

Experimental study on Cr(VI) reduction by *Pseudomonas aeruginosa*

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Abstract: Investigation on Cr(VI) reduction was conducted using *Pseudomonas aeruginosa*. The study demonstrated that the Cr(VI) can be effectively reduced to Cr(III) by *Pseudomonas aeruginosa*. The effects of the factors affecting Cr(VI) reduction rate including carbon source type, pH, initial Cr(VI) concentration and amount of cells inoculum were thoroughly studied. Malate was found to yield maximum biotransformation, followed by succinate and glucose, with the reduction rate of 60.86%, 43.76% and 28.86% respectively. The optimum pH for Cr(VI) reduction was 7.0, with reduction efficiency of 61.71% being achieved. With the increase of initial Cr(VI) concentration, the rate of Cr(VI) reduction decreased. The reduction was inhibited strongly when the initial Cr(VI) concentration increased to 157 mg/L. As the amount of cells inoculum increased, the rate of Cr(VI) reduction also increased. The mechanism of Cr(VI) reduction and final products were also analysed. The results suggested that the soluble enzymes appear to be responsible for Cr(VI) reduction by *Pseudomonas aeruginosa*, and the reduced Cr(III) was not precipitated in the form of Cr(OH)₃.

Keywords: *Pseudomonas aeruginosa*; Cr(VI) reduction; reduction rate; Cr(VI) remediation

Introduction

Chromium is released into the environment by a large number of industrial operations including chrome plating, petroleum refining, leather tanning, wood preserving, textile manufacturing, and pulp processing. Consequently, chromium contamination of soil and groundwater has been a significant problem worldwide. It exists both in hexavalent and trivalent forms. It has well recognized that Cr(VI) is very toxic, carcinogenic, and mutagenic both in humans and animals, whereas Cr(III) is less toxic, less soluble, and thus a lesser problem (Guha, 2001).

Conventional methods for the treatment of chromate include chemical reduction by using a reducing agent such as sodium sulfate, and adsorption on the ion exchange and chelating resins. These methods are effective in treatment of chromium pollution, but they consume high amounts of energy and large quantities of chemical reagent. Biological treatment of Cr(VI)-containing wastes could be one of the most cost-effective remediation technologies in both on-site and off-site treatment options (Evans, 1997).

Many microorganisms have been isolated that can reduce Cr(VI) to Cr(III) (Amanda, 2002), and a lot of researches on microbial decontamination of chromium have been done, especially in recent years scientists have realized that understanding the genetic makeup of microbes that thrive in chromium polluted environments may help them engineer bacteria that can clean the contaminants from environments and have made much effort on the studies about gene and genetically engineered microorganisms in microbial remediation of chromium. Microbial tolerance mechanisms for chromium have been identified and described in detail in some reports. Nies *et al.* described a small family of

proteins, CHR, which contains members that function in chromate transport. They consist of about 400 amino acyl residues, appear to have 10 transmembrane α -helical segments in an unusual 4 + 6 arrangement, and arose by an intragenic duplication event (Nies, 1998). In addition, special attention is given to the application of genes in bioremediation. Through genetic engineering, scientists have purified some chromate reductase and cloned or transferred the chromium-resistant gene or chromate reductase-encoding gene from one microbe into other microbes that decompose organic pollutants or other contaminants. Alternatively, scientists think that they could use chromium-reducing microbe as a host for other bacterial genes that would enable it to break down a variety of pollutants (Park, 2000; Spain, 2003; Nies, 1990; Shen, 1993). In another potential application, scientists cloned parts of chromate-resistant cells plasmid, which contains determinants encoding inducible resistance to chromate and studied the mutation so as to develop metal-sensing bacterial strains used as bacterial chromate sensor (Peitzsch, 1998).

To enhance the probability for success of a bioremediation strategy, knowledge of the biochemistry involved in the Cr(VI) reduction and an understanding of the conditions under which the microbial populations have greatest specific chromate reduction rates are needed. The main objective of this study is to evaluate the potential of *Pseudomonas aeruginosa* that is known to be a Cr(VI) reducing bacterium (Gvozdyak, 1986) for the biotransformation of Cr(VI) to Cr(III) under different conditions, and to initially analyze the mechanisms, while providing the fundamental data for the microbial application in the bioremediation of chromium.

