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Isolation and characterization of a Cr(VI)-reduction *Ochrobactrum* sp. strain CSCr-3 from chromium landfill

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ABSTRACT

A strain CSCr-3 with high Cr(VI)-reducing ability under alkaline conditions was isolated from a chromium landfill and identified as *Ochrobactrum* sp. on the basis of 16S rRNA gene sequence analysis. The cells were rod shaped, Gram-negative and motile. The physiological characteristics and Cr(VI)-reduction of the strain were also studied. The results showed that the *Ochrobactrum* sp. strain CSCr-3 was tolerant to very high concentration of Cr(VI) (800 mg/L) and capable of reducing different forms of Cr(VI) (chromate and dichromate), under a wide range of temperatures (25–40 °C) and pH (7–11) with optimum at 35 °C and initial pH 10. Higher rates of Cr(VI)-reduction were observed with higher initial cell and Cr(VI) concentrations. Strain CSCr-3 could reduce Cr(VI) very efficiently over a wide range of Cr(VI) concentrations (100–800 mg/L). The addition of glucose caused a dramatic increase in Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3, while the presence of sulfate or nitrate had no influence. The presence of other metals, such as Cu, Co, Mn, etc., significantly stimulated Cr(VI)-reduction ability by the strain CSCr-3. The results obtained in this study have significance for the bioremediation of chromate pollution.

1. Introduction

Chromium (Cr) has increased rapidly since the industrial revolution. Chromium is widely used in industrial operations such as leather tanning, electroplating, paints, pigment production, steel manufacture and others [1,2]. In the environment, Cr(VI) contamination alters the structure of soil microbial communities. As a result of reduced microbial growth and activities, organic matter accumulates Cr(VI) in soils. In humans, several traumata are associated with Cr(VI) exposure, including nasal irritation and ulceration, skin irritation, eardrum perforation and lung carcinoma. Furthermore, Cr(VI) can accumulate in the placenta, impairing fetal development in mammals [2,3]. Cr(VI) is taken up through the membrane sulfate transport channels in cells of sulfate-utilizing organisms. Inside the cell, Cr(VI) can oxidatively damage DNA and other cell components via the production of more reactive intermediate species Cr(V) and Cr(IV) to produce its toxic, mutagenic and carcinogenic effects on biological systems [4]. Cr(VI) from these industries has become a well-recognized bio-hazard.

Conventional methods for removing chromium usually involve reduction and separation from the water phase. Traditionally, physico-chemical processes are used to reduce Cr(VI) concentrations to levels that comply with statutory standards. Most commonly used processes include reduction–precipitation, ion exchange and reverse osmosis. However, the costs to set up the required equipment and to operate these processes are prohibitively high for large-scale treatment [5]. The cell membrane is nearly impermeable to Cr(III) and thus Cr(III) has only approx. one-thousandth of the toxicity of Cr(VI). Because the insolubility of Cr(III) facilitates its precipitation and removal, the biotransformation of Cr(VI) to Cr(III) has been considered as an alternative process for treating Cr(VI)-contaminated wastes [6,7]. Since the discovery of the first microbe capable of reducing Cr(VI) in the 1970s [8], the search for Cr(VI)-reducing microorganisms (both aerobic and anaerobic) has been enthusiastically pursued, with numerous strains being isolated [9–11]. Up to now many bacterial strains such as *Bacillus* [12], *Shewanella* [15], *Desulfovibrio* [16], *Microbacterium* [1] and so on, have been reported to reduce the toxic Cr(VI) to the less toxic Cr(III), indicating an important bio-remedial step in detoxification of Cr(VI)-contaminated wastes. However, the availability of an effective Cr(VI)-removing bacterial strain is an essential pre-requisite for developing a bioremediation process aimed at the detoxification of Cr(VI)-contaminated waste waters.

The objective of this study was to characterize the Cr(VI) resistance and reduction potential of strain CSCr-3 isolated from a chromium landfill where the level of hexavalent chromium is very high.

