

Bioreduction of Chromate by an Isolated *Bacillus anthracis* Cr-4 with Soluble Cr(III) Product

Wei-Hua Xu · Hao Jian · Yun-Guo Liu ·
Guang-Ming Zeng · Xin Li · Wei Zhang

Received: 1 December 2014 / Accepted: 6 February 2015 / Published online: 25 February 2015
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Abstract Microbial Cr(VI) reduction is a significant process in detoxification of Cr(VI) pollution. In this study, a new Cr(VI)-reducing bacterial strain, Cr-4, was isolated from soil around the chromium-containing slag. The analysis of the 16S ribosomal RNA (rRNA) gene sequence revealed that the newly isolated strain was closely related to *Bacillus anthracis*. The response to Cr(VI) stress and reduction capacity of the isolate were investigated. Cell growth decreased with the increase of Cr(VI) concentration. Cell morphology varied and cell growth was inhibited remarkably in the presence of 125 mg/L Cr(VI). The strain grew well and removed Cr(VI) effectively at a Cr(VI) concentration lower than 50 mg/L. Cr(VI)-reducing activity was inhibited by Zn^{2+} , while significantly stimulated by Cu^{2+} . The activity of Cr(VI) reduction by cell-free extract was demonstrated. Total chromium analysis and the energy-dispersive X-ray analysis (EDAX) spectrum revealed that Cr(VI) removal was caused mainly by microbial reduction rather than by biosorption and the main part of the reduced Cr(III) existed as soluble form in solutions.

Keywords *Bacillus anthracis* · Bioremediation · Cr(VI) reduction · Cr(VI) stress · Cr(III)

1 Introduction

Biotransformation of heavy metals is a promising technique to convert toxic heavy metals to less toxic forms (Contreras et al. 2011). Microorganisms play an important role in transformation of heavy metals from toxic form to less toxic form. Many microorganisms have been found to be able to convert high-valence metals, e.g., Cr(VI), U(VI), Hg(II), and Ag(I), to a lower oxidation state, and thus reduce the toxic effect of heavy metals. Cr(VI) is commonly present in the environment due to its extensive application in industry, and a sustainable way is needed to clean up Cr(VI)-contaminated environments (He et al. 2011; Sahinkaya et al. 2012).

The toxicity and mobility of chromium are highly dependent on its oxidation state (Martins et al. 2010). Trivalent chromium is a micronutrient for living organisms including plants, animals, and human beings, while Cr(VI) is a toxin for living cells, as well as a class A carcinogenic substance by inhalation (Accornero et al. 2010). On the other hand, Cr(III) has a strong affinity for negatively charged ions and colloids in soils and can be precipitated as $Cr(OH)_3$ at neutral pH and, thus, is relatively immobile and less available for biological uptake. Conversely, hexavalent chromium has a higher solubility and mobility, and is more bioavailable than Cr(III) (Contreras et al. 2011). Therefore, transformation of Cr(VI) to Cr(III) is an effective measure to reduce the

W.-H. Xu (✉) · H. Jian · Y.-G. Liu · G.-M. Zeng · X. Li ·
W. Zhang
College of Environmental Science and Engineering, Hunan
University, Changsha, China
e-mail: whxu@hnu.edu.cn

W.-H. Xu · H. Jian · Y.-G. Liu · G.-M. Zeng · X. Li ·
W. Zhang
Key Laboratory of Environmental Biology and Pollution
Control, Hunan University, Ministry of Education,
Changsha 410082 Hunan, China

hazardous effect of Cr(VI) on our environment. Reduction of Cr(VI) to Cr(III) is generally considered as a significant step in treatment of Cr(VI) pollution due to the lower toxicity of Cr(III) than Cr(VI).