1 Materials and methods

1.1 Preparation of media

The general growth medium for bacteria consisted of peptone(5 g), NaCl(5 g), yeast extract(3 g), and carbon source(1 g) in 1 L of distilled water. The pH of the medium was adjusted to 7.0 with 10% (w/v) HCl and 10% (w/v) NaOH. All media were autoclaved at 120°C for 20 min before Cr(VI) reduction experiments. Three different carbon sources, namely glucose, malate, and succinate were incorporated in the media.

1.2 Bacterial strain and cultivation conditions

Pseudomonas aeruginosa (CCTCC AB 91095) was purchased from China Center for Type Culture Collection.

Pure cultures of *P. aeruginosa* were first grown in agar slants by incubating at 37°C for 24 h. Liquid cultures were then enriched by transferring one loop of cells from the slants to 100 ml of previously sterilized nutrient broth in 250 ml flasks and incubating at 37°C by shaking at 150 r/m for 30 h in an constant temperature shaker incubator(SKL-3F).

1.3 Cr(VI) solution

Cr(VI) stock solution was prepared from $K_2Cr_2O_7$ and autoclaved at 120°C for 20 min for Cr(VI) reduction experiments.

1.4 Cr(VI) reduction experiments

The 250 ml flasks containing 100 ml media were inoculated with 5 ml cultures at logarithmic-phase, then adding Cr(VI) from a sterilized stock $K_2Cr_2O_7$ solution to the flasks at desired concentration and incubated in the same way described above. The experiments were conducted by varying the carbon sources, pH, initial Cr(VI) concentration and amount of cells inoculum respectively to determine the best conditions for Cr(VI) reduction.

To further understand the mechanism of Cr(VI) reduction, another series of experiments were also carried out: (1) The cells of *Pseudomonas aeruginosa* were treated with dinitrophenol (DNP), a decoupler of oxidative phosphorylation, and killed by heating respectively. (2) Cell free extracts were prepared according to a modified procedure of Bopp and Ehrlich (Bopp, 1988). The cultures were harvested by centrifugation at 10000 r/min for 20 min, washed and resuspended in phosphate buffer (pH = 7.0), then disrupted by an ultrasonic probe(VC100). A portion(20 ml) of the sonicate was then centrifugated at 15000 r/min for 20 min. The supernatant obtained was used as soluble fraction and sediment was used as cell membrane fraction for Cr(VI) reduction experiments. Cell free extracts(supernatant or sediment) were dispensed into sterile 100 ml flasks containing 20 mg/L Cr(VI) and electron donors compounds, and incubated with shaking at 150 r/m, 37°C in a shaker incubator to determine the components responsible for Cr(VI) reduction by *P. aeruginosa*.

1.5 Analytical methods

Samples were drawn from reaction bottles at specified time intervals with a sterile pipette and then centrifugated at 10000 r/min for 20 min with centrifugal machine(TGL-16G). The concentration of Cr(VI) in the supernatant was determined colorimetrically at 540 nm using diphenylcarbazide reagent in acid solution with 721 spectrophotometer. 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. The Cr(VI) reduction rate was calculated based on the following formula:

$$\text{Reduction rate} = \frac{C_i - C_f}{C_i} \times 100\%$$

Where C_i is the initial Cr(VI) concentration(mg/L); C_f is the final Cr(VI) concentration(mg/L).

The analysis about $Cr(OH)_3$ precipitate was based on the mass balance calculations between total Cr and initial Cr(VI) added to the flasks, and Cr(III), was determined from difference between total Cr and Cr(VI).

2 Results and discussion

2.1 Screening of carbon source

Previous studies have shown that microorganism can use organic matters as electron donor for Cr(VI) reduction and the type of carbon source(electron donor) has effect on the reduction rate(Guha, 2001). Screening of carbon source for chromium reduction was conducted with glucose, malate, and succinate respectively. Fig.1 shows the kinetics and rates of Cr(VI) reduction by *P. aeruginosa* in the presence of different carbon sources. Chromium reduction was observed in the presence of each electron donors, although the extent of reduction varied significantly. Malate was the best effective as an electron donor for bacterial Cr(VI) reduction with Cr(VI) concentration dropped from 87.5 mg/L to 34.25 mg/L and the reduction rate of 60.86% was obtained. In contrast, Cr(VI) concentration only decreased to 49.25 mg/L, 69.25 mg/L with the reduction rate of 43.76%, 28.86% at the termination of the experiments when succinate and glucose were applied respectively.

