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ScienceDirect
Trans. Nonferrous Met. Soc. China 19(2009) 1336−1341
Transactions of Nonferrous Metals Society of China
www.tnmsc.cn

Characterization of Cr(Ⅵ) resistance and reduction by *Pseudomonas aeruginosa*

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Received 24 November 2008; accepted 24 February 2009

Abstract: The experiments were conducted to evaluate the Cr(Ⅵ) resistance and reduction by *Pseudomonas aeruginosa*. After this bacterium tolerated 40 mg/L Cr(Ⅵ), the growth of cells was observed. The bacterial growth was obviously lower than the controls over 24 h and the binary cell fission was observed in cell morphology by scanning electron microscope. *P. aeruginosa* was found to be able to reduce Cr(Ⅵ) although Cr(Ⅵ) had toxic effects on the cells. The results demonstrate that Cr(Ⅵ) is reduced from 40 mg/L to about 18 mg /L in 72 h. The value of pH drops from 7.02 to around 5.65 after 72 h. A significant increase in the value of redox potential occurs during Cr(Ⅵ) reduction and Cr(Ⅵ) reduction can be observed over a range of redox potential from +3 mV to +91 mV. Both of SO_4^{2-} and NO_3^- have no effect on Cr(Ⅵ) reduction. The presence of Zn^{2+} has a notable inhibitory effect on Cr(Ⅵ) reduction while Cu^{2+} substantially stimulates Cr(Ⅵ) reduction. In the presence of Zn^{2+}, Cr(Ⅵ) decreases from 40 mg/L to only 26−27 mg/L, whereas Cr(Ⅵ) drops to 1−2 mg/L after 48 h in the presence of Cu^{2+}.
Key words: *Pseudomonas aeruginosa*; Cr(Ⅵ) reduction; Cr(Ⅵ) resistance; bacterial growth

1 Introduction

Heavy metal pollution has become a serious environmental problem and caused great threat to environmental safety and human health. Chromium is one of the most hazardous heavy metals. Its wide application in industry has resulted in its occurrence as a potential pollutant in water and soil.

Chromium commonly exists in two oxidation states, Cr(Ⅵ) and Cr(Ⅲ). The hexavalent form of chromium is water-soluble, highly toxic, mutagenic and carcinogenic; while the trivalent form of chromium is less soluble, therefore less mobile, and much less toxic than Cr(Ⅵ) [1−2]. Due to the vastly differing toxicity of the two oxidation states, the approaches to reduce Cr(Ⅵ) to Cr(Ⅲ) are of great interests for detoxification of Cr(Ⅵ)-polluted wastes. The conventional methods for Cr(Ⅵ) reduction is to apply chemical reductants such as ferrous iron, sulfides. But chemical technologies consume large quantities of chemical reagents and easily produce secondary wastes that require remediation. In recent years, bioremediation using microorganisms and alga has been regarded as an alternative strategy for Cr(Ⅵ) contamination since biosorption, bioaccumulation and bioreduction of Cr(Ⅵ) by bacterial, fungal and algal biomass have been recognized[3−5].

In previous studies, the capability of Cr(Ⅵ) reduction has been found in a variety of microorganisms, including strains of *Pseudomonas*[6−8], *Bacillus*[9−11], *Escherichia*[12−14], *Enterobacter*[15−17] and *Shewanella* [18−20]. It has been reported that enzymes in cells are responsible for microbial Cr(Ⅵ) reduction [21−22]. Cr(Ⅵ) reductase in *Bacillus* sp.[9−10] and *Escherichia coli*[12] has been shown to be soluble enzymes, and Cr(Ⅵ) reductase in *Pseudomonas* sp. was associated with the cytosolic fractions but not with the membrane fractions[8], while in cells of *Shewanella oneidensis*[18] and *Enterobacter cloacae*[16] Cr(Ⅵ) reductase has been found to be membrane associated.

