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ORIGINAL ARTICLE

Bioreduction of Cr(VI) by *Bacillus* sp. QH-1 isolated from soil under chromium-containing slag heap in high altitude area

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Abstract A chromium-reducing strain QH-1, identified as *Bacillus* sp., was isolated from soil under chromium-containing slag heap in Qinghai high altitude area, China. The strain was found to resist 200 mg/L Cr(VI), and Cr(VI) negatively affects the metabolic activity of the cells, as well as the cell morphology of *Bacillus* sp. QH-1. The reduction efficiency of Cr(VI) at concentrations of Cr(VI) 25 mg/L, 50 mg/L, 100 mg/L and 200 mg/L were 99.48 %, 65.99 %, 23.22 % and 6.99 %, respectively, decreasing with increasing initial Cr(VI) concentration. This indicates that the toxicity of Cr(VI) increased with concentration. Energy dispersive X-ray analysis revealed that there was insoluble Cr(III) generated during Cr(VI) reduction. In order to apply strain QH-1 to remove Cr(VI) from groundwater, factors of concentration of electron donors (glucose) and temperature were investigated in a synthetic medium. The results demonstrated that glucose could promote the reduction of Cr(VI) by this strain, and the general trend of Cr(VI) reduction increased with temperature within the range of 4 to 37 °C. Cr(VI) was reduced effectively at 25 °C and 37 °C, and all of Cr(VI) was reduced after 96 h at 37 °C, while the reduction was slow at 4 °C and 15 °C, and it almost ceased after about 120 h. These results could be potentially useful for the bioremediation of Cr(VI) in groundwater.

Keywords Cr(VI) reduction · *Bacillus* sp. QH-1 · Temperature · Glucose

Introduction

Chromium is a common contaminant, extensively distributed in the environment and widely used in various industrial processes, including tanneries, wood processing, chemical manufacturing, electroplating, and so on (Kotas and Stasicka 2000; Rehr et al. 2010). It is present in groundwater, mainly in two oxidation states as Cr(III) and Cr(VI) (Miretzky and Cirelli 2010; Zink et al. 2010). Cr(III), at very low concentration, is an essential nutrient for growth and metabolic activities of microorganisms. By contrast, Cr(VI) is more hazardous due to its mutagenic and carcinogenic properties. It has been reported that Cr(VI) is nearly 1,000 times more mutagenic and 100 times more toxic than Cr(III) (Morales-Barrera et al. 2010; Unceta et al. 2010). Therefore, conversion of Cr(VI) to Cr(III) is an effective way of combating Cr(VI) pollution.

Conventional methods for Cr(VI) removal consist of chemical reduction, adsorption, precipitation, ion exchange and electrodialysis (Zhu et al. 2008; Chaudhuri and Bin Azizan 2012; Murugavelh and Mohanty 2012). Compared to these means, microbial reduction of Cr(VI) is a more effective method, due to lower operation costs, shorter period and fairly smaller quantities of produced sludge (Lee et al. 2008a; Shi et al. 2012). Variety of chromium reducing strains in environment have been reported, including *Halomonas* sp. (Focardi et al. 2012), *Acinetobacter* sp. (Shetty et al. 2012), *Bacillus* sp. (Liu et al. 2006; Cheng and Li 2009; Kathiravan et al. 2011), *Clostridium* (Nguema and Luo 2012), strains belonging to the genus *Pseudomonas* (Dogan et al. 2011), the species *Enterococcus gallinarum* (Sayel et al. 2012) and so on. However, previous studies focus on using bacteria to treat Cr(VI)-contaminated soil and wastewater; little attention has been paid to removal Cr(VI) in groundwater by microbial reduction.

In the present study, the Cr(VI) resistance and reduction of an aerobic bacterial strain were characterized with the presence of growing cells in LB medium. The toxic effects of Cr(VI) on this strain were observed by scanning electron microscopy, and the precipitate generated during reduction was analyzed with energy dispersive X-ray.