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2. Materials and methods

2.1. Isolation and identification of bacterial strains

Cr(VI)-reducing bacterial strain CSCr-3 was isolated from the chromium landfill, located at a chromate factory in Changsha, China. Bacteria were maintained in a liquid medium which contained (g/L): peptone 10.0; yeast extract 5.0; NaCl 5.0; $MgSO_4{\cdot}7H_2O$ 0.2; K_2HPO_4 0.05 (adjusted to pH 9.5–10.0 with NaOH) supplemented with $K_2Cr_2O_7$, and incubated at 30 °C with 160–180 rpm shaking. Cultures were purified by isolating single colonies grown on solid medium containing 1.2% (w/v) agar.

Isolated strains were characterized morphologically, biochemically and physiologically following Gerhardt et al. [17]. The taxonomic identity of the strain CSCr-3 was confirmed by 16S rRNA gene sequencing. The 16S rRNA gene (about 1200 bp) was amplified and the extension product was then sequenced on an automated DNA sequencer. The data were compared to the sequences in NCBI GenBank database.

2.2. Cr(VI) resistance and reduction experiment

Resistance of strain CSCr-3 to Cr(VI) was determined in nutrient medium with $K_2Cr_2O_7$. Culture medium (100 mL) in 250 mL conical flasks was supplemented with the desired Cr(VI) concentration, inoculated with 1% exponential growth phase bacterial culture and incubated at 30 °C with 160 rpm shaking. Growth was monitored at specific time intervals and determined by direct microscopic count using optical microscopy (OLYMPUS CX31RTSF, Olympus Corporation, Tokyo, Japan).

The isolate was inoculated in 100 mL of medium in flasks supplemented with 100 and 200 mg/L of Cr(VI) as K_2CrO_4, $K_2Cr_2O_7$ and incubated at 30 °C with shaking. Samples were drawn at regular time intervals and centrifuged at 10,000 rpm for 15 min. Cr(VI) concentration in the supernatant was determined colorimetrically using diphenylcarbazide reagent in acid solution. The absorbance was measured at 540 nm by UV 754N model spectrophotometer. Samples for total chromium analysis were first digested with a mixture of sulfuric–nitric acids and oxidized with potassium permanganate before reacting with diphenylcarbazide and determined colorimetrically [18]. Cr(VI)-reduction was calculated from the difference between total chromium and Cr(VI).

2.3. Factors affecting Cr(VI)-reduction

To characterize the Cr(VI)-reduction efficiency of strain CSCr-3, the effects of temperature (25, 30, 35, 40, 45 °C), initial pH (5, 7, 9, 10, 11), inoculated cell concentration (0.08×10^8 to 1×10^8 cells/mL) and initial Cr(VI) concentration (200–800 mg/L) were investigated. Cr(VI)-reduction was studied in aerobic batch cultures. Autoclaved nutrient medium (100 mL) in 250 mL culture flasks was supplemented with Cr(VI), inoculated from log phase bacterial culture (with the desired number of cells) and incubated at the appropriate temperature with shaking (160–180 rpm). Samples were drawn at regular time intervals and analyzed for disappearance of Cr(VI) as described above. In order to monitor any abiotic Cr(VI)-reduction, cell-free controls were also used for each Cr(VI)-reduction assay. Samples were aseptically drawn at defined times, centrifuged at $7378 \times g$ for 10 min and the supernatant analyzed for residual Cr(VI) by using the standard diphenyl carbazide method [19].

The effects of glucose (supplemented 0.1% glucose (w/v) as the electron donor) and heavy metals to final concentrations of 20 mg/L (Co^{2+}) or 100 mg/L (Cu^{2+}, Mn^{2+}, Ni^{2+}, Mo^{2+} and Zn^{2+}) on Cr(VI)-reduction by strain SCCr-3 were also investigated. Nutrient medium (100 mL) in culture flasks was supplemented with Cr(VI) to a final concentration of 400 mg/L and incubated at 35 °C with shaking. The experiments were performed as described above and the mean values were reported.

2.4. Statistical analysis

All experiments were done in triplicates. The results were subjected to statistical analyzes and standard error of the means (S.E.M.) and least significant difference (LSD) were calculated [20].