Biological reduction of Cr(VI) is an eco-friendly and cost-effective method for remediation of Cr(VI) contamination (Sugiyama et al. 2012; Cirik et al. 2013). Many new microorganisms that catalyze Cr(VI) to Cr(III) have been isolated from sludge, wastewater, landfill, and soil since the capability of Cr(VI) reduction was first reported on *Pseudomonas* species in 1977 (Romanenko and Koreňkov 1977). Microbial Cr(VI) reduction is mediated by direct enzymatic reduction or by indirect chemical reduction (Chen et al. 2011). Some microorganisms, mainly including the sulfate-reducing bacteria and dissimilatory Fe(III)-reducing strains, reduce Cr(VI) to Cr(III) indirectly by byproducts such as S(II) (Cheung and Gu 2003; Pagnanelli et al. 2012) and Fe(II) (Xu et al. 2005; Cummings et al. 2007). Most of reported Cr(VI)-reducing strains directly enzymatically reduce Cr(VI) to Cr(III) (Camargo et al. 2003; Thacker et al. 2007; Mistry et al. 2010; Chen et al. 2012; Focardi et al. 2012). Chromate reductase activity is associated with the soluble cell fraction (Pal et al. 2005; Sultan and Hasnain 2012) or membrane cell fraction (Wang et al. 1990; Mangaiyarkarasi et al. 2011). The purified soluble flavoproteins showed the activity of enzymatic Cr(VI) reduction in *Pseudomonas putida* and *Escherichia coli* (Ackerley et al. 2004). Hydrogenase (Lovley and Phillips 1994; Michel et al. 2001; Chardin et al. 2003) and cytochrome (Lovley and Phillips 1994; Michel et al. 2001) were also found to function as chromate reductase. Although a lot of researches about microbial Cr(VI) reduction are carried out, finding more new appropriate microbial species used for controlling chromate pollution is always of interest.

In the present study, a new chromate-reducing bacterial strain Cr-4 was isolated and identified by 16S ribosomal RNA (rRNA) gene sequence analysis. Some biochemical and chromate-reducing properties of the isolated strain were researched, so as to evaluate the Cr(VI) detoxification capacity of the strain.

2 Materials and Methods

2.1 Preparation of Culture Medium

Nutrient broth (NB) medium composed of beef extract (5 g/L), peptone (10 g/L), and NaCl (5 g/L) was

prepared for the culture of the Cr(VI)-reducing strains. The NB medium used in Cr(VI) reduction experiments was supplemented with glucose (1 g/L). The pH value of the medium was 7.2–7.4.

2.2 Isolation of the Cr(VI)-Reducing Strain

The Cr(VI)-reducing strain was isolated from the soil around the chromium-containing slag in a previous Chromate Factory of Changsha, China. The soil sample (10 g) was mixed with 90 mL sterile distilled water in a 250-mL flask; then, 1 mL of the soil solution was transferred to the test tube containing 9 mL sterile distilled water. The diluted solution (1 mL) was then transferred to another test tube containing 9 mL sterile distilled water. This above operation was repeated to obtain the serial of tenfold dilutions of sample solution (10^{-1} – 10^{-8}). Every dilution was drawn and dropped, respectively, to the culture plates, followed by pouring the NB agar medium. The agar plates were incubated at 37 °C for 1–2 days. The colonies with different morphologies were selected and streaked on agar plates to obtain homogeneous colonies, then incubated for 1–2 days. The isolated strains were inoculated from the plates onto the agar slants and stored at 4 °C until needed for further experiments.

2.3 SEM of the Isolated Strain

The isolated strain was detected by scanning electron microscopy (SEM) after being grown in liquid medium with 0, 50, and 125 mg/L Cr(VI), respectively, for 36 h. The strain was fixed, dehydrated, and lyophilized as previously reported (Xu et al. 2009), then was detected by a JSM-6360LV scanning electron microscope (JEOL).

2.4 Identification of the Isolated Strain

Biochemical characteristics and genetic analysis techniques were employed to identify the isolated strain. The genomic DNA was extracted by alkaline lysis method and amplified using bacterial-specific forward primer F27 (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer R1522 (5'-AAGGAGGTGATCCAGCCGCA-3'). Purification of the PCR products and determination of 16S rRNA gene sequence were performed by Top (Guangzhou, China). The sequence was compared with the NCBI database. Sequence alignment was

performed with ClustalX, and phylogenetic analysis was accomplished by using the MEGA 5.01 software.