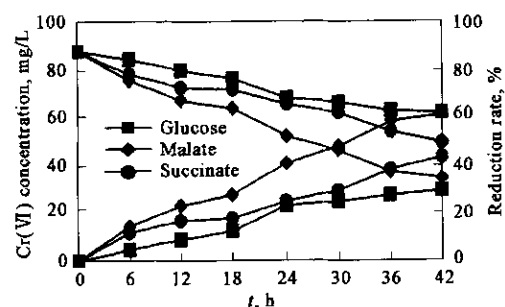


Fig.1 Effect of different carbon source on Cr(VI) reduction

In the course of organic matters' catabolism, one group

of reactions that may be set apart as the major reaction sequence that provides electrons is known as TCA cycle. Glucose has to be catabolized to pyruvate to enter TCA cycle, whereas malate, succinate are intermediates of this cycle (Wei, 1996). Hence, it can be expected that glucose would be the least effective when compared with the other two electron donor compounds. In addition, malate reduction leads to the formation of NADH, whereas succinate conversion to formate yields FADH₂. NADH has been demonstrated to be a more preferable electron donor than FADH₂ for cellular reactions (Wei, 1996). This may be the reason for better Cr(VI) reduction in the presence of malate.

The effect of carbon source concentration on Cr(VI) reduction is listed in Table 1. It is clear that the rate of Cr(VI) reduction and biomass increased with the increase of malate concentration, and it is also clear that the reduction rate of Cr(VI) only showed slight increase when the concentration of malate increased to 1500 mg/L from 1000 mg/L. Therefore, 1000 mg/L of malate was relatively reasonable for Cr(VI) reduction.

Table 1 Effect of malate concentration on Cr(VI) reduction

| Malate concentration, mg/L | Residual Cr(VI) after 42 h, mg/L | Final cell density, mg/L | Cr(VI) reduction rate, % |
|----------------------------|----------------------------------|--------------------------|--------------------------|
| 0 | 85.15 | 225.05 | < 5 |
| 500 | 47.00 | 590.00 | 46.28 |
| 1000 | 35.50 | 1204.60 | 59.42 |
| 1500 | 33.75 | 1536.83 | 61.43 |

Notes: initial Cr(VI) concentration = 87.5 mg/L; initial cell concentration = 225 mg/L; the cell density was determined from cell dry weight

2.2 Effect of pH

Cr(VI) reduction by *P. aeruginosa* was evaluated within a pH range of 4–8 and the results are presented in

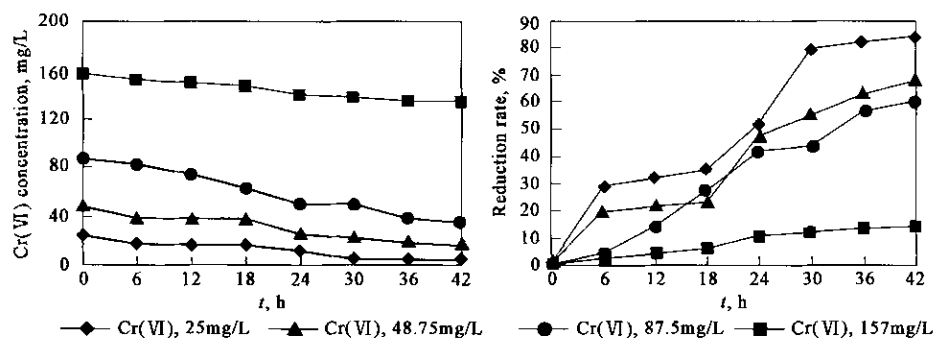


Fig. 3 Effect of initial Cr(VI) concentration on Cr(VI) reduction

2.4 Effect of amount of cells inoculum on Cr(VI) reduction

The amount of inoculum was varied to determine its effect on the ability of *P. aeruginosa* to transform Cr(VI) to Cr(III) at the initial Cr(VI) concentration of 87.5 mg/L. The results are listed in Table 2. It is concluded that a greater amount of inoculum will help in more efficient processing of the Cr(VI) reduction. The increase of amount

of cells inoculum resulted in the enhancement of reduction rate.

