1 mL of CFE, and 100 mM of electron donor (except NADH (1 mM)) incubated at 30 °C for 60 min. **b** Effect of various concentration of sodium pyruvate (electron donor) on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0) and 0.1 mL of CFE, incubated at 30 °C for 60 min. **c** Effect of various concentration of NADH (electron donor) on chromium reductase activity. The assay mixture (1.0 mL) contained 38.46 μM of Cr(VI) in 50 mM phosphate buffer (pH 6.0) and 0.1 mL of CFE, incubated at 30 °C for 60 min


a
Specific Cr(VI) reduction (nM of Cr(VI)/(mg of protein.min))
With enzyme
Without enzyme
Propionic acid
Sodium lactate
Formic acid
Sodium pyruvate
NADH
Sodium oxalate
Maltose
Lactose
Dextose
b
Specific Cr(VI) reduction (nM of Cr(VI)/(mg of protein.min))
With enzyme
Without enzyme
0.5mM
1mM
5mM
10mM
50mM
100mM
200mM
300m M
c
Specific Cr(VI) reduction (nM of Cr(VI)/(mg of protein.min))
With enzyme
Without enzyme
0.5mM
1mM
2mM
3mM
4mM

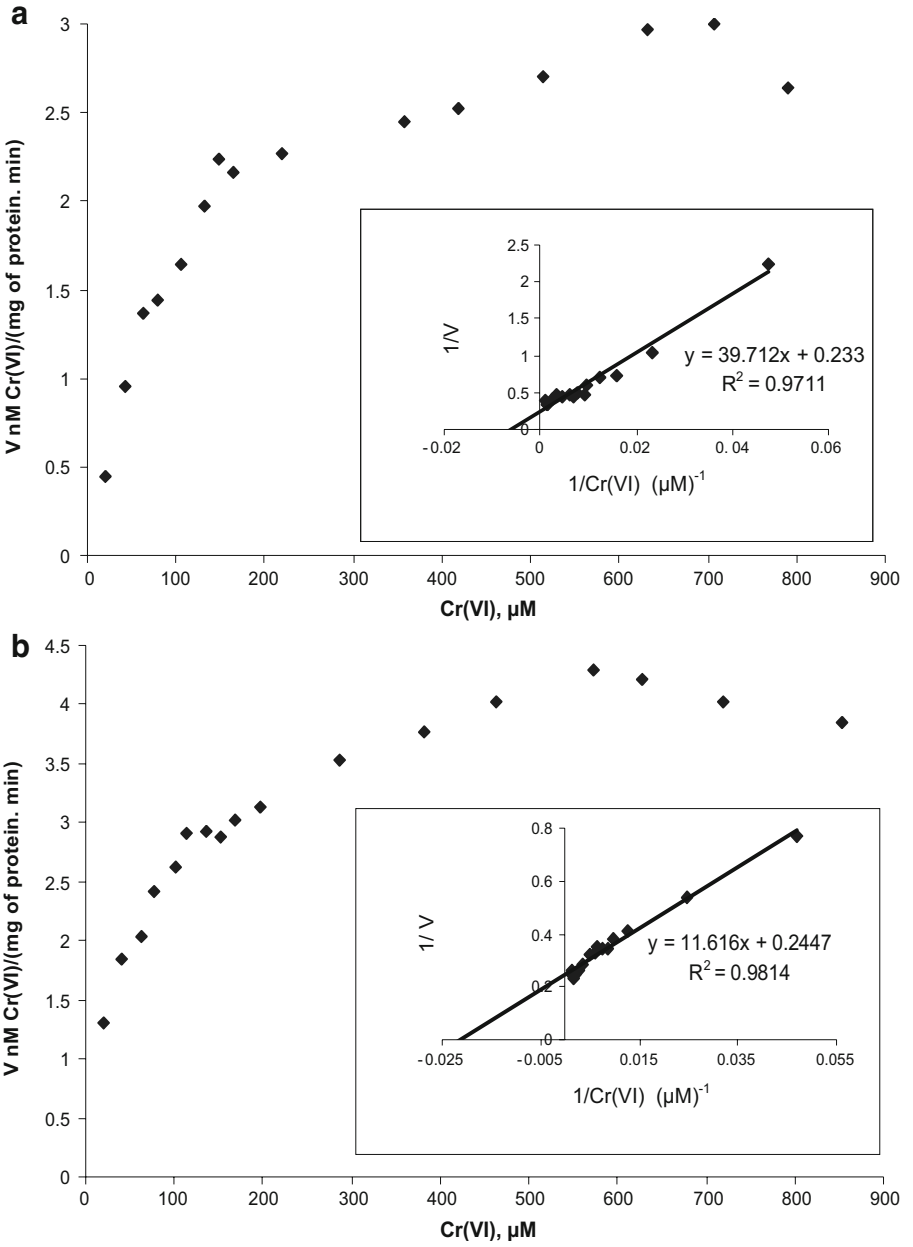


Fig. 6 **a** Effect of initial Cr(VI) concentration on chromate reduction in presence of sodium pyruvate. The *inset* denotes liberalized Lineweaver–Burk plot for Cr(VI) reduction. The assay mixture (1.0 mL) contained increasing concentration of Cr(VI) in 50 mM acetate buffer (pH 5.5) and 0.1 mL of CFE, incubated at 30 °C for 30 min. **b** Effect of initial Cr(VI) concentration on chromate reduction in presence of NADH. The *inset* denotes liberalized Lineweaver–Burk plot for Cr(VI) reduction. The assay mixture (1.0 mL) contained increasing concentration of Cr(VI) in 50 mM acetate buffer (pH 5.5) and 0.1 mL of CFE, incubated at 30 °C for 30 min

Table 1 Kinetic parameters (K_m and V_{max}) for Cr(VI) reduction by cell-free extracts of various microorganisms.

Microorganism	Electron donor	V_{max} (nM of Cr(VI)/min/mg protein)	K_m (μM of Cr(VI))	Reference
Pseudomonas putida	NADH	6 as CrO_4^{2-}	40 as CrO_4^{2-}	[29]
Bacillus sp. QC1-2	NADH	416 as CrO_4^{2-}	1.25 as CrO_4^{2-}	[30]
Bacillus sp. ES 29	NADH	171	7.09	[16]
GST–EcNfsA–chromium reductase	NADH	3.8	11.8	[39]
GST–EcNfsB	NADH	3.9	23.5	
GST–VhNfsA	NADH	10.7	15.4	