2.5 Cr(VI) Reduction by the Isolate

Batch experiments were carried out to determine the Cr(VI)-reducing capacity of the isolated strain. The isolated strain was enriched by transferring one loop of the cells from the agar slants to 100 mL liquid medium in a 250-mL flask, incubated at 37 °C by shaking at 150 rpm in an orbital incubator. The overnight-grown cells were harvested by centrifugation at 4000 rpm for 15 min, washed, and suspended with phosphate buffer (pH 7.0). The cell suspension (1 mL) was inoculated to 250-mL flasks containing 100 mL liquid medium and the desired Cr(VI) concentration. The flasks were then incubated at 37 °C by shaking at 150 rpm in an orbital incubator. A sample was drawn at regular intervals and centrifuged at 10,000 rpm for 10 min, and the supernatant was used to determine the Cr(VI) concentration. The effect of Zn²⁺ (25, 100 mg/L) and Cu²⁺ (25, 100 mg/L) was determined by adding sterile ZnSO₄ and CuCl₂, respectively, to the reaction flasks containing 100 mL liquid medium and 40 mg/L Cr(VI); then, the isolated strain was inoculated to the flask. The flasks were incubated as described above, and the Cr(VI) concentration was measured at regular times. The experiments were carried out in triplicate and average values are reported.

2.6 Cr(VI) Reduction by Cell-Free Extract

Cell-free extract was prepared and used in Cr(VI) reduction experiments. The cell suspension was sonicated by an ultrasonic probe in an ice bath. The sonicate was centrifuged at 8000 rpm for 20 min, and the supernatant was collected and used as the cell-free extract (CFE). Cr(VI) reduction was initiated by adding the desired Cr(VI) concentration into the CFE. Cr(VI) concentration in the mixture was determined at regular intervals.

2.7 Chromium Assay and Cell Growth Measurement

The concentration of Cr(VI) was determined colorimetrically by the diphenylcarbazide method. The

absorbance was measured at 540 nm with a UV 754 N model spectrophotometer. In order to estimate the property of the reduced product Cr(III) after reduction of Cr(VI), total chromium was tested with diphenylcarbazide after acidic oxidation of chromium(III) to chromium(VI). Cr(III) was determined from the difference between total Cr and Cr(VI), and Cr(OH)₃ was estimated according to the variation of the concentration of total Cr in the supernatant of the liquid medium.

In order to determine the cell growth curve under Cr(VI) stress, the isolated strain was cultured in liquid medium containing different Cr(VI) concentrations ranging from 0 to 125 mg/L. A sample was drawn at regular intervals, and the cell growth was determined by measuring the optical density of the sample at 600 nm (OD₆₀₀) with a UV 754 N model spectrophotometer.

2.8 EDAX of the Cell Pellet

In order to determine whether chromium was adsorbed on cells, energy-dispersive X-ray analysis (EDAX) was used to analyze the cells. The strains were harvested by centrifugation at 4000 rpm for 15 min at the end of Cr(VI) reduction experiments. The harvested strains were dried at 60 °C for 48 h and sprayed gold, then were examined with the EDAX microanalysis system (Genesis).

3 Results and Discussion

3.1 Identification and Characterization of the Cr(VI)-Reducing Strain

One group of colonies was obtained from agar plates. The ability of Cr(VI) reduction of different colonies was tested and one of colony Cr-4 with the highest Cr(VI) reduction efficiency was identified. The colony of Cr-4 was circular (diameter 1–2 mm), off-white with regular edges and a rough surface. Scanning electron microscope inspection revealed that the cell of Cr-4 was long-rod shaped (2.4–3.3 μm long, 0.9–1.18 μm wide), and the flagellums were observed on the cells (Fig. 1a, b). Some biochemical characteristics of the isolated Cr-4 were determined, and the results are listed in Table 1.

The 16S rRNA gene sequence and phylogenetic analysis indicate that the isolated strain Cr-4 is affiliated