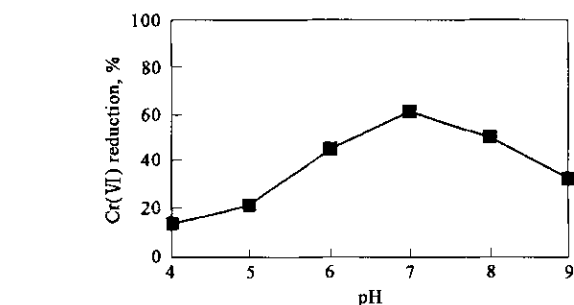


Fig. 2 Effect of pH on Cr(VI) reduction

2.3 Effect of initial Cr(VI) concentration

The effect of initial Cr(VI) concentration on the rate of Cr(VI) reduction was investigated over a concentration range of 25–200 mg/L. Fig. 3 shows that Cr(VI) reduction by *P. aeruginosa* occurred even at the highest Cr(VI) concentration of 157 mg/L, but the rate of Cr(VI) reduction decreased with time and eventually ceased at the highest Cr(VI) concentration. Cr(VI) concentration dropped to lower than 5 mg/L, 16 mg/L and the reduction rate of 84%, 67.69% was obtained at initial concentration of 25 mg/L, 48.75 mg/L. This indicated the existence of a finite Cr(VI) reduction capability that is possibly due to Cr(VI) toxicity to cells (Evans, 1997; Shakoori, 2000; Guha, 2001).

of cells inoculum resulted in the enhancement of reduction rate.

Table 2 Effect of amount of cells inoculum on Cr(VI) reduction

| Amount of inoculum, ml | Residual Cr(VI) concentration after 42 h, mg/L | Cr(VI) reduction rate, % |
|------------------------|--|--------------------------|
| 0.5 | 61.15 | 30.11 |
| 1 | 50.18 | 42.65 |
| 5 | 32.08 | 63.34 |
| 10 | 30.52 | 65.12 |

2.5 Mechanism of Cr(VI) reduction

The reduction experiments were carried out with cells of *P. aeruginosa* subjected to different treatments along with the controls (no cells). The Cr(VI) reduction rate is presented in Fig. 4. It is obvious that the normal cells subjected to no treatments reduced the greatest amount of Cr(VI) with the reduction rate of 60.24% being achieved, whereas cells destroyed by heat only accounted for 12.47% reduction. This may be due to the complexation of Cr(VI) with the denatured organic matter of biomass. No measurable reduction was detected (< 5%) with controls, which confirmed again the ability of *P. aeruginosa* to reduce Cr(VI). Cells treated with DNP exhibited Cr(VI) reduction with a decreased efficiency of 30.48% in comparison with the free cell. The reason may be that DNP is a decoupler of oxidative phosphorylation and can reduce or even stop the reaction which produces ATP. Thus, the normal activity of microorganisms is disturbed (Zhou, 2000). This indicated that the normal physiological activity of bacteria is necessary for effective microbial reduction of Cr(VI).

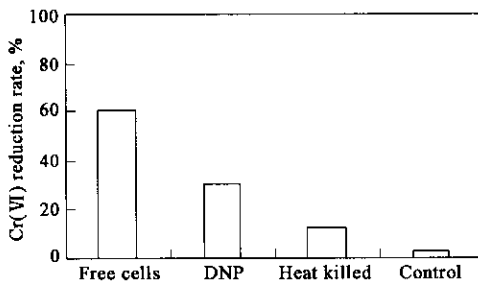


Fig. 4 Effect of different treatments to *Pseudomonas aeruginosa* on Cr(VI) reduction

Enzymes capable of Cr(VI) reduction are often referred to in the literature as "chromate reductases". These enzymes have been purified to various degrees from many cells such as *Pseudomonas putida* MK1, *P. putida* PRS2000, *P. ambigua* G-1, *Enterobacter Cloacae* HO1, and *S. oneidensis* MR-1. The purified "chromate reductases" of the *Pseudomonas* strains have been shown to be soluble proteins whereas those of *S. oneidensis* and *E. cloacae* have been found to be membrane associated (Sridhar, 2002). Experiments were conducted with cell free extracts of *P. aeruginosa* to understand the components responsible for Cr(VI) reduction and malate was used as electron donor. As shown in Fig. 5, Cr(VI) reduction occurred in the case of soluble fraction, and that contributed by membrane fraction was negligible. The results suggest that soluble enzymes are responsible for Cr(VI) reduction by *P. aeruginosa*.

The mechanisms of resistance to chromate involve efflux of chromate by *P. aeruginosa* cells expressing the ChrA protein, which contains 416 amino acid residues, and the genes for mechanisms have been found on plasmids (Nies, 1998; Cervantes, 1990; Spain, 2003). The researchers have studied the cloning of *chr* gene which is induced when

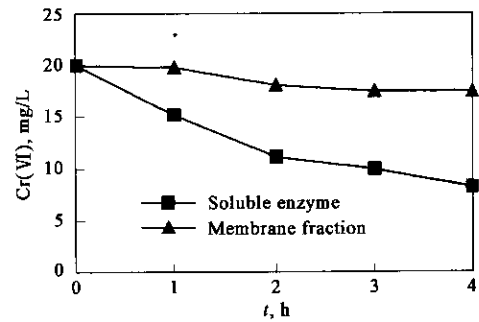


Fig. 5 Cr(VI) reduction efficiency by different components of *Pseudomonas aeruginosa*

chromate accumulates and becomes toxic, nucleotide sequence, and expression of chromate resistance determinant of *P. aeruginosa* plasmid. Pimentel *et al.* have cloned *chr* gene of *P. aeruginosa* plasmid pUM505 into another plasmid and found that cells harboring recombinant plasmid pEPL1, which expresses the ChrA protein, showed accelerated efflux of chromate as compared to the plasmidless chromate-sensitive derivative (Pimentel, 2002). Alvarez *et al.* also have got the findings that everted membrane vesicles of *P. aeruginosa* PA01 harboring plasmid pCR0616, expressing the ChrA chromate resistance protein, accumulated four times more chromate than vesicles from plasmidless cells (Alvarez, 1999). But Cr(VI) reduction and Cr(VI) resistance have been considered to be unrelated (Pattanapitpaisal, 2001). These analysis discussed above combined with our results indicate that *P. aeruginosa* has the ability to reduce Cr(VI) coupled with the resistance to chromate.

2.6 Analysis of final products

The reduced Cr(III) by bacteria can be precipitated out at neutral pH and eventually be removed from the medium in the form of chromium hydroxide (Yamamoto, 1993; Campos, 1995). This study analyzed the ending products at neutral pH. We did not measure Cr(OH)₃ precipitate from spectroscopic studies, but from mass balance calculations (initial Cr(VI) versus total chromium balance in Fig. 6), we found that the mass of total Cr(Cr(III) + Cr(VI)) was equal to the mass of Cr(VI) added in the flasks. Thus we deduced that, after Cr(VI) was transformed Cr(III) (initial Cr(VI) versus final reduced Cr(III) in Fig. 6), the reduced Cr(III) remained in the liquid phase, i. e. there was no precipitate. Some researches have thought that this may be due to the high organic content of the medium allowing for Cr(III) complexation, and thus keeping Cr(III) in the solution and preventing any precipitation (Turick, 1996; Guha, 2001; Amanda, 2002). Therefore, further work is expected to determine the effect of organic matters or other factors on Cr(OH)₃ precipitate.

3 Conclusions

Pseudomonas aeruginosa was capable of reducing Cr

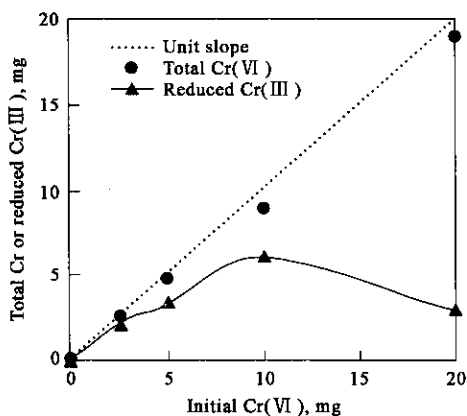


Fig. 6 Relationship between initial Cr(VI) and total Cr or reduced Cr(III)

(VI) by utilizing three different carbon sources as electron donors. Malate was the best one among the three carbon sources studied and the rate of Cr(VI) reduction increased with the increase of malate concentration but which was finite.

The optimum pH for Cr(VI) reduction was 7.0. Cr(VI) reduction rate decreased with the increase of initial Cr(VI) concentration, although Cr(VI) reduction was observed at four different initial concentration. The increase of amount of cells inoculum resulted in the enhancement of Cr(VI) reduction rate.

Requirement of viability of cells and normal physiological activity of bacteria was important for achieving high reduction rate of Cr(VI). The soluble enzymes were responsible for Cr(VI) reduction by *P. aeruginosa*, and the contribution of cell membrane in the process of Cr(VI) reduction was negligible.

The reduced Cr(III) was not precipitated out in form of Cr(OH)₃ under the present experimental conditions. The reason is not very clear. Therefore, further study should be conducted in the future to determine the factors which have effect on Cr(OH)₃ precipitate.

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