

## IN-VITRO BIOREDUCTION OF HEXAVALENT CHROMIUM BY VIABLE WHOLE CELLS OF *Arthrobacter* sp. SUK 1201

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### ABSTRACT

A chromium resistant and reducing bacterium *Arthrobacter* sp. SUK 1201 was isolated from chromite mine overburden dumps of Orissa, India. Viable whole cells of this isolate was capable of completely reducing 100  $\mu$ M Cr(VI) in chemically defined MS medium within 28 h of incubation under batch cultivation. Reduction of chromate increased with increased cell density and was maximum at a density of  $10^{10}$  cells/ml, but the reduction potential of the suspended cells decreased with increase in Cr(VI) concentration in the medium. Chromate reducing efficiency was promoted when glycerol and glucose was used as electron donors, while the optimum pH and temperature of Cr(VI) reduction was found to be 7.0 and 35°C respectively. The reduction process was inhibited by divalent cations Ni, Co and Cd, but not by Cu and Fe. Similarly, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), N,N-Di cyclohexyl carboimide (DCC), sodium azide and sodium fluoride were inhibitory to chromate reduction, while in presence of 2,4 dinitrophenol (2,4 DNP) chromate reduction by SUK 1201 cells remained unaffected.

**Keywords:** *Arthrobacter* sp., chromate reduction, chromite mine overburden, bioreduction, bioremediation

### INTRODUCTION

In nature, the transition metal chromium is found to occur as trivalent [Cr(III)] and hexavalent [Cr(VI)] forms as they represent the most stable oxidation states. Mobilization of Cr(III) is slow unless dissolved in acidic environment or complexed with organic compounds (Rai *et al.*, 1987) and is less bioavailable in natural environment, whereas Cr(VI) is highly toxic, carcinogenic and mutagenic (Bagchi *et al.*, 2002) due to its high degree of solubility and membrane permeability leading to oxidative stress, DNA damage and altered gene expression.

Reduction of toxic Cr(VI) to less toxic Cr(III) from and its subsequent precipitation at neutral pH is one of the cost-effective and eco-friendly method for detoxification of Cr(VI) in contaminated wastes. On the contrary, the traditional physico-chemical processes of detoxification such as precipitation, ion exchange and adsorption are not only expensive but also generate secondary pollutants (Camargo *et al.*, 2003). Pure cultures of a wide variety of bacterial strains (Camargo *et al.*, 2003; Asianti *et al.*, 2004) as well as consortium (Molokwane *et al.*, 2008) have been reported to reduce Cr(VI) under both aerobic (Megharaj *et al.*, 2003; Thacker *et al.*, 2007) and anaerobic conditions (Cervantes *et al.*, 2001). In addition, chromium resistant and reducing bacteria isolated from chromite mining environments have attracted increased interest for potential application in bioremediation of Cr(VI) polluted waste water (Dhal *et al.*, 2010; Dey and Paul, 2012). *Arthrobacter*, the common Gram-positive bacteria having characteristics rod to cocci cell cycle are capable of surviving in various chromium contaminated industrial areas such as tannery (Meghraj *et al.*, 2003), chromite mine (Molokwane *et al.*, 2008; Dey and Paul, 2012) and Department of Energy (DOE) waste sites (Asianti *et al.*, 2004) and have been explored for their chromate reducing potentials. These isolates were able to reduce hexavalent chromium during growth, by whole cells and also by cell-free extracts. Asianti *et al.*, (2004) and Meghraj *et al.*, (2003) have demonstrated that *Arthrobacter* strains were able to reduce nearly 35 and 30  $\mu$ g/ml of Cr(VI) in 10 day and 46 h respectively. Similarly, Camargo *et al.*, (2004) have evaluated the chromate reducing efficiency of *Arthrobacter crystallopoites* ES 32 during growth.

During the course of screening of bacterial strains capable of reducing hexavalent chromium, we have reported the isolation of *Arthrobacter* sp. SUK 1201 from chromite mine overburden of Orissa, India and have evaluated its chromate reducing potential during growth (Dey and Paul, 2012). The present study concentrates on the assessment of Cr(VI) reduction ability of the viable whole

cells of *Arthrobacter* sp. SUK 1201 under batch culture and determination of optimum conditions for such reductions.