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In order to apply this bacteria to remediate Cr(VI) effectively in groundwater, factors of temperature and concentration of electron donors were investigated in laboratory experiments, which may provide useful information to pilot plants in future studies.

Materials and methods

Bacterial strains and cultural conditions

The Cr(VI)-reducing strain was isolated from soil under a chromium-containing slag heap with 2,432 mg/kg soluble Cr(VI) in the Qinghai high altitude area, China. One gram of the contaminated soil was added to 100 mL sterile water, and then shaken at 150 rpm for 30 min. Standard serial dilutions followed to obtain serial ten-fold dilutions of the soil suspension (10^{-1}–10^{-7}). Aliquots of 0.1 mL from the 10^{-4}–10^{-7} soil suspension dilutions were spread on LB agar plates. The plates were incubated at 30 °C for 48 h. Colonies of different morphologies were selected and then streaked on separate plates and incubated at 30 °C for 48 h. After testing the reduction capacity of the bacteria to Cr(VI), the strain that reduced Cr(VI) the most was selected and stored on agar slants at 4 °C until needed for further experiments.

The bacterial colony was cultured on LB agar plates; it was pale white and smooth. The cells were long rods (diameter about 2.5 μm) under a HITACHI S-3000 N scanning electron microscope (SEM) (Fig. 3a). The 16S rRNA gene fragment of this strain was amplified with the bacterial specific primers 27 F (5'-AGAGTTTGATCMTGGCTCAG-3') – 1492R (5'-TACGGTTACCTTGTTACGACTT-3') (Webster et al. 2003) in a 25 μL PCR mixture that consisted of 10×PCR buffer, 100 μmol dNTP mixture, 0.2 μmol primer, 50 ng template and 1 U of Taq DNA polymerase (TaKaRa, Japan), according to the following program: initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s, with a final extension step of 10 min at 72 °C. PCR products were purified and ligated into the pMD18-T vector (Takara) following the instructions given by the manufacturer, and then commercially sequenced using ABI model 3730 sequencer(Applied Biosystems). The retrieved 16S rRNA gene fragment nucleotide sequence was edited in MEGA 5.05, and then was deposited in the NCBI GenBank database with accession number KF574820. Furthermore, the retrieved sequence was identified using the Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp) program in Ribosomal Data base Project. This strain was identified as *Bacillus* sp., and nominated as QH-1.

Cr(VI) resistance and reduction were carried out in Luria Broth (LB) medium containing peptone (10.0 g), yeast extract (5.0 g), NaCl (10.0 g) in 1 L distilled water at pH 7.0.

The experiments of factors on Cr(VI) reduction were carried out in a synthetic media consisting of $CaCl_2$ (38 mg), NH_4Cl (250 mg), KCl (500 mg), $MgCl_2$ (250 mg), KH_2PO_4 (200 mg), NaCl (1000 mg), yeast extract (300 mg) in 1 L distilled water at pH 7.0 (Lee et al. 2008b). When yeast extract was excluded from the medium, no growth occurred. All media were autoclaved at 121 °C for 15 min before use.

Cr(VI) resistance and reduction

A loop of cells was transferred from the agar slants to 100 mL of sterilized Luria Broth (LB) medium (pH 7.0) with different concentrations of Cr(VI) (0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L and 200 mg/L), and incubated at 37 °C with shaking at 150 rpm. Cr(VI) was added as K_2CrO_4 from a filter-sterilized stock solution. Cell growth was monitored by measuring absorbance of culture at 600 nm at different incubation times. All experiments were performed in duplicate.

The procedure for Cr(VI) reduction was the same as the resistance experiments, except the initial density of bacteria was 2×10^8 cell/mL. After incubating for 24 h, 5 mL culture from each flask were filtered for Cr(VI) concentration measurement of the supernatant. All experiments were performed in duplicate.

