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Evaluation of *in vitro* Reduction of Hexavalent Chromium by Cell-Free Extract of *Arthrobacter* sp. SUK 1201

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Authors' contributions

This work was carried out in collaboration between both authors. Authors SD and AKP have jointly shared the responsibility of designing the concept and protocol, performing and analyzing the experimental results and preparing the manuscript including literature searches. Authors have also read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: This study aims at to evaluate the hexavalent chromium [Cr(VI)] reduction potential of crude cell-free extracts of chromium resistant and reducing bacterium *Arthrobacter* sp. SUK 1201 and determination of optimum conditions for Cr(VI) reduction for possible bioremediation of Cr pollutants.
Place and Duration of Study: Chromium reduction studies with *Arthrobacter* sp. SUK 1201, was undertaken in the Microbiology Laboratory, Department of Botany, University of Calcutta, Kolkata during 2010-2012.
Methodology: Cell-free extract was prepared from freshly grown cell mass of *Arthrobacter* sp. SUK 1201 following the standard procedure. Cell mass suspended in Tris-HCl was sonicated (120 KHz for 30 min), centrifuged (12,000×g at 4ºC for 10 min) and the supernatant (S_{12}) was used as the cell- free extract (CFE). Chromate reductase activity of the CFE was assayed colorimetrically using 1, 5-diphenylcarbazide as the complexing reagent.
Results: Chromate reductase activity of CFE of *Arthrobacter* sp. SUK 1201 was constitutive in nature and reduced Cr(VI) with decreasing efficiency as the concentration of Cr(VI) was increased. Its K_m and V_{max} were 263.45 μM Cr(VI) and 17.5 U mg^{-1} protein respectively. Reduction of Cr(VI) was optimal at pH 7 and 32ºC but was extremely

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thermolabile. NADH was the most suitable electron donor, and the chromate reduction was enhanced by Cu(II) and Fe(III), but inhibited by Hg(II). Among the different inhibitors tested, 2, 4-dinitrophenol (DNP) restored nearly 96.4% reductase activity, while carbonyl cyanide-m-chloro phenyl hydrazone (CCCP) was most inhibitory to the process.
Conclusion: It has been established that the Cr(VI) reduction potential of the cell-free extract of *Arthrobacter* sp. SUK 1201 is promising and could be exploited in the bioremediation of toxic hexavalent chromium.

Keywords: Arthrobacter sp. SUK 1201; cell-free extract; aerobic chromate reduction; enzyme kinetics; chromate reductase; Cr pollution; Cr(VI) bioremediation.

1. INTRODUCTION

Reduction of toxic hexavalent chromium [Cr(VI)] to relatively non-toxic trivalent chromium [Cr(III)] by a wide variety of indigenous microbial cultures as well as microbial consortium under both aerobic [1-3] and anaerobic conditions [4,5] have been identified as a potential tool for bioremediation of Cr pollutants. The advantages of such bioreduction process over those of the physico-chemical methods have been highlighted by several others [6,7].

During aerobic growth, bacterial soluble proteins are involved in Cr(VI) reduction and utilize NADH as electron donor, while under anaerobic condition membrane bound chromate reductase reduces Cr(VI) through respiratory chains involving cytochromes [4,5]. Bacteria of genera *Pseudomonas*, *Arthrobacter*, *Escherichia, Brucella*, *Ochrobacterium* and *Bacillus* are known to reduce Cr(VI) through soluble chromate reductases [1-3,6-9]. However, cytoplasmic fractions of isolate *Pannonibacter phragmitetus* LSSE-09 [10] were found to reduce hexavalent chromium in both aerobic as well as in anaerobic conditions. Moreover, bacterial enzymes such as hydrogenases [11], nitroreductases [12] and quinone reductases [13] have been reported to exhibit chromate reductase activity.