### MATERIAL AND METHODS

#### Source and maintenance of bacterial culture

The chromate reducing bacterium, *Arthrobacter* sp. SUK 1201 (MTCC accession number 8728 and NCBI Gen Bank accession No. JQ312665) used in this study was isolated from mine overburden materials collected from Sukinda chromite mining areas of Orissa, India (Dey and Paul, 2012). The strain was routinely maintained on slopes of peptone, yeast extract and glucose (PYEG) agar medium (Wang and Xiao, 1995) containing (g/L) peptone, 10.0; yeast extract, 5.0; glucose, 3.0 and agar agar, 20.0 (pH 7.0). The medium was supplemented with 2 mM Cr(VI) and the over-night grown cultures were stored at 4°C for short term preservation, while for long-term preservation, the freshly grown cells were suspended in sterile 30% glycerol in cryo vials and stored at - 80°C for future use.

#### Preparation of cell mass for chromate reduction studies

Whole cells of *Arthrobacter* sp. SUK 1201 were harvested from overnight grown cultures incubated at 35°C under continuous shaking in MS medium. The MS medium contained (g/L): NH<sub>4</sub>Cl, 0.03; K<sub>2</sub>HPO<sub>4</sub>, 0.03; KH<sub>2</sub>PO<sub>4</sub>, 0.05; NaCl, 0.01; MgSO<sub>4</sub>, 7H<sub>2</sub>O, 0.01 (pH 7.0). The cell mass was obtained by centrifugation (10,000 $\times$ g) at 4°C for 10 min and washed 2-3 times with ice cold Tris buffer (pH 7.2) following the method of Wang and Xiao (1995). The cell mass thus obtained was diluted to a particular cell density and used for reduction studies. Viability of the cells was determined by serial dilution of the cell suspension and plating on PYEG agar medium supplemented with 2 mM Cr(VI). Viable cell numbers /mL were calculated from the colony forming units (c.f.u)/mL after 24 h of incubation at 35°C.

#### Chromate reduction by suspended whole cells

Chromate reduction by viable whole cells of *Arthrobacter* sp. SUK 1201 was carried out in 25 mL of Minimal salts (MS) medium (in 100 mL Erlenmeyer flasks) supplemented with 100  $\mu$ M Cr(VI). Flasks were inoculated with viable cells at a density of  $10^8$  cells/mL and incubated at 35°C under continuous shaking (120 rpm) in a rotary shaker. Samples were withdrawn aseptically at regular

intervals and reduction of chromate was estimated by measuring the residual hexavalent chromium in the reduction medium using diphenylcarbazide method (Park et al., 2000). The growth of the cells was determined by counting total number of cells/mL of culture using a haemocytometer (Neubauer, Fein-Optik Jena, Germany) and a phase contrast microscope (Zeiss Winkel Model no. 148786, Germany).

**Effect of different media on chromate reduction**

Effect of different media on chromate reduction capability of viable whole cells of *Arthrobacter* sp. SUK 1201 was determined using mineral salts (MS) medium, half-strength peptone yeast extract glucose (PYEG) medium and Vogel Bonner (V. B.) broth. The half-strength PYEG medium was prepared by diluting the PYEG medium with distilled water in the ratio of 1:1. Vogel Bonner broth was made up of 2.0% sterile stock solution of V. B. concentrate. The V. B. concentrate contained (g/L): K<sub>2</sub>HPO<sub>4</sub>, 500.0; Na(NH<sub>4</sub>)HPO<sub>4</sub>·4H<sub>2</sub>O, 175.0; citric acid, 100.0; MgSO<sub>4</sub>·7H<sub>2</sub>O, 10.0 and 2.0 % of 25% D-glucose (pH 7.0) (Wang and Xiao, 1995). The media were supplemented with 100 µM Cr(VI) using separately sterilized chromate solution and inoculated with viable cells at a density of 10<sup>8</sup> cells/mL. Conditions of growth, measurement of growth and residual hexavalent chromium in the reduction medium were same as described earlier.

**Effect of electron donors on chromate reduction**

Chromate reduction by viable whole cells of *Arthrobacter* sp. SUK 1201 was studied in presence of various electron donors, which include glycerol, glucose, sucrose, acetate, citrate, propionate, glycine, peptone, tryptone and yeast extract. The reduction medium (25 mL of MS medium/100 mL) was supplemented with 100 µM Cr(VI) along with the electron donors at 0.1% (w/v). Other experimental conditions were same as described above.