P. ambigua	NADH	27	13	
Bacillus sphaericus AND 303	NADH	1.432×10^3	158.12	[20]
Escherichia coli ATCC 33456	NADPH	322.2×10^3	47.5	[18]
	NADH	130.7×10^3	17.2	
Thermus scotoductus SA-01	NADH	$6.2\pm0.2\times10^3$	3.5±0.3	[40]
	NADPH	$16\pm0.4\times10^3$	8.4±0.1	
Arthrobacter rhombi-RE	NADH	4.09	48	Present study
	Sodium pyruvate	4.29	170.5	

employed, presence of Ca^{2+} enhanced the activity of chromate reductase by 8%. Mg^{2+}, K^+, and Co^{2+} did not have any effect on chromate reductase activity, whereas other metal ions like Hg^{2+}, Cd^{2+}, Ba^{2+}, and Zn^{2+} inhibited the chromate reductase activity by different amounts (Fig. 4a). The enzyme activity was indeed greatly inhibited by Ag^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+}. Hg^{2+} and Cd^{2+} are known to form a mercaptide bond with sulfhydryl group(s) of the enzyme molecule, thus inhibiting enzyme activity. Thus, the inhibitory effect by heavy metal ions is probably related to the oxidation of sulfhydryl group(s) on the surface of the enzyme molecule. The inhibitory effect of Hg^{2+} was expected, because mercury binds to a variety of enzyme systems with a specific affinity for ligands containing SH^- systems. Effect of Hg^{2+} on Cr(VI) reduction by *P. putida* and other chromate reductases were reported [15, 17, 29]. Cadmium metal was selected to determine the inhibition mechanism of sulfur seeking metals on chromium reductase. Concentration of metal was varied between 1 and 10 mM, while the substrate concentration was varied between 19.23 and 38.46 μM. When $1/v$ (reciprocal of reaction velocity, 1/μmol/min/mg protein) was plotted against $1/[S]$ (Lineweaver–Burk plot) for three different substrate concentrations, the three lines crossed approximately at the same point on the *X*-axis. This indicated that Michaelis constant (K_m) remained constant for different concentrations of cadmium, while V_{max} (maximum velocity) decreased with increase in cadmium concentrations (Fig. 4b). $1/v$ was also plotted against inhibitor concentration [*I*] (Dixon plot) since Dixon plot is used frequently to identify the type of inhibition