Attempts have also been made to isolate the chromate reductases particularly from soluble cell-free extracts of *Pseudomonas putida* MK 1. Suzuki et al. [14] purified it to homogeneity using ammonium sulphate, anion exchange chromatography and gel filtration. The Cr(VI)-reductase coding gene (chrR) from *P. putida* MK1 has been identified and sequenced [14]. Chromate reduction via the nitroreductases (NfsA) of *Pseudomonas ambigua*, *Escherichia coli* and *Vibrio harveyi* has also been demonstrated [12,14,15]. In *Thermus scotoductus* [16] a membrane bound chromate reductase was demonstrated, isolated and purified. Sequence homology identified the protein as a dihydrolipoamide dehydrogenase which is part of the multisubunit pyruvate dehydrogenase complex (PDH).

In the present study, Cr(VI) reduction potential of crude cell-free extracts obtained from freshly harvested *Arthrobacter* sp. SUK 1201 cells was evaluated. The optimal conditions for Cr(VI) reduction and the effect of various metals on chromium reductase activity were also evaluated.

2. MATERIALS AND METHODS

2.1 Bacterial Strain and Cultural Conditions

Arthrobacter sp. SUK 1201 (MTCC accession number 8728 and NCBI Gen Bank accession No. JQ312665), the chromium resistant and reducing bacterium isolated from chromite mine overburden materials of Sukinda valley, Orissa, India [17] was used throughout this study. The strain was grown on slopes of peptone, yeast-extract and glucose (PYEG) agar [18] containing (g l^{-1}): peptone 10.0, yeast-extract 5.0 and glucose 3.0 (pH 7.0) and stored at 4ºC for future use.

2.2 Preparation of Cell-Free Extract

Cell-free extract of *Arthrobacter* sp. SUK 1201 was prepared following the procedure of Wang and Xiao [18] and Camargo et al. [19]. The strain was grown in peptone, yeast-extract and glucose broth (PYEG) for 24 h at 35ºC under continuous shaking (120 rpm) and the cell mass was harvested by centrifugation (10,000×g) at 4ºC for 10 min. As and when required, the growth medium was supplemented with 1 mM Cr(VI). The cell mass was washed with Tris-HCl buffer and re-suspended in the same buffer at 5% the original culture volume. The suspended cells were sonicated keeping in an ice bath (120 KHz for 30 min) and centrifuged (12,000×g) at 4ºC for 10 min. The supernatant (S_{12}) was used as the cell-free extract (CFE) and the pellet fraction (P_{12}) was suspended in Tris-HCl buffer.

2.3 Chromate Reduction by Cell-Free Extract

Chromate reductase activity of the cell-free extract (CFE) was assayed following the procedure as described by Park et al. [8]. The assay mixture (1.0 ml) contained 50 μM Cr(VI) (as K_2CrO_4) in 0.2 M Tris buffer (pH 7.0), 0.1 mM NADH and the reaction was initiated by adding 0.2 ml of CFE as the enzyme. Cr(VI) reduction was measured by estimating the decrease in Cr(VI) in the reaction mixture after definite period of incubation at 32ºC.

3. RESULTS AND DISCUSSION

Chromate reduction by cell-free extract (CFE) of *Arthrobacter* sp. SUK 1201 cells grown in PYEG medium without supplementation of Cr(VI) was quite effective in reducing chromate. It reduced nearly 64% of 50 μM Cr(VI) in 60 min. The cell-free extract was found to contain a reductase activity of about 4.96 U mg-1 protein and was efficient for Cr(VI) reduction under aerobic condition. The Cr(VI) reductase enzyme was mainly localized in the soluble fraction (S_{12}) of the cells. The pellet fraction (P_{12}) of these actively growing cells, however, has failed to show any chromate reduction activity (Table 1).

Table 1. Chromate reductase activity of cell fractions of *Arthrobacter* sp. SUK 1201 grown in PYEG medium with and without Cr(VI)

Cell fraction	Growth conditions			
	Medium - Cr(VI)		Medium + 1 mM Cr(VI)	
	Cr(VI) reductase activity, U mg^{-1} protein	% Cr(VI) reduction	Cr(VI) reductase activity, U mg^{-1} protein	% Cr(VI) reduction
Soluble fraction (S_{12})	4.96 ± 0.04	63.53	4.81 ± 0.04	62.6
Pellet fraction (P_{12})	Nil	Nil	Nil	Nil

The assay mixture (1.0 ml) contained 50 µM Cr(VI) in 0.2 M Tris HCl buffer (pH 7.0), 0.2 ml of CFE and incubated at 32°C. 0.1 mM of NADH was used. Residual Cr(VI) was estimated following diphenylcarbazide method after 60 min of incubation. One unit (U) of Cr(VI) reductase activity was defined as the amount of enzyme that convert 1.0 µM Cr(VI) per min at 32°C. Results represent mean of triplicate experiments ± standard error.

