OUKA
The University of Osaka
Institutional Knowledge Archive

Title	Isolation and characterization of novel antimonate-reducing bacteria for effective antimony-containing wastewater treatment
Author(s)	楊, 子然
Citation	大阪大学, 2021, 博士論文
Version Type	VoR
URL	https://doi.org/10.18910/85249
rights	
Note	

The University of Osaka Institutional Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

The University of Osaka

Doctoral Dissertation

Isolation and characterization of novel antimonate-reducing bacteria for effective antimony-containing wastewater treatment

アンチモン含有廃水処理に向けた新規アンチモン還元細菌の単離と特徴づけ

YANG　ZIRAN

（楊　子然）

February 2021

Division of Sustainable Energy and Environmental Engineering

Graduate School of Engineering,

Osaka University

Content

Abbreviations

As: Arsenic

As(V): Arsenate

As(III): Arsenite

AMM: Anoxic minimal medium

BSR: Business for social responsibility

CAPS: *N*-Cyclohexyl-3-aminopropanesulfonic acid

CHES: *N*-Cyclohexyl-2-aminoethanesulfonic acid

EDX: Energy dispersive X-ray spectroscopy

EU: European Union

Fe: Iron

Fe(III): Ferric ion

Fe(II): Ferrous ion

HEPES: 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid

HPLC: High-performance liquid chromatography

HPLC-HG-AFS: High-performance liquid chromatography-hydride generation-atomic fluorescence spectrometry

ICP-AES: Inductively coupled plasma-atomic emission spectrometry

ICP-MS: Inductively coupled plasma mass spectrometry

MES: 2-(*N*-Morpholino)ethanesulfonic acid

MMM: Minimal salt medium

OD_{600}: Optical density at 600 nm

OTUs: Operational taxonomic units

PCR: Polymerase chain reaction

PHA: Polyhydroxyalkanoates

Se: Selenium

Se(VI): Selenate

Se(IV): Selenite

Sb: Antimony

Sb(V): Antimonate

Sb(III): Antimonite

SEM: Scanning electron microscope

SRB: Sulfate reducing bacteria

TEM: Transmission electron microscope

TSB: Tryptic soy broth

USEPA: United States Environmental Protection Agency

VS: Volatile solids

WHO: World Health Organization

XRD: X-ray diffraction

List of Figures

during anaerobic Sb(V) reduction by strains AR-2 (a) and AR-3 (b).

Fig. 3-6. Positive correlations between cell growth represented by the increase of protein concentration and acetate consumption (ΔAcetate) (a and b) and between cell growth and Sb(V) decline (ΔSb(V)) (c and d) during anaerobic Sb(V) reduction by strains AR-2 (a and c, respectively) and AR-3 (b and d, respectively).

Fig. 3-7. Time course of soluble Sb(V) and Sb(III) (a,c) and protein concentrations (b,d) during Sb(V) reduction experiments under anaerobic, microaerobic, and aerobic conditions by strains AR-2 (a and b, respectively) and AR-3 (c and d, respectively). Error bars represent the standard deviation (n = 3).

Fig.3-8. Acetate consumption during anaerobic, microaerobic and aerobic Sb(V) reduction experiments by strains AR-2 (a) and AR-3 (b). Error bars indicate the standard deviation (n = 3).

Fig. 3-9 Scanning electron micrographs (a,b), EDX spectra (c,d), and X-ray diffraction patterns (e and f) of precipitates formed during anaerobic Sb(V) reduction for 7 d by strains AR-2 (a, c and e, respectively) and AR-3 (b, d, and f, respectively).

Fig. 3-10. Scanning electron micrographs of precipitates after 35 d in anaerobic Sb(V) reduction experiments using strains AR-2 (a) and AR-3 (b).

Fig. 4-1 Time course of Sb(V) and total Sb in aqueous phase under different temperature during Sb(V) reduction by AR-2.

Fig. 4-2 Time course of Sb(V) and total Sb in aqueous phase under different temperature during Sb(V) reduction by AR-3.

Fig. 4-3 Sb(V) reduction efficiency and Sb removal efficiency of strains AR-2 (a) and AR-3 (b) under different temperature conditions. Error bars represent the standard deviation (n = 3)

Fig. 4-4 Time course of Sb(V) and total Sb in aqueous phase with different initial pH during Sb(V) reduction by AR-2.