Scanning electron microscope of *Bacillus* sp. QH-1

Bacteria cells inoculated in LB medium (pH 7.0) containing different concentrations of Cr(VI) (0 mg/L, 25 mg/L, 100 mg/L and 200 mg/L) for 48 h were harvested with centrifugation (5,000 rpm, 4 °C, 20 min). Cell pellets were washed with distilled water, fixed in phosphate buffer (pH 7.0) with 2.5 % (v/v) glutaraldehyde for 2 h, and then washed with phosphate buffer (pH 7.0) three times. Finally, the cell pellets were dehydrated using gradient alcohol (30 %, 50 %, 70 %, 90 % and 100 %). The samples were dried at critical point and coated with sputtering gold. Specimens were detected using a HITACHI S-3000 N scanning electron microscope (SEM).

Energy dispersive X-ray analysis of reduced precipitate

The reduced precipitates in LB medium containing 0 mg/L and 100 mg/L Cr(VI) by *Bacillus* sp. QH-1 were collected, washed with distilled water three times and dried for 48 h at 60 °C for energy dispersive X-ray analysis (EDXA)

Factors affecting on Cr(VI) reduction

Here, glucose was used as the electron donor, and the influence of concentration of electron donors (0 mg/L, 18 mg/L, 180 mg/L, 450 mg/L and 1,800 mg/L) and temperature (4 °C,

15 °C, 25 °C and 37 °C) on Cr(VI) reduction was investigated in a synthetic media instead of groundwater with 2.8 mg/L Cr(VI), and the initial density of bacteria was 2×10^8 cell/mL. All experiments were carried out in batch reactors using 500 mL conical flasks with 200 mL synthetic media, and incubated with shaking (150 rpm). All experiments were performed in duplicate.

Analysis Method

Hexavalent chromium was determined colorimetrically using the 1, 5-diphenylcarbazied method at 540 nm using a spectrophotometer (Ackerley et al. 2004). Cell growth was monitored by measuring optical density at 600 nm.

Results and Discussion

Cr(VI) resistance and reduction

Batch cultures of *Bacillus* sp. QH-1 were conducted in LB media with initial Cr(VI) concentrations ranging from 25 to 200 mg/L, and no Cr(VI) added was as control experiment. The growth curves of the cells at different concentrations of Cr(VI) are shown in Fig. 1. There were significant differences between media with and without Cr(VI). Strain *Bacillus* sp. QH-1 was slightly inhibited by Cr(VI) at 25 mg/L, while there was a significant inhibition effect at 100 mg/L and 200 mg/L. This indicates that Cr(VI) negatively affects the metabolic activity of the cells of *Bacillus* sp. QH-1. The general trend of cells in medium with Cr(VI) increased at the initial stages (10 h), then decreased. After the cell decrease, there was an increase. The phenomenon may be caused by the resistance of bacteria to the toxicity of Cr(VI). Furthermore, the reduction efficiencies were 99.48 %, 65.99 %, 23.22 % and 6.99 % under 25 mg/L, 50 mg/L, 100 mg/L and 200 mg/L Cr(VI) after incubating for 24 h, respectively. As shown in Fig. 2, the reduction efficiency of Cr(VI) reduction exhibited by *Bacillus* sp. QH-1 decreased with increasing initial Cr(VI) concentration.

Scanning electron microscope of *Bacillus* sp. QH-1

To further understand Cr(VI) reduction by *Bacillus* sp. QH-1 at the microscopic scale, the residual cells of *Bacillus* sp. QH-1 separated from culturing in LB medium with four different concentrations of Cr(VI) (0 mg/L, 25 mg/L, 100 mg/L and 200 mg/L) were collected for SEM analysis. The presence of Cr(VI) had a significant impact on the morphology of the cells, as shown in Fig. 3. Without adding Cr(VI), the cells were long rods, with smooth surfaces and relatively regular shapes (Fig. 3a), while part of the cells surface were wrinkled at 25 mg/L Cr(VI) (Fig. 3b). Besides, there was obvious division and punch on the cells surface at 100 mg/L (Fig. 3c) and 200 mg/L Cr(VI) (Fig. 3d). The change in cell morphology is possibly caused by the stress of Cr(VI), and the variation in cell morphology might be an adopted mechanism to resist the toxicity of Cr(VI). A similar observation was also reported by Garg et al. (2013). They noted that there were differences in the morphology of *Pseudomonas putida* cells without and with 500 mg/L Cr(VI) by SEM analysis. The *P. putida* cells are smooth rods in the absence of Cr(VI), whereas, upon cultivation in 500 mg/L Cr(VI), few cells are distorted with broken cell walls.

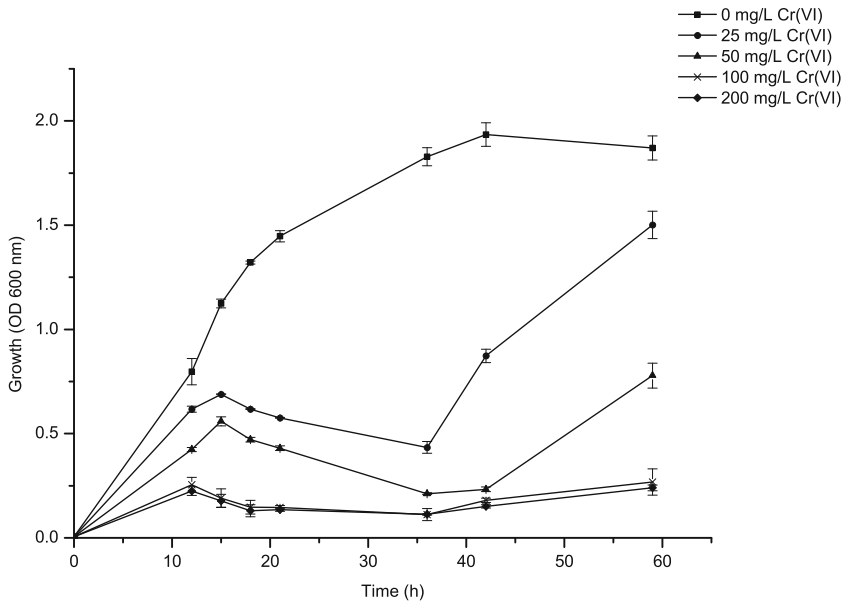

Fig. 1 Growth of *Bacillus* sp. QH-1 in LB medium containing different concentrations of Cr(VI)

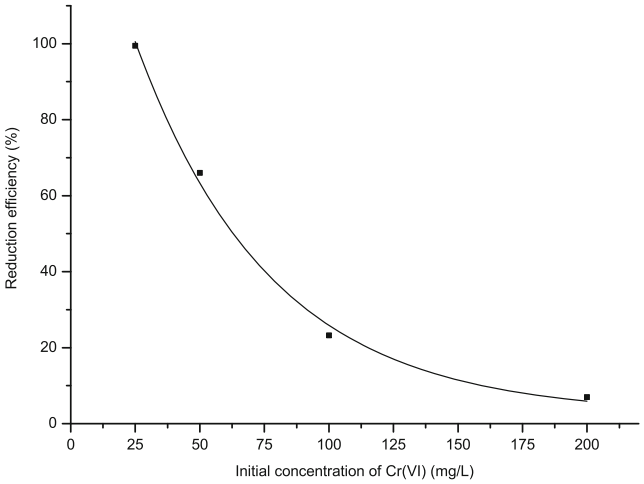

Fig. 2 Relationship between Cr(VI) reduction efficiency and initial Cr(VI) concentration