Chromate reduction efficiency of CFEs obtained from cells pre-grown in medium supplemented with 1 mM Cr(VI) when compared was found to be almost equal in terms of % Cr(VI) reduction and reductase activity, suggesting that the chromate reductase activity of *Arthrobacter* sp. SUK 1201 is constitutive and do not require the presence of Cr(VI) for induction of such enzyme. The CFE of the Cr(VI) induced cells showed 62.6% chromate reduction with 4.81 U of reductase activity per mg of protein. Similar constitutive chromate reductases have also been reported by several others using a number of different bacterial systems [8,9,19,20,21]. Reductase activity of pellet fraction of these Cr(VI) treated cells as usual was devoid of any reductase activity (Table 1).

3.1 Time Course of Cr(VI) Reduction

Time course of Cr(VI) reduction by the untreated and autoclaved cell-free extracts (S_{12}) of *Arthrobacter* sp. SUK 1201 cells grown in Cr-free medium was tested in reaction mixture with an initial Cr(VI) concentration of 50 µM. The pattern of reduction as shown in Fig. 1 indicated that the extent of Cr(VI) reduction was rapid during the first 10 min of incubation where nearly 19.5 µM of Cr(VI) i.e. nearly 61% of chromate was reduced and the process continued till 30 min of incubation. Beyond this, there was no significant increase in reduction. The specific activity was 4.96 U mg^{-1} protein at 32ºC and pH 7.0 after 30 min incubation. However, no reduction was evident when autoclaved cell-free extract (S_{12}) and pellet (P_{12}) were used. This shows that the autoclaved fraction was devoid of reductase activity indicating that chromate reduction by *Arthrobacter* sp. SUK 1201 was enzymatically-driven (Fig. 1) which undergo denaturation at high temperature. Identical results were also obtained with the untreated pellet fraction.

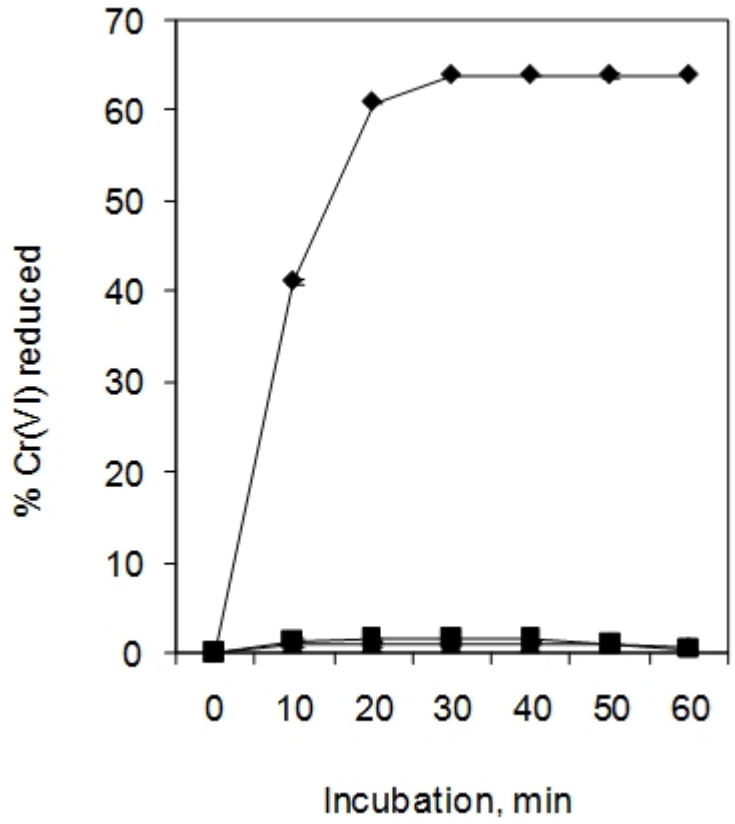

Fig. 1. Reduction of Cr(VI) by cell-free extract (-♦-), pellet fraction (-■-) and autoclaved cell-free extract (-▲-) of *Arthrobacter* sp. SUK 1201