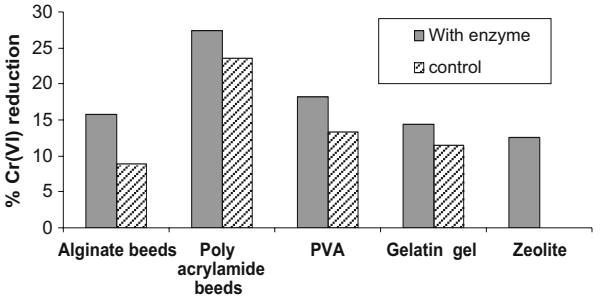


Fig. 7 Screening of various enzyme-immobilized matrices (40 mL working volume contained 38.46 μM of Cr(VI) in 50 mM acetate buffer (pH 5.5) and 10 mM of electron donor (pyruvate), incubated at 150 rpm and at 30 °C for 60 min)

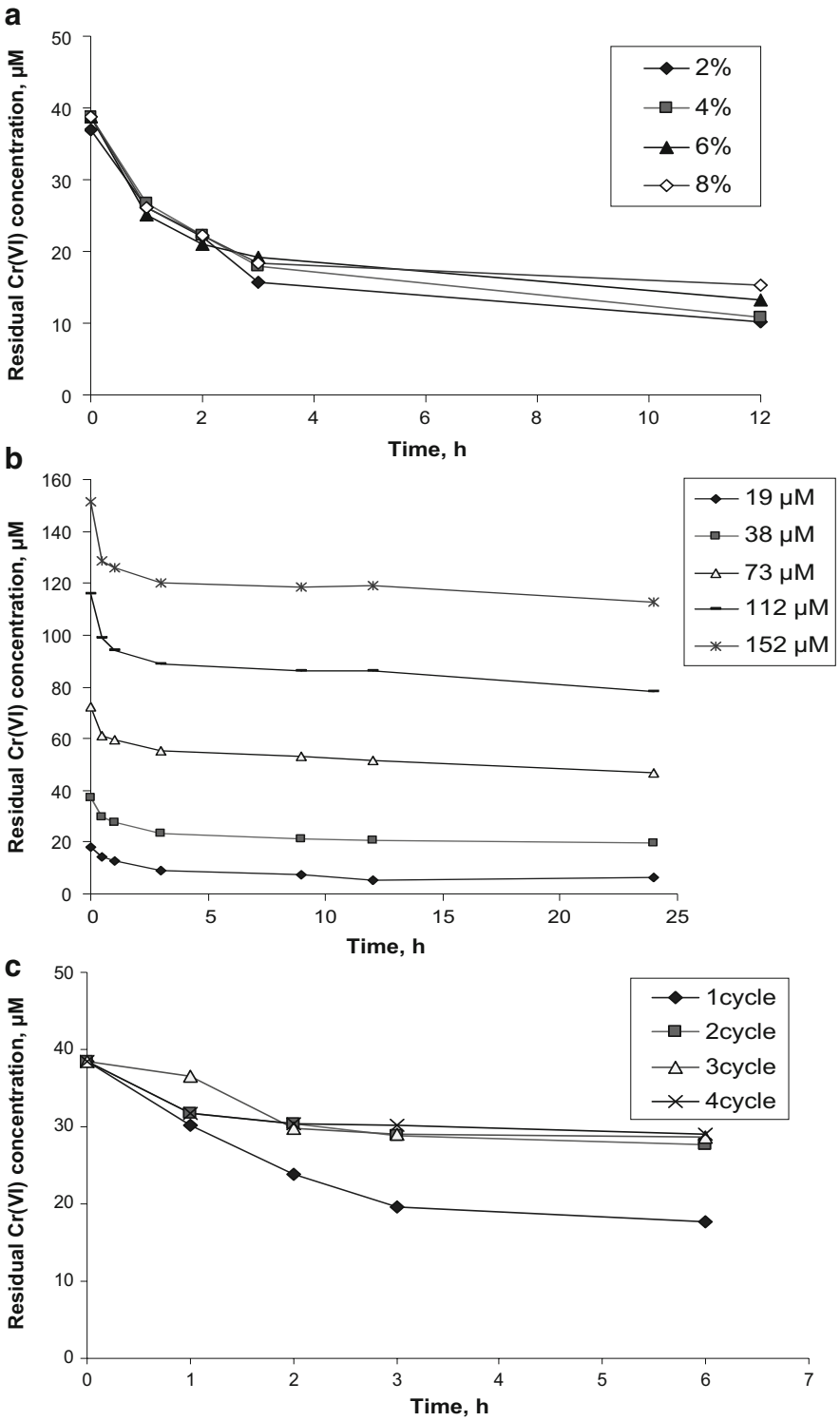
and to determine the K_i value (dissociation constant for inhibitor binding). Both of these plots suggest that the inhibition by cadmium is apparently noncompetitive. From the intersecting point of the three lines, the K_i value was determined as 21.5 mM. The noncompetitive type inhibition by heavy metal ions suggest that the binding site for the heavy metal differs from the catalytic site of the enzyme. It probably corresponds to a sulfhydryl group or imidazole group on the surface of the enzyme molecule [38]. Camargo et al. [16] reported that mercury was a noncompetitive inhibitor for Cr(VI) reduction by a cell-free extract of *Bacillus sp. ES 29*.