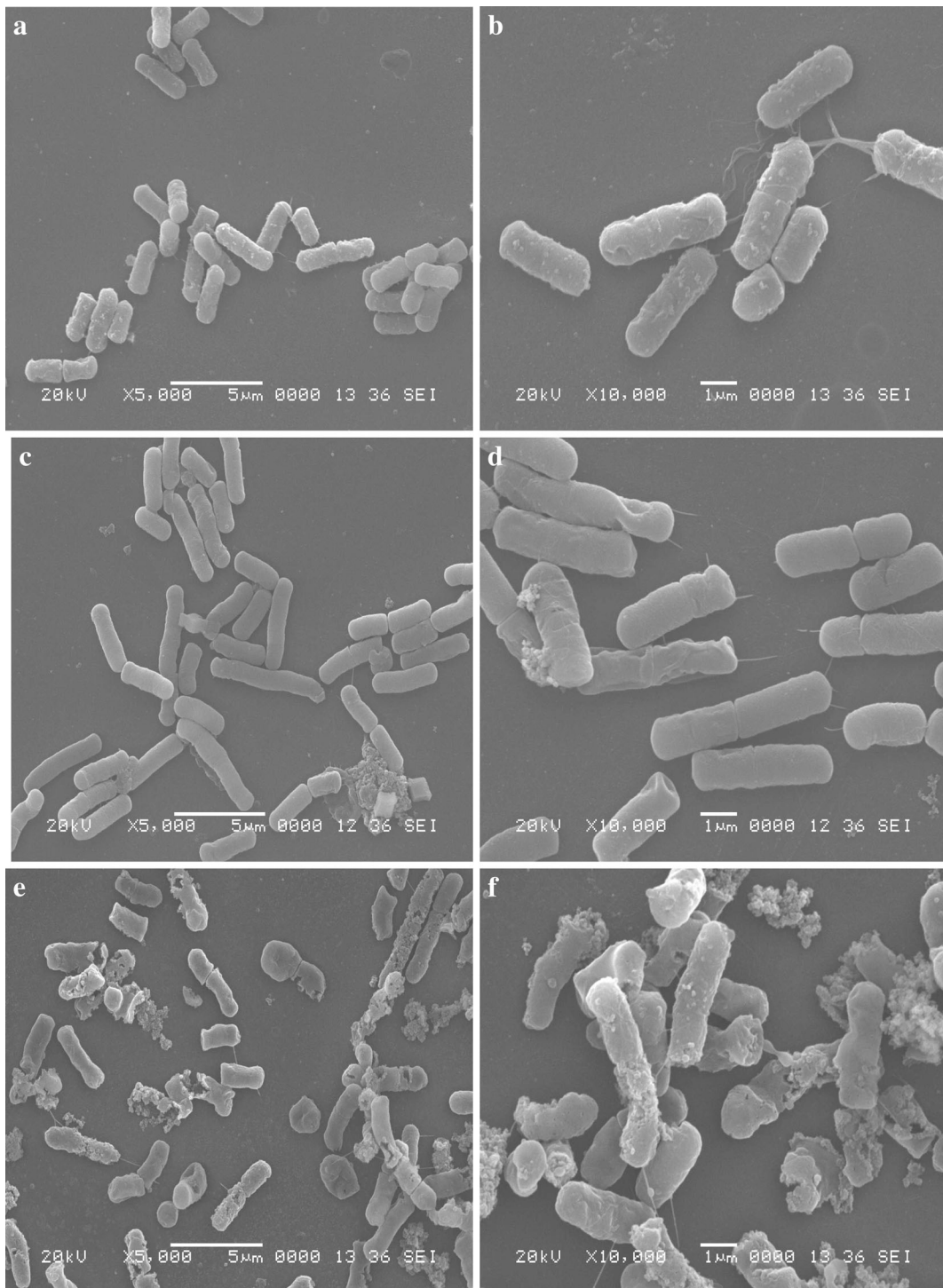


Fig. 1 SEM images of the isolated *Bacillus anthracis* Cr-4 grown in liquid medium containing **a, b** 0 mg/L Cr(VI), **c, d** 50 mg/L Cr(VI), and **e, f** 125 mg/L Cr(VI). The cells were harvested after being incubated by shaking at 150 rpm and 37 °C for 36 h

to *Bacillus* sp (Fig. 2). The 16S rRNA gene sequence of the strain Cr-4 showed a sequence homology of 99.2 %

with the sequence of *Bacillus anthracis* 16S rRNA gene (C9401).