Cr(VI) reduction assay mixture (1.0 ml) containingd 50 µM Cr(VI) and 0.2 ml of CFE (0.2 mg protein/ml) in 0.2 M Tris HCl buffer (pH 7.0) and 0.1 mM of NADH was incubated at 32°C. Residual Cr(VI) was estimated following diphenylcarbazide method at regular interval. Results represent mean of triplicate experiments ± standard error.

3.2 Effect of Cell-Free Extract Concentration

The effect of increasing concentration of crude CFE of *Arthrobacter* sp. SUK 1201 was tested for a range of 0.2 to 1.0 mg protein ml^{-1} on chromate reduction and the results (Fig. 2) show that as the concentration of CFE was increased, the amount of Cr(VI) reduced also increased. With maximum concentration of CFE i.e. 1 mg protein ml^{-1}, complete reduction of 50 µM Cr(VI) occurred within 20 min of incubation indicating a high degree of chromate reducing efficiency of the cell-free extract. This generalised pattern of chromate reductase activity of isolate SUK 1201 corroborates the findings of Camargo et al. [22] and Elangovan et al. [23] with bacterial isolates *Arthrobacter crystallopoites* and *A. rhombi* respectively.

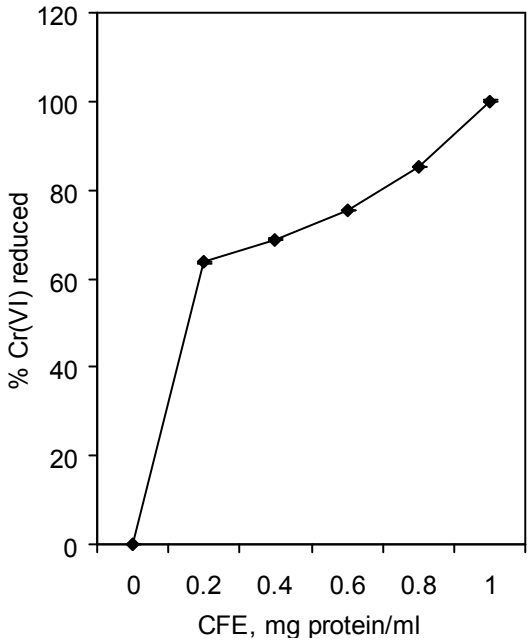

Fig. 2. Effect of concentration of cell-free extract of *Arthrobacter* sp. SUK 1201 on chromate reduction

Experimental details are same as explained under Fig. 1, the concentration of CFE in the reaction mixture ranged from 0.2-1.0 mg protein/ml. Results represent mean of triplicate experiments ± standard error.

3.3 Effect of Cr(VI) Concentration

The specific Cr(VI) reduction activity of crude cell-free extract (S_{12}) of *Arthrobacter* sp. SUK 1201 was found to increase with increase in initial Cr(VI) concentration up to 250 µM, beyond which the activity slowed down but continued till 400 µM Cr(VI) (Fig. 3a). It was also observed that the kinetics of Cr(VI) reductase activity fit well with the linearised Lineweaver-Burk plot. The Michelis-Menten constant (K_m) and the maximum specific velocity (V_{max}) were calculated to be 263.45 µM Cr(VI) and 17.5 U mg^{-1} protein respectively (Fig. 3b). Comparison of enzyme kinetics revealed that the K_m value [263.45 µM Cr(VI)] obtained with the cell-free extract (Fig. 3b) of *Arthrobacter* sp. SUK 1201 was much higher than that of other *Arthrobacter* sp. reported previously by Elangovan et al. [23] and Camargo et al. [22] showing a high affinity for Cr(VI) ions in the present isolate.