Pseudomonas aeruginosa has been previously reported to be able to reduce Cr(Ⅵ)[6−7], but the

Foundation item: Project(IRT0719) supported by the Program for Changjiang Scholars and Innovative Research Team in University, China; Projects (2006BAD03A1704, 2006BAD03A1706) supported by the National Science & Technology Pillar Program during the 11th Five-year Plan Period of China
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DOI: 10.1016/S1003-6326(08)60446-X

potential of Cr(Ⅵ) reduction by this strain has not been thoroughly investigated. In this study, the Cr(Ⅵ) resistance was evaluated by detecting the morphology and growth of cells after the strain was exposed to Cr(Ⅵ). Some characteristics such as bacterial growth, the variation of redox potential(ORP) and pH during Cr(Ⅵ) reduction and the effects of coexistent anions and heavy metals on Cr(Ⅵ) reduction by *P. aeruginosa* were researched, so as to determine the Cr(Ⅵ) reduction potential of this strain more roundly.

2 Experimental

2.1 Nutrient medium

The nutrient medium used for cells cultivation consisted of 3 g beef extract, 5 g peptone and 5 g NaCl in 1 L distilled water. The pH value of the medium was adjusted to 7.0±0.2 with 10% NaOH and 10% HCl. The media were autoclaved at 120 ℃ for 20 min before using for cells cultivation. The nutrient medium used for Cr(Ⅵ) reduction was supplemented with 1% (mass to volume ratio) glucose and was autoclaved at 115 ℃ for 30 min.

2.2 Strains and cultivation conditions

Pseudomonas aeruginosa (CCTCC AB 91095) was purchased from China Center for Type Culture Collection. This strain was first grown on agar slant and stored at 4 ℃ before using. The cells were enriched by transferring one loop of cells from agar slant to 100 mL of previously sterilized liquid nutrient medium in 250 mL flasks and then incubated at 37 ℃ by shaking at 150 r/min in an orbital incubator.

2.3 Cr(Ⅵ) reduction experiments

The cells grown for 24 h were centrifuged at 4 000 r/min for 15 min, and the cells pellet was then washed three times with phosphate buffer (pH=7.0) and suspended with the same buffer. This cell suspension was then inoculated (1%, volume fraction) to 250 mL flasks containing 100 mL nutrient medium and desired Cr(Ⅵ) ($K_2Cr_2O_7$) concentration. In the controls, the same amount of phosphate buffer (without cells) was added as the inoculated cell suspension. The flasks were then incubated at 37 ℃ by shaking at 150 r/min. In the experiments for determining the effects of SO_4^{2-}, NO_3^-, Zn^{2+} and Cu^{2+} on Cr(Ⅵ) reduction, a desired amounts of SO_4^{2-} (Na_2SO_4), NO_3^- (KNO_3), Zn^{2+} ($ZnSO_4$) and Cu^{2+} ($CuCl_2$) were respectively added to the flasks before inoculation of cells suspension. The solutions of $K_2Cr_2O_7$, Na_2SO_4, KNO_3, $ZnSO_4$, $CuCl_2$ and phosphate buffer were autoclaved at 120 ℃ for 20 min before using.

The experiments were carried out in triplicate. The results were presented as the mean values of triplicates.

2.4 Analytical methods

Samples (1 mL) were drawn from the flasks at interval times and centrifuged at 10 000 r/min for 10 min. The supernatant was used to determine Cr(Ⅵ) concentration. Cr(Ⅵ) was determined colorimetrically by reaction with diphenylcarbazide in acid solution. The absorbance was measured at 540 nm with a UV 754N model spectrophotometer. Total Cr was also determined at regular time. The samples for total chromium analysis were first digested with a mixture of sulphuric-nitric acids and oxidized with potassium permanganate before reacting with diphenylcarbazide and determined colorimetrically. Bacterial growth in the liquid culture was determined by measuring the absorbance of 1 cm cuvette at 600 nm with a UV 754N model spectrophotometer. Redox potential and pH of bacterial cultures were measured electrometically by a pH meter (pHS−25, Shanghai, China) equipped with a glass electrode as the measuring electrode and a calomel electrode as the reference electrode.