Effect of Electron Donors

In most of the biological reactions, electron transfer from organic substrate is mediated through NADH and NADPH. Fractionation and assay of the CFE of *Arthrobacter rhombi-RE* revealed that a soluble NADH-dependent enzyme is responsible for the catalytic reduction of Cr(VI). However, NADH is not a cost-effective electron donor for large-scale wastewater treatments systems. Hence, the effects of various electron donors, on Cr(VI) reduction by CFE of *Arthrobacter rhombi-RE*, were evaluated. Electron donors employed in the present study were propionic acid, sodium lactate, formic acid, sodium pyruvate, NADH, dextrose, lactose, and maltose. Figure 5a shows the Cr(VI) reduction by CFE in presence of different electron donors. For uniformity, concentrations of all electron donors were maintained at 100 mM of electron donor (except NADH (1 mM)). As shown in Fig. 5a, Cr(VI) reduction rate was high (1.1 nM of Cr(VI)/mg protein/min) when sodium pyruvate was used as the electron donor. This system exhibited a two-fold increase in Cr (VI) reduction compared to blank (without CFE). Though CFE was able to reduce Cr(VI), the presence of electron donors improved the Cr(VI) reduction. Among the ten electron donors screened, sodium pyruvate has shown maximum specific Cr(VI) reduction followed by NADH. Though propionic acid was an effective electron donor, it caused a significant reduction in system pH. Hence, propionic acid was not used for further studies. As sodium pyruvate is much economical compared to NADH as an electron donor, it was used as the electron donor for the remaining studies. High concentration of NADH alone (in absence of enzyme) was reducing Cr(VI) completely. Hence, a lower concentration of NADH was employed in the present study.

To optimize the Cr(VI) reduction, it is critical to determine the concentration of electron donor required to achieve the desired treatment level. The minimization of electron donor supply decreases the residual electron donor concentration in the treated effluent, thereby reducing treatment cost and post-treatment requirements. An experiment was performed to determine the minimum concentration of electron donor required to achieve and sustain complete removal of 38.46 μM Cr(VI) at pH 6.0 and 30 °C. Different concentrations of sodium pyruvate (0.5–300 mM) and NADH (0.5–4 mM) were employed to reduce Cr(VI) and the results are presented in Fig. 5b and c. It is clear that both pyruvate and NADH showed almost the same net specific Cr(VI) reduction capacity. One millimolar of sodium