Table 1 Biochemical characteristics of the isolate Cr-4

Test	Result
Gram staining	Positive
Spore staining	Positive
Oxidase test	Negative
Catalase test	Positive
Hydrolysis of starch	Positive
Hydrolysis of gelatin	Positive
NO ₃ ⁻ reduced to NO ₂ ⁻	Positive
V-P test	Positive
Indole test	Negative

3.2 Cell Morphology and Growth of the Isolate Under Cr(VI) Stress

The toxic effect of Cr(VI) on the strains increases with the increase of Cr(VI) concentration. The cell form of the isolate Cr-4 was intact but less full when grown in Cr(VI)-added (50 mg/L) liquid medium (Fig. 1c, d). The cell size decreased and the deformation and rupture were observed in cells when the strain Cr-4 was exposed to 125 mg/L Cr(VI) (Fig. 1e, f). In our previous study on *Pseudomonas aeruginosa*, the deformation and rupture were observed in cells only exposed to 40 mg/L Cr(VI) (Xu et al. 2009). The strain Cr-4 was isolated from Cr(VI)-contaminated environment, and the higher Cr(VI)-resistant ability of the cells may be induced by

long-time Cr(VI) stress; thus, the isolated Cr-4 is able to tolerate a higher concentration of Cr(VI) than *P. aeruginosa*. The thickening of the cell wall and new bacteria capsules were observed in *Arthrobacter* K-4 in the presence of a high concentration of Cr(VI) (≥ 200 ppm) (Lin et al. 2006).

The diagram of the cell growth curve indicates that the inhibitory effect of Cr(VI) on cell growth increased with increasing Cr(VI) concentration from 12.5 up to 125 mg/L (Fig. 3). A very slight reduction of cell growth was observed in the presence of 12.5 and 25 mg/L Cr(VI). The growth trend was favorable in the presence of 50 mg/L Cr(VI), although an obvious reduction of cell growth appeared. The cell growth was significantly inhibited by 100 and 125 mg/L Cr(VI) and only a little cell growth was observed.

3.3 Effect of Zn²⁺ and Cu²⁺ on Cr(VI) Reduction

Figure 4 shows that the effect of Zn²⁺ on bacterial Cr(VI) reduction is quite distinct from that of Cu²⁺. The effect of 25 mg/L Zn²⁺ was negligible, but 100 mg/L Zn²⁺ had an inhibitory effect on bacterial Cr(VI) reduction. However, both of 25 and 100 mg/L Cu²⁺ substantially promoted bacterial Cr(VI) reduction. As shown in Fig. 4, Cr(VI) concentration decreased from initial 40 to 20 mg/L in the absence of Zn²⁺ and Cu²⁺, and Cr(VI) concentration dropped only to 26 mg/L in the presence of 100 mg/L Zn²⁺ after 24 h, while

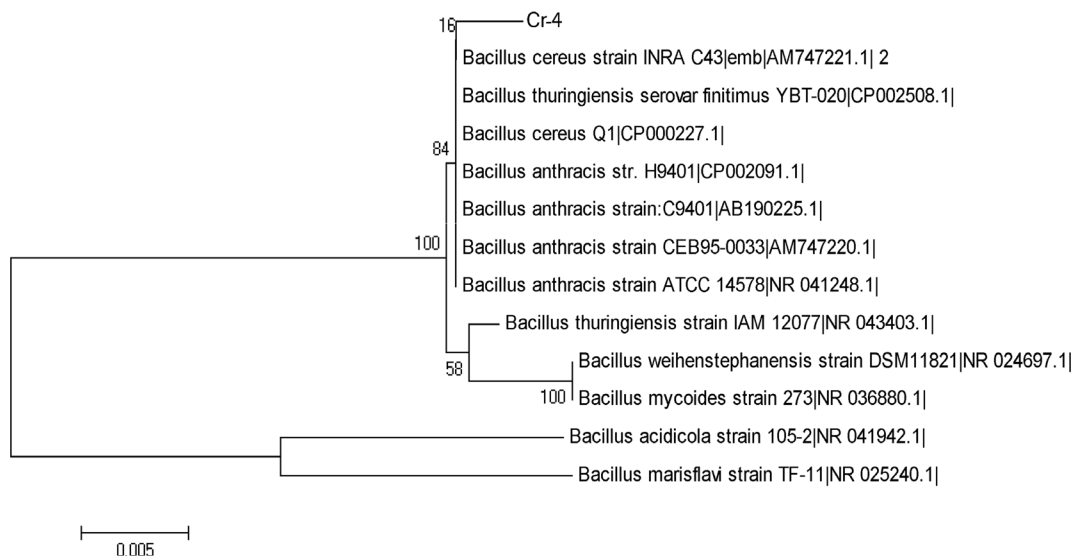


Fig. 2 Phylogenetic tree derived from 16S rRNA gene sequence of *Bacillus anthracis* Cr-4. The tree was constructed using MEGA 5.01 software