Energy dispersive X-ray analysis of reduced precipitate

During the process of Cr(VI) reduction by *Bacillus* sp. QH-1 in LB medium, the color of the medium changed from yellow to gray, and finally blue. Apart from that, the turbidity of the LB medium increased followed by the decrease of concentration of Cr(VI). A similar phenomenon was observed in *Achromobacter* sp. strain Chi1 (Zhu et al. 2006). Furthermore, energy dispersive X-ray analysis was performed to determine the elemental composition of precipitate after the cell growth in LB medium containing 100 mg/L Cr(VI). As shown in Fig. 4b, the main elements of the precipitate were C, O, P, K, Cr and S. Combined with the color change of medium, it can be assumed that there was insoluble Cr(III) generated during Cr(VI) reduction. Additionally, previous studies have proven that Cr may be present at the cell surface as organic complex or as Cr(III)-phosphate phase, or both (Rai et al. 1987; McLean and Beveridge 2001; Neal et al. 2002). Among them, organic complex Cr(III) usually exists in soluble form, while Cr(III)-phosphate exists as a precipitate Compared to the control experiment (Fig. 4a), the peak intensity of P and K waned after reduction (Fig. 4b), which indicates that the reduced product of Cr(VI) by *Bacillus* sp. QH-1 may be combined with phosphate as precipitation. The decrease of K may be due to a small amount of soluble Cr(III) adsorbed by microbes through the cation exchange with K.

Effect of concentration of electron donors on Cr(VI) reduction

Cr(VI)-reducing strains utilize a variety of organic compounds as electron donors, and most of the known electron donors are low molecular weight carbohydrates (Philip et al. 1998). Here, glucose was used as the electron donor. Experiments with different glucose concentrations (0 mg/L, 18 mg/L, 180 mg/L, 450 mg/L, 1,800 mg/L) were conducted at 37 °C. Plots of Cr(VI) concentration versus time are given in Fig. 5. Cr(VI) reduction occurred in the control experiment without glucose, which indicates that an unintended source of electron donor was present. The masses of cells present in our experiments should have been enough to support reduction by endogenous decay. The endogenous decay that caused Cr(VI) reduction in our control experiments has been observed by others in similar experiments, and is normal for this type of experiment. Sikora

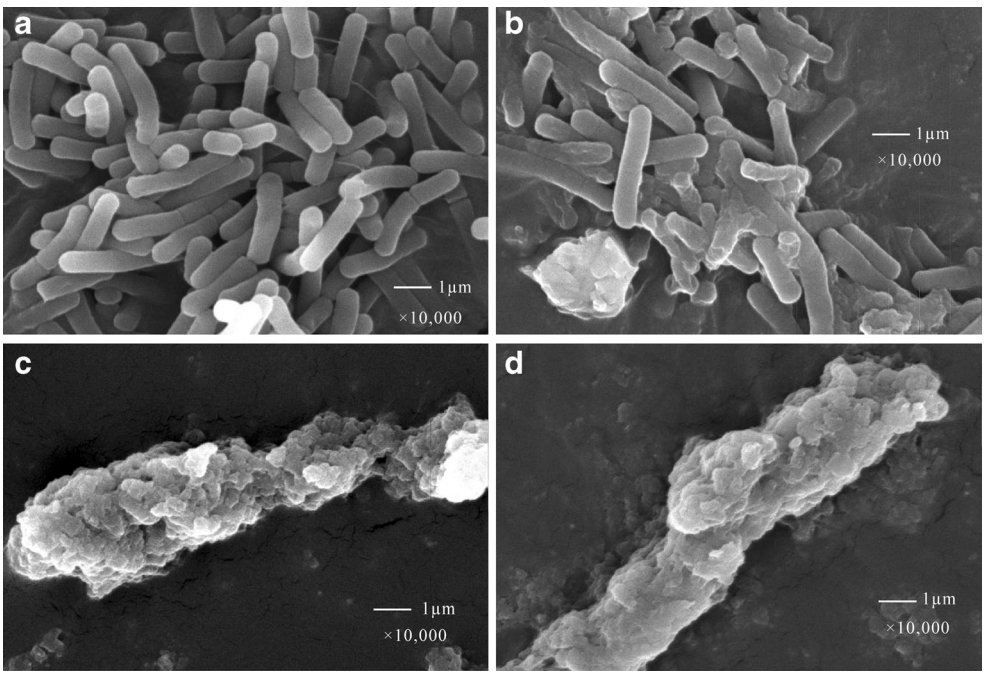

Fig. 3 SEM photos of *Bacillus* sp. QH-1 cells after growth 48 h in LB medium containing different concentration of Cr(VI) [(**a**) without Cr(VI), (**b**) with 25 mg/L Cr(VI), (**c**) with 100 mg/L Cr(VI), (**d**) with 200 mg/L Cr(VI)]. Scale bars: 1 μm

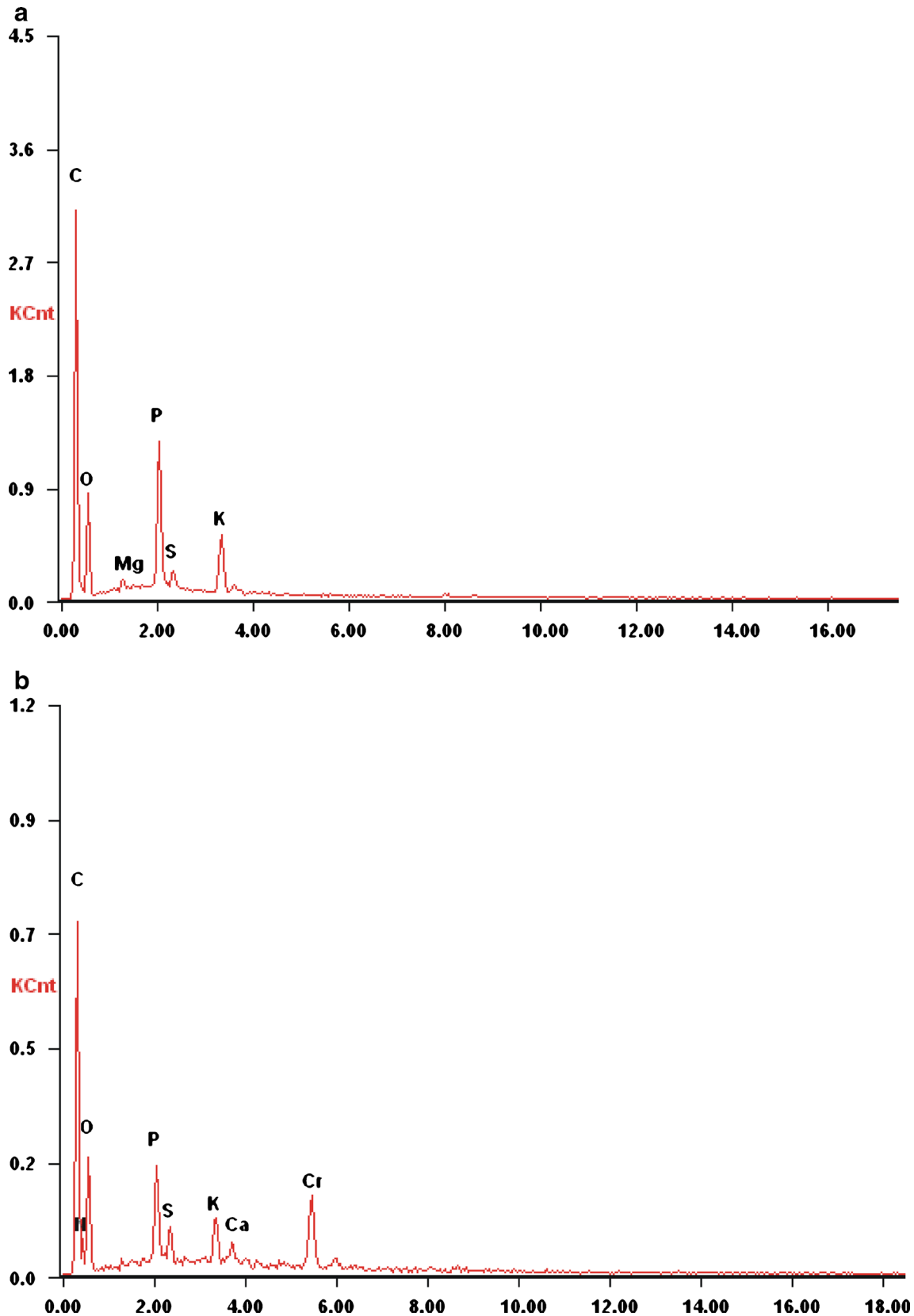

Fig. 4 EDXA spectrum of the precipitate obtained during the Cr(VI) reduction by strain *Bacillus* sp. QH-1 [(**a**) control, without Cr(VI), (**b**) with 100 mg/L Cr(VI)]

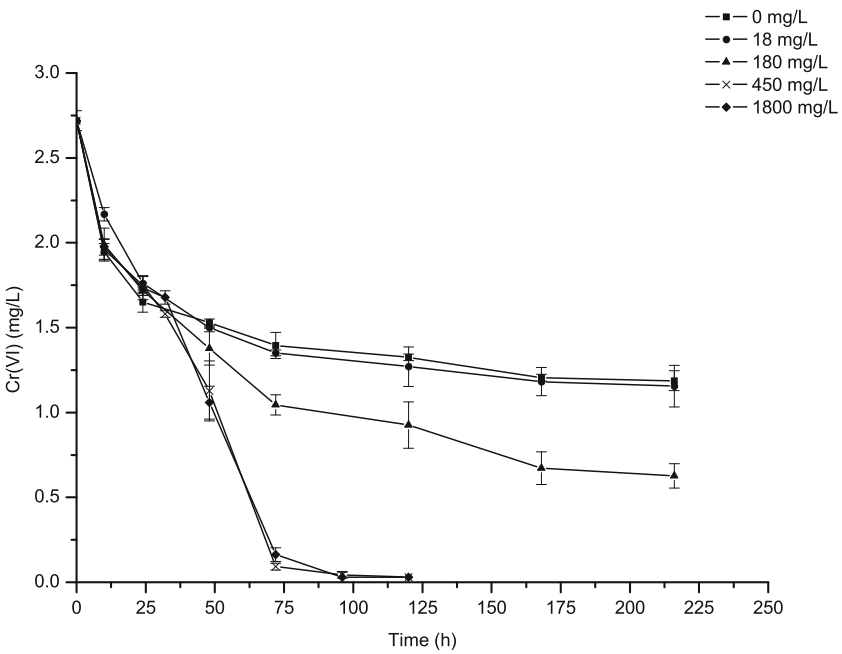

Fig. 5 Effect of concentration of carbon source on Cr(VI) reduction

et al. obtained similar results from similar electron donor-absent experiments with *Shewanella oneidensis* MR-1 (Sikora et al. 2008). Apart from that, the reduction was slow in the control experiment (no glucose) and 18 mg/L glucose, and it ceased entirely after about 120 h, presumably because electron donor concentrations were small. The curves of 450 mg/L and 1,800 mg/L glucose are similar, and almost all of the dissolved Cr(VI) was reduced after 120 h. This indicated that increasing the concentration of electron donors had no significant effect on the reduction as it reached the maximum level. In this study, the optimum level was 450 mg/L. This suggests that the concentrations of glucose had significant effect on Cr(VI) reduction, and could promote Cr(VI) reduction. A similar result has been reported in a previous study; Sayel et al. (2012) reported that the addition of glucose caused a light increase in Cr(VI) reduction by *Enterococcus gallinarum*.

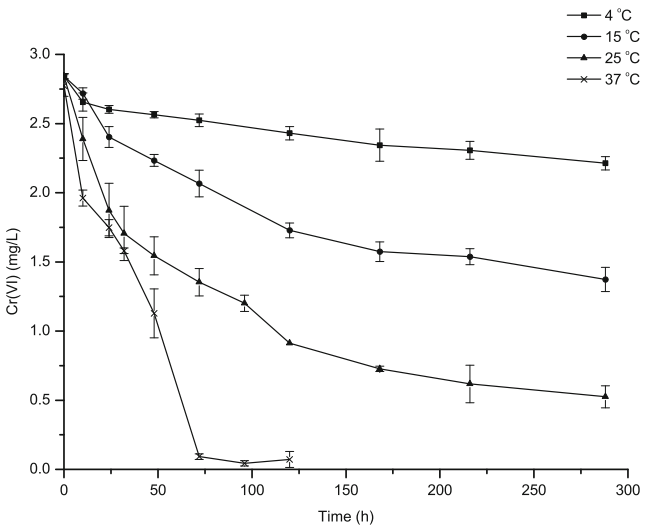

Fig. 6 Effect of temperature on Cr(VI) reduction

Effect of temperature on Cr(VI) reduction

The bacterial strain *Bacillus* sp. QH-1 was isolated from soil under a chromium-containing slag heap, which is aerobic. It is generally accepted that microbial Cr(VI) reduction is a kind of enzymatic reaction under aerobic conditions, and temperature plays an important role in the activity of enzyme. Therefore, it is necessary to consider the influence of temperature on Cr(VI) reduction by microbes in groundwater. The optimum growth temperature for *Bacillus* sp. QH-1 is 37 °C (data not shown here), higher than the temperature of groundwater. The experiments were conducted at four different temperatures, 4 °C, 15 °C, 25 °C and 37 °C, and the concentration of electron donors (glucose) was 450 mg/L. The results are given in Fig. 6. Cr(VI) was reduced effectively at 25 °C and 37 °C, and all of the Cr(VI) was reduced after 96 h at 37 °C. While the reduction rate was slow at 4 °C and 15 °C, it almost ceased after about 120 h. The deviation from the optimum temperature (37 °C), which decreased Cr(VI) reduction, may be due to the change in cell membrane structure, or the metabolism of denaturation of chromium reductase enzyme (Kathiravan et al. 2011). From the plot, we can draw a conclusion that temperature plays a significant role in Cr(VI) reduction. It suggested that temperature should be considered when applying this strain to remediate Cr(VI) in groundwater.

Conclusion

It is reported that several *Bacillus* sp. can reduce Cr(VI), with a maximum tolerance of Cr(VI) at 104 mg/L (Garbisu et al. 1998; Megharaj et al. 2004; Elangovan et al. 2006; Sarangi and Krishnan 2008). However, the tolerance level of Cr(VI) for *Bacillus* sp. QH–1was 200 mg/L, which indicated that this strain has the potential for remediating groundwater with Cr(VI) concentration up to 200 mg/L. The present study showed that concentration of electron donors (glucose) and temperature have significant effects on microbial Cr(VI) reduction. Increase of glucose concentration promoted the reduction of Cr(VI) by *Bacillus* sp. QH–1. Furthermore, it implied that temperature played a significant role in Cr(VI) reduction; compared with at 25 °C and 37 °C, the reduction was slow at 4 °C and 15 °C, suggesting that temperature must be considered for Cr(VI)-contaminated groundwater treated by this strain. The results obtained in this study may provide valuable information for the bioremediation of Cr(VI)-contaminated groundwater. Further studies will be conducted on the mechanisms by which the bacteria reduce Cr(VI).

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