Fig. 8 **a** Effect of alginate concentration on chromium reductase immobilization (40 mL working volume contained 38.46 μM of Cr(VI) in 50 mM acetate buffer (pH 5.5) and 10 mM of electron donor (pyruvate), incubated at 150 rpm and at 30 °C). **b** Effect of Cr(VI) concentration of enzyme immobilized in calcium alginate beads. The assay mixture 40 mL contained 38.46 μM of Cr(VI) in 50 mM acetate buffer (pH 5.5), 10 mM of electron donor (pyruvate), and 5 g wet weight (approximate) enzyme-immobilized beads, incubated at 150 rpm and at 30 °C. **c** Reusability studies of enzyme immobilized in calcium alginate beads (40 mL working volume contained 38.46 μM of Cr(VI) in 50 mM acetate buffer (pH 5.5), 10 mM of electron donor (pyruvate), and 5 g wet weight (approximate) of enzyme-immobilized beads, incubated at 150 rpm and at 30 °C)


a
Residual Cr(VI) concentration, μM
Time, h
2%
4%
6%
8%
b
Residual Cr(VI) concentration, μM
Time, h
19 μM
38 μM
73 μM
112 μM
152 μM
c
Residual Cr(VI) concentration, μM
Time, h
1cycle
2cycle
3cycle
4cycle

pyruvate showed a net specific Cr(VI) reduction of 0.725 nM Cr(VI) reduced/mg protein/min and same concentration of NADH showed a net specific Cr(VI) reduction of 0.74 nM Cr(VI) reduced/mg protein/min. A considerable amount of Cr(VI) reduction was observed in both sodium pyruvate and NADH abiotic blanks (Fig. 5b, c). It was also observed that 1 mM was the minimum sodium pyruvate concentration required for maximum reduction of 38.46 μM of Cr(VI) (Fig. 5b). From the results, it is clear that sodium pyruvate is cost effective compared to NADH and it can be used to treat large volumes of industrial wastewater containing Cr(VI).

Kinetics of Cr(VI) Reduction by Chromium Reductase

To determine the kinetic parameters, Cr(VI) reduction by the CFE was assessed for different Cr(VI) concentrations (0–854 μM) with sodium pyruvate as well as NADH as electron donors. The assay mixture was incubated at 30 °C for 30 min. Kinetic parameters (K_m and V_{max}) were determined using the Michaelis–Menten equation [35].

$$V_o = \frac{V_{max}}{K_m + S} \tag{1}$$

where V_{max} is the maximum nonspecific rate or maximum specific velocity and K_m is the Michaelis–Menten constant. Figure 6a shows that the specific chromium reduction rate (sodium pyruvate as electron donor) increased rapidly with an increase in initial Cr(VI) concentrations up to 707 μM, beyond which the rate was reduced. Kinetics of Cr(VI) reduction by CFE fitted well with the linearized Lineweaver–Burk plot (Fig. 6a). K_m and V_{max} values obtained from the Lineweaver–Burk plot are 170.5 μM and 4.29 nM/min/mg protein, respectively.

Even in the case of NADH as electron donor, specific Cr(VI) reduction increased rapidly (Fig. 6b) with an increase in initial Cr(VI) concentrations up to 574 μM, beyond which the rate was reduced. K_m and V_{max} values for this case are 48 μM and 4.09 nM/min/mg protein, respectively. Table 1 presents the various values for K_m and V_{max} reported in the literature for CFE from different microorganisms. Behavior of electron donors varies with molecular structure and environmental conditions. As a result, Cr(VI) reduction rates and biokinetic parameters (V_{max} and K_m) of the system also vary depending on the electron donors [41]. V_{max} for sodium pyruvate system is slightly higher than that of NADH system, though K_m was higher for pyruvate.

Cr(VI) Reduction Using Immobilized Enzyme

The Cr(VI) reduction by CFE-immobilized matrices was evaluated in batch experiments and the results are presented in Fig. 7. The activity of enzyme immobilized on alginate beads and Zerolite resin was higher than that on gelatin gel, polyacrylamide gel, and PVA. It was also observed that, except Zerolite resin (cation exchange resin), all other matrices adsorbed Cr(VI).