**Effect of pH on chromate reduction**

Effect of different pH on the Cr(VI) reducing capability of whole cells of *Arthrobacter* sp. SUK 1201 was determined by adjusting the pH of Minimal salts (MS) medium in the range of pH 4-8 using 0.1 (N) NaOH and 0.1 (N) HCl. Chromate concentration, cell density/mL and incubation conditions were same as described earlier.

**Effect of additional metal ions on chromate reduction**

Chromate reduction by *Arthrobacter* sp. SUK 1201 was studied in presence of additional metal ions such as Mn(II), Co(II), Zn(II), Fe(III), Cu(II), Ni(II) and Cd(II). The metals were used as chloride salts, sterilized separately and added to MS medium at equimolar (100 µM) level of Cr(VI). Other experimental conditions were same as described above.

**Effect of metabolic inhibitors on chromate reduction**

The effect of metabolic inhibitors on chromate reduction by viable whole cells of *Arthrobacter* sp. SUK 1201 was investigated using the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ATPase inhibitor N,N-Di cyclohexyl carboiimide (DCC), artificial electron acceptor sodium azide, enolase inhibitor sodium fluoride and 2,4 dinitrophenol (2,4 DNP). The inhibitors were separately sterilized and added to the reduction medium at equimolar concentration.

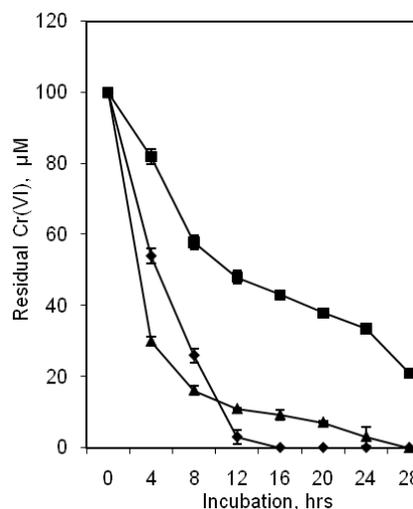
**Statistical analysis**

All experiments were carried out in triplicate and results represent mean ± standard error.

**RESULTS**

**Effect of different media on chromate reduction**

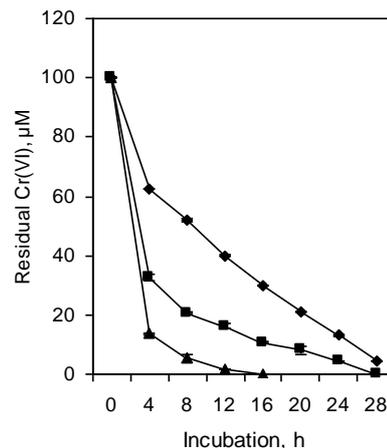
Freshly grown viable cells of *Arthrobacter* sp. SUK 1201 were used to determine the effect of different media on chromate reduction. The test media include mineral salts (MS) medium, half strength peptone yeast extract glucose (PYEG) medium and Vogel Bonner (V. B.) broth. Results indicate that whole cells of *Arthrobacter* sp. SUK 1201 in half strength PYEG medium was the most effective one as complete reduction of initial Cr(VI) occurred in 16 h. This was followed by MS medium, where complete reduction of 100 µM Cr(VI) was achieved within 28 h. However, in V. B. broth nearly 79% of Cr(VI) was reduced by cells of SUK 1201 (Figure 1). Considering the efficacy of reduction as well as to avoid chances of interference of the organic constituents of PYEG medium in the process of reduction, the MS medium was selected and used as the base for subsequent chromate reduction studies with whole cells of *Arthrobacter* sp. SUK 1201.



**Figure 1** Effect of different media on hexavalent chromium reduction by whole cells of *Arthrobacter* sp. SUK 1201 (- ♦ - PYEG medium, - ■ - V.B. broth and - ▲ - MS medium)

**Effect of cell density**

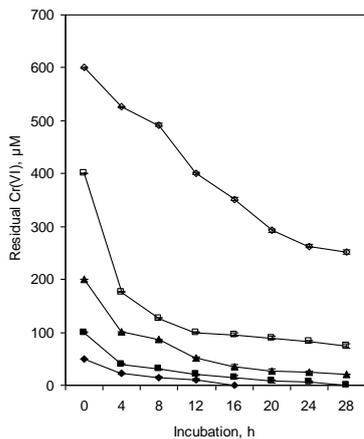
Chromate reducing capacity of the whole cells of the isolate SUK 1201 was tested at a cell density of 10<sup>8</sup> – 10<sup>10</sup> cells/mL in MS medium. Results as represented in Figure 2 clearly indicate that 10<sup>10</sup> cells/mL was the level of cell concentration where complete reduction of chromate occurred within 16 h of incubation. However, whole cells at a density of 10<sup>8</sup> cells/mL were comparatively less efficient in reducing the chromate and require prolong incubation for equivalent reduction.