3.4 Effect of pH

The influence of pH on Cr(VI) reduction by crude CFE (S_{12}) of *Arthrobacter* sp. SUK 1201 was determined at a wide pH range of 4.0-8.0 using citrate (4.0 to 6.5), phosphate (6.0 to 7.5) and Tris-HCl (7.0 to 8.0) buffers with overlapping pH range. Results as expressed in Fig. 4 indicate that the Cr(VI) reductase activity of cell-free extract of SUK 1201 was maximum at pH 7.0 irrespective of the type of buffer used and it declined sharply on either side of the pH scale. Farrell and Ranallo [24] postulated that the pH of the reaction medium affects the degree of ionization of the enzyme and changes the protein conformation.

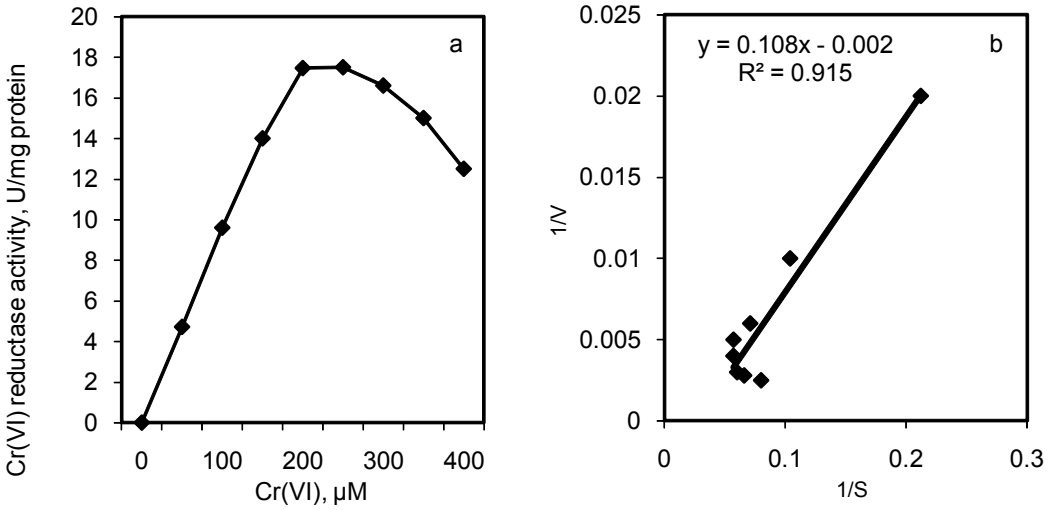

Fig. 3. Effect of initial Cr(VI) concentration on chromate reduction (a) by cell-free extract of *Arthrobacter* sp. SUK 1201 and Lineweaver-Burke plot (b) for Cr(VI) reduction by cell-free extract

Experimental details are same as explained under Fig. 1, the concentration of Cr(VI) in the reaction mixture ranged from 50-400 µM. Results represent mean of triplicate experiments ± standard error.

Further, different types of buffer with overlapping pH gave similar results, which clearly indicated that it is the pH not the chemical composition of the buffer that influences the enzyme activity (Fig. 4). Similar optimum pH was also reported in *Streptomyces* sp. MC1, but the reductase activity at pH 6 was almost 90% of the optimum [21] unlike the present strain.

3.5 Effect of Temperature

The reductase activity of CFE of *Arthrobacter* sp. SUK 1201 was found to be expressed within the whole range of temperature (30-40ºC) tested and it was found that the reductase activity was highly sensitive to temperature and tends to denature at higher temperature (Fig. 5). The optimal reductase activity, however, was evident at 32ºC which was 4.86 U mg^{-1} protein. The reductase activity was found to be extremely sensitive to temperature as there was a sharp decline in the activity of the crude cell-free extract beyond 32ºC (Fig. 5). Similar heat sensitivity of chromate reductase was also evident from experimental findings of Sarangi and Krishnan [25]. However, *E. coli* ATCC 33456 and *Bacillus* sp. presented an optimum growth and activity temperature of 37ºC [2,26].