Fig. 4-5 Time course of Sb(V) and total Sb in aqueous phase with different initial pH during Sb(V) reduction by AR-3.

Fig. 4-6 Sb(V) reduction efficiency and Sb removal efficiency of strains AR-2 (a) and AR-3 (b) under different pH conditions. Error bars represent the standard deviation (n = 3)

Fig. 4-7 Sb(V) reduction by the isolated strains with various carbon source. (a) AR-2 lactate; (b) AR-3 lactate; (c) AR-2 acetate; (d) AR-3 acetate; (e) AR-2 maltose; (f) AR-3 maltose; (g) AR-2 TSB; (h) AR-3 TSB.

Fig. 4-8 The ability of strains AR-2 and AR-3 to utilize various electron accepters. (a) arsenate; (b) selenate; (c) Fe(III); (d) nitrate; (e) sulfate. Error bars represent the standard deviation (n = 3).

Fig. 4-9 (a) Sb(V) reduction and Sb removal by strain AR-2 in the coexistence of nitrate and (b) the change in coexisting nitrate during Sb(V) reduction. Error bars represent the standard deviation (n = 3)

Fig. 4-10 Sb(V) reduction and Sb removal by strain AR-3 under the coexistence of other electron acceptors (a: As; c: nitrate) and the change in coexisting electron acceptors during Sb(V) reduction (b: As; d: nitrate). Error bars represent the standard deviation (n = 3)

List of Tables

Chapter 1
Introduction

1.1 Uses of antimony

Antimony (Sb) is a kind of metalloid which belongs to group 15 of the Periodic Table along with pnictogen including nitrogen (N), phosphorus (P), arsenic (As), bismuth (Bi) and perhaps the uncharacterized synthetic element moscovium (Mc). It has an atomic weight of 122 and a density of 6.697 kg/m^3 at 26 ℃ (Anderson, 2012). Sb can exist in a variety of oxidation states (-III, 0, III, V) but mainly found in two oxidation states III (antimonite (Sb(III)) and V (antimonate (Sb(V)) in environment (Filella et al., 2002a). Since antimony is a strong chalcophile element, it mainly occurs in nature as Sb_2S_3 (stibnite) and Sb_2O_3 (valentinite).

Sb and its compounds were found as early as 4000 BCE and have been used in medicines and cosmetics since ancient times. Besides, Sb was used for purifying gold from copper and silver up in 18th century (Filella et al., 2002a). As to modern industry, Sb is frequently used in the production of alloys for years. In addition, Sb and its compounds are widely utilized as semiconductor materials, a catalyst for polyethylene terephthalate synthesis, battery manufacture and in various operations such as production of paint pigments, flame retardants, brake linings, plastics, glassware and ceramics (Ungreanu et al., 2015).

World reserves of Sb are over 2 million tons in 2006 and are mainly located in Bolivia, China, Russia, South Africa and Mexico (Carlin, 2000). Nowadays, Sb is the ninth-most mined metal worldwide (Scheinost et al., 2006) and current world production of Sb is over 140,000 tons per year (Table 1-1). Such huge annual production and consumption of Sb may generate a mass of Sb-contaminated wastes, which probably cause serious environmental pollution.

Table 1-1 Major Sb mine production in the world from 2010 to 2015 (in metric tons) (Survey, U.S.G., 2012, 2013, 2014, 2015, 2016)

Country	Antimony mine production / Metric tons					
	Year					
	2010	2011	2012	2013	2014	2015
China	120,000	150,000	145,000	120,000	120,000	115,000
Russia	3000	3300	6500	7000	9000	9000
Australia	-	-	-	-	5800	5500
Bolivia	3000	3900	4000	5000	5500	5000
Tajikistan	2000	2000	2000	4700	4700	4700
Turkey	-	-	-	-	4500	4500
Burma	-	-	-	9000	3300	3500
South Africa	3000	4700	3800	3100	1600	-

