Bioreduction of Selenium by Sulfate Reducing Bacterium and its Influence on Selenium Transport in Geological Environment

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Abstract: Selenium (Se) reduction was studied with sulfate reducing bacteria, Desulfovibrio desulfuricans, in the presence and absence of pyrite under anaerobic condition. Concentration of Se in medium was analyzed by ICP-AES. Precipitate of Se and Se accumulated on the bacteria were analyzed by XRD, XANES techniques. Reduction of soluble Se occurred rapidly with the bacteria when hydrogen gas serves as the electron donor both in the presence and the absence of pyrite. Oxidation states of Se precipitate and Se accumulated on the bacteria were identified to be elemental Se in the absence of pyrite and Fe-bearing selenide dominantly in the presence of pyrite by XANES analyses. These results suggest that the bioreduction of Se by Desulfovibrio desulfuricans causes the reduction of soluble Se to be elemental Se which has low solubility in water and the reduced Se further incorporated into pyrite to be Fe-bearing selenide, a more stable form of Se. Such process would be an essential immobilization mechanism of Se in geological environment.

Keywords: Bioreduction, Selenium, Sulfate reducing bacterium, HLW disposal, Radionuclide transport

1. INTRODUCTION

Selenium contamination in environment is caused by various human activities, ranging from basic agricultural practices, mining, fly-ash emission to high-tech industrial processes [1]. Although selenium is a necessary nutrient in life cycling [2], excessive selenium may pose the most serious long-term risk to environment, especially aquatic habits and fishery resources [1] [3] [4]. Additionally, radionuclide selenium is a byproduct in the application of nuclear energy. Selenium-79 contained in high level radioactive waste (HLW) has been indicated to be an important radionuclide in the safety assessment of HLW disposal [5] due to its relatively long half-life and complicated chemical behavior. The increasing interest is noticed in the techniques respective to the decontamination or remediation of the environment contaminated by metals including selenium recently. One of the potential techniques is bio-immobilization. Microbial reduction of many metallic species has been well documented [6]. These metallic species are microbial-reduced to be species at relatively low oxidation states when they serve as electron acceptors in the microbial reduction processes.

It is well known that there are four species of inorganic selenium, selenates, selenites, elemental selenium and selenides present in environment. Among these species, selenates and selenites are soluble in water, toxic, and easily transport with water in environment because their dominant species in water are selenious and selenic anions which usually have very low affinity to natural media. They are commonly stable in aerobic environments, while elemental selenium and selenides with relatively solubility appear under anaerobic conditions. Since the low affinity of anions of selenium, sorption mechanism is unlikely to be a good way for immobilization of selenium in environment. Microbial reduction is expected as a potential way to the immobilization of selenium. Recent studies have been shown the potential of sulfate reducing bacteria for on-site remediation of a selenium-contaminated mine site [7], and effective removal selenate from wastewater [8]. However, information on the microbial reduction in the presence of a mineral is insufficient, especially for iron minerals under a reducing condition. Understanding the reduction process and its influence on selenium transport are crucial for selenium immobilization in geological environment, in which generally known as a reducing condition.

In this study, selenium reduction was studied with sulfate reducing bacteria, Desulfovibrio desulfuricans, in the presence and absence of pyrite under anaerobic condition to understand the reduction process. The implication of such process was discussed from the viewpoint of environmental issues.

2. MATERIALS AND METHOD

2.1. Cell cultivation

Stocks of Sulfate reducing bacteria, Desulfovibrio desulfuricans strains, were maintained in 20 cm³ aliquots of culture medium A, which containing: peptone 5.0g, meat extract 3.0 g, yeast extract, 0.2 g, glucose 5.0 g, MgSO₄ 1.5 g, Fe₂(SO₄)₃ 0.05 g, (NH₄)₂SO₄ 0.05 g, distilled water 1.0 dm³, with pH adjusted to 7.0 ± 0.2 with 1 mol·dm⁻³ NaOH solution. The medium was pre-degassed with high purity nitrogen gas (N₂) and sterilized by autoclave at 121°C for 20 min. The cells were cultured in the medium in serum bottles sealed with a butyl rubber stopper alu-

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amine crimp caps under the \( \text{N}_2 \) atmosphere, with continuous shaking with a rotary-type shaker at 100 rpm in an incubator controlled at 37°C. The growth of the cells was confirmed by optical density method which the absorbance of sample at 600 nm (OD600) is measured, supplied with optical spectroscopy. The strains were re-subcultured every 2-3 weeks (10 vol% inocula).

Cells used in the selenium reduction experiment were sub-cultured in medium B, which contained the same composition as medium A, except for \( \text{Fe}_2(\text{SO}_4)_3 \) to reach a low concentration of iron for avoiding possible precipitation of an iron compound in the culture. The supplement of the amount of sulfate due to exclusion of \( \text{Fe}_2(\text{SO}_4)_3 \) was made by increase \( (\text{NH}_4)_2\text{SO}_4 \). These cells were repeatedly sub-cultured in the sealed serum bottles in the same measure as that used for the cultures of the stocks of the Desulfovibrio desulfuricans strains, until negligible iron compound precipitate, carried over from medium B repeatedly. The cells in the medium with negligible iron compound were further pre-grown in medium B for two weeks prior to the selenium reduction experiment. Additionally, partial of these pre-grown cells were sterilized by autoclave at 121°C for 20 min to be dead cells for the experiment.

2.2. Exposure to selenium

The reduction of selenium was studied with the sulfate reducing bacteria, Desulfovibrio desulfuricans, in the presence and absence of pyrite under anaerobic condition. Hydrogen gas (4.8 vol% \( \text{H}_2 \)) was used as the electron donor in the reduction process. Hydrogen is a key compound for Desulfovibrio desulfuricans, since it is one of its major energy sources in natural habitats and also an intermediate in the energy metabolism [9]. Hydrogen gas was also used to keep the reducing condition in a glove box.

The pyrite was grind in an agate mortar into fine powder in the glove box to avoid the oxidation of pyrite. The amount of 0.2 g pyrite powder was sealed in the serum bottle in the glove box, and then transferred out of the glove box for sterilization by autoclave at 121°C for 20 min prior to use.

The pre-grown cells were harvested by centrifugation at 3500 rpm for 15 min, and washed 3 times in 20 mmol⋅dm\(^{-3}\) HEPES-NaOH buffer at pH 7.0 pre-equilibrated with \( \text{N}_2 \) to displace \( \text{O}_2 \) in the buffer. The harvested cells were re-suspended to a biomass density of 0.2 g⋅dm\(^{-3}\) in 1 mmol⋅dm\(^{-3}\) HEPES-NaOH buffer also pre-equilibrated with \( \text{N}_2 \), and separated into several sub-samples, each contained 10 cm\(^3\).

Four samples were prepared. These samples were: (a) only selenium in the buffer solution (SetIV) only); (b) cells suspension spiked with selenium (SetIV+cells); (c) cells suspension spiked with selenium and added pyrite (SetIV+cells+FeS\(_2\)); (d) dead cells suspension spiked with selenium (SetIV+dead cells). Selenite stock solution was spiked into each subsample via the rubber stopper using a hypodermic syringe fitted with a sterilized needle. The finally initial concentration of selenium was 0.37 mmol⋅dm\(^{-3}\), then the subsamples were supplemented with \( \text{H}_2 \) replacing the \( \text{N}_2 \) gas in the headspace. Hydrogen gas severed as the electron donor for selenium reduction.

Finally, the cells were incubated in the incubator under conditions of shaking with a rotary-type shaker at 100 rpm in an incubator controlled at 37°C for 15 days. Timed samples of cell suspensions were taken for each sample for multi-analyses including selenium concentration in media, morphology of cells and the precipitate, and oxidation states of selenium. The redox potential (Eh) and pH values were measured before the sampling.

2.3. Analytical techniques

The activity of the bacteria was analyzed with the optical density method and supplemented by optical microscope method with a counting chamber. Timed samples of cell suspensions were taken for each sample. For analyses of the selenium concentrations, samples were taken and centrifuged at 6000 rpm for 10 min, followed by filtration the supernatant using 0.2µm Millipore filter in the glove box. The filtrate was used for analyses of selenium concentrations using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (ICPS-7000, Shimazu). To avoid the effects of organic matters on ICP-AES, the filtrate was digested by microwave digester (MARS5, USA) according to the standard digestion menu with volume ratio of 1:1 sample to concentrated nitric acid. The microwave digestion in a sealed Teflon vessel could avoid loss of selenium, which usually occurs in a high-temperature digestion method. The samples after digested were diluted in 0.1 mol⋅dm\(^{-3}\) nitric acid for ICP-AES measurement.

For analyses of X-ray diffraction (XRD), the cell suspensions were centrifuged at 3500 rpm for 15 min, and the cell washed with deionized water pre-purged with \( \text{H}_2 \) gas for 3 times. In order to decrease the effect of incompletely centrifugation and the cells was further suction filtrated through filter with pore size of 0.2 µm. The cells and/or the precipitate on the filters were collected by rising with deionized water pre-purged with the \( \text{H}_2 \) gas to avoid the oxidation of the reduced selenium. The collected cells and/or precipitate were separated into 2 sub-samples for analysis by XRD and X-ray absorption near edge structure (XANES), respectively. Sample for XRD analysis was dried in the glove box and then mounted on a low-background XRD sample holder. The XRD analysis was conducted on the precipitates on the cells and/or on the mineral surface using XRD (MiniFlex, Rigaku) operated at a power of 30 kV, 15 mA, using Cu Kα radiation at room temperature. The samples for XANES analyses were sealed with de-oxygen reagent in the glove box and then transferred out of the glove box for analysis. Selenium K-edge XANES analyses were carried out at the beam line 27B of Photon Facility of High Energy Research Organization, Tsukuba, Japan. The details on the measurement and the standard samples have been described in our previous study [10].

3. RESULTS AND DISCUSSION

3.1. Se concentrations in media

Rapid decreases in the selenium concentrations in the media were observed both in the presence and the absence of pyrite, when \( \text{H}_2 \) served as the electron donor. Fig. 1 showed the selenium concentrations in the media as a function of time post the selenium spike. The concentrations in samples (b) and (c) decreased almost linearly from the initial concentrations of 0.35 mmol⋅dm\(^{-3}\) to
around 0.08 mmol.dm\(^{-3}\) at 48 hours, and further to 0.05 mmol.dm\(^{-3}\) in the observation period. Finally, the concentrations decreased to be less than the detection limit of ICP-MS for selenium measurement; whereas only slight decreases in selenium concentrations were observed for sample (a), in which no cells was added. For sample (d), although the dead cells were added, opposite to those for samples (b) and (c), in which the resting cells were presented, the decrease in the selenium concentrations showed similar tendency to that of sample (a). These implies that the decreases in the concentrations are closely associated with the resting cells, no matter if pyrite exist or not. Red precipitate was observed for sample (b) in the absence of pyrite after the spike of selenium, but none in sample (c) which was found to be black-colored. The precipitates were analyzed by XRD to identify their difference.

3.2. Crystalline of selenium precipitates proved by XRD analyses

The XRD patterns indicated that amorphous elemental selenium formed with cells in the absence of pyrite, but no evidence showing its existence in the presence of pyrite. Fig. 2 showed the XRD patterns for samples (b) and (c). The XRD patterns for sample (b)' and (c)' were obtained originally from samples (b) and (c), respectively, but dried at 70°C for 12 hours to investigate the stabilities of the precipitate. On the XRD pattern for sample (b), a broad peak at low angle suggested an amorphous material. It is interested that the XRD pattern for sample (b)' completely differed from that for the sample (b). The pattern for sample (b)' was identified to be hexagonal crystal of elemental selenium.

Selenium, known like sulfur, exists in at least three allotropic forms, or modifications: crystalline, metallic, and amorphous. The hexagonal crystal is the most stable crystalline form. Compared with the XRD pattern for pyrite before reaction with the cells, the XRD patterns, especially the diffraction peak positions for samples (c) and (c)' showed no obvious difference, possibly suggesting no elemental selenium were precipitated in the presence of pyrite. The results of XRD analyses proved partly that the selenium spiked in the initial state, tetravalent selenium Se(IV) has been microbially reduced to be amorphous elemental selenium Se(0).

![Graph](image)

Fig. 1 Time dependences of Se concentration in the media with hydrogen gas as the electron donor in the Se reduction process by Desulfovibrio desulfuricans.

3.3. XANEX analyses results

Oxidation states of selenium precipitates and Se accumulated on the bacteria were identified to be elemental Se in the absence of pyrite and to be Fe-bearing selenide dominantly by XANES analyses. Fig. 3 gave the selenium K-edge XANES spectra of samples (b) and (c). The absorption peak on the spectrum of sample (b) was identified to be consistent with that of standard sample for elemental selenium Se(0) given in previously[10] after energy correction for the spectrum. This result was consistent with that given by XRD analyses. The absorption peak on the spectrum of sample (c), however, was found to be left-shifted around 1.6 eV compared with that for sample (b), suggesting lower oxidation states of selenium, Se(-II), existed in sample (c). The peak position on XANES spectra for some selenium compounds were discussed in previous study[11], in which it was mentioned that only 1.6 eV difference between oxidation state Se(0) and Se(-II).

![Graph](image)

Fig. 3 Selenium K-edge XANES spectra of samples: (b) Se(IV)+cells; (c) Se(IV)+cells+FeS\(_2\)
in the absence of pyrite; (c) in the presence of pyrite.

3.4. Dissimilatory reduction process

Reduction of selenite by *Desulfovibrio desulfuricans* is dissimilatory sulfate reduction processes, in which selenium is reduced to be elemental selenium and selenides. Such mechanism is well supported by the rapid decreases in the selenium concentrations in the media with cells, the analytical results of XRD and XANES for the selenium precipitates above-mentioned.

Bioreduction of selenite occurred rapidly with the bacteria when H₂ served as the electron donor both in the presence and the absence of pyrite. Selenium reduction has been attributed to hydrogenase activity [12]. Selenide H₂Se, as analog of sulfide H₂S can also be produced by enzymes of bacteria cytoplasm. As byproduct, selenide H₂Se can low the redox potential in the media to result in an inorganic reduction of selenite, which is thought to be depended on the redox potential (Eh). For sample (a), the redox potential was measured to be -159 - -187 mV (vs. SHE) and the pH was 6.8 - 7.1 in the experimental period. The dominant species would be elemental selenium Se(0) in this Eh and pH range based on the thermodynamic data on selenium. In the presence of pyrite, however, much lower Eh -197 - -215 mV (vs. SHE) was observed. In this range, the dominant species is predicted thermodynamically to be selenide presenting in form of HSe⁻. The presence of pyrite enhances the formation of iron-bearing selenide. Although the solubility of pyrite is low under reducing condition, dissolution and re-precipitation of new pyrite process is always available. In such process, both amorphous elemental selenium Se(0) and selenide HSe⁻ can incorporated into the new precipitate. Even though there was no direct evidence on the crystal structure associated with iron-bearing selenides because of their limit amount for XRD analyses, the results given by XANES clearly showed the existence of selenides. It has been reported that Se(0) could be incorporated into solid amorphous FeS, or Fe₅S₄, to be Fe₅S₄Se, or forming the analog structure of FeS or FeS₂ as FeSe FeSe₂[13]. Moreover, Metal ions are much more reactive to dis-solved sulfide when adsorbed onto cell walls than in solution [14] [15], and bacteria cell walls are a preferred nucleation site for metal sulfide [16] [17].

3.5. Implications for selenium transport in the subsurface environment

The present study showed that selenite can be reduced to be elemental selenium and selenide HSe⁻, or incorporated into pyrite to be iron-bearing selenides when H₂ served as the electron donor in the dissimilatory process of sulfate reduction by *Desulfovibrio desulfuricans*. The combination of *Desulfovibrio desulfuricans* activities and inorganic chemical reactions can be expected to be a potential treatment method for decontamination or remediation of the environment contaminated by selenium, as well as a major immobilization mechanism of radioisotope selenium highly concerned in current safety assessment of HLW geological disposal. This could be interpreted based on the large differences in the solubility among difference species of selenium. As mentioned in the introduction, four species of inorganic selenium, selenates, selenites, elemental selenium and selenides present in environment. Selenate and selenite are soluble, while selenide H₂Se has solubility of in the order of 10⁻⁵ mol·dm⁻³ calculated based on the thermodynamic data[18], and elemental selenium has much more low solubility reported to be around 10⁻⁷ mol·dm⁻³ [19], and selenide FeSe₂ has the lowest solubility of around 10⁻¹⁰ mol·dm⁻³ [19].

Iron-bearing minerals are especially essential to the selenium reduction by sulfate reducing bacteria such as *Desulfovibrio desulfuricans*. Even though selenium in the forms of H₂Se and FeSe₂ has the same oxidation state, Se(-II), the solubility of selenium associated with iron like FeSe₂ is three orders of magnitudes lower than H₂Se. The lower solubility represents less transport. Although some other metal ions such as Ba²⁺, Pb²⁺ and Mn²⁺ also can be involved in the processes, but iron ion is the most preferred. Because it is known that iron is the 4th most abundant element in the earth’s crust. Iron can be supplied by dissolving various forms of iron-bearing minerals. Among these iron-bearing minerals, in fact, iron sulfide is usually associated with microbial activity. Its mineralization is most often attributed to microbial activity, and more specifically to the activity of the dissimilatory sulfate reducing bacteria; because selenium is commonly found associated with sulfur and sulfides in nature. The usual sources of selenium are the native sulfides such as pyrite, chalcopyrite, and zinc blende.

As partner of iron-bearing minerals, sulfate reducing bacteria are one kind of the most important bacteria that can be survived in subsurface environment. The presence of the sulfate reducing bacteria enhances the formation of stable iron-bearing selenides. It has been reported that pyrite formation from monoselenide could accelerated in the presence of sulfate reducing bacteria, compared to abiotic processes, an apparent consequence of nucleation of pyrite on inner and outer surfaces of the cell envelope [20]. Similarly, the sulfide reducing bacteria can be expected to accelerate the formation of iron-bearing selenide such as FeSe or FeSe₂.

Combining microbial reduction by using sulfate reducing bacteria and iron-bearing minerals or compounds may be a preferred treatment technology for longer-term stabilization of selenium in the contaminated environment, especially in environment that are not likely to be exposed to surface weathering such as HLW repository.

4. CONCLUSIONS

On the basis of the results given in the present study, it can be concluded that sulfate reducing bacteria combined with iron-bearing minerals such as pyrite used in the present study are useful to be applied in the decontamination or remediation of the selenium-contaminated environment. Reduction of selenite by *Desulfovibrio desulfuricans* is dissimilatory sulfate reduction processes, in which selenium is reduced to be elemental selenium and selenides. The bioreduction of selenium by *Desulfovibrio desulfuricans* causes the reduction of soluble selenium to be elemental selenium which has low solubility in water and the reduced selenium further incorporated into pyrite to be Fe-bearing selenide, a more stable form of selenium. Such process would be an essential immobilization mechanism of selenium in geological environment.
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