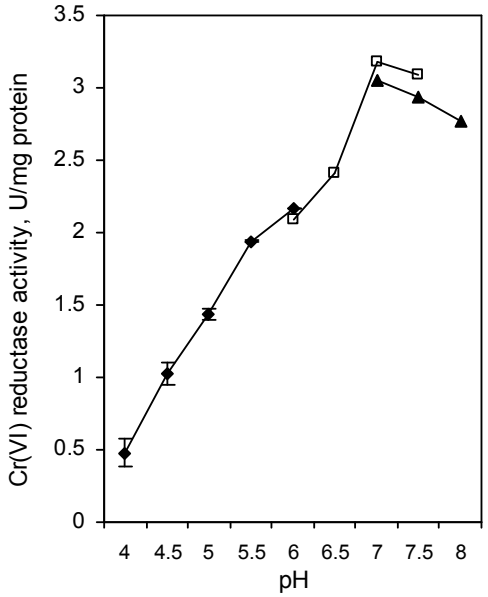

Fig. 4. Effect of pH on chromate reductase activity of cell-free extract of *Arthrobacter* sp. SUK 1201 (-♦- citrate, -□- phosphate and -▲- Tris-HCl buffer)

Experimental details are same as explained under Fig. 1, the pH of the reaction mixture was adjusted in the range of pH 4.0-8.0. Results represent mean of triplicate experiments ± standard error.

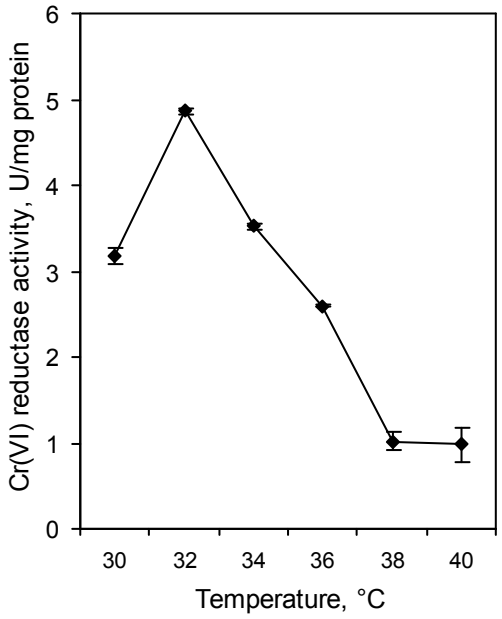

Fig. 5. Effect of temperature on chromate reductase activity of cell-free extract of *Arthrobacter* sp. SUK 1201

Experimental details are same as explained under Fig. 1, the incubation temperature of the reaction mixture ranged between 30-40°C. Results represent mean of triplicate experiments ± standard error.

3.6 Determination of Thermostability

Thermostability of crude cell-free extract of *Arthrobacter* sp. SUK 1201 was tested following its pre-treatment at 40ºC, 60ºC and 80ºC for 5 min in a thermostatically controlled water bath. Following the temperature treatments, the cell-free extracts were cooled to room temperature and their activity was assayed in the usual manner. Results (Table 2) clearly indicate that the Cr(VI) reductase activity of the CFE (S_{12}) was extremely thermolabile as it retained only 9.5% activity when exposed to 40ºC for 5 min. At higher temperature (80ºC) treatment the reductase activity of CFE was almost nil.

Similar thermolabile nature of chromium reducing enzymes has also been reported for *Bacillus subtilis* as it failed to show any Cr(VI) reductase activity after heating at 100ºC for 10 min [27]. However, CFE of *Pseudomonas putida* MK1 could have retained the reductase activity when pre-treated at 80ºC for 5 min, but was very low as compared to that at 50ºC [8]. As usual, cell-free extract of *Streptomyces* sp. MC1 pre-treated at 100ºC for 5 min failed to show any chromate reductase activity [21].

Table 2. Thermostability of the crude cell-free extract of *Arthrobacter* sp. SUK 1201

Pre-treatment of CFE at, ºC	Cr(VI) reductase activity, U mg^{-1} protein[a]	% Relative activity
40	0.46 ± 0.04	9.55
60	0.41 ± 0.02	8.50
80	0.07 ± 0.01	1.59
Control (without pre-treatment)	4.89 ± 0.01	100

Crude CFE was pre-treated for 5 min at different temperatures before being used for chromate reduction studies. Cr(VI) reductase activity was estimated using an initial Cr(VI) concentration of 50 µM. Residual Cr(VI) was estimated following diphenylcarbazide method after 30 min of incubation. % Relative activity = Activity of chromate reductase relative to control set. Results represent mean of triplicate experiments ± standard error.