3. Results and discussion

3.1. Identification and characteristics of strains

A strain CSCr-3 with high Cr(VI)-reduction ability under alkaline conditions was isolated from a chromium landfill. The strain belongs to genus *Ochrobactrum* sp. (98.8% similarity with *Ochrobactrum* sp. 1605) on the basis of 16S rRNA gene sequence analyzes (about 1200 bp). The cells of the strain were rod-shaped, Gram-negative, and motile.

3.2. Cr(VI) resistance and reduction experiment

Cr(VI) resistance of *Ochrobactrum* sp. CSCr-3 was evaluated by the growth response of the strain under different concentrations of Cr(VI). Fig. 1 shows the relationship between the growth of cells and initial Cr(VI) concentrations. The growth of cells was heavily influenced by Cr(VI) at a concentration of 800 mg/L, while Cr(VI) at 200, 400 and 600 mg/L had only slight effect on the growth. The lag phase was 12 h for all studied Cr(VI) concentrations below 600 mg/L. The exponential phase extended to 30 and 42 h with 0–200 and 400–600 mg/L, respectively. Garbisu et al. [21] reported that chromate at 52 mg/L significantly affected cell growth of *Bacillus subtilis* and the cells failed to grow and reduce chromate at 104 mg/L chromate. The strains isolated by Megharaj et al. from Cr contaminated soil could grow up to 100 mg/L in minimal medium [13]. *O. intermedium* CrT-1 and *Brevibacterium* sp. CrT-13 tolerated 10,000 mg/L chromate in acetate minimal medium [14].

Cr(VI)-reduction potential of *Ochrobactrum* sp. CSCr-3 was assessed with two kinds of different Cr(VI) salts, K_2CrO_4 and $K_2Cr_2O_7$. Strain CSCr-3 had good Cr(VI)-reduction potential with both Cr(VI) salts as seen in Fig. 2. At 200 mg/L Cr(VI) concentration, Cr(VI) was reduced up to 80% by strain CSCr-3

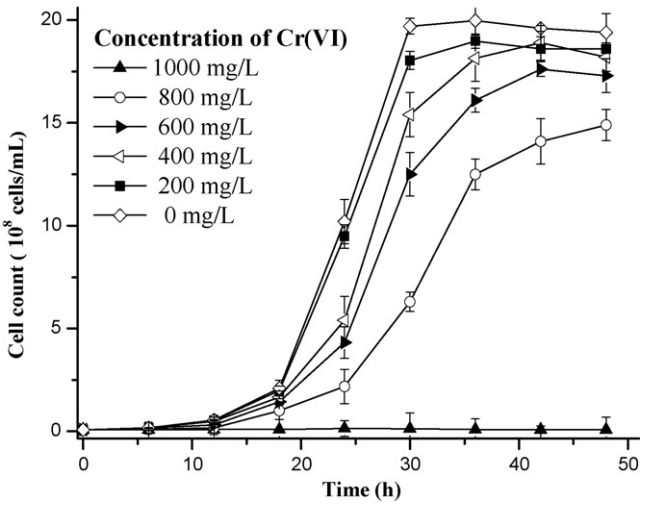

Fig. 1. Growth curves of *Ochrobactrum* sp. CSCr-3 at varying Cr(VI) concentration.

