Original Paper**Biological Oxidation of Arsenite in Strong Acid Water**Hiroshi NAKAZAWA¹ and Wataru HAREYAMA¹¹Graduate School of Engineering, Iwate University, Morioka, Iwate 019-8551, Japan**Abstract**

Arsenic occurs mostly as arsenite (As (III)) or arsenate (As (V)) in natural water. As(III) is more difficult to be removed than As(V), and it is necessary to oxidize As(III) to As(V) for effective removal. Arsenite-oxidizing population, named IWAS, was selected from acid mine drainage. IWAS oxidized arsenite to arsenate in the pH range from 2.1 to 1.2. IWAS can gain the energy for the growth through oxidation of As(III) to As(V). In this study, we examined the effects of factors such as organic matter, As(III) concentration and so forth on microbial oxidation of arsenite.

Key words: arsenite, arsenate, biological oxidation, strong acid, pH

1. Introduction

Arsenic, a potential carcinogenic element, is present in natural water systems because of both natural and anthropogenic activities. The environmental standard and effluent standard of arsenic are 0.01 mg/L and 0.1 mg/L respectively in Japan. Arsenic occurs mostly as arsenite (As (III)) or arsenate (As (V)) in natural water. As(III) is more toxic than As(V)¹⁾, and As(V) is removed more effectively than As(III) by the conventional treatment processes²⁾. Some mine drainages, hot spring waters and ground waters contain arsenic. Arsenic in the mine drainages and hot spring waters often occurs as As(III). The oxidation of As (III) to As (V) is necessary to achieve effective removal of arsenic.

The oxidation of arsenite may be catalyzed biologically. The first identified arsenite-oxidizing bacterium isolated from an arsenical cattle dip, named *Bacillus arsenoxydans*, was reported in 1918³⁾. Turner⁴⁾ also isolated 15 arsenite-oxidizing bacteria strains from cattle dippings. Since the work of Turner, several arsenite-oxidizing bacteria have been found and isolated from various environments. *Alcaligenes faecalis* has been isolated from raw sewage⁵⁾ and soil⁶⁾. The arsenite-oxidizing bacteria that Turner found are considered synonymous with *A. faecalis*. Wakao, et al.⁷⁾ isolated heterotrophic arsenite-oxidizing bacteria from an

acid river near an abandoned sulfur mine. Chemolithoautotrophic arsenite-oxidizing bacteria, NT-26⁸⁾ and *Psuedomonas arsenitoxidans*⁹⁾ were isolated from gold mines. These bacteria can gain energy for growth from the oxidation of arsenite. Battaglia-Brunet, et al.¹⁰⁾ selected an arsenite-oxidizing population, named CASO1, from mine drainage. CASO1 exhibited significant arsenite-oxidizing activity between pH3 and pH8. The bacteria that were described above, however, oxidized As (III) in environments above pH2.

The mine drainage in the abandoned Horobetsu mine, in addition to very high acidity (pH 1.8), is characterized by the high concentration of ferrous iron (on average, 266 ppm) and arsenic (on average, 10 ppm). As (III) is a predominant species of arsenic in the acid mine drainage, but about 15% of total arsenic occurred as As(V). The indigenous microorganisms in the acid mine drainage might be relevant to the occurrence of As(V). We examined an arsenite oxidizing activity of indigenous microorganisms and selected an arsenite-oxidizing population from the acid mine drainage, named IWAS. IWAS can oxidize As(III) below pH2.0. This is the first report about arsenite-oxidizing population below pH2.0. In this study, we examined the effects of factors such as pH, organic matter, As(III) concentration and so forth on microbial oxidation of arsenite.

2. Materials and Methods

The standard arsenite stock solution (As 1000 ppm) was prepared by dissolving arsenic trioxide As_2O_3 (Kanto Kagaku Co.) in 1N sodium hydroxide solution followed by adjusting pH value to 7.0 with sulfuric acid and potassium hydroxide. Other reagents used in this study were of analytical grade.

In this study, two kinds of mediums were used. One was prepared by adding standard arsenite solution to distilled water, referred to as DAS medium. The other was a 10 fold diluted 9K medium, without FeSO_4 , reported by Silverman and Lundgren⁽¹¹⁾, and referred to as D9K medium. The ingredients of D9K medium were 0.3 g/L of $(\text{NH}_4)_2\text{SO}_4$, 0.01 g/L of KCl, 0.005 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.001 g/L of $\text{Ca}(\text{NO}_3)_2$. The pH value in two mediums was adjusted to 1.8 with sulfuric acid and potassium hydroxide.