1.2 Sb pollution in aquatic environment

Recent years, Sb pollution was detected in several rivers (Wilson et al., 2009; Zhang et al., 2009; Wu et al., 2011; Asaoka et al., 2012). Although Sb also occurs in aquatic environment as a result of rock weathering or soil runoff, the release caused by human activities can reach up to 100 times natural levels (Filella et al., 2002a). Historical and current mining and smelting activities are the main reason that expedites the diffusion of Sb in aquatic and soil systems through the discharge of mine wastes (Lin et al., 2018; Sh et al., 2012). Since China has the largest reserves of Sb in the world with 114 Sb mines located within 18 provinces or autonomous regions (He et al., 2012), China plays an important role in global anthropogenic Sb emissions (Tian et al., 2012). For example, Gao et al. (2012) found that Sb concentration in the sediments of the Beijiang River (in south China) reached 39.0 mg/kg mainly due to the metal smelting industries and local mining activities in the upper regions of the river. High

concentration of Sb was also observed in the water flowing through Xikuangshan mine area (Hunan, China), which is the world's largest Sb mine, as a result of Sb release from waste heaps and the tailing ponds (Zhou et al., 2017). Water samples from Xikuangshan mine area showed higher level of Sb (2-6384 μg/L) than the world average level (1 μg/L) (Wang et al., 2011), and the Sb concentration in surrounding water area was 53.6 ± 46.7 μg/L (Fu et al., 2010). Sb contamination was also found in many other aquatic environment near mining area (Culioli et al., 2009; Resongles et al., 2013; Ritchie et al., 2013; Cidu et al., 2018).

1.3 Toxicity and water quality guidelines of Sb

Sb and its compounds have been considered as highly toxic chemicals, and inorganic compounds of Sb are more toxic than its organic species (Herath et at., 2017). Inhalational exposure to Sb compounds may cause respiratory effects, cardiovascular effects, gastrointestinal effects, dermal effects and reproductive effects on humans (Sundar and Chakravarty, 2010). Although there is inadequate evidence for carcinogenicity of Sb in human, it has been proved that Sb_2S_3 and Sb_2O_3 may cause lung tumours in rats. Besides, *in vitro* genotoxicity studies showed positive results in chromosome breakage in human leukocytes tests, bacterial mutation tests and chromosomal aberration tests in cultured mammalian cells (Paton et al., 1972; Asakura et al., 2009). In addition, Sb has emetic properties, and as low as 0.529 mg/kg will bring about vomiting (Sundar and Chakravarty, 2010). Sb and it compounds also have negative impacts on other creatures. For example, Sb(V) and Sb(III) show significant inhibition on culturable soil bacterial populations and stimulating effects on actinomycetes (Herath et at., 2017). The increasing Sb concentration in water, sediment and soil environments can change the diversity and structure of microbial communities (Sun et al., 2017; Wang et al., 2018; Xiao et al., 2016). Although the plants are more tolerant to Sb compared with animals, the accumulation of high concentrations of Sb may result in the significant suppression of leaf

and root biomass production (Shtangeeva et al., 2011). Since 1970s, Sb and its compounds have been labeled as priority pollutants by the United States Environmental Protection Agency (USEPA) (USEPA, 1979) and the European Union (EU) (EU, 1976) (Ungreanu et al., 2015; He et al., 2018). USEPA allows the maximum Sb contamination level in drinking water at 6 μg/L (USEPA, 2009). The maximum allowable concentration of Sb in drinking water set by World Health Organization (WHO) is 5 μg/L (WHO, 2003). In addition, other organizations and countries also have their standards for antimony (Table 1-2). Considering the high toxicity of Sb, its emission standards for wastewater have been set by some countries and organizations. For example, emission standards for Sb in China, which has the largest reserves of Sb in the world, has been set at 1 mg/L (Emission standards of pollutants for stannum, antimony, mercury and industry, 2014).