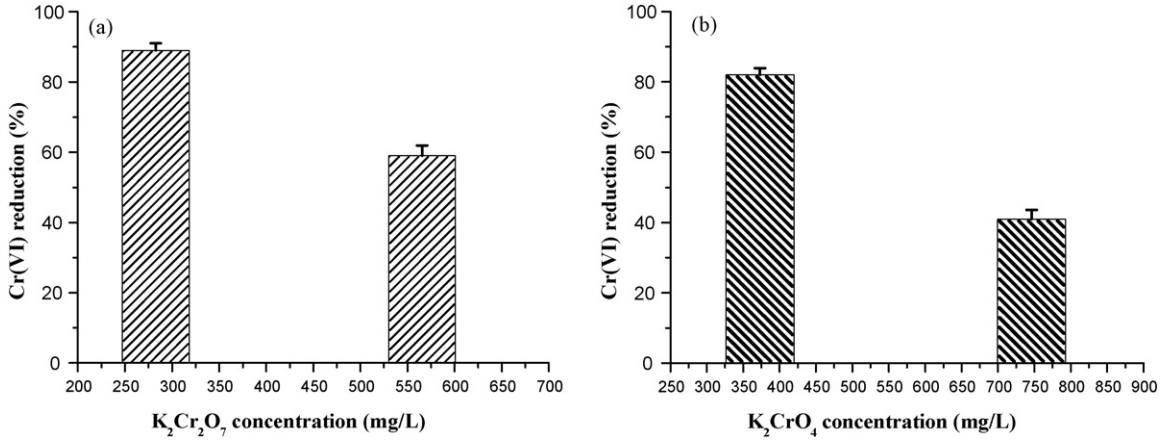

Fig. 2. Cr(VI)-reduction yield by *Ochrobactrum* sp. CSCr-3 exposed to different forms of Cr(VI) ($Cr_2O_7^{2-}$ (a) and CrO_4^{2-} (b)) in 30 h. 282.7 mg/L of $K_2Cr_2O_7$ or 373.1 mg/L of K_2CrO_4 is equally to 100 mg/L of Cr(VI).

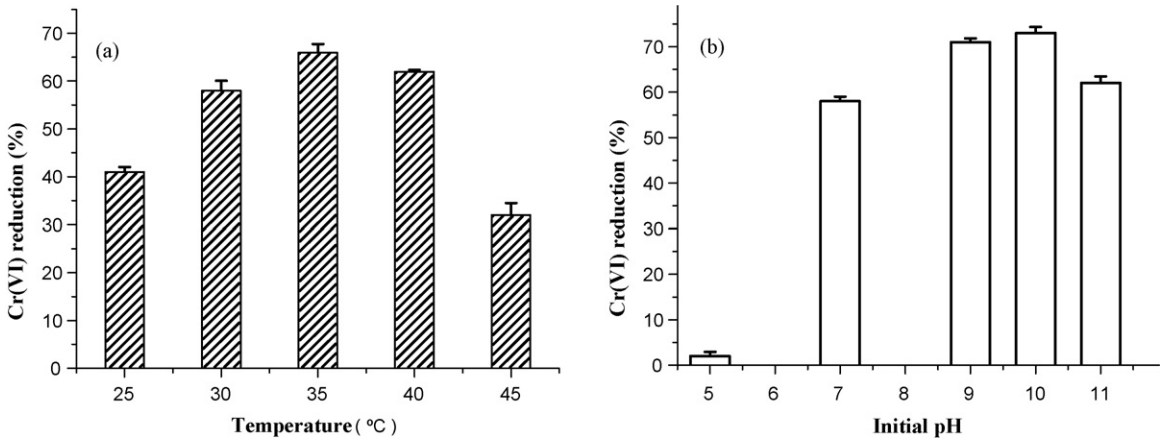

Fig. 3. Influence of temperature (a) and initial pH (b) on Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3 in 30 h.

within 30 h. At Cr(VI) 400 mg/L, over 40% Cr(VI)-reduction was obtained within 30 h. Cr(VI) occurs in aquatic environment either as CrO_4^{2-} or $Cr_2O_7^{2-}$ [4,21] and strain CSCr-3 isolated in this study was able to reduce both forms of hexavalent chromium.

3.3. Factors affecting Cr(VI)-reduction

3.3.1. Effects of temperature and pH

Temperature is an important factor that has an effect on microbial Cr(VI)-reduction. Cr(VI)-reduction by the strain CSCr-3 was evaluated under five different temperatures: 25, 30, 35, 40 and 45 °C within 30 h. The strain CSCr-3 reduced Cr(VI) fairly well (32–66%) from 25 to 45 °C with an optimum at 35 °C as shown in Fig. 3(a). The optimum temperature for Cr(VI) reduction by *Bacillus* sp. [1] and *Pseudomonas* strain CRB5 was 30 °C [22].