Effect of Sodium Alginate Concentration on Enzyme Activity

It has been reported that the porosity of the calcium alginate beads depends upon the alginate type and the gelling agent concentration [42]. Hence, various quantities of sodium alginate were used for the preparation of calcium alginate beads in order to vary the relative degree of

cross linking, which would create different pore size, and the results are presented in Fig. 8a. Maximum immobilization efficiency was achieved (72.55%) for a 2% (*w*/*v*) sodium alginate solution and lowest (60%) for 8% (*w*/*v*) sodium alginate solution. Leakage of the CFE (protein) was observed at different alginate concentrations up to 6% (*w*/*v*). However, beads prepared by using 8% sodium alginate solution did not show any enzyme leakage. At 4% of sodium alginate, there was no breakage and good agglomeration of beads was observed.

Kinetics of Cr(VI) reduction by CFE-immobilized alginate beads was carried out with different Cr(VI) concentrations (19, 38, 73, 112, and 152 μM) using sodium pyruvate as electron donor and the result is presented in Fig. 8b. After 3 h, 52% of the Cr(VI) was reduced in the reaction mixture with an initial Cr(VI) concentration of 19 μM. At 9 h of incubation, Cr(VI) reduction was increased to 64%. However, Cr(VI) reduction ceased after 9 h of incubation irrespective of initial Cr(VI) concentration.

The reusability of CFE-immobilized alginate beads was determined by using the same beads for four reaction cycles with an initial Cr(VI) concentration of 38 μM. Cr(VI) reduction activity was measured after each cycle of incubation, and the beads were recovered and washed with phosphate buffer (pH 6) before reuse. Results (Fig. 8c) show that enzyme immobilized in alginate beads can be reused in many cycles of operation though there was 37% reduction in efficiency by the end of the fourth cycle.

Continuous Reactor Studies

The efficiency and longevity of immobilized chromate reductase were also evaluated using continuous flow reactors. Columns were packed with chromium reductase-immobilized alginate beads and a solution containing 38.46 μM of Cr(VI), 10 mM of sodium pyruvate with a pH of 5.5 was pumped at a flow rate of 10 mL/h (0.088 $m^3/m^2/h$). Enzyme-immobilized system was operated at a HRT of 2 h with an influent Cr(VI) concentration of 38.46 μM, which corresponds to a chromium loading rate of 462 mM of Cr(VI)/m^3/day. The columns were able to achieve a Cr(VI) reduction of 75% in 1 h. However, the Cr(VI) reduction efficiency reduced and stabilized at 40% after 8 h of operation (Fig. 9). These results revealed that the immobilized enzyme system was ineffective in reducing Cr(VI) in a continuous mode.

Conclusions

Cr(VI) reductase activity of *Arthrobacter rhombi-RE* was associated with the soluble fraction of the cells and the contribution of extracellular enzymes on Cr(VI) reduction was

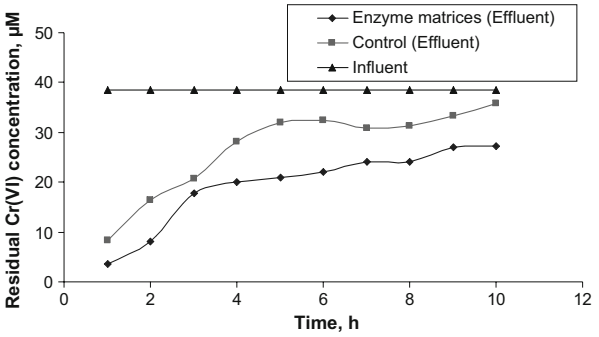


Fig. 9 Continuous studies on Cr(VI) reduction using chromium reductase immobilized in calcium alginate beads. Influent solution—38.46 μM of Cr(VI), 1 mM of sodium pyruvate in 50 mM acetate buffer with a pH of 5.5 and HRT of 2 h

negligible. The specific Cr(VI) reduction activity of the cell-free extract was 0.46 nM Cr (VI) reduction/mg protein/min at pH 6.0 and 30 °C. Addition of NADH significantly enhanced the chromate reductase activity (0.77 nM Cr(VI) reduced/mg protein/min). K_m and V_{max} values were 48 μM and 4.09 nM/min/mg protein, respectively, in presence of NADH as electron donor. Among the various electron donors screened, sodium pyruvate was found to be the most effective, followed by NADH, propionic acid, formic acid, and sodium lactate. It was observed that the minimum sodium pyruvate (electron donor) concentration required for the complete reduction of 38.46 μM Cr(VI) at pH 6.0 and 30 °C was 1 mM. K_m and V_{max} were 170.5 μM and 4.29 nM/mg protein/min, respectively, when sodium pyruvate was used as electron donor. The enzyme activity was enhanced in the presence of Ca^{2+}, while it was inhibited by Hg^{2+}, Cd^{2+}, Ba^{2+}, and Zn^{2+}. Inhibition of chromium reductase by Cd^{2+} was noncompetitive in nature, with a K_i of 21.5 mM. Alginate beads appear to be a better immobilization medium for crude chromium reductase. The efficiency and longevity of immobilized chromium reductase was also evaluated using continuous flow reactors.