3.7 Effect of Electron Donors

The cell-free extract of *Arthrobacter* sp. SUK 1201 was able to utilize a variety of organic compounds as electron donors during Cr(VI) reduction, but their reduction efficiency varied considerably (Table 3). The activity of crude CFE was maximum when NADH was used as electron donor followed by glycerol, glycine and glucose showing a specific activity of 4.8, 3.9, 3.4 and 3.2 U mg^{-1} of protein respectively.

Similar dependency on NADH was also seen in isolates *Pseudomonas ambigua* G-1 [14], *P. putida* MK 1 [8], *P. aeruginosa* [28], *Bacillus* ES 29 [19], *Bacillus* AND 303 [9] and *Streptomyces* sp. [21]. Suzuki et al. [14] has reported that NADH donates an electron to Cr(VI), reducing it to an intermediate form, Cr(V), which in turn accepts two electrons from two molecules of the same coenzyme to produce Cr(III). The isolate SUK 1201 can also utilize exogenous glucose as electron donor similar to that reported by Bopp and Ehrlich [29], Garbisu et al. [27] and Pal et al. [9] in *P. fluorescens*, *Bacillus subtilis* and *B. sphaericus* AND 303 respectively. Sodium propionate was the least efficient electron donor showing an activity of 0.8 U mg-1 of protein.

Table 3. Effect of electron donors on Cr(VI) reductase activity of cell-free extract of *Arthrobacter* sp. SUK 1201

Electron donor (0.1 mM)	Cr(VI) reductase activity, U mg^{-1} protein	% Relative activity
Glycerol	3.9 ± 0.03	78.62
Glucose	3.2 ± 0.01	64.71
Na acetate	2.5 ± 0.04	50.60
Na propionate	0.8 ± 0.01	16.85
Glycine	3.4 ± 0.02	68.54
Maltose	2.0 ± 0.00	40.52
Lactose	1.7 ± 0.02	34.67
Control / NADH	4.8 ± 0.00	100.0

Cr(VI) reductase activity was estimated using an initial 50 µM Cr(VI), residual Cr(VI) was estimated following diphenylcarbazide method after 30 min of incubation. % Relative activity = Activity of chromate reductase relative to control set. Results represent mean of triplicate experiments ± standard error.

3.8 Effect of Different Cations

The presence of additional elements affected Cr(VI) reduction activity of CFE of *Arthrobacter* sp. SUK 1201. There was a distinct increase in the activity of chromate reductase in presence of metal like Fe (III) and Cu(II) (Fig. 6). The reductase activity was found to be 7.82 U mg^{-1} protein in both cases. Stimulatory effect of Cu(II) is not uncommon and has been reported previously in *Bacillus* sp. [19], *Arthrobacter crystallopoites* [22], *Bacillus* sp. [3], *Leucobacter* sp. [25] and *Ochrobactrum anthropi* [30]. Cu(II) is a prosthetic group for many reductase enzymes and its main function has been reported to be related to electron transport protection or acting as a single-electron redox centre and in some cases, as shuttle for electrons between protein subunits [31]. Also it may be possible that Cu(II) is indirectly involved in the protection of chromate reductase from O_2 [32]. Addition of exogenous iron was also found to increases the Cr(VI) reduction rate [33].

Mg(II) and Ni(II) ions showed a specific activity of 4.26 U mg^{-1} protein and 3.38 U mg^{-1} protein respectively. Hg(II) was the most inhibitory one showing nearly 85% loss of specific activity owing to its action as disulphide reducers causing denaturation of reductase protein. Similar cases of inhibition of Cr(VI) reductase activity in presence of Hg(II) were also documented by several researchers [3,10,19,34].