2.5 Scanning electron microscopy

Cell morphology was examined by SEM. Bacteria grown in liquid medium and Cr(Ⅵ)-amended liquid medium were respectively harvested by centrifugation at 4 000 r/min for 30 min. The cells were first fixed in 3% glutaraldehyde at 4 ℃ for 2 h; washed with phosphate buffer three times at 4 ℃; and then fixed with 1% osmic acid for 1 h and washed with phosphate buffer three times. The fixed cells were then dehydrated through a 30%−100% ethanol series for 10 min at each stage and two changes in 100% ethanol. Thereafter, cells were immersed in tert-butyl alcohol at least for 2 h, critical point dried in freeze drier for 3−4 h, and sputter coated with gold. The prepared samples were then examined with a JSM−6360LV scanning electron microscope (JEOL).

3 Results and discussion

3.1 Cr(Ⅵ) resistance

Bacterial growth was measured within 24 h for *P. aeruginosa* as the strains were grown in liquid medium amended with 0, 10, 20, 40, 80 and 100 mg/L of Cr(Ⅵ), respectively. *P. aeruginosa* tolerated 40 mg/L Cr(Ⅵ), which is indicated by a slow growth of cells under this Cr(Ⅵ) concentration (Fig.1). In Fig.6, OD_{600} was obtained by measuring the obsorbance of cells culture at 600 nm.

Fig.1 also shows that bacterial growth decreased with the increase of Cr(Ⅵ) concentration and a lag period of bacterial growth was observed as *P. aeruginosa* was exposed to Cr(Ⅵ) concentration higher than 20 mg/L. It was found that 10 mg/L Cr(Ⅵ) had a slight

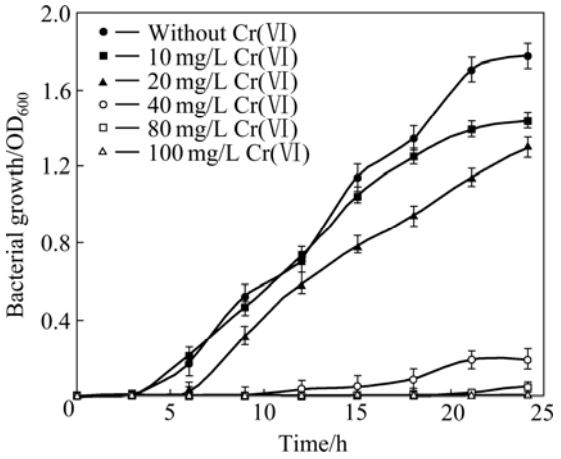

Fig.1 Effect of Cr(Ⅵ) on growth of *P. aeruginosa* (Cells cultured by shaking at 150 r/min and 37 ℃)

effect on bacterial growth, but the presence of 40 mg/L Cr(Ⅵ) caused a significant decrease in bacterial growth. As Cr(Ⅵ) concentration was up to 80 or 100 mg/L, only a negligible growth was observed. This indicates that bacterial growth of *P. aeruginosa* is heavily inhibited due to the toxicity of Cr(Ⅵ) at higher concentrations. It was reported for *Escherichia coli* that the specific growth rate decreased and lag period increased as Cr(Ⅵ) concentration in culture medium increased[23].

In order to further determine the effects of Cr(Ⅵ) on the cells of *P. aeruginosa*, the cell morphology was examined by SEM after this strain was grown in liquid medium amended without Cr(Ⅵ) and with 40 mg/L Cr(Ⅵ), respectively, for 36 h. Fig.2 shows that the morphologies of cells are not intact, the cell size increases in the presence of 40 mg/L Cr(Ⅵ); and the binary cell fission is observed as the strain is exposed to Cr(Ⅵ). This indicates that Cr(Ⅵ) has toxic effects on the cells of *P. aeruginosa*. For some Cr(Ⅵ)-resistant bacteria, more bacterium capsules were observed in cell surface and the thickness of cell wall increased as they were treated with higher concentration of Cr(Ⅵ)[24], while the cells of *Acinetobacter haemolyticus* were found to lose their shape and increase in size, and their cell wall became thinner when this strain was exposed to Cr(Ⅵ) [25].