References

1. Alves, M. M., Ceca, C. G. G., Carvalho, R. G. D., Castanheira, J. M., Periera, M. C. S., & Vasconcelos, L. A. T. (1993). *Water Research*, *27*, 1333–1338. doi:10.1016/0043-1354(93)90220-C.
2. Shen, H., & Wang, Y. T. (1993). *Applied and Environmental Microbiology*, *59*, 3771–3777.
3. Volesky, B., & Holan, Z. R. (1995). *Biotechnology Progress*, *11*, 235–250. doi:10.1021/bp00033a001.
4. Philip, L., Iyengar, L., & Venkobachar, C. (1998). *Journal of Environmental Engineering*, *124*, 1165–1170. doi:10.1061/(ASCE)0733-9372(1998)124:12(1165).
5. Guha, H., Jayachandran, K., & Maurrasse, F. (2001). *Environmental Pollution*, *115*, 209–218. doi:10.1016/S0269-7491(01)00108-7.
6. Schmieman, E. A., Yonge, D. R., Rege, M. A., Petersen, J. N., Turick, C. E., Johnstone, D. L., et al. (1998). *Journal of Environmental Engineering*, *124*, 449–455. doi:10.1061/(ASCE)0733-9372(1998)124:5(449).
7. Chirwa, E. N., & Wang, Y. T. (2000). *Water Research*, *34*, 2376–2384. doi:10.1016/S0043-1354(99)00363-2.
8. Smith, W. A., Apel, W. A., Petersen, J. N., & Peyton, B. M. (2002). *Bioremediation Journal*, *6*, 205–215. doi:10.1080/10889860290777567.
9. Wang, Y., & Xiao, C. (1995). *Water Research*, *29*, 2467–2474. doi:10.1016/0043-1354(95)00093-Z.
10. Chen, J. M., & Hao, O. J. (1997). *Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire)*, *69*, 70–76. doi:10.1002/(SICI)1097-4660(199705)69:1<70::AID-JCTB665>3.0.CO;2-4.
11. Philip, L., Iyengar, L., & Venkobachar, C. (1999). *International Journal of Environment and Pollution*, *11*, 202–210. doi:10.1504/IJEP.1999.002258.
12. Chirwa, E. N., & Wang, Y. T. (2001). *Water Research*, *35*(8), 1921–1932. doi:10.1016/S0043-1354(00)00472-3.
13. Dermou, E., Velissariou, A., Xenos, D., & Vayenas, D. V. (2007). *Desalination*, *211*, 156–163. doi:10.1016/j.desal.2006.02.090.
14. Zakaria, Z. A., Zakaria, Z., Surif, S., & Ahmad, W. A. (2007). *Journal of Hazardous Materials*, *148*, 164–171. doi:10.1016/j.jhazmat.2007.02.029.
15. Park, C. H., Keyhan, M., Wielinga, B., Fendorf, S., & Matin, A. (2000). *Applied and Environmental Microbiology*, *66*, 1788–1795. doi:10.1128/AEM.66.5.1788-1795.2000.
16. Camargo, F. A., Okeke, B. C., Bento, F. M., & Frankenberger, W. T. (2003). *Applied Microbiology and Biotechnology*, *62*, 569–573. doi:10.1007/s00253-003-1291-x.
17. Megharaj, M., Avudainayagam, S., & Naidu, R. (2004). *Current Microbiology*, *47*, 51–54. doi:10.1007/s00284-002-3889-0.
18. Bae, W. C., Lee, H. K., Choe, Y. C., Jahng, D. J., Lee, S. H., Kim, S. J., et al. (2005). *Journal of Microbiology (Seoul, Korea)*, *43*, 21–27.
19. Elangovan, R., Abipsha, S., Rohit, B., Philip, L., & Chandraraj, K. (2006). *Biotechnology Letters*, *28*(4), 247–252. doi:10.1007/s10529-005-5526-z.