Table 1-2 Standards and guideline for Sb in drinking water and effluent

Organization/ Country	Limit	Category	Reference
World Health Organization (WHO)	5 μg/L	Guideline value in drinking water	WHO (2003)
United States	6 μg/L	Maximum contaminant level goal for the safe drinking water	USEPA (2009)
Canada	6 μg/L	Maximum acceptable concentration in drinking water	Guidelines for Canadian drinking water quality (1997)
European Union (EU)	5 μg/L	Drinking water standards	EU (1998)
China	5 μg/L	Drinking water standards	National standards of China for drinking water quality (2006)
	1 mg/L	Emission standards for effluent	Emission standards of pollutants for stannum, antimony and mercury industries (2014)
Japan	0.02 mg/L	Guideline value in aquatic environments	Ministry of the environment of Japan (2004)
Business for social responsibility (BSR)-sustainable water group	0.5 mg/L	Limit value for wastewater	Sustainable water group water quality guidelines (2010)
International Finance Corporation, World Bank Group	0.3 mg/L	Guideline value in effluent of glass manufacturing	Environmental, health, and safety guidelines for glass manufacturing (2007)

1.4 Present removal technologies of soluble Sb

As mentioned above, since Sb has become recognized as an important pollutant in aquatic environment, it is necessary to remove soluble Sb from industrial wastewater and/or contaminated water, i.e. water phase. Current Sb removal technologies are based on physicochemical principles such as adsorption, coagulation, membrane separation, electrochemical treatment, ion exchange, and extraction (Ungreanu et al., 2015; Li et al., 2018). However, these technologies have some weak points. In general, physicochemical treatment technologies possess the problem of high-cost and huge energy/resource consumption. Also by-products generated by the physicochemical treatment technologies, e.g., adsorbents after adsorption treatment, inorganic sludge of coagulation, need further treatment or disposal, and may cause secondary Sb pollution (Li et al., 2018). In addition, current physicochemical technologies are not necessarily effective: removal efficiency is considerably varied depending on chemical form of Sb (Li et al., 2018). In aqueous phase, Sb mainly exists in two oxidation states (III and V) (Filella et al., 2002a, 2002b). Although Sb(III) can be relatively easily removed (Filella et al., 2002a, 2002b), the present technologies cannot well function against Sb(V). Therefore, conversion of redox state from Sb(V) to Sb(III) should be the key for establishing the technologies to remove soluble Sb.

1.5 Possibility of biological removal of soluble Sb

Though biological wastewater technologies generally have advantages of low-cost and energy/resource-saving properties compared with physicochemical technologies, a typical biological treatment technology, conventional activated sludge process, cannot reduce the Sb concentration in sewage at all (Hargreaves et al., 2016). However, some researchers are focusing on biological methods for Sb removal using specific microbial reactions, namely microbial Sb(V) reduction. Recently, it has been reported that some microorganisms can reduce

Sb(V) to Sb(III) especially under anaerobic conditions, and resultant Sb(III) is subsequently removed from aqueous phase by forming precipitates like Sb_2O_3 and Sb_2S_3 via naturally occurring chemical reactions. However, despite myriads of previous reports on microbial Sb(III) oxidation (Hamamura et al., 2013; Han et al., 2016; Terry et al., 2015), knowledge on microbial Sb(V) reduction is limited. From the limited studies on microbial reduction of S(V), it has become known to proceed via indirect and direct mechanisms (Figs. 1-1, 1-2).