3.2 Cr(Ⅵ) reduction

The variation of Cr(Ⅵ) concentration is shown in Fig.3. It is found that Cr(Ⅵ) concentration decreases from 40 mg/L to about 18 mg/L after 72 h when the bacteria are inoculated, while Cr(Ⅵ) reduction is not observed in the uninoculated controls (Fig.3(a)). This indicates the ability of *P. aeruginosa* to reduce Cr(Ⅵ) although cell morphology is damaged and bacterial growth is inhibited by Cr(Ⅵ). Total Cr in solutions was also measured. The results in Fig.3(b) demonstrate that there is only a slight decrease in the concentration of total Cr and more than 85% of chromium is in solutions. This may indicate that only a small amount of chromium is adsorbed on the cells and the decrease of Cr(Ⅵ) concentration is not caused by adsorption on cells, but mainly by the reduction activity of *P. aeruginosa*.

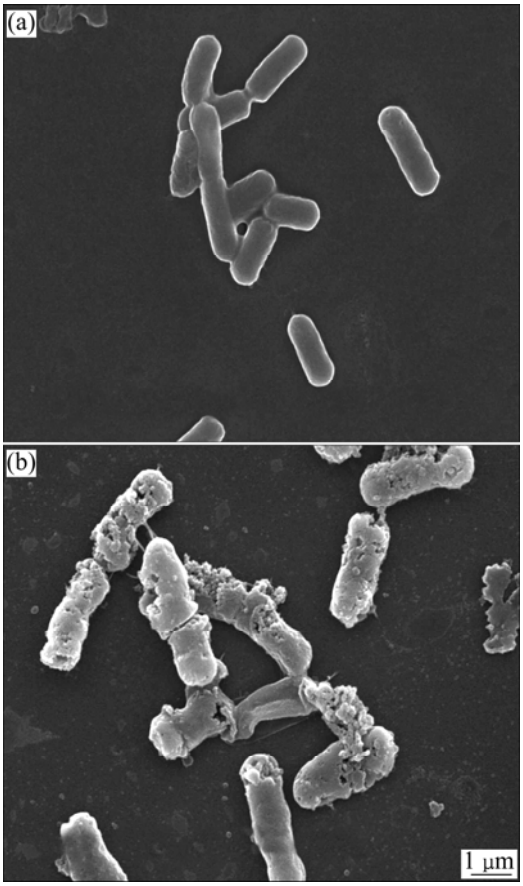

Fig.2 SEM images of *P. aeruginosa*: (a) Controls (untreated with Cr(Ⅵ)); (b) Cells treated with 40 mg/L Cr(Ⅵ) (Cells cultured by shaking at 150 r/min and 37 ℃ for 36 h)

3.3 Relationship between Cr(Ⅵ) reduction and bacterial growth

Cr(Ⅵ) concentration and bacterial growth were simultaneously examined to evaluate the relationship between Cr(Ⅵ) reduction and bacterial growth. Fig.4 shows that Cr(Ⅵ) reduction activity is basically correlated with bacterial growth. Cr(Ⅵ) concentration gradually decreases with the increase of bacterial growth, and there is a significant increase in bacterial growth as Cr(Ⅵ) is reduced more efficiently. This may indicate that Cr(Ⅵ) reduction and bacterial growth affect each other. The growth of cells stimulates Cr(Ⅵ) reduction and efficient Cr(Ⅵ) reduction conversely promotes bacterial growth.