3.9 Effect of Metabolic Inhibitors

The effect of metabolic inhibitors on Cr(VI) reduction by cell-free extract of *Arthrobacter* sp. SUK 1201 was investigated using inhibitors of different types. The inhibitors were separately sterilized and added to the reaction mixture in an equimolecular concentration. The inhibitors that were tested include ATPase inhibitor DCCP (N,N,-Dicyclohexyl carboiimide), protonophores like CCCP (Carbonyl cyanide-m-chloro phenyl hydrazone) and 2,4-DNP (2,4-Di nitrophenol), artificial electron acceptors like sodium azide and enolase inhibitors sodium fluoride. The presence of metabolic inhibitors such as 2, 4-dinitrophenol, DCCP, sodium azide, CCCP and NaF affected the chromate reductase activity of the CFE of *Arthrobacter* sp. SUK 1201 and chromate reduction was severely affected in presence of CCCP, which impaired the enolase acticity and showed a specific activity of 0.99 U mg^{-1} protein (Table 4).

In case of 2,4-DNP nearly 96.4% chromate reductase activity of the crude CFE was restored indicating minimum susceptibility to this agent which also acts as a uncoupler which might have accelerated the respiratory chain linked electron transport mechanism [35] and thereby showed chromate reductase activity more or less similar to control.

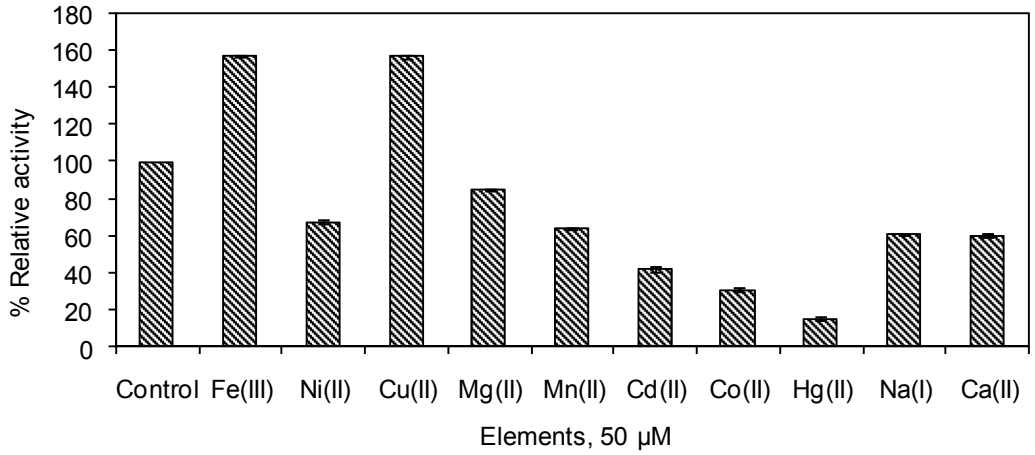

Fig. 6. Effect of different cations on chromate reductase activity of cell-free extract of *Arthrobacter* sp. SUK 1201

Experimental details are same as explained under Fig. 1, the concentration of the elements were kept at 50 µM. Results represent mean of triplicate experiments ± standard error.

Table 4. Effect of inhibitors on chromate reduction by cell-free extract of *Arthrobacter* sp. SUK 1201

Inhibitors, (50 µM)	Cr(VI) reductase activity, U mg^{-1} protein	% Relative activity
Control (- Inhibitor)	4.76 ± 0.02	100.0
2,4-DNP	4.65 ± 0.00	96.40
DCC	2.96 ± 0.00	32.13
NaN_3	3.73 ± 0.00	41.54
NaF	2.89 ± 0.04	39.27
CCCP	0.99 ± 0.00	20.94

The Cr(VI) and inhibitor content of all sets was maintained at 50 µM, residual Cr(VI) was estimated following diphenylcarbazide method after 30 min of incubation. % Relative activity = Activity of chromate reductase relative to control set. Results represent mean of triplicate experiments ± standard error. DNP = 2,4-Di nitrophenol, DCC = N,N,-Dicyclohexyl carboiimide, NaN_3 = Sodium azide, NaF = Sodium fluoride, CCCP = Carbonyl cyanide-m-chloro phenyl hydrazone.

4. CONCLUSION

The optimization of Cr(VI) reduction under different experimental conditions by cell-free extract of *Arthrobacter* sp. SUK 1201 clearly established the biotechnological potential of transformation of Cr(VI) to less toxic Cr(III) and thus could be useful in detoxification of chromium pollutants particularly in aquatic systems.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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