20. Pal, A., Dutta, S., & Paul, A. K. (2005). *Current Microbiology*, *51*, 327–330. doi:10.1007/s00284-005-0048-4.
21. Garbisu, C., Alkorta, I., Llama, M. J., & Serra, J. L. (1998). *Biodegradation*, *9*, 133–141. doi:10.1023/A:1008358816529.
22. McLean, J., & Beveridge, T. J. (2001). *Applied and Environmental Microbiology*, *67*, 1076–1084. doi:10.1128/AEM.67.3.1076-1084.2001.
23. White, C. A., & Kennedy, J. F. (1985). In A. Wiseman (Ed.), *Handbook of enzyme biotechnology* pp. 147–380. Chichester: Horwood.
24. Munjal, N., & Sawhney, S. K. (2002). *Enzyme and Microbial Technology*, *30*, 613–619. doi:10.1016/S0141-0229(02)00019-4.
25. Mabbett, A. N., Yong, P., Farr, J. P. G., & Macaskie, L. E. (2004). *Biotechnology and Bioengineering*, *87*, 104–109. doi:10.1002/bit.20105.
26. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *The Journal of Biological Chemistry*, *193*, 265–275.
27. APHA (1998). *Standard methods for the examination of water and wastewater* (20th ed.). Washington, DC: American Public Health Association.
28. Lovley, D. R., & Phillips, E. J. P. (1994). *Applied and Environmental Microbiology*, *60*, 726–728.
29. Ishibashi, Y., Cervantes, C., & Silver, S. (1990). *Applied and Environmental Microbiology*, *56*, 2268–2270.
30. Campos, J., Martinez-Pacheco, M., & Cervantes, C. (1995). *Antonie Van Leeuwenhoek*, *68*, 203–208. doi:10.1007/BF00871816.
31. Bopp, L. H., & Ehrlich, H. L. (1988). *Archives of Microbiology*, *150*, 426–431. doi:10.1007/BF00422281.
32. Wang, P. C., Mori, T., Toda, K., & Ohtake, H. (1990). *Journal of Bacteriology*, *172*, 1670–1672.
33. Wang, P., Mori, T., Komori, K., Sasatsu, M., Toda, K., & Ohtake, H. (1989). *Applied and Environmental Microbiology*, *55*, 1665–1669.
34. Bhinde, J. V., Dhakephalkar, P. K., & Paknikar, K. M. (1996). *Biotechnology Letters*, *18*, 667–672. doi:10.1007/BF00130763.
35. Farrell, S. O., & Ranallo, R. T. (2000). *Experiments in biochemistry. a hands-on approach*. Orlando: Saunders College Publications.
36. Losi, M. E., Amrhein, C., & Frankenberger, W. T. (1994). *Reviews of Environmental Contamination and Toxicology*, *36*, 91–121.
37. Camargo, F. A., Bento, F. M., Okeke, B. C., & Frankenberger, W. T. (2004). *Biological Trace Element Research*, *97*, 183–194. doi:10.1385/BTER:97:2:183.
38. Hiraoka, B. Y., Fukasawa, K., & Harada, M. (1987). *Molecular and Cellular Biochemistry*, *73*, 111–115. doi:10.1007/BF00219425.
39. Kwak, Y. H., Lee, D. S., & Kim, H. B. (2003). *Applied and Environmental Microbiology*, *69*, 4390–4395. doi:10.1128/AEM.69.8.4390-4395.2003.
40. Opperman, D. J., Piater, L. A., & Heerden, E. V. (2008). *Journal of Bacteriology*, *8*, 3076–3082.
41. Birke, R. L., & Lombardi, J. R. (1988). In R. J. Gale (Ed.), *Surface enhanced Raman scattering in spectroelectrochemistry: theory and practice*. Plenum, New York.
42. Longo, M. A., Novella, I. S., García, L. A., & Díaz, M. (1992). *Enzyme and Microbial Technology*, *14*, 586–590. doi:10.1016/0141-0229(92)90131-7.