**Figure 2** Hexavalent chromium reduction by whole cells of *Arthrobacter* sp. SUK 1201 as influenced by cell density (- ♦ -10<sup>8</sup>, - ■ -10<sup>9</sup>, - ▲ -10<sup>10</sup> cells/mL)

**Effect of Cr(VI) concentration**

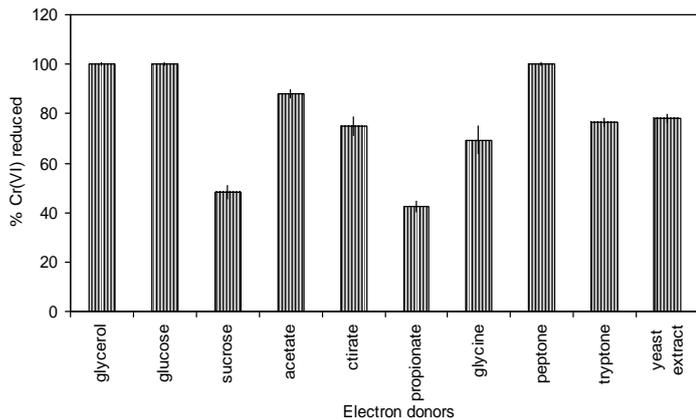
Chromate reducing capacity of the whole cells of isolate SUK 1201 was evaluated at Cr(VI) concentrations ranging from 50–600 µM. Reduction efficiency of *Arthrobacter* sp. SUK 1201 cells was found to increase with increase in Cr(VI) concentration up to 100 µM. Although complete reduction of 50 and 100 µM Cr(VI) occurred within 16 and 28 h respectively, nearly 89, 82 and 58% of initial chromium was reduced in 28 h when the initial chromium concentration of the reduction medium was maintained at 200, 400 and 600 µM respectively (Figure 3).



**Figure 3** Hexavalent chromium reduction by whole cells of *Arthrobacter* sp. SUK 1201 as influenced by Cr(VI) concentration (-♦- 50, -■- 100, -▲- 200, -□- 400, -◇- 600 µM)

**Effect of electron donor**

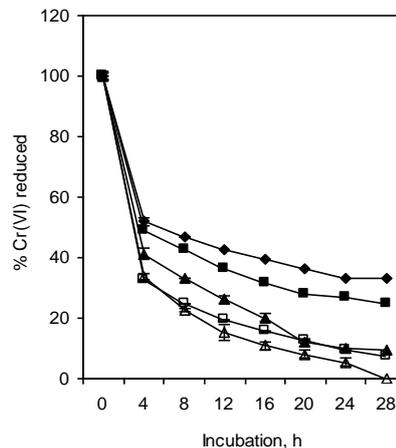
Chromate reduction by whole cells of *Arthrobacter* sp. SUK 1201 was studied in presence of various electron donors (0.1% w/v). The electron donors include glycerol, glucose, sucrose, acetate, citrate, propionate, glycine, peptone, tryptone and yeast extract. Results as demonstrated in Figure 4 clearly indicate that Cr(VI) reduction was very much dependent on the electron donor present in the medium. Maximum Cr(VI) reduction was evident when glycerol, glucose and peptone was used as the electron donor. In presence of these electron donors, complete reduction of Cr(VI) occurred. Propionate was found to be least efficient as electron donor for chromate reduction by SUK 1201 cells.



**Figure 4** Effect of electron donor on Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201

**Effect of pH**

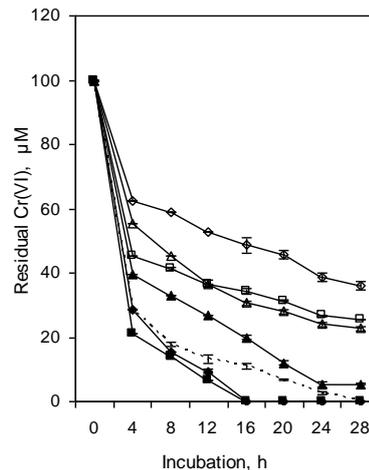
Effect of pH on chromate reduction was carried out in MS medium adjusted to different pH values. Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201 was greatly dependent on the pH of the medium. Results show that maximum Cr(VI) reduction occurred at pH 7.0, which was retarded both by the acidic and alkaline pH of the medium (Figure 5).