Fig. 3(b) shows the effect of initial pH (5–11) on the Cr(VI)-reduction yield of strain CSCr-3 in 30 h. The strain CSCr-3 reduced Cr(VI) from pH 7 to 11 with an optimum at pH 10. There was no obvious difference in Cr(VI)-reduction yield when pH ranged from 9 to 11, but Cr(VI)-reduction almost ceased at acidic pH 5. Shakoori et al. [23] reported that the optimum pH was 9 for Cr(VI) reduction by a Gram-positive bacterium, but Liu et al. [1,24] found that the optimum pH was 7 for *Pseudomonas aeruginosa* and *Bacillus coagulans*.

3.3.2. Effect of cell concentration on Cr(VI)-reduction

The effect of initial cell concentration from 0.08×10^8 to 8×10^8 cells/mL on Cr(VI) reduction is shown in Fig. 4. Cr(VI)-reduction by strain CSCr-3 increased with an increase in an initial cell concentration from 0.08×10^8 to 1×10^8 cells/mL as

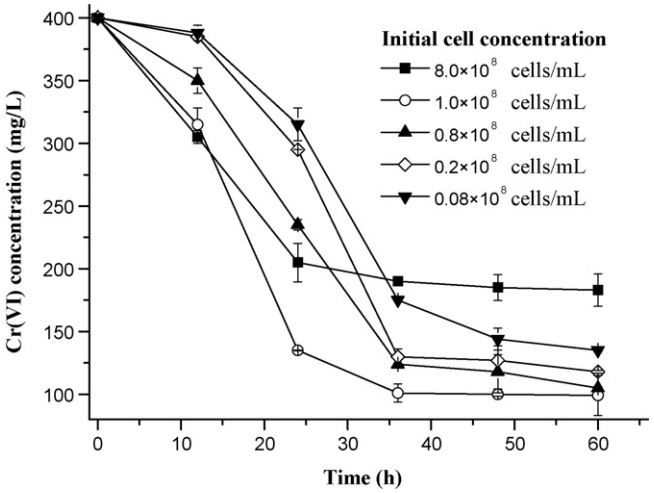

Fig. 4. Effect of initial cell concentration on Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3 at pH 10 and 35 °C.

observed by other workers [2]. At the initial cell concentration of 1×10^8 cells/mL, 74% of the initial Cr(VI) was reduced within 36 h, but with lower cell concentration the same yield was obtained within 48 h. A similar trend was also observed with *Pseudomonas* CRB5 [22]. At the highest cell concentration used (8×10^8 cells/mL), Cr(VI) reduction yield within 60 h was lower than at all other cell concentrations. Hence, the exhibited Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3 was performed in the exponential phase in Fig. 4.

3.3.3. Effect of initial Cr(VI) concentration

The effect of initial Cr(VI) concentration on Cr(VI)-reduction was investigated over a range of 100–800 mg/L. As shown in Fig. 5, Cr(VI)-reduction occurred even at the highest concentration of 800 mg/L, but complete Cr(VI)-reduction was not observed at this initial concentration in 96 h. Complete Cr(VI)-reduction was observed for 100 and 200 mg/L at 48 h. At initial Cr(VI) concentrations of 400, 600 and 800 mg/L, strain CSCr-3 reduced 303 mg/L (75%), 334 mg/L (51%) and 247 mg/L (30%) Cr(VI), respectively, within 96 h. DeLeo and Ehrlich [25] reported 99.7% reduction of Cr(VI) by *P. fluorescens* LB300 at an initial concentration of 112.5 mg/L within a period of 289 h. *Microbacterium* sp. completely reduced 20 mg/L of Cr(VI) within 72 h [2]. The *Pseudomonad* strain CRB5, however, showed complete reduction of 20 mg/L of chromate after 120 h [4]. *B. sphaericus* AND303 failed to completely reduce 10 mg/L of Cr(VI) [26]. In the present study in the range of 100–600 mg/L Cr(VI), more Cr(VI) was reduced with higher initial Cr(VI) concentrations (Fig. 5.). Megharaj et al. [13] also reported that the time taken for total reduction of Cr(VI) increased with increasing concentrations of Cr(VI). The Cr(VI)-reduction rate by strain CSCr-3 increased with increasing Cr(VI) concentration up to 600 mg/L and the highest rate was observed over the initial 24 h (exponential phase) of incubation.

3.3.4. Effect of glucose on Cr(VI)-reduction

It has previously been reported that chromate-reducing microorganisms may utilize a variety of organic compounds as electron donors for Cr(VI) reduction [1,18,24,27,28]. In this study, the effect of glucose on Cr(VI)-reduction was studied. Fig. 6 shows that the concentration of Cr(VI) decreased from 400 to 84 mg/L when 1 g/L glucose was applied, while in the medium without glucose the Cr(VI) concentration dropped only to 105 mg/L after incubation for 64 h, which implies that glucose slightly promoted the bacterial Cr(VI)-reduction. Wang et al. [1] found that glucose also promoted Cr(VI) reduction by *Penicillium* sp. Garbisu et al. [21] reported that addition of glucose caused a dramatic increase in the rate of chromate reduction catalyzed by the soluble fraction of cell-free extracts of *Bacillus subtilis*. From Fig. 6, we also can see that no Cr(VI)-reduction was observed in the medium without any cells inoculated, which confirms the ability of bacteria, CSCr-3, to reduce Cr(VI).

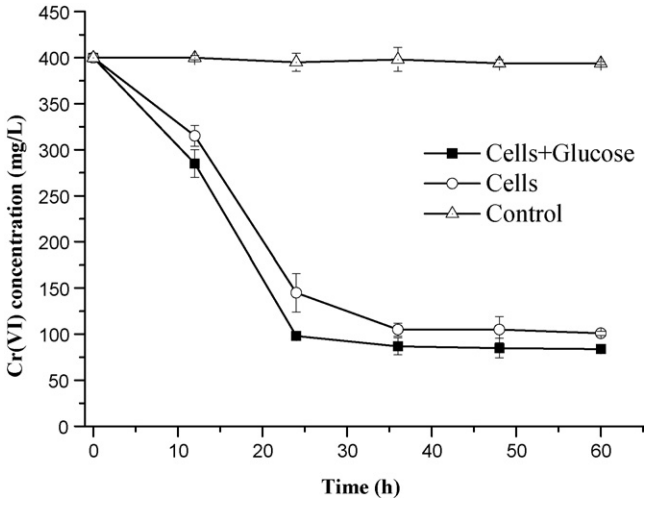

Fig. 6. Effect of glucose (1 g/L) on Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3.

3.3.5. Effects of other metals on Cr(VI)-reduction

As other metals can also be present in industrial effluents, effects of other heavy metal cations on Cr(VI)-reduction by the strain CSCr-3 was also studied in this work. As shown in Fig. 7, the presence of Cu^{2+}, Co^{2+} and Mn^{2+} slightly increased Cr(VI)-reduction while Ni^{2+}, Mo^{2+} and Zn^{2+} inhibited Cr(VI)-reduction by strain CSCr-3. Camargo et al. [29] also reported stimulatory effect of Cu^{2+}, Co^{2+} and Mn^{2+} on Cr(VI) reduction activity by cell-free extract of *Bacillus* sp. ES 29. Chromate reduction by *B. sphaericus* was inhibited by the presence of metal ions like Ni^{2+}, Co^{2+} and Pb^{2+}, both at low (20 mg/L) and high (100 mg/L) concentrations [26]. The stimulatory mechanism of Cr(VI) reduction activity by Cu^{2+} and other metals is not clear. However, Cu^{2+} is a prosthetic group for many reductase enzymes.