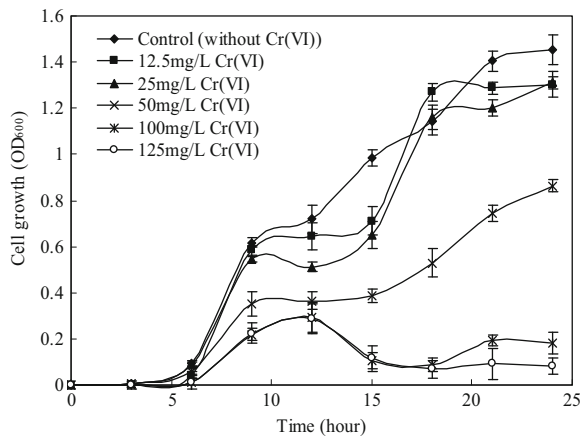


Fig. 3 Cell growth of the isolated *Bacillus anthracis* Cr-4 under Cr(VI) stress. The cells were incubated by shaking at 37 °C and 150 rpm

Cr(VI) concentration fell below 2 mg/L in the presence of 25 or 100 mg/L Cu^{2+} . Our previous study on bacterial Cr(VI) reduction with *P. aeruginosa* demonstrated that Cr(VI) reduction was significantly inhibited by Zn^{2+} but promoted by Cu^{2+} (Xu et al. 2009). The same result was found in bacterial Cr(VI) reduction by *Ochrobactrum intermedium* SDCr-5 (Sultan and Hasnain 2007). Cr(VI) reductase activity was also stimulated by Cu^{2+} (Camargo et al. 2003; Desai et al. 2008). This may be related to the fact that Cu^{2+} is a prosthetic group for many reductase enzymes and plays a role in electron-transport protection or acts as a single-electron redox center and a shuttle for electrons between protein subunits (Abe et al. 2001; Camargo et al. 2003).

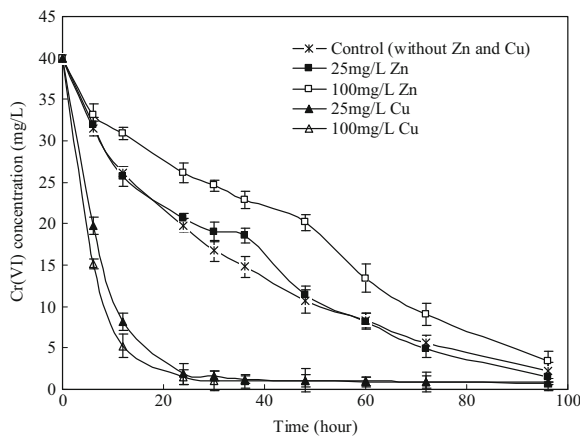


Fig. 4 Effects of Zn^{2+} and Cu^{2+} on Cr(VI) reduction by the isolated *Bacillus anthracis* Cr-4 (shaking speed=150 rpm, temperature=37 °C, pH=7.2)

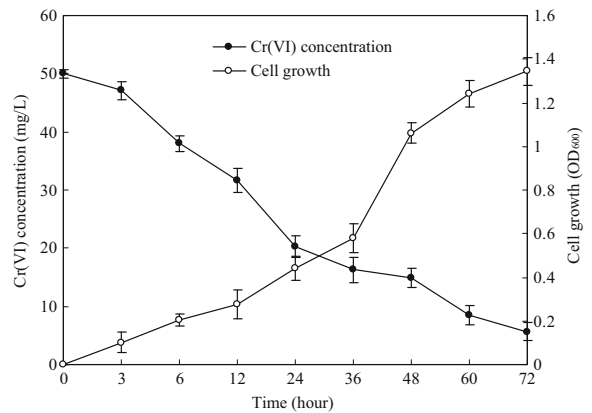


Fig. 5 Variation of Cr(VI) concentration and cell growth in liquid medium (shaking speed=150 rpm, temperature=37 °C, pH=7.2)