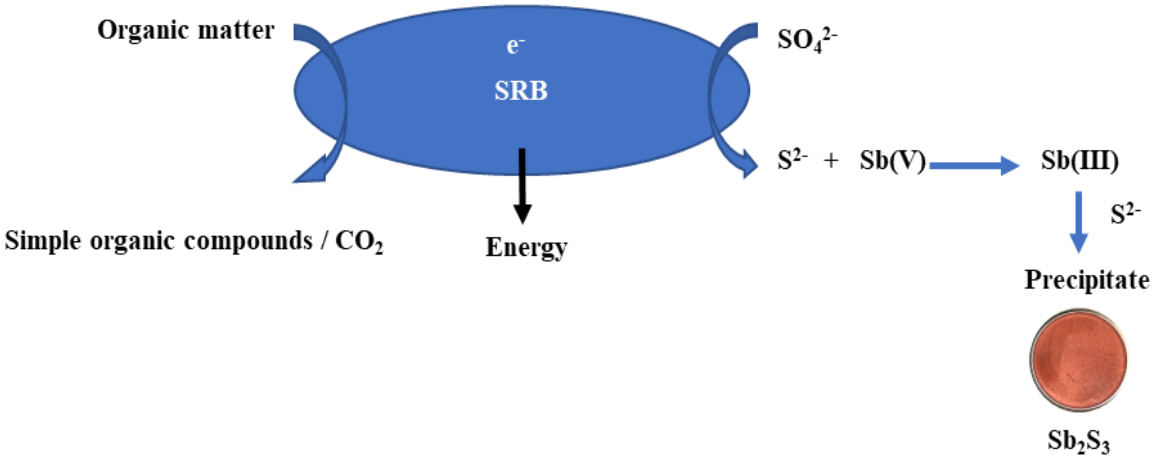


Fig. 1-1 Sb(V) reduction by sulfide produced by SRB (indirect mechanism)

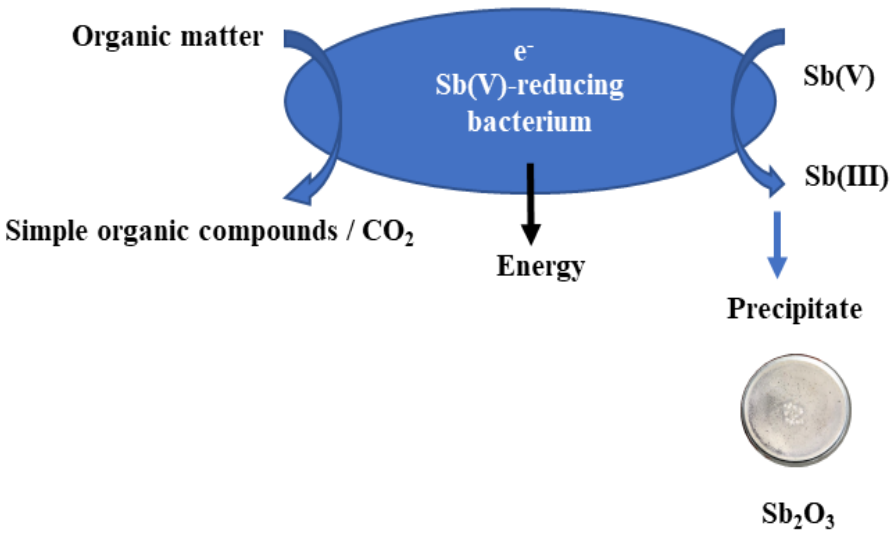


Fig. 1-2 Microbiological Sb(V) reduction by anaerobic respiration (direct mechanism)

1.5.1 Sb(V) reduction by indirect pathway

In the indirect pathway, Sb(V) is chemically reduced to Sb(III) by sulfide produced by sulfate-reducing bacteria (SRB). Study by Wang et al. (2013) noted that SRB enriched from anaerobic sludge could effectively remove Sb(V) and soluble Sb from water under anaerobic condition. SRB can reduce sulfate to sulfide while oxidizing some carbon source (Eq. (1-1)). Then Sb(V) was reduced to Sb(III) chemically by H_2S (Eq. (1-2)). The reduction product Sb(III) is combined with S^{2-} to form stibnite (Sb_2S_3) precipitate (Eq. (1-3)), leading to the decline of soluble Sb. According to this study, reduction of Sb(V) and the removal of soluble Sb in the SRB-precipitation system highly depended on solution pH and SRB activity. The maximum removal efficiency of both Sb(V) and soluble Sb was observed at pH 7. Under the circumstance, 20 mg/L Sb(V) was reduced to 0.16 mg/L by SBR system within 7 d.

$$2CH_3CHOHCOOH + 3SO_4^{2-} \rightarrow 6HCO_3^- + 3H_2S \quad (1\text{-}1)$$

$$Sb(OH)_6^- + H_2S + H^+ \rightarrow Sb(OH)_3 + S_{(s)} + 3H_2O \quad (1\text{-}2)$$

