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Enhancing effect of iron on chromate reduction by *Cellulomonas flavigena*

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Abstract

Cr(VI) is considerably toxic and the detoxification of Cr(VI) is of great importance. This study investigated the effect of iron on Cr(VI) reduction by *Cellulomonas flavigena*. The results demonstrated that addition of $FeCl_3$ or lepidocrocite promoted Cr(VI) reduction, with the reduction ratio of above 90 and 80% achieved, respectively, but addition of hematite did not lead to the increase of reduction ratio, which suggests that the effect of iron on chromate reduction appears different with the diversity of iron-oxides. In this study, the effect of initial Cr(VI) and Fe(III) concentration on Cr(VI) reduction and the change of pH value were also investigated. The reduction ratio increased with the increase of the initial concentration ratio of Fe(III)/Cr(VI). The value of pH gradually increased from 7.0 to around 9.0.

Keywords: Reduction; *Cellulomonas flavigena*; Hexavalent chromium; Reduction ratio; Ferric iron

1. Introduction

Chromium has been recognized as one of the most serious pollutants among heavy metals in environment, thus remediation of chromate pollution receives much more concern. It is well known that chromium exists mainly as two stable oxidation states, Cr(VI) and Cr(III), which have widely contrasting toxicity and transport characteristics. Cr(VI) is quite toxic and mobile, and can be easily absorbed by living organisms. In contrast, Cr(III) is relatively less toxic, and has a limited hydroxide solubility and forms strong complexes with soil minerals resulting in relatively immobile and less available for biological uptake. Consequently, Cr(VI) poses greater threat to public health, environment and ecosystem, compared with Cr(III) [1], and how to effectively reduce Cr(VI) to Cr(III) is the crucial problem in the remediation of chromate pollution. Therefore, understanding processes that promote the reduction of Cr(VI) to Cr(III) with the subsequent detoxification and immobilization is of considerable importance.

Chromium(VI) can be reduced by biological and chemical means. Bioremediation is currently regarded as an alternative strategy supported by the discovery that a great deal of microorganisms with the ability of chromate resistance and chromate reduction have been identified and screened out from contaminated and non-contaminated environment [2–4]. However, microbial reduction of Cr(VI) to Cr(III) is relatively slow and the reduction efficiency is not absolutely satisfactory [5]. The investigation on finding useful measures to enhance the chromate reduction is significant. On the other hand, the reduction of Cr(VI) can be achieved by chemical reagents. Among the possible chemical reductants in natural environments, ferrous iron and dissolved sulfide are the predominant reducing agent controlling the reduction of hexavalent chromium [6]. Nevertheless, production of Fe(II) and S(−II) mainly depends on microbial activity because the reduction of ferric iron and sulfate takes place primarily via microbial dissimilatory reduction, and many bacteria are found to be able to couple the oxi-

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dation of organic compounds or H_2 to the reduction of iron-oxides and sulfates [7–9]. Published reports have also implied that dissimilatory iron-reduction is very important for mediating numerous biogeochemical processes [10–13]. The pathway of chromate reduction by dissimilatory iron-reducing bacteria can be expressed by the following reactions [14]:

$$\tfrac{3}{4}C_3H_5O_3^- + 3Fe(OH)_3 \rightarrow \tfrac{3}{4}C_2H_3O_2^- + 3Fe^{2+} + \tfrac{3}{4}HCO_3^- + 2H_2O + 5\tfrac{1}{4}OH^- \quad (1)$$

$$3Fe^{2+} + HCrO_4^- + 8H_2O \rightarrow 3Fe(OH)_3 + Cr(OH)_3 + 5H^+ \quad (2)$$

From reactions (1) and (2), we can draw some significant implications. Iron is cyclic between the bacteria and chromium. Fe(II) generated by dissimilatory reduction of Fe(III) in reaction (1) is oxidized back to Fe(III) by Cr(VI) in reaction (2), thereby acting as an electron shuttle (a catalytic role) between the bacteria and chromium. This reaction scheme suggests a continual regeneration of the primary terminal electron acceptor. Thus, by cycling minor amounts of iron present in the environment a significant amount of Cr(VI) could be potentially reduced even in systems having limited available Fe. Based on these implications, this study was conducted to evaluate the role of microbial dissimilatory iron-reduction in the chromate reduction, while providing the fundamental data for the promotion of the remediation of chromium pollution.

Cellulomonas flavigen was used in this study. This kind of bacteria is able to reduce dissimilatorily Cr(VI), Fe(III) and U(VI) into Cr(III), Fe(II) and U(IV) [15], thus it is suitable to microbially reduce Fe(III), followed by Cr(VI) reduction by the reduced Fe(II). Some other species are also effective for reduction of Cr(VI) and Fe(III), but we cannot obtain them. Additionally, there are few studies about Cr(VI) reduction, especially about the effect of iron on Cr(VI) reduction by *C. flavigen*. This study may provide some useful evidence for the application of this kind of bacteria into the Cr(VI) reduction.

2. Materials and methods

2.1. Media and cultivation

The dissimilatory iron-reducing bacteria, *C. flavigena* (CCTCC AB 90023), was purchased from China Center for Type Culture Collection. Bacterial strain was first grown on agar slants containing NA media, which consisted of beef extract 3 g, peptone 5 g, agar 20 g in 1 l of distilled water, and the pH was adjusted to 6.8 ± 0.2. After the incubation of cultures at 37 °C for 24 h in slants, pure cultures was then grown in 250 ml serum bottles containing 100 ml nutrient broth under anaerobic conditions by transferring one loop of cultures from the slants to the serum bottles. The anaerobic conditions were monitored according to previous reports [15]. The nutrient broth contained (per liter distilled water): yeast extract 5 g, malate 1 g, NH_4Cl 0.03 g, K_2HPO_4 0.03 g, KH_2PO_4 0.05 g, NaCl 0.01 g, $MgSO_4{\cdot}7H_2O$ 0.01 g, and the pH of the medium was adjusted to 7.0 ± 0.1 [16]. The serum bottles was then incubated at 37 °C by shaking at 150 rpm for 24 h in an constant temperature shaker incubator (SKL-3F).

2.2. Preparation of cells and chromate reduction experiments

Cells grown for 24 h in nutrient broth, were harvested by centrifugation in the presence of O_2-free N_2:CO_2 (80:20) at 5000 rpm for 15 min (LD5-10). The supernatant was discarded and the cell pellets were washed three times and suspended in phosphate buffer (1/15 mol/l KH_2PO_4, 1/15 mol/l Na_2HPO_4, pH 7.0) before used in chromate reduction experiments.

All chromate reduction experiments were carried out in 250 ml serum bottles containing 100 ml nutrient broth medium. The serum bottles were inoculated with cells, and Cr(VI) was then added from the stock solution of K_2CrO_4. Direct reduction of Cr(VI) by *C. flavigena* was tested without any Fe(III) added, and direct reduction of Fe(III) by *C. flavigena* was also tested with no Cr(VI) added. In addition, another series of experiments were done with Fe(III) added from the stock solution of $FeCl_3$, so as to test the effect of microbial dissimilatory iron-reduction on chromate reduction. In reduction experiments, anoxic conditions were guaranteed according to previous description [15].

All of media and stock solutions were autoclaved at 121 °C for 20 min before used in chromate reduction experiments. The experiments were performed in replicate and the mean values were taken into account.

2.3. Analytical methods

In order to analyze the change of Cr(VI) and Fe(II) concentration, samples were drawn from serum bottles at intervals of 6 h and then centrifuged at 13,000 rpm for 15 min in sorvall biofuge fresco. The concentration of Cr(VI) was measured spectrophotometrically at 540 nm by the diphenyl carbazide method [17]. Production of Fe(II) was determined spectrophotometrically at 510 nm by 1,10-phenanthroline method [17]. Cr(VI) reduction ratio was calculated according to the following equation:

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

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

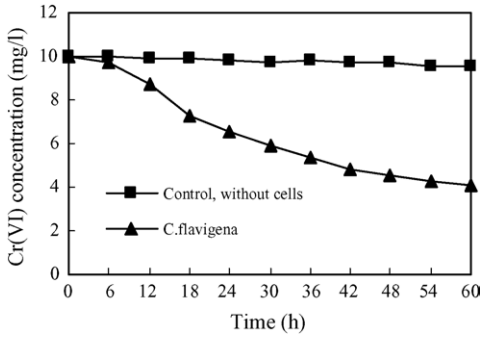

Fig. 1. Direct Cr(VI) reduction by *C. flavigena*.

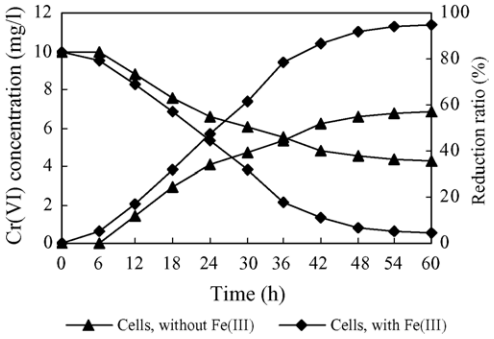

Fig. 3. Effect of Fe(III) on Cr(VI) reduction.

3. Results and discussion

3.1. Direct microbial reduction of Cr(VI) and Fe(III)

Microorganisms can reduce Cr(VI) and Fe(III), utilizing organic compounds as electron donors. In this study, malate was applied as electron donor for microbial reduction of Cr(VI) and Fe(III). Fig. 1 shows the change of Cr(VI) concentration in reaction bottles. Cr(VI) concentration gradually decreased from initial concentration of 10 mg/l to final concentration of 4.13 mg/l. Fig. 2 indicates that the concentration of Fe(II) increased, suggesting the dissimilatory reduction of Fe(III) by *C. flavigena*. It is clear from the picture that no obvious reduction of Cr(VI) or Fe(III) occurred in the controls (no cells).

3.2. Effect of Fe(III) on Cr(VI) reduction

$FeCl_3$ was added to the reaction bottles to investigate the effect of Fe(III) on Cr(VI) reduction. The kinetics and the rate of Cr(VI) reduction are presented in Fig. 3. More amounts of Cr(VI) were reduced when Fe(III) was added. Cr(VI) concentration dropped from initial concentration of 10 mg/l to final concentration of below 1 mg/l, and the reduction ratio above 90% was obtained. Fig. 3 also shows that 78.3% of Cr(VI) was reduced at time of 36 h. In contrast, Cr(VI) concentration just decreased to 4.31 mg/l, with the reduction ratio of 56.9% being achieved in Fe(III)-free reaction bottles, and only 44.7% of Cr(VI) was converted into Cr(III) at time of 36 h. These results imply that addition of Fe(III) stimulated the reduction of Cr(VI), coupling the elevation of reduction ratio to the acceleration of reduction process.

This may be due to the toxicity of hexavalent chromium to the cells for the relatively slower direct reduction and lower reduction ratio in the system without Fe(III). When $FeCl_3$ was added, iron played a significant role in chromium reduction as described above. Fe(III) was reduced to Fe(II) through microbial respiratory activity, and the reduced Fe(II) then served as the reductant for chromium reduction, thus alleviating the toxicity of Cr(VI) to the cells. This may be the reason that a more ideal chromium reduction was obtained in the presence of Fe(III). The results are consistent with the conclusions that Wielinga et al. have drawn from Cr(VI) reduction by *Shewanella* alga. Wielinga et al. reported that no viable cells were recovered at high Cr(VI) concentration, while viable cells of 5×10^7 CFU/ml were observed in iron amended system. This indicated that iron provided an indirect resistance mechanism for microbes against the toxicity of hexavalent chromium [14]. Meanwhile, iron was cycled between microbes and chromium, suggesting that great amounts of chromate could be reduced even there are not so many irons available. The results suggest that the ability of iron to be cycled and act as a catalyst for Cr(VI) reduction should be considered when accessing the capacity of soils or waters to effectively reduce Cr(VI) to Cr(III). The reduction pathway also indicates the probability of simultaneous removal of chromate and organic contaminants under microbial activity.

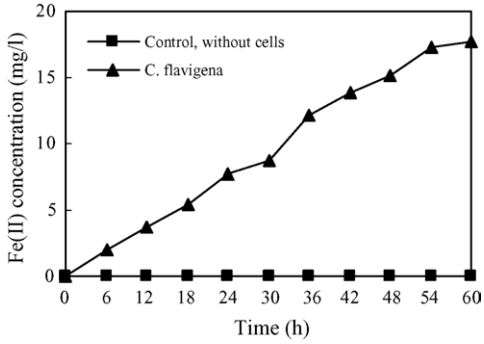

Fig. 2. Direct Fe(III) reduction by *C. flavigena*.

3.3. Effect of Fe(III) speciation on Cr(VI) reduction

In above experiments, Fe(III) was added in the form of $FeCl_3$, and it is soluble in the medium. But, in natural environments, especially in sediments, majority of Fe(III) is present as insoluble iron-oxides [7]. The experiments about studying the effect of Fe(III) in different speciation on Cr(VI) reduction was carried out in the presence of three different states of Fe(III), namely $FeCl_3$, lepidocrocite, hematite. The Cr(VI) reduction and the change of Fe(II) concentration are presented in Figs. 4 and 5, respectively. As shown in Fig. 4, the system amended $FeCl_3$ acquired the best results of Cr(VI)

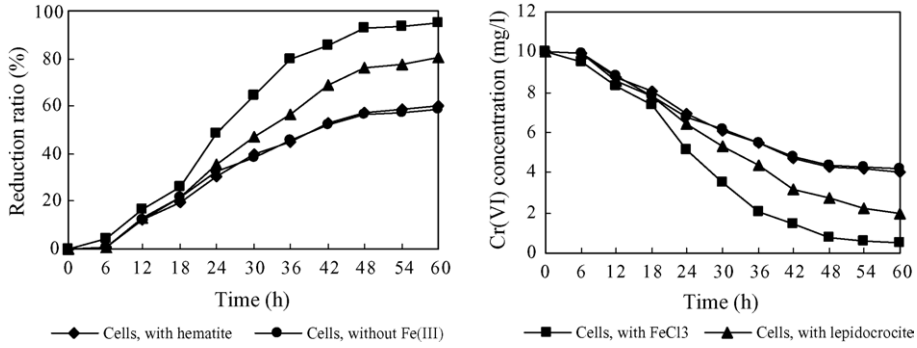

Fig. 4. Cr(VI) reduction with different speciation of Fe(III) added.

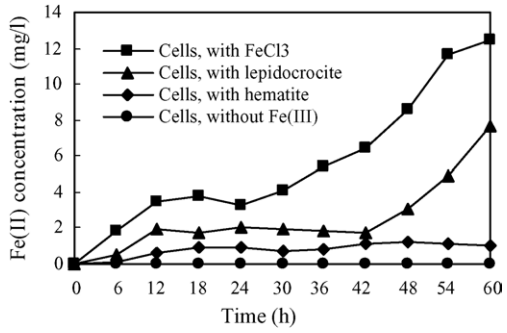

Fig. 5. Change of Fe(II) concentration with different speciation of Fe(III) added.

reduction, with the reduction ratio of 94.9% being obtained, and the reduction of Cr(VI) in this system was faster than the other two. Compared with the controls (no Fe(III)), whose reduction ratio was only 58.4%, the system amended lepidocrocite had a higher reduction ratio of 80.3%, indicating that the addition of lepidocrocite also had enhancing effect on Cr(VI) reduction, but that was not as good as $FeCl_3$. As for hematite, the reduction ratio was only 59.8%, which was close to the reduction ratio of the control.

The ability of microbes to reduce iron-oxides differs with the diversity of iron-oxides, and the crystallization and surface area of iron-oxides are the main factors affecting the microbial reduction of iron-oxides [7,18]. Fig. 5 shows that Fe(II) concentration increased in the system amended by $FeCl_3$ or lepidocrocite, whereas in system amended by hematite a small quantity of Fe(II) was detected, providing the evidence that $FeCl_3$ and lepidocrocite are more favorable than hematite for *C. flavigena*. This maybe lies in the difference of crystallization and surface area between lepidocrocite and hematite. Qu et al. also found that the strains, *Geobacter metallireducens* GS-15 reduced more amounts of lepidocrocite and faster than hematite [18]. In addition, because $FeCl_3$ is soluble in the medium, the cells have more opportunities to contact with Fe(III), and Fe(III) is more accessible for microbial reduction. Lovley et al. have reported that Fe(III) reducers can reduce soluble chelated Fe(III) much faster than insoluble Fe(III) oxides [19]. What has been discussed above can provide some clues for explaining why $FeCl_3$ is the best additive, followed by lepidocrocite, hematite. Additionally, from Fig. 5, we can see that in $FeCl_3$-amended system, the increase of Fe(II) concentration was not obvious in 12–36 h, but after 36 h, Fe(II) concentration increased gradually and more obviously. This is related to the Cr(VI) reduction. At 36 h, most of Cr(VI) was reduced and iron was cycled, whereas later, because the rest of Cr(VI) to be reduced was becoming less, Fe(II) could be accumulated in the medium.

3.4. Effect of initial Cr(VI) and Fe(III) concentration on Cr(VI) reduction

Previous studies have indicated that initial Cr(VI) concentration has effect on Cr(VI) reduction ratio [20–22]. This study investigated the effect of both of initial Cr(VI) and Fe(III) ($FeCl_3$) concentration on Cr(VI) reduction at three levels of initial concentration. The experimental design and results are listed in Table 1.

Table 1
Effect of initial Cr(VI) and Fe(III) concentration on Cr(VI) reduction

Initial Cr(VI) content (mg/l)	Initial Fe(III) content (mg/l)	Final Cr(VI) content (mg/l)	Final Fe(II) content (mg/l)	Cr(VI) reduction ratio (%)
5	5	0.87	3.41	82.60
5	10	0.45	6.23	91.00
5	30	0.26	12.64	94.80
10	5	1.86	3.15	81.40
10	10	1.02	8.62	89.80
10	30	0.57	11.59	94.3
30	5	8.39	2.97	72.03
30	10	6.02	7.65	79.93
30	30	3.78	12.72	87.4

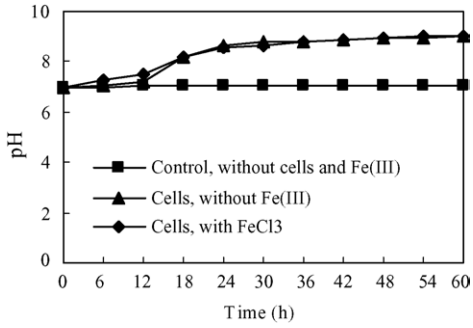

Fig. 6. Change of pH.

At the same initial Fe(III) concentration, the reduction ratio decreased with the increase of initial Cr(VI) concentration. This may be due to the toxicity of Cr(VI) to the cells. At the same initial Cr(VI) concentration, the reduction ratio increased with the increase of initial Fe(III) concentration. When the initial Fe(III) concentration was 30 mg/l, and initial Cr(VI) concentration was 5 or 10 mg/l, with the initial concentration ratio of Fe(III)/Cr(VI) being 6 and 3, the reduction ratio reaches 94.8 and 94.3%, respectively. However, when the initial Fe(III) concentration was 5 mg/l, and the initial Cr(VI) concentration was 5, 10 or 30 mg/l, with the initial concentration ratio of Fe(III)/Cr(VI) being 1, 1/2 and 1/6, the reduction ratio reaches only 82.6, 81.4 and 72.03%, respectively. As a consequence, it can be concluded that the higher initial concentration ratio of Fe(III)/Cr(VI) resulted in higher reduction ratio. This is probably because that there were more amounts of Fe(III) to be used by microbes when the initial Fe(III) concentration increased, thus helping the Cr(VI) reduction.

3.5. Change of pH

The pH value is an important index reflecting the microbial activity. Investigating the change of pH is helpful to understand the reduction mechanisms. The change of pH in reaction bottles is demonstrated in Fig. 6. All of the pH values increased from 7.0 to about 9.0 at the end of the experiments, except for the controls (no cells). The pH value in reaction bottles inoculated with cells increased much more in 0–36 h, followed by slow and slight increase. This seems to be corresponding to the process of Cr(VI) reduction (Fig. 3, most of Cr(VI) was reduced in 0–36 h). So the change of pH is correlative to the reduction activity. Chen et al. found that adding Cr(VI) to soil resulted in the increase of pH in soil, and explained that by the reaction:

$$Cr_2O_7^{2-} + 14H^+ + 6e \rightarrow 2Cr^{3+} + 7H_2O \quad (3)$$

The consumption of H^+ by Cr(VI) reduction diminished the amount of H^+, thus leading to the rise of pH. Chen et al. also found that adding Cr(III) to soil resulted in the decrease of pH in soil, and explained that by the reactions:

$$[Cr(H_2O)_6]^{3+} + H_2O \rightarrow [Cr(OH)(H_2O)_5]^{2+} + H_3O^+ \quad (4)$$

$$[Cr(H_2O)_6]^{3+} + 3OH^- \rightarrow Cr(OH)_3\downarrow + 6H_2O \quad (5)$$

Hydrolyzation of Cr(III) in reaction (4) releases some H^+, and precipitation of $Cr(OH)_3$ in reaction (5) consumes some OH^-, thus leading to the decrease of pH [1]. All of these reactions, plus reaction (1) and (2), probably have contribution to the change of pH in this study. Furthermore, maybe, reaction (1) and (3) are more active than reaction (2), (4) and (5), finally resulting in the increase of pH in this study. Also, the microbial metabolic products and other reactions may contribute to the increase of pH, so further research work is necessary to find out the truth.

4. Conclusions

Adding Fe(III) could catalyze the Cr(VI) reduction via a two-step, closely coupled, biotic-abiotic reaction pathway by the dissimilatory bacteria, *C. flavigena*. The enhancing effect of Fe(III) appeared differently with the diversity of Fe(III) speciation. Among the three iron compounds employed, soluble $FeCl_3$ gave the greatest promotion to the Cr(VI) reduction, followed by insoluble iron-oxides, lepidocrocite. But, addition of another insoluble iron-oxides, hematite appeared to make no contribution to the Cr(VI) reduction. In addition, the initial Cr(VI) and Fe(III) concentration had effect on Cr(VI) reduction ratio. The reduction ratio increased with the increase of initial concentration ratio of Fe(III)/Cr(VI). Finally, the value of pH in the reaction bottles rose up from 7.0 to about 9.0. The reason for that is not very clear, therefore, more experiments are required in order to further understand the process and mechanisms of Cr(VI) and Fe(III) reduction.

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