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Stabilisation/Solidification Treatment and Remediation

Advances in S/S for Waste and Contaminated Land

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Stabilization of chromium by reductase enzyme treatment

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ABSTRACT: Hexavalent chromium (Cr(VI)) is highly toxic, and a major heavy metal contaminant in the environment. An important strategy for bioremediating Cr(VI) is to microbiologically reduce it to less toxic Cr(III). One of the major routes of bacterial chromate reduction is enzymatic reduction mediated by chromate reductase after its uptake inside the cell. While the enzymatic pathway is common in aerobic bacteria, it probably also occurs anaerobically. To gain more insight about this pathway, we investigated the reduction of Cr(VI) by a highly resistant Bacillus species. Reduction depended on which of three organic substrates was used, i.e., glucose, lactate and acetate at 0.1 to 2.0 mM, and the rate of reduction decreased with increasing concentration of chromate. Because sulfhydryl sites are known to be active sites for enzyme reductase, bacterial growth and reduction of Cr(VI) by Bacillus in the presence of varying concentrations of sulfate and thiosulfate were investigated. While changes in sulfate did not affect the reduction rate, raising the thiosulfate concentration in the medium from 0.05 to 1.0 mM markedly increased the reduction rate. Thiosulfate enhanced the reduction of Cr(VI), probably by accelerating the biosynthesis of chromate reductase enzymes, although other mechanisms may be involved. Our results show that the enzymes or other substances mediating the reduction reside mostly in the cytoplasm. This reductase enzyme could be extracted and applied sites contaminated with Cr(VI), to convert it to Cr(III), which would prevent leaching of the pollutant to groundwater.

1 INTRODUCTION

Chromium is one of the major heavy-metal contaminants in the environment. Many industries release chromium during their operations, including the metal-finishing industry, petroleum refining, leather tanning, iron- and steel-production, inorganic-chemical production, textile manufacturing, and pulp-producing processes. In general, the toxicity of heavy-metal contaminants may be related to their chemical speciation. For chromium, the oxidized hexavalent species present in complex anions, such as chromate (CrO$_4^{2-}$), dichromate (Cr$_2$O$_7^{2-}$), and dichromate (Cr$_2$O$_7^{2-}$), is highly toxic, and is likely to be a carcinogen and a mutagen (Connor & Wetterhahn, 1983). However, reduced Cr(III), which readily forms insoluble oxides and hydroxides, [Cr(OH)$_3$], is regarded as less toxic or nontoxic to organisms (Tindon et al. 1978, Rai et al. 1987).

Consequently, the environmental toxicity of chromium arises mainly from the presence of Cr(VI). Because the Cr(III) species is not poisonous to humans, the reduction of Cr(VI) to Cr(III) may constitute a potentially valuable mechanism for remediating Cr(VI) toxicity.

Both abiotic and biotic processes can reduce environmental Cr(VI) to Cr(III) (Smillie et al. 1981, Wang & Xiao 1995, Lovley & Phillips 1994). The redox potential (Eh) of the immediate environment, its pH, and the presence of reductive molecules are some important factors controlling the rate of abiotic reduction. Reactive sulfur species, such as hydrogen sulfide and thios, probably play a crucial role in reducing Cr(VI) under reducing conditions (Smilie et al. 1981).

Bacterial reduction is a significant pathway for reducing Cr(VI) to Cr(III), both anaerobically and aerobically. Since the first report in the 1970s of the isolation of chromium-reducing Pseudomonas strains from chromate-contaminated sewage sludge (Romanenko & Koren’kov 1977), several more chromate-reducing bacteria have been identified. They include additional strains of Pseudomonas, as well as strains of Micrococcus, Escherichia, Enterobacter, Bacillus, Aeromonas, and Achromobacter species (Kavasnikov et al. 1985, Gvozdyak et al. 1986, Horitsu et al. 1987, Bopp & Ehrlch 1988, Wang et al. 1989, Ihebashi et al. 1990). The mechanisms by which these microorganisms reduce Cr(VI) vary and are species-dependent. Essentially, there are three major routes of reduction. The first two are anaerobic only; the third occurs mostly under aerobic conditions, but also can occur under anaerobic conditions.

In one anaerobic pathway, bacteria use Cr(VI) as a terminal electron acceptor in their respiratory chains, with organic substrates as the reductants; the list encompasses many facultative anaerobes, including...
in the presence of blast furnace slag, Cr(VI) was reduced to Cr(III), which can precipitate a stable and insoluble form of Cr(OH)₃. According to Allan & KuKacka (1995), blast furnace slag seems to be able to exert a solubility control on chromium. Otorosono et al. (1995) studied the S/S mechanism involving chromium and tricalcium silicate. Dormatas & Meng (2003) effectively immobilised Cr(III) by quicklime-fly ash treatment. So far no biological method for Cr/S has been reported.

Enzyme technology is an established technique, and enzymes produced by microorganisms could be used for this purpose without introducing microorganisms into the environment. The latter is a particular concern for genetically modified microorganisms.

2 MATERIALS AND METHODS

2.1 Media

A mineral salt medium (MSM) was formulated (modified from Guha et al. 2001) and optimized for use under the conditions of the Cr(VI) reduction experiments. The MSM had the following composition: 6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 0.1 mL of 1 M MgCl₂, 1 mL of 1 M Na₂SO₄, 0.5 g Tryptone, 1 L distilled water, adjusted to pH 7.

2.2 Microorganism

Bacillus sp. ATCC 700729 was used in the experiment. The cells to be used as inocula were routinely sub-cultured in shake flasks containing MSM with glucose as the energy source.

2.3 Reduction experiments with different substrates

The aerobic reduction experiments were carried out in 1 L Erlenmeyer flasks containing 500 mL MSM. Cell suspensions (ca. 7 X 10⁵ cells/mL) were added to these flasks along with 1% glucose, 1% sodium citrate or 1% sodium lactate, and various concentrations of chromium (0.1, 0.2, 0.5, 0.75, 1.0, and 2.0 mM). Cultures were incubated at 37°C without shaking. Samples were taken for Cr(VI) analysis and for estimating bacterial growth at various times afterwards. Culture aliquots were plated on nutrient agar (Sigma) without Cr(VI) to assess cell viability. For each treatment, cell-free control flasks were prepared to monitor whether abiotic chromate reduction occurred. All experiments, including the abiotic controls were conducted in duplicate.

Reduction of Cr(VI) in the presence of different concentrations of sulfate and thiosulfate was investigated by adding 0.05 or 1.0 mM of the sulfur compounds to the culture. Because thiosulfate forms a precipitate with
Cr(VI) during autoclaving, we first filter-sterilized the thiosulfate and then added it to the medium and the Cr(VI) that had been routinely autoclaved.

2.4 Bacterial growth

Bacteria were counted by the Pour plate technique. The samples were serially diluted and plated on nutrient agar and incubated at 37°C for 24 h. The number of bacteria growing on the agar surface was counted and expressed as colony Forming Units (CFU) per mL.

2.5 Cr(VI) estimation

Chromate [Cr(VI)] in the liquid fraction of each treatment was analyzed at various times by aseptically removing an aliquot of the liquid followed by centrifugation at 13,000 Xg for 5 minutes in 2.0 mL microcentrifuge tubes. Cr(VI) in the supernatant was then measured colorimetrically at 540 nm using the diphenyl carbazide method (APHA 1989).

2.6 Cell fractionation

Cells were grown in MSM at 37°C and harvested at mid-exponential phase by centrifugation at 4000 Xg. They were washed three times by centrifugation in HEPES buffer (pH 7), resuspended in 10 mL of the same buffer, and kept in an ice bath. Cells were mechanically ruptured using an ultrasonicator (Cole-Parmer, model 8845-5). The suspension then was centrifuged at 12,000 Xg to pelletize the unbroken cells. The supernatant was spun at 150,000 Xg for 2 h at 4°C. This high speed supernatant (S15o) was retained as the cytoplasmic fraction, while the pellet obtained from this step was resuspended in 10 mL of buffer and used as the membrane fraction. The Cr(VI) concentration in the cell membrane and cytoplasmic fractions were adjusted to 0.1 and 0.2 mM, and Cr(VI) reduction was estimated at 0 h and 12 h after adding Cr(VI).

3 RESULTS AND DISCUSSION

3.1 Growth and chromium reduction with different organic substrates

Figure 1 gives the time-series measurements for the growth of the bacterium with different organic substrates in the presence of different concentrations of Cr(VI).

At concentrations of 0.1 and 0.2 mM, Cr(VI) was completely reduced after 96 and 168 h respectively.

The kinetics of growth and Cr(VI) reduction in the lactate medium were similar to those in the glucose medium with an increase of the population from $7 \times 10^3$ CFU/mL to $2.8 \times 10^5$ in the first 24 h period with no added Cr(VI). The rates of Cr(VI) reduction varied with the initial level of added Cr(VI). At 0.1 mM initial Cr(VI), complete reduction was achieved within 72 h, whereas it took 168 h to completely reduce 0.2 mM Cr(VI). With acetate as the organic electron acceptor, the bacterial count also increased to a maximum of $3.9 \times 10^5$ during the first 24 h incubation period with no Cr(VI) present.

However, when Cr(VI) was added, Cr reduction was noticeably lower than when the microbe was cultured on glucose and lactate. We calculated approximate relative rates for Cr(VI) reduction at different intervals using a polynomial fit for the curves for time vs percent reduction of Cr(VI) (Table 1).

As shown in Figure 1 and this table, the maximum efficiency of reduction was observed in glucose followed by lactate and then acetate. Schmieman et al. (1997) observed the growth of a mixed bacterial culture in the presence of Cr(VI) with acetate as the substrate, but recorded no reduction of Cr(VI). By contrast, the species of Bacillus we used tolerated Cr(VI) and grew on a substrate of acetate but the reduction rate was less compared to that of other substrates.

Overall, the extent of Cr(VI) reduction correlated directly with bacterial growth. Furthermore, the rate of reduction of Cr(VI) generally decreased with time, regardless of the initial cell concentration. The decrease in the concentration of the utilisable organic substrate probably was an important reason for the decline in chromium reduction. In fact, respiaking the same organic substrates (1% glucose, 1% lactate or 1% acetate) at 168 h during stationary phase increased the rate of growth in bacterial cultures containing 0.5 mM Cr(VI) (Figure 2).

As Figure 1 shows, we investigated the effect of initial Cr(VI) concentration on its rate of reduction over a Cr(VI) concentration range of 0.1 to 2.0 mM. Although the reduction of Cr(VI) by Bacillus sp. occurred even under the highest Cr(VI) concentration, it was not complete when the initial concentration was higher than 0.2 mM. The highest rate of Cr(VI) reduction was observed with initial concentrations between 0.1 to 0.2 mM with 1% glucose or 1% lactate as the substrate; at these levels, Cr(VI) was completely reduced within 100 h (Figure 1).

Raising the concentration of Cr(VI) in the medium from 200 µM to 2.0 mM lowered the microbes' capacity for reduction and also lengthened the time taken for reduction to occur. Others also observed a similar trend with Escherichia coli (Shen & Wang 1994), A. radiobacter (Llovera et al. 1993), and E. cloacae...
(Wang et al. 1989). The chromate ions must be transported by an active ion-pump mechanism inside the cell for the reduction to occur. This transport mechanism probably depends on some active membrane carriers which may become saturated or affected at higher concentrations of Cr(VI) affecting its transport. However, there may be other rate-limiting steps in the reductive process.

3.2 Reduction of chromium (VI) by Bacillus strain in the presence of different concentrations of sulfate and thiosulfate

We examined the growth and Cr(VI) reduction by Bacillus in the presence of two major sulfur species, sulfate and thiosulfate. The experiments were conducted at two different chromate values, 0.5 and 1.0 mM. Figure 3 compares bacterial growth and Cr(VI)

![Graphs showing bacterial growth and Cr(VI) reduction](image)

Figure 1. Growth of Bacillus sp. with different organic substrates in the presence of different concentrations of Cr(VI), A - Growth with glucose, B - Cr(VI) reduction with glucose, C - Growth with lactate, D - Cr(VI) reduction with lactate, E - Growth with acetate, F - Cr(VI) reduction with acetate.
reduction at two different sulfate concentrations of 0.05 mM and 1.0 mM in chromate concentration of 1.0 mM. Cr(VI) in the medium. As Figure 3 shows, varying the sulfate concentration in the media did not significantly affect the rate of reduction of Cr(VI). Similar results were obtained for 0.5 mM Cr(VI) in the medium. In contrast to sulfate, raising the thiosulfate concentration in the medium from 0.05 to 1.0 mM doubled the reduction rate under similar conditions (Figure 3). The bacteria reduced Cr(VI) more with thiosulfate present in the medium than with sulfate.

Overall, adding 1.0 mM thiosulfate greatly enhanced Cr(VI) reduction. Quiñones et al. (2001) reported that adding elemental sulfur enhanced Cr(VI) reduction by *Thiobacillus ferrooxidans*. They also demonstrated that the reducing compounds associated with colloidal sulfur reduce Cr(VI), even though their concentration in solution is low.

The reason for enhanced reduction of Cr(VI) in the presence of thiosulfate is not clear. In general, sulfur nucleophiles (e.g. thiols) are known to reduce Cr(VI) to Cr(III). Thus, the reduction of Cr(VI) can occur through a chemical reaction with thiosulfate.

<table>
<thead>
<tr>
<th>Concentration of Cr(VI) (mM)</th>
<th>Relative rate of reduction ± standard error (µM/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.85 ± 0.14 0.83 ± 0.20 0.75 ± 0.27</td>
</tr>
<tr>
<td>0.2</td>
<td>1.48 ± 0.46 1.38 ± 0.54 1.14 ± 0.13</td>
</tr>
<tr>
<td>0.5</td>
<td>2.50 ± 0.13 2.15 ± 0.18 0.70 ± 0.16</td>
</tr>
<tr>
<td>0.75</td>
<td>2.70 ± 0.24 2.02 ± 0.31 0.82 ± 0.12</td>
</tr>
<tr>
<td>1.0</td>
<td>2.60 ± 0.31 0.90 ± 0.23 0.50 ± 0.12</td>
</tr>
<tr>
<td>2.0</td>
<td>2.20 ± 0.48 1.60 ± 0.46 0.20 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 2. Effect of respiro with organic substrates during the stationary phase (~168 h) of growth and Cr(VI) reduction in comparison with a control that was not respiro. A – Growth curves with glucose; B – Cr(VI) reduction with glucose; C – Growth curves with lactate; D – Cr(VI) reduction with lactate.
However, as Figure 4 shows, the reduction of Cr(VI) was relatively insignificant in abiotic controls when compared to bacterial cultures (Figure 3). This result strongly suggests that biochemical reduction was the primary mechanism for the reduction of Cr(VI) in the presence of thiosulfate.

A possible mechanism for enhanced Cr(VI) reduction in the presence of thiosulfate may involve the uptake of thiosulfate and Cr(VI) by the bacteria, followed by a biochemically mediated reduction of the Cr(VI) by thiosulfate or any other reduced sulfur species generated from it. However, in general, biochemical reductions occur through mediation with reductase enzymes, which are mainly distributed in the cytoplasm. Because thiol and thiolate sites are known to be active sites of several enzymes that mediate redox reactions (Miller et al. 1989, Rabuck et al. 1990, Ellis & Poole 1997), we suggest that these groups may also play a role in chromate reductases which convert Cr(VI) to Cr(III).

If this is the primary mechanism for Cr(VI) reduction in Bacillus, then our results may imply that when the bacteria grow in the presence of thiosulfate, the biosynthesis of chromate reductase enzymes is enhanced.

3.3 Site of biochemical reduction of Cr(VI)

We examined the cellular distribution of the reductases in the Bacillus sp. by monitoring the Cr(VI)-reducing...
activity of isolated cell membranes and the cytoplasmic fractions. Table 2 gives the relative efficiencies for Cr(VI) reduction by these different fractions at two different concentrations of Cr(VI). These results show that the enzymes mediating the reduction were mostly in the cytoplasm, although the cell membrane fractions show some activity.

Possibly, the membrane fraction was not completely separated from the soluble enzymes, rendering this effect. Enzymatic reduction of Cr(VI) has already been reported by many authors with different strains of microorganisms, such as *Bacillus* strain (Campos et al. 1995), *E. coli* (Shen & Wang 1994), *Pseudomonas maltophilia* (Blake et al. 1993), *Pseudomonas ambiguus* (Suzuki et al. 1992), and in a species of *Pseudomonas* (McLean & Beveridge 2001). Our results obtained with a cytoplasmic and cell membrane fractions demonstrate that Cr(VI) reduction in *Bacillus* sp. reside mostly in soluble species localized in the cytoplasm, which probably are soluble reductases.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>0.1 mM Cr(VI)</th>
<th>0.2 mM Cr(VI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reduction at 0.5 h</td>
<td>Reduction after 12 h</td>
</tr>
<tr>
<td>Membrane</td>
<td>6.4 ± 0.4*</td>
<td>10.3 ± 0.9</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>8.8 ± 0.9</td>
<td>16.9 ± 1.7</td>
</tr>
</tbody>
</table>

* Standard error

3.4 Complex mechanism for Cr(VI) reduction

The reduction of Cr(VI) mediated by reductase enzymes in aerobic bacteria such as *Bacillus* sp. seems to be a complex multistep process. Figure 5 summarizes a scheme of potential pathways for this reduction in the presence of thiosulfate.

It probably proceeds through a series of steps, of which the first should be an active uptake of the chromate ion by the bacteria. Uptake probably is concentration dependent and might be an important factor limiting the rate of reduction. Once Cr(VI) enters the cytoplasm, it may go through a chain of biochemical carriers, including the chromate reductase, to effect the reduction. Thiosulfate may enhance the reduction of Cr(VI) by accelerating the biosynthesis of chromate reductase enzymes, although there may be other mechanisms. The reduced species generated in the cytoplasm must be a soluble organic complex because the common Cr(III) species, Cr(III)-hydroxide, is insoluble and cannot be transported across the membrane. The Cr(III)-complex expelled into the extracellular medium then transforms to the insoluble hydroxide form.

Figure 5. Scheme of possible pathways for Cr(VI) reduction in the presence of thiosulfate.

4 CONCLUSIONS

This work has shown that reductase enzyme extracted from the cell free broth can potentially be used as a stabilisation agent. Reductase enzyme reduces the toxic, soluble Cr(VI) to non-toxic insoluble Cr(III). The application of reductase enzyme in chromium contaminated sites for solidification would be a sustainable solution for remediation. Further, reductase enzyme production by bacteria could be enhanced using genetic manipulation techniques and research is needed to investigate its compatibility with conventional S/S using cement.

ACKNOWLEDGEMENTS

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