An arsenite-oxidizing population IWAS was selected from mine drainage in the abandoned Horobetsu mine as follows. 5 mL of the mine drainage or its filtrate through the membrane filter (pore size $0.45 \mu\text{m}$) was transferred to a 300 ml Erlenmeyer containing 200 ml of arsenite solution (pH 1.8) with the concentration of about 5 ppm. After the incubation of 30 days at 25°C , As (III) was oxidized to As (V) completely with the addition of the mine drainage while the oxidation did not occur with the addition of the filtrate, indicating the biological oxidation of As(III). In order to subculture, at intervals of about 3 weeks, 10 ml of the latest subculture was transferred to a 300 ml Erlenmeyer containing 200 ml of arsenite solution of about 10 ppm (pH 1.8). The subculture has been repeated.

Oxidation experiments were carried out using 300 ml Erlenmeyer flasks with 200 ml of DAS medium or D9K medium. A designated amount of the latest enrichment medium was transferred to flasks. Flasks were incubated at 25°C and agitated aerobically at 130 rpm on a reciprocating shaker. Sample solutions were withdrawn periodically for analysis. Sample solutions were filtered through $0.45 \mu\text{m}$ pore size membrane filter (Toyo Roshi Kaisha, Ltd.) and acidified highly with addition of 6N hydrochloric acid in order to prevent further arsenite oxidation. Bacteria concentration was determined by counting under an optical microscope.

Arsenite was determined by the method developed by Yokoyama, et al.⁽¹²⁾. It consists of a flow system using ion-exclusion chromatography, a continuous hydride generator (Hitachi HFS-3) and

an atomic absorption spectrophotometer (Hitachi Z-5000). Arsenite and arsenate were separated using an anion-exchange resin, SP-Sephadex C-25 (Pharmacia Fine Chemicals Co.). 0.1 mol/L $\text{NH}_3\text{-NH}_4\text{Cl}$ (pH 8.3) was used as an eluent. Total arsenic (As(III)+As(V)) was determined by the graphite furnace atomic absorption spectrometry (Hitachi Z-5000).

3. Experimental results

The oxidation experiments were carried out by adding 5 mL or 15 mL of latest enrichment medium of IWAS to DAS medium. The results obtained from the experiments are shown in Figure 1 together with the experimental results under sterile condition. No decrease in As (III) concentration was observed without addition of the enrichment medium until the end of the experiment. The As (III) concentration decreased after a lag phase of 5 days with the addition of the enrichment medium. With the addition of 15 mL, the As (III) concentration decreased from initial concentration of 4.2 ppm to 0.1 ppm after 12 days of incubation, but the total arsenic concentration did not change during the experiment period, indicating the arsenite-oxidizing activity of IWAS. The addition of 5 mL of the enrichment medium also caused the decrease in As(III) concentration, and there was no significant difference in As(III) oxidation rate between amount of inoculums added, 5 mL and 15 mL. It is noticeable that IWAS can oxidize As(III) without any organic matter in the medium.

After the complete oxidation of arsenite in the experiments described above, the standard arsenite stock solution (As 1000 ppm) was added into the medium and the oxidation tests were conducted 3 times. The experimental results are shown in

Fig. 1 Change in the As(III) concentration with the addition of enrichment medium.

Fig. 2 Change in the As(III) concentration with repeated addition of arsenite solution after complete oxidation of arsenite.

Fig. 3 Effect of initial concentration of arsenite on the oxidation of arsenite.

Figure 2. In the first oxidation test (Run 1st) it took 12 days to oxidize As(III) to As(V) completely. In the second oxidation test (Run 2nd), As(III) concentration decreased without the lag phase and the rate of As(III) oxidation increased remarkably. The As(III) concentration decreased from the initial concentration of 4.2 ppm to 0.1 ppm after one day of the incubation in the third oxidation test (Run 3rd).

In order to examine the effect of mediums, oxidation experiments were carried out using DAS medium and D9K medium. As(III) concentration decreased from the initial concentration of 11.7 ppm to 0.2 ppm in the D9K medium after 25 days while from 9.1 ppm to 4.2 ppm in the DAS medium. The oxidation rate of As(III) in the D9K medium was higher than that in the DAS medium. It may be because the D9K medium contains essential elements such as nitrogen and phosphate for the growth of bacteria. In the further experiments and subculture, D9K medium was used as medium.

Fig. 4 Effect of initial cell concentration on the arsenite oxidation.

Arsenite oxidation by IWAS was studied at different initial As(III) concentration. The initial As(III) concentrations were 10.2, 20.6 and 33.0 ppm. Figure 3 shows the effect of initial As(III) concentration on the biological oxidation of As(III). As(III) concentration decreased gradually until the day three, and thereafter the oxidation rate increased at higher initial As(III) concentration (20.6 ppm, 33.0 ppm). The oxidation rates of arsenite were calculated by performing a linear regression on the phase during which the decrease in As(III) concentration was linear-like. The arsenite oxidation rates were 1.90, 2.95 and 3.00 mgL⁻¹ day⁻¹ at the initial As(III) concentrations of 10.2, 20.6 and 33.0 ppm, respectively. After complete oxidation of arsenite in the medium of the initial As(III) concentration of 33.0 ppm, As(III) solution was added to be 102 ppm of As(III) concentration and the oxidation test was carried out. Arsenite was completely oxidized in 18 days, indicating that IWAS is tolerant to high As(III) concentration.

Figure 4 shows the effect of initial concentration of IWAS on the oxidation of As(III). The oxidation rate increased with increase in the initial cell concentration. With cell concentration of 2.3×10^6 cells/ml, arsenite concentration decreased from 10.4 ppm to 0.3 ppm in 4 days of incubation.

The effect of initial pH on the microbial oxidation of arsenite was examined by varying initial pH values in the range from 1.2 to 2.1. The results are shown in Figure 5. At pH 2.1, As(III) concentration decreased from 10.7 to 0.4 in the incubation of 6 days. With decrease in the initial pH, the oxidation rate decreased. In the case of pH 1.2, it took 15 days to oxidize more than 90% of arsenite.

In order to evaluate the effect of organic matter on the arsenite oxidation and cell growth, oxidation experiments were conducted by adding yeast

Fig. 5 Effect of initial pH on the oxidation rate of arsenite.

extract as organic matter. The results are shown in Figure 6. With yeast extract concentration of 0.01w/v%, arsenite concentration decreased at the initial experiment stage gradually followed remarkable decrease after the day four. Cell concentration increased with decrease in the As(III) concentration. Without addition of yeast extract, however, increase in cell concentration coincided with decrease in As(III) concentration, suggesting that IWAS can obtain the energy for the growth through the oxidation of arsenite to arsenate. It is noticeable that yeast extract did not accelerate the oxidation of As(III) by IWAS.

As the As(III) concentration decreased in the experiments, the formation of microbial aggregates was observed. The effect of the microbial aggregates on the arsenite oxidation was examined. The enrichment medium was filtrated through No.5C paper filter (Toyo Roshi Kaisha, Ltd.) to remove microbial aggregates. The oxidation experiments were conducted by adding 15 mL of the filtrate or enrichment medium into the medium. The results are shown in Figure 7. Without microbial aggregates the arsenite oxidation occurred, but the oxidation rate of arsenite was lower than that with microbial aggregates.

Discussion

Several arsenite-oxidizing bacteria have been found and isolated. These bacteria have their own optimum pH for arsenite oxidation and growth. Osborne and Ehrlich⁶⁾ found the optimum pH for arsenite oxidation by *Alcaligenes faecalis* to be 7. The chemolithoautotrophic bacterium NT-26 exhibited maximum activity at pH 5.5⁸⁾. Wakao, et al.⁷⁾ showed that As(III)-resistant bacteria isolated from in an acid environment grew well between pH 3 and pH 4. Lesser or no growth was observed

Fig. 6 Effect of yeast extract concentration on arsenate oxidation and cell growth.

Fig. 7 Effect of the filtration of inoculums on the oxidation of arsenite.

at pH 2 and below, and at pH 5.5 and above. The CASO1 population oxidized arsenite in the pH range from pH 2 to pH 10¹⁰⁾. The optimum pH for oxidation was at pH 6.0. The arsenite oxidation rate decreased with the decrease in pH from pH 6 to pH 2. The oxidation rate of arsenite at pH 2 was one fifteenth of that at pH 6.0. We reported that bacteria selected from abandoned gold mine with pH value of 7.5 oxidized As(III) in the pH range between 4 and 8.5. At pH 3.0, the arsenite oxidation did not occur during the experimental period¹³⁾. The arsenite-oxidizing activities of these

bacteria under strong acid conditions such as below the pH value of 2 have not been examined.

We found that indigenous microorganisms in the acid mine drainage (pH 1.8) might be relevant to the oxidation of As(III) in the mine drainage and selected arsenite-oxidizing population, named IWAS. IWAS can oxidize As(III) to As(V) between pH 1.2 and pH 2.1 in the absence of organic matter, but the oxidation rate decreased remarkably at pH 1.2. Even at high concentration of arsenite (102 ppm), arsenite was completely oxidized in 18 days at pH 1.8.

Cell concentration increased with decrease in As(III) concentration in diluted ferrous ion-free 9K medium, suggesting that IWAS can gain energy for growth through arsenite oxidation. The oxidation reaction of arsenite to arsenate depends on pH. At pH < 2.0, the oxidation reaction is represented as follows¹⁰⁾.

The standard free-energy change for this reaction is $-129.65 \text{ kJmol}^{-1}$. Theoretically, therefore, arsenite may be a potential source of metabolic energy for bacterial growth.

Battaglia-Brunet, et al.¹⁰⁾ found that the biofilm on the internal surface of the glass reactor developed as CASO1 oxidized arsenite, and considered that the high oxidation rate may have resulted from the presence of this biofilm. In this study, the formation of aggregates was observed as the arsenite oxidation proceeded. However, it is not clear how the aggregates affect the arsenite oxidation.

References

- 1) J.M. Neff: Ecotoxicology of arsenic in marine environment. *Environ. Toxicol. Chem.*, **16**, pp.917–927 (1997)
- 2) O. Miyamoto, S. Suginoara, T. Yanagase: *Nippon Kogyokaishi*, **89**, pp.101–106 (1973)
- 3) H.H. Green: Description of a bacterium which oxidizes arsenite to arsenate, and of one which reduces arsenate to arsenite, isolated from a cattle-dipping tank. *S. Afr. J. Sci.*, **14**, pp.465–467 (1918)
- 4) A.W. Turner: Bacterial oxidation of arsenite, *Nature (London)*, **164**, pp.76–77 (1949)
- 5) S.E. Phillips and M.L. Taylor: Oxidation of arsenite to arsenate by *Alcaligenes faecalis*, *Appl. Environ. Microbiol.*, **32**, pp.392–399 (1976)
- 6) F.H. Osborne and H.L. Ehrlich: Oxidation of arsenite by a soil isolate of *Alcaligenes*, *J. Appl. Bacteriol.*, **41**, pp.295–305 (1976)
- 7) N. Wakao, H. Koyatsu, Y. Komai, H. Shimokawara, Y. Sakurai, H. Shiota: Microbial oxidation of arsenite and occurrence of arsenite-oxidizing bacteria in acid mine water from a sulfur-pyrite mine. *Geomicrobiology Journal*, **6**, pp.11–24 (1988)
- 8) J.M. Santini, L.I. Sly, R.D. Chnagl and J.M. Macy: A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: physiological, and preliminary biochemical studies. *Appl. Environ. Microbiol.*, **66**, pp.92–97 (2000)
- 9) A.N. Ilyaletdinov and S.A. Abdrashitova: Autotrophic oxidation of arsenic by a culture of *Pseudomonas arsenitoxidans*. *Mikrobiologiya*, **50**, pp.197–204 (1981)
- 10) F. Battaglia-Brunet, M.C. Dictor, F. Garrido, C. Crouzet, D. Morin, K. Dekeyser, M. Clarens and P. Baranger: An arsenic(III)-oxidizing bacterial population: selection, characterization, and performance in reactors, *J. Appl. Microbiol.*, **93**, pp.656–667 (2002)
- 11) M.P. Silverman and G. Lundgren: *J. Bacteriol.*, **77**, pp.642–647 (1959)
- 12) T. Yokoyama, Y. Takahashi and T. Tarutani: Simultaneous determination of arsenic and arsenious acids in geothermal water, *Chemical Geology*, **103**, pp.103–111 (1993)
- 13) H. Nakazawa, H. Sato, Y. Kudo, K. Yokomoto, M. Komori, S. Okubo: Oxidation of arsenite by bacteria isolated from mine drainage, Shigen-to-Sozai, **117**, pp.133–137 (2001)