3.4 Relationship Between Cell Growth and Cr(VI) Reduction

In order to determine the relationship between Cr(VI) reduction and cell growth, cell growth and Cr(VI) concentration were simultaneously measured at regular intervals in Cr(VI) reduction experiments. The variation of cell growth and Cr(VI) concentration is shown in Fig. 5. There is a certain correlation between cell growth and Cr(VI) reduction. It was observed that more than 65 % of 50 mg/L Cr(VI) was reduced in the early 36 h with the growth of Cr-4 cells, but the increase of cell growth was relatively slower in the early 36 h. This is related to the toxicity of Cr(VI) and the cells are in lag phase during this period. In the subsequent 36 h, an obvious increase of cell growth appeared due to the

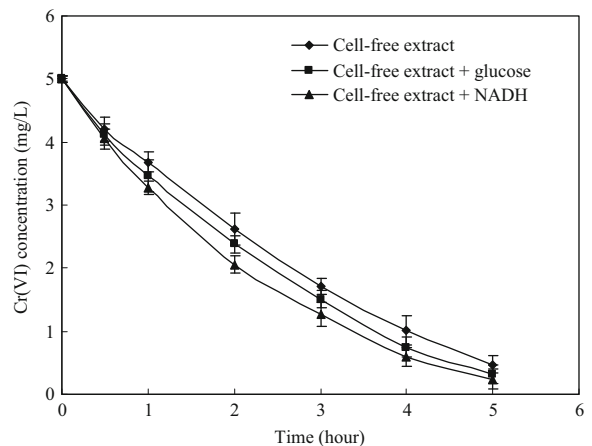


Fig. 6 Cr(VI) reduction by cell-free extract of the isolated *Bacillus anthracis* Cr-4

decrease of Cr(VI) toxicity as most of Cr(VI) was reduced.

3.5 Cr(VI) Reduction by CFE

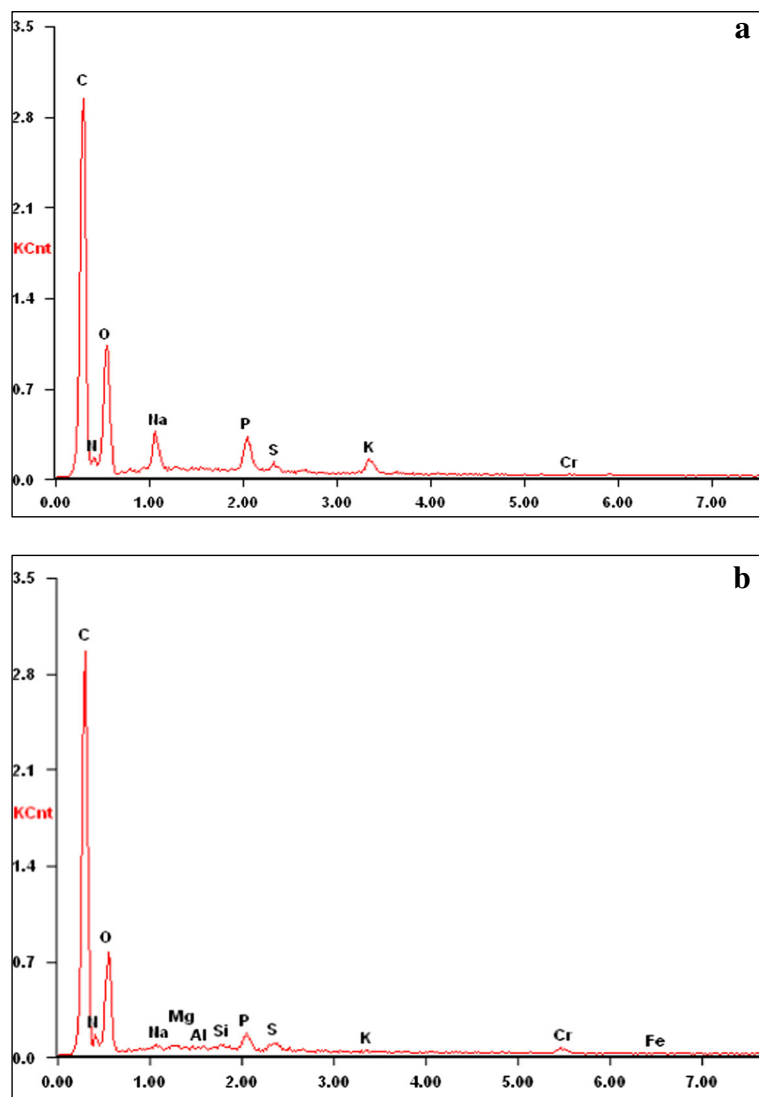
Cell-free extract (CFE) was used to determine the chromate reductase activity in the isolate Cr-4. Cr(VI) reduction was observed in the solutions of CFE (Fig. 6), suggesting the enzymatic mechanism of Cr(VI) reduction in the isolated strain. Figure 6 also shows that chromate reductase activity was increased with supplementation of glucose or NADH. It has been reported that NADH and NADPH serve as electron donors and promote