$$2Sb(OH)_3 + 3H_2S \rightarrow Sb_2S_{3(s)} + 6H_2O \quad (1\text{-}3)$$

$$H_2S \rightarrow H^+ + HS^- \quad (1\text{-}4)$$

$$HS^- \rightarrow H^+ + S^{2-} \quad (1\text{-}5)$$

The bio-precipitation of soluble Sb by a mixed batch culture of SRB was also achieved in the study of Zhang et al. (2016). 93% of the total Sb in simulated wastewater was removed during 11-d period of batch experiment at an initial pH 7 at 20 ℃. The scanning electron microscope (SEM) examination showed that the formed precipitates were a mixture of amorphous phases of the metal precipitates and bacteria, and the strongest peaks of energy-dispersive X-ray spectroscopy (EDX) spectrum were Sb and S, suggesting that Sb_2S_3 was the predominant product of the bio-precipitation of Sb by SRB. This study also indicated that in addition to the formation of Sb_2S_3, Sb sorption on the biomass of SRB also contributed slightly to the removal of Sb from the simulated wastewater.

Xi et al. (2020) have reported that the coexisting ferrous ions (Fe(II)) in SRB system could increase the metabolic activity of SRB and accelerate the bio-precipitation of Sb(III) via the indirect pathway. Characterization of the precipitate suggested that the soluble Sb was mainly converted to Sb_2S_3 and Sb_2O_3 in this situation.

1.5.2 Sb(V) reduction by direct pathway

In addition to indirect pathway, direct Sb(V) reduction is caused via heterotrophic anaerobic respiration by dissimilatory Sb(V)-reducing microorganisms using appropriate organics as electron donors (direct pathway). Wang et al. (2018) enriched a Sb(V) reducing microbiota from the surface water near an active Sb mine. After the enrichment with feeding 2mM $K[Sb(OH)_6]$, the Sb(V) reducing microbiota was dominated by *Alkaliphilus* (18−36%), *Clostridiaceae* (17−18%), *Tissierella* (24−27%), and *Lysinibacillus* (16−37%). This enrichment reduced 88% of the soluble Sb(V) to Sb(III) utilizing lactate as carbon source following Eq. (1-6). Formed Sb(III) could be immobilized as $Sb(OH)_3$ and/or Sb_2O_3 into the solid phase.

$$3CH_3CHOHCOO^- + 4Sb(OH)_6^- + H^+ \rightarrow 3CH_3COO^- + 3HCO_3^- + 4Sb(OH)_3 \qquad (1\text{-}6)$$

Microbial community enriched by Zhu et al. (2018) also could reduce Sb(V) in absence of sulfate with acetate as electron donor, indicating the occurrence of Sb(V) reduction via direct pathway. Nearly 100% of the initial Sb(V) (1 mM) was reduced within 12 h and over 50% of the aqueous Sb was removed. The microbial consortium was also able to reduce Sb(V) using H_2 as the sole electron donor (Lai et al., 2016). Sb(V) reduction by the H_2-fed culture had a similar pattern and rate of Sb(V) reduction to that observed when using lactate as the sole electron donor. During the Sb(V) reduction, Sb_2O_3 was generated as the precipitates. Other enriched microbial communities that can reduce aqueous Sb(V) in direct pathway utilizing organics as electron donors were reported (Nguyen et al., 2018; Lai et al., 2018a).

1.6 Isolation of Sb(V)-reducing bacteria

In order to clarify the detailed mechanisms of microbial Sb(V) reduction and to obtain useful biocatalysts for treatment/remediation of aquatic Sb contamination, Sb(V)-reducing bacteria have been isolated successfully and characterized in the recent previous studies. Although Sb(V) reduction by an isolated strain of SRB was reported, its reduction performance was extremely inefficient as reported by Li et al. (2019). On the other hand, some bacterial strains capable of efficiently reducing of Sb(V) by anaerobic respiration have been isolated and clearly described as followings.

An novel anaerobic, Gram-negative bacterium, designated strain MLFW-2^T was isolated as the first Sb(V)-reducing bacterium by Abin and Hollibaugh (2014), and is the most-well characterized Sb(V)-reducing bacterium. This isolated strain could reduce 2 mM Sb(V) in approximately 80 h at 30 ℃ with 1 mM lactate as the sole carbon source. Lactate was consumed and oxidized to acetate accompanied with Sb(V) reduction. The cell density increased to an extent during the cultivation, suggesting that strain *Desulfuribacillus stibiiarsenatis* MLFW-2^T obtained energy from this reaction. The formation of white precipitate was observed as Sb(V) reduction proceeded, and its EDX analysis showed that the precipitate was comprised of Sb and O in the atomic ratio consistent with Sb_2O_3. The visualization by SEM showed that the precipitate occurred in two different structures, cubic and “bowtie” types; the former was identified as sénarmontite, while the latter as valentinite by X-ray diffraction (XRD) analysis.