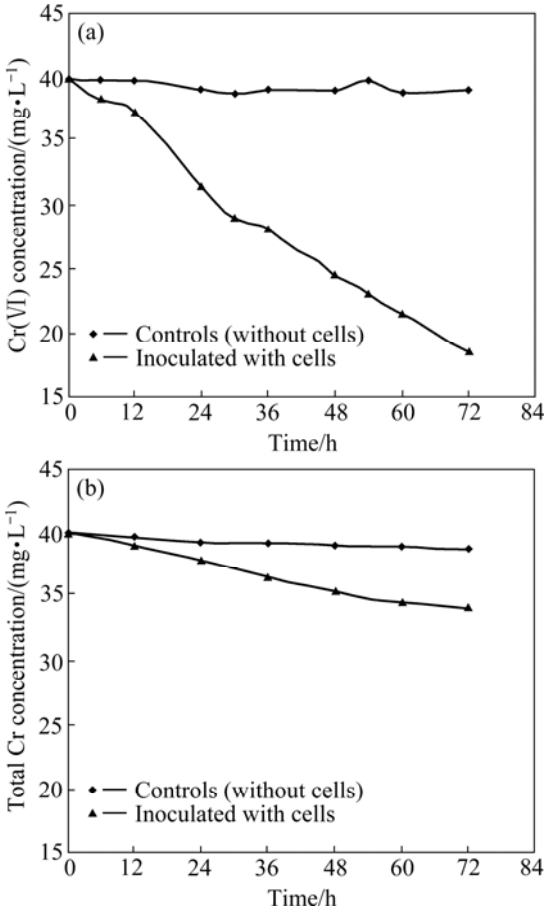

Fig.3 Cr(Ⅵ) reduction by *P. aeruginosa*: (a) Variation of Cr(Ⅵ) concentration; (b) Variation of total Cr concentration during Cr(Ⅵ) reduction (Cr(Ⅵ) reduced by shaking at 150 r/min and 37 ℃)

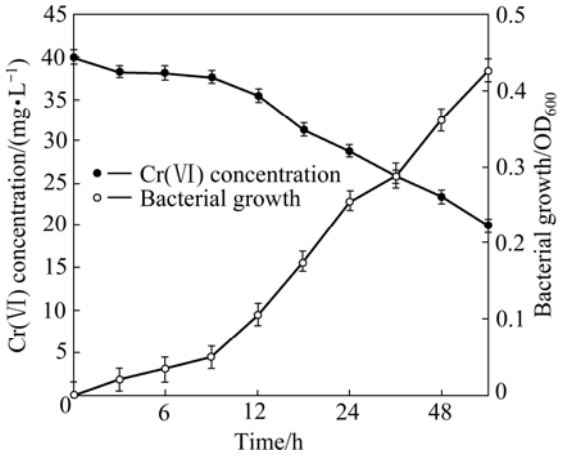

Fig.4 Relationship between Cr(Ⅵ) reduction and bacterial growth of *P. aeruginosa* (Cr(Ⅵ) reduced by shaking at 150 r/min and 37 ℃)

3.4 Variation of pH and redox potential during Cr(Ⅵ) reduction

As shown in Fig.5(a), there is an obvious decrease in pH value and pH decreases from 7.02 to 5.46 during the first 24 h, but after 24 h, the variation of pH value is quite small and pH is about 5.65 at 72 h. Fig.5(a) also shows that Cr(Ⅵ) reduction activity occurs during the whole process of pH variation.

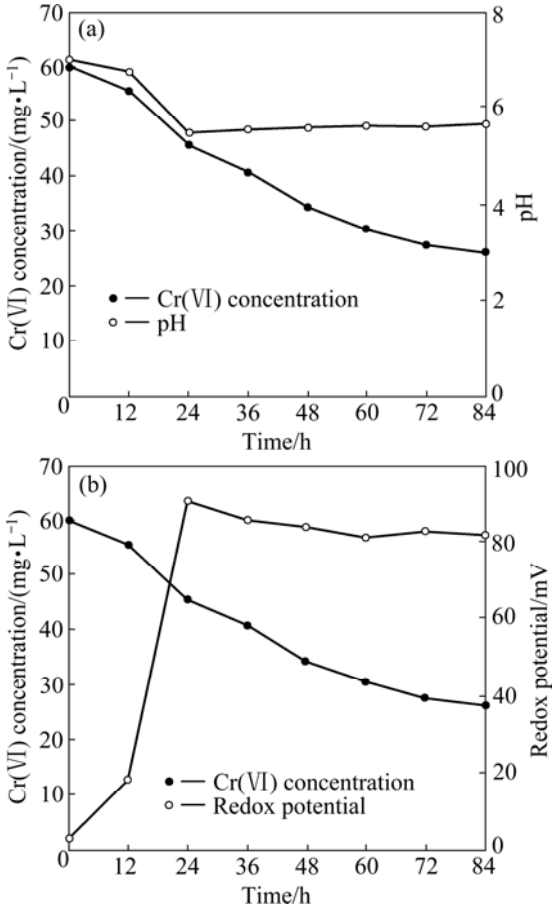

Fig.5 Variation of pH (a) and redox potential (b) during Cr(Ⅵ) reduction by *P. aeruginosa* (Cr(Ⅵ) reduced by shaking at 150 r/min and 37 ℃)

The variation of redox potential(ORP) was examined during Cr(Ⅵ) reduction. Fig.5(b) shows that a rapid increase of ORP from +3 mV to +91 mV occurs during the first 24 h. There is a small decrease in the value of ORP after 24 h. Cr(Ⅵ) reduction was observed throughout the variation of ORP. Accordingly, no particular correlation is found between ORP and Cr(Ⅵ) reduction. This is similar to the results found by WANG and XIAO[26]. However, some other studies have demonstrated that a low ORP was required for Cr(Ⅵ) reduction. The rate of Cr(Ⅵ) reduction by resting cells of *Agrobacterium radiobacter* was found to be greater in cell suspensions with −240 mV than with −198 mV[27]. When the redox potential of *E. coli* cultures was more than −140 mV, no Cr(Ⅵ) reduction was observed within the first 1 h[28]. In this study, Cr(Ⅵ) reduction was observed over the whole range from +3 mV to +91 mV. This indicates that a low value of ORP is not necessarily required for Cr(Ⅵ) reduction by *P. aeruginosa*.

3.5 Effect of sulfate and nitrate on Cr(Ⅵ) reduction

The effects of anions, SO_4^{2-} and NO_3^- on Cr(Ⅵ) reduction were determined. The results are presented in Fig.6. Two concentrations, 40 mg/L SO_4^{2-} and 80 mg/L SO_4^{2-} have no effects on Cr(Ⅵ) reduction, and Cr(Ⅵ) reduction is also not influenced by NO_3^-, whether the concentration is 40 mg/L or the concentration is 80 mg/L. Aerobic chromate reduction was generally not affected by sulfate and nitrate[9, 11, 26]. Anaerobic chromate reduction was, however, sensitive to nitrate and sulfate ions due to the fact that nitrate and sulfate ions may compete with chromate as anaerobic electron acceptors[9].

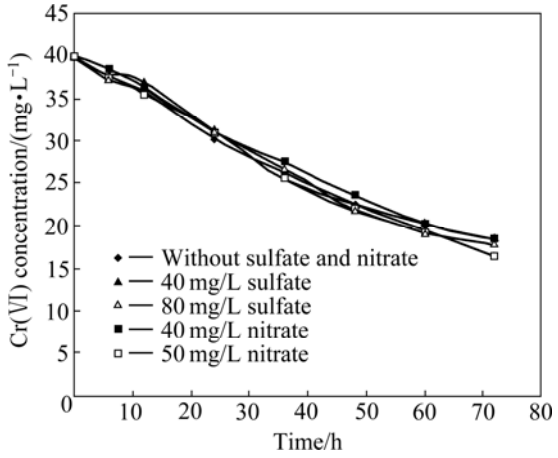

Fig.6 Effects of sulfate and nitrate on Cr(Ⅵ) reduction by *P. aeruginosa* (Cr(Ⅵ) reduced by shaking at 150 r/min and 37 ℃)

3.6 Effect of Zn^{2+} and Cu^{2+} on Cr(Ⅵ) reduction

The effect of Zn^{2+} on Cr(Ⅵ) reduction was completely different from that of Cu^{2+}. The data in Fig.7 show that Cr(Ⅵ) reduction is significantly inhibited by Zn^{2+}, even the concentration of Zn^{2+} drops from 100 mg/L to 25 mg/L. In contrast, Cu^{2+} at both concentrations, 25 mg/L and 100 mg/L, substantially stimulates Cr(Ⅵ) reduction. From Fig.7, we can see that Cr(Ⅵ) concentration in the controls (without Zn^{2+} and Cu^{2+}) drops from 40 mg/L to less than 1 mg/L after 96 h; while in the presence of Zn^{2+}, Cr(Ⅵ) decreases to only 26−27 mg/L, whereas Cr(Ⅵ) drops to 1−2 mg/L only after 48 h in the presence of Cu^{2+}.