**Figure 5** Effect of pH on Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201 (-♦- 4.0, -■- 5.0, -▲- 6.0, -△- 7.0, -□- 8.0)

**Effect of additional metal ions**

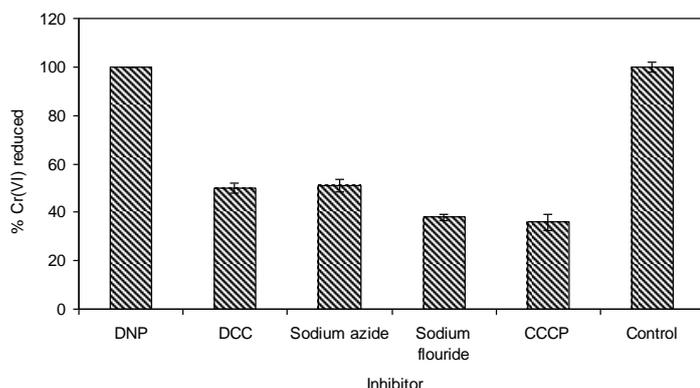
Chromate reduction by whole cells of the isolate *Arthrobacter* sp. SUK 1201 showed that supplementation of Mn(II), Co(II), Zn(II) and Ni(II) in the MS medium were in general inhibitory to chromate reduction. Mn(II) was most inhibitory showing nearly 42.6% inhibition of hexavalent chromium reduction when compared with the control. Co(II) showed 36% inhibition followed by Zn(II) and Ni(II) showing 34 and 18% inhibition respectively. Presence of Fe(III) and Cu(II) however, appeared to be promotive in nature showing complete reduction of 100 µM of Cr(VI) with 16 h of incubation (Figure 6).



**Figure 6** Effect of metal ions on Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201 [Metals: -♦- Fe(III), -■- Cu(II), -▲- Ni(II), -△- Co(II), -□- Zn(II), -◇- Mn(II), --- Control]

**Effect of metabolic inhibitors**

Chromate reduction by free whole cells of SUK 1201 was affected by the presence of inhibitors in reduction medium. The response of 2,4-di nitrophenol (2,4 DNP) on chromate reduction by whole cells of SUK 1201 was neither inhibitory nor promotive as the extent of Cr(VI) reduction in presence of 2,4 DNP was more or less identical with the control. Protonophores like sodium fluoride was the strongest inhibitor causing 42.6% Cr(VI) reduction followed by carbonyl cyanide-*m*-chloro phenyl hydrazone (CCCP), sodium azide and N,N-Dicyclohexyl carboimide (DCC) (Figure 7).



**Figure 7** Effect of inhibitors on Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201

## DISCUSSION

Aerobic reduction of hexavalent chromium by viable whole cells in suspended mode have been studied quite extensively using a variety of microorganisms (Farag and Zaki, 2010; Ezaka and Anyanwu, 2011). During the present investigation, chromate reduction by viable whole cells of *Arthrobacter* sp. SUK 1201 (MTCC accession number 8728 and NCBI Gen Bank accession No. JQ312665) has been studied under batch culture using a chemically defined MS medium.

The composition of the suspending media greatly influenced the efficiency of chromate reduction by SUK 1201 cells under laboratory conditions (Figure 1). The half strength peptone yeast extract glucose (PYEG) medium, though showed complete reduction of chromate but was not considered for further experiments as it is reported to reduce the availability of hexavalent chromium due to its complexation with organic constituents. The minimal salts (MS) medium supplemented with 0.1% (w/v) glucose was considered as the most effective one for Cr(VI) reduction by *Arthrobacter* sp. SUK 1201. MS medium being a completely inorganic one, minimized the possible complexation of Cr(VI) with media components and allowed the assessment of Cr(VI) reduction potential more accurately. Meghraj et al. (2003) in their study also reported similar results with *Arthrobacter* sp.

A high cell density ( $10^{10}$  cells/mL) of *Arthrobacter* sp. SUK 1201 was essentially required for complete reduction of 100  $\mu$ M Cr(VI) in 16 h (Figure 2). Similar results were also found with *Microbacterium* MP 30 (Pattanapitpaisal et al., 2001), where the rate of Cr(VI) reduction increased with increase in cell concentration ranging from  $10^7$ - $10^{10}$  cells/mL. Likewise, with increase in cell density, the rate of Cr(VI) reduction was also found to increase in *Bacillus sphaericus* AND 303 (Pal and Paul, 2004).

The rate of chromate reduction by suspended whole cells of wide variety of bacteria has been reported to be greatly influenced by the initial Cr(VI) concentration (He et al., 2009; Pattanapitpaisal et al., 2001; Wang and Xiao, 1995). In the present study complete reduction by the cells of SUK 1201 occurred only when the Cr(VI) concentration was maintained at 50 and 100  $\mu$ M, but at higher concentration complete reduction has failed to occur (Figure 3).

Chromate reducing organisms in general utilize a variety of organic compounds as electron donors for Cr(VI) reduction. Aerobic Cr(VI) reduction by whole cells of *Pseudomonas* sp. (Mc Lean and Beveridge, 2000), *Bacillus* sp. (Pal and Paul, 2004) and *Ochrobactrum* sp. (He et al., 2009) has been reported to be enhanced in the presence of glucose. Marbrouk (2008) also reported that peptone and yeast extract favoured chromate reduction in *Streptomyces* sp. MS-2. Likewise, glycerol, glucose and peptone served as efficient electron donors for Cr(VI) reduction by *Arthrobacter* sp. SUK 1201 (Figure 4).

The optimum pH for Cr(VI) reduction by cells of *Arthrobacter* sp. SUK 1201 appear to be identical with the optimum pH for growth of the isolate (Dey and Paul, 2012). Moreover, the process of Cr(VI) reduction by SUK 1201 cells was severely affected by acidic and alkaline pH (Figure 5). Cells of *Arthrobacter* sp. SUK 1201 effectively reduced chromate in a narrow range of pH with an optimum of pH 7.0 similar to that of *Arthrobacter crystallopoites* (Camargo et al., 2003). Such an influence of pH on the chromate reduction by whole cells of *Arthrobacter* sp. SUK 1201, may possibly be correlated with their growth as well as metabolic activity. Further, the pH mediated alteration of the reductase activity may be the result of the modification of the structure or conformation of chromate reductase of *Arthrobacter* sp. SUK 1201.

Chromium reduction by SUK 1201 cells and its stimulation by Cu(II) (Figure 6) might be due to the fact that Cu is a prosthetic group for many reductase enzymes including those of chromium reductases of microbial origin. The copper ions may act as an electron redox center and help in the shuttle of electron between subunits of the enzyme (Sultan and Hasnain, 2006). Similar enhancement of Cr(VI) reduction potential was also evident with *Ochrobactrum intermedium*, (Sultan and Hasnain, 2007), *Amphibacillus* sp. KSUCr3 and *Bacillus* sp. KSUCr5 (Ibrahim et al., 2011, 2012).

The uncoupler, 2,4 DNP mediated acceleration of chromate reduction by whole cell suspension of *Arthrobacter* sp. SUK 1201 was supported by enhancement of Cr(VI) reduction by 2,4 DNP in *E. coli* (Shen and Wang, 1993), *Burkholderia cepacia* (Wani et al., 2007) and *Staphylococcus gallinarum* (Alam and Ahmed, 2011) (Figure 7). On the contrary, sodium azide, CCCP and DCC mediated inhibitory effect on the process of chromate reduction could be due to inhibition of cytochrome oxidase, disruption of chemiosmotic gradient and inhibition of ATPase activity respectively. Similar to *Arthrobacter* sp. SUK 1201, inhibition of Cr(VI) reduction in presence of  $\text{NaN}_3$  was also evident with *Stenotrophomonas maltophilia*, *Staphylococcus gallinarum*, *Pantoea* sp. and *Aeromonas* sp. (Alam and Ahmed, 2011). Thus optimization of different physico-chemical conditions for Cr(VI) reduction by viable whole cell suspension of *Arthrobacter* sp. SUK 1201 establishes its potential for biotechnological application in the transformation of highly toxic and mutagenic Cr(VI) to less toxic Cr(III) and thus could be identified as an ideal tool for detoxification of chromium pollutants.

## CONCLUSION

Optimization of conditions for Cr(VI) reduction by whole cells of *Arthrobacter* sp. SUK 1201 established its biotechnological potential for transformation of highly toxic and carcinogenic Cr(VI) to less toxic and insoluble Cr(III) and thus could be an effective tool in bioremediation of chromium pollutants.

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