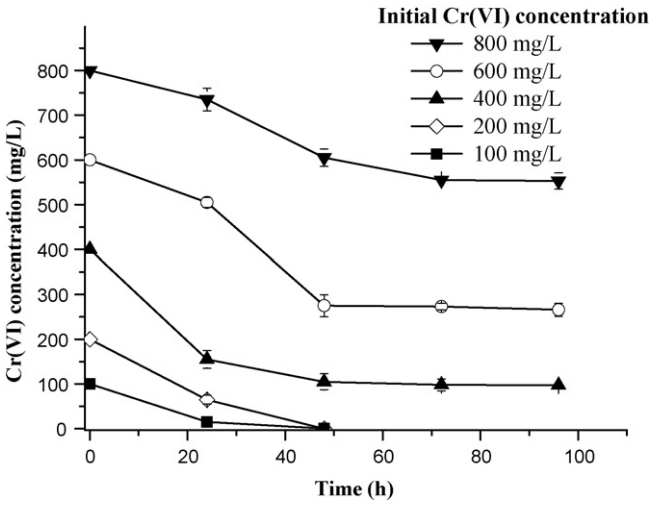

Fig. 5. Effect of initial Cr(VI) concentration (100–800 mg/L) on Cr(VI)-reduction by *Ochrobactrum* sp. CSCr-3 at pH 10 and 35 °C over a period of 96 h.

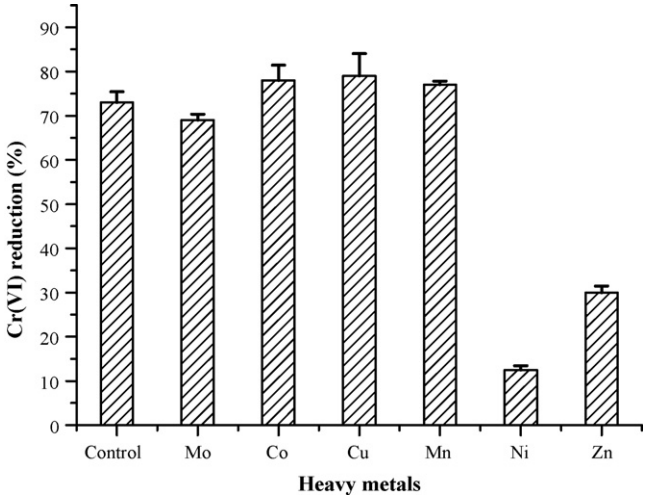

Fig. 7. Effects of heavy metals supplementation on Cr(VI)-reduction yield by *Ochrobactrum* sp. CSCr-3. Final concentrations of heavy metals: 20 mg/L (Co^{2+}) or 100 mg/L (Cu^{2+}, Mn^{2+}, Ni^{2+}, Mo^{2+} and Zn^{2+}).

The main function of Cu^{2+} has been reported to be related to electron transport protection or to act as an electron redox center and, in some cases, as a shuttle for electrons between protein subunits [30]. However, some other studies reported that the presence of heavy metals (Ni, Mn, Zn, Cu, Co) did not significantly affect the reduction potential of *O. intermedium* CrT-1 [14].

In the presence of oxygen, bacterial Cr(VI) reduction commonly occurs as a two- or three-step process with Cr(VI) initially reduced to the short-lived intermediates Cr(V) and/or Cr(IV) before further reduction to the thermodynamically stable end product, Cr(III). Nevertheless, it is at present unclear as to whether the reduction of Cr(V) to Cr(IV) and Cr(IV) to Cr(III) was spontaneous or enzyme mediated. NADH, NADPH and electron from the endogenous reserve are implicated as electron donors in the Cr(VI) reduction process [31]. The exact route how electrons be transferred from glucose to Cr(VI) is also still unclear.

4. Conclusions

We first isolated *Ochrobactrum* sp. strain CSCr-3 from chromium landfill which can effectively reduce Cr(VI) to Cr(III) under a wide range of temperatures, pH values and Cr(VI) concentrations. The addition of glucose and the presence of other metals especially Cu^{2+}, Co^{2+} and Mn^{2+} caused a slight increase in Cr(VI)-reduction. These results obtained in this study may provide useful information for the bioremediation of chromate under a wide range of environmental conditions. The exact mechanism of aerobic Cr(VI) reduction by *Ochrobactrum* sp. strain CSCr-3 needs to be further researched.

Acknowledgments

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