SULTAN and HASNAIN[29] also found that Zn^{2+} had an inhibitory effect while Cu^{2+} had a stimulatory effect on Cr(Ⅵ) reduction by *Ochrobactrum intermedium* strain SDCr-5. It had been reported that 1 mmol/L Zn^{2+} caused a 32% decrease in Cr(Ⅵ) reduction by *E. coli*[23]. Cr(Ⅵ) reduction by cell-free extract of *Bacillus* sp. ES 29 was stimulated by Cu^{2+}[10]. The presence of Cu^{2+} also stimulated the Cr(Ⅵ) reductase activity of *Pseudomonas* sp. G1DM21 by 33%[8]. However, some other studies have demonstrated

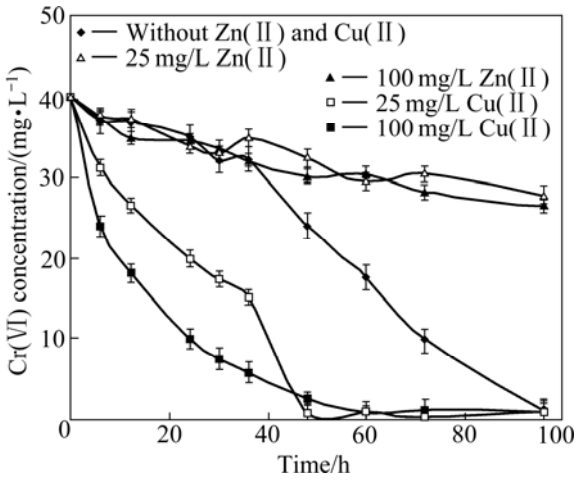

Fig.7 Effects of Zn^{2+} and Cu^{2+} on Cr(Ⅵ) reduction by *P. aeruginosa* (Cr(Ⅵ) reduced by shaking at 150 r/min and 37 ℃)

the inhibitory effect of Cu^{2+} on Cr(Ⅵ) reduction. OHTAKE et al[15] found that 0.5 mmol/L Cu^{2+} inhibited 32% of Cr(Ⅵ) reduction by a membrane-associated chromate reductase under anaerobic conditions. The presence of Cu^{2+} (100 mg/L) caused a 16%−33% decrease in Cr(Ⅵ) reduction by *E.coli* but not at lower concentration[13]. The stimulatory mechanism of Cr(Ⅵ) reduction activity by Cu^{2+} is not clear. But, it has been reported that Cu^{2+} is a transition metal that is a prosthetic group for many reductase enzymes and the main function of Cu^{2+} is related to electron-transport protection or acts as a single-electron redox center and, in some cases, as a shuttle for electrons between protein subunits[10, 30].

4 Conclusions

1) The strain of *Pseudomonas aeruginosa* is resistant to 40 mg/L Cr(Ⅵ), but the binary cell fission is observed in cell morphology and cell size increases as the strain is exposed to 40 mg/L Cr(Ⅵ), and the growth of cells decreases with the increase of Cr(Ⅵ) concentration.

2) Although Cr(Ⅵ) has toxic effects on the cells, the strain reduces 40 mg/L Cr(Ⅵ) to about 18 mg/L Cr(Ⅵ) in 72 h.

3) There is a good correlation between Cr(Ⅵ) reduction activity and bacterial growth. A drop of pH value and a remarkable increase of ORP are observed during Cr(Ⅵ) reduction. Cr(Ⅵ) is efficiently reduced by *P. aeruginosa* over a ORP range from +3 mV to +91 mV.

4) Cr(Ⅵ) reduction is not affected by sulfate and nitrate, but significantly influenced by Zn^{2+} and Cu^{2+}. Both 25 mg/L Zn^{2+} and 100 mg/L Zn^{2+} significantly inhibite Cr(Ⅵ) reduction, while both 25 mg/L Cu^{2+} and

100 mg/L Cu^{2+} substantially stimulate Cr(Ⅵ) reduction.

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(Edited by YANG Hua)