bacterial Cr(VI) reduction (Focardi et al. 2012; Sau et al. 2010).

3.6 Chromium Characterization

Cr(VI) can be removed by microbial reduction and adsorption. In the present study, total chromium was measured at the end of Cr(VI) reduction experiments. It was found that more than 80 % of total chromium (data not shown) was present in the supernatant of the liquid medium after Cr(VI) reduction, and there was no more than 20 % of total chromium in the cell pellet. Thus, the decrease of Cr(VI) concentration (Fig. 5) was caused not by adsorption but mainly by microbial

Fig. 7 EDAX spectrum of **a** the cell pellet before Cr(VI) reduction and **b** the cell pellet after Cr(VI) reduction



reduction. The EDAX analysis spectrum of the cell pellet is shown in Fig. 7. The EDAX spectrum identified the presence of C, N, O, Na, P, S, K, and Cr in the strains with relative weights of 60.55 % for C, 8.20 % for N, 23.38 % for O, 2.71 % for Na, 2.60 % for P, 0.57 % for S, 1.93 % for K, and 0.05 % for Cr. The elements of C, N, and O are the main cellular components in the strains. Comparing the spectrum of the cell pellet treated with Cr(VI) with that of untreated cells, a weak Cr peak appeared and the relative weight of Cr only increased to 1.42 %. This further indicates that the amount of chromium adsorbed on the strains was small.

On the other hand, more than 80 % of total chromium was found in the supernatant of the liquid medium after Cr(VI) reduction, which also indicates that the reduced Cr(III) was not precipitated from solutions but existed as soluble form. The reduced Cr(III) is commonly precipitated as Cr(OH)₃ under alkaline pH conditions, but the highly soluble organo-Cr(III) complexes can also be formed in the presence of organic ligands (Kantar et al. 2011). In this study, the pH value of the liquid medium decreased from the initial 7.2 to 5.0–5.5 in the process of Cr(VI) reduction and the liquid medium contained organic materials. These could inhibit the precipitation of Cr(III) from solutions.

4 Conclusions

A Cr(VI)-reducing strain, Cr-4, was isolated from soil, and the strain showed a sequence homology of 99.2 % to *B. anthracis* (C9401). The toxic effects of Cr(VI) on cells increased with the increase of Cr(VI) concentration from 12.5 to 125 mg/L. The variation of cell morphology was observed, and cell growth was apparently inhibited at the higher Cr(VI) concentration of 125 mg/L. The isolated strain showed an effective Cr(VI) reduction ability as Cr(VI) concentration was lower than 50 mg/L. More than 80 % of total chromium was present in the supernatant of the liquid medium, which indicates that the stain removed Cr(VI) mainly by microbial reduction rather than by biosorption and the reduced Cr(III) was not precipitated from solutions. The EDAX spectrum also showed that only a small amount of chromium was adsorbed by the cells. Zn²⁺ had inhibitory effects while Cu²⁺ had enhancing effects on microbial Cr(VI) reduction. The activity of Cr(VI) reduction was also observed in CFE and promoted by glucose and NADH. The results indicate that the isolated strain

has the Cr(VI)-resistant and Cr(VI)-reducing ability, and this strain could be used for biotransformation of toxic Cr(VI) to less toxic Cr(III).

Acknowledgments This work was supported by the National Natural Science Foundation of China (51108167) and the Fundamental Research Funds for the Central Universities, Hunan University.

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