Follow-up studies were performed to further characterize strain MLFW-2^T (Abin and Hollibaugh, 2017; 2019). It was shown that the optimal growth of strain MLFW-2^T was achieved at 34 ℃ and pH 8.25-8.50 in medium containing 0.75 % (w/v) NaCl (Abin and Hollibaugh, 2017). Lactate, pyruvate, formate and H_2 could be utilized as electron donors to support the growth. In addition to Sb(V), strain MLFW-2^T was able to reduce nitrate, nitrite, DMSO, arsenate, antimonate, selenate and selenite. Enzymes of the dimethyl sulfoxide

reductase (DMSOR) family found in strain MLFW-2^T were considered to contribute to the dissimilatory metabolism of these compounds (Abin and Hollibaugh, 2019). The results of gene transcriptional assay in strain MLFW-2^T indicated that respiratory antimonate reductase (*anrA*), respiratory arsenate reductase (*arrA*), periplasmic nitrate reductase (*napA*) and membrane-bound selenate reductase (*srdA*) were induced by the presence of antimonate, arsenate, nitrate and selenate, respectively.

The second Sb(V)-reducing bacterium, *Sinorhizobium* sp. JUK-1, was isolated from the water outlet of an antimony factory in Korea by Nguyen and Lee (2014). 5 mM Sb(V) was reduced to Sb(III) during the incubation at 30 ℃ with an initial pH 7.7. Acetate was used as electron donor in this study and the reaction was described as follows:

$$CH_3COO^- + 4Sb(OH)_6^- + 3H^+ \rightarrow 2HCO_3^- + 4Sb(OH)_3 + 8H_2O$$

The soluble Sb decreased during the Sb(V) reduction by this strain as a result of the production of precipitates. According to the result of EDX analysis, it was clarified that the precipitate was composed of Sb, O, C, Na and Mg. The inductively coupled plasma mass spectrometry (ICP-MS) analysis of the digested precipitate showed that it contained 53% Sb, 0.3% Ca and 0.4% Mg by weight, suggesting the main component of the precipitates should be Sb.

In addition, very recent studies have demonstrated that some *Shewanella* strains possess the capability to reduce Sb(V). *Shewanella* sp. CNZ-1, which was isolated from marine sediments, could reduce and remove Sb(V) under anoxic conditions (Zhang et al., 2019). However, the efficiency of Sb(V) reduction by CNZ-1 was much lower than the above-mentioned two strains, and it was estimated that the biosorption mainly contributed to the removal of Sb(V). *Shewanella* sp. ANA-3 was also capable of Sb(V) reduction by using it as electron acceptor with lactate as sole carbon source in dissimilatory metabolism (Wang et al., 2020). All of the Sb(V)-reducing bacterial strains above could reduce Sb(V) only under strict anaerobic conditions.

1.7 Objective of this study

It seems possible to develop biological technologies for removing soluble Sb in wastewater using microbial Sb(V) reduction, which can give the benefits of low-cost and environmental-friendliness compared with existing physicochemical technologies. However, the knowledge about the microorganisms which can reduce Sb(V) and remove aqueous Sb is still very limited, and this lack of the knowledge may be an obstacle of progress in the technology development. Therefore, it is necessary to heap up the knowledge on the microbial Sb(V) reduction, especially focusing on the acquisition of effective Sb(V)-reducing bacteria applicable to treatment of Sb-containing wastewater. The objective of this study is to isolate and characterize effective Sb(V)-reducing bacteria for developing microbial technologies for removing Sb(V) in aqueous phase.

This chapter, Chapter 1, described the present situation of pollution of aquatic environment by Sb, and emphasized the importance of development of biological wastewater treatment technologies for Sb-containing wastewater as the background of this study. Based on the review of the previous related research articles, the object of this study was set as mentioned above.

In Chapter 2, microbial Sb(V) reduction and removal potentials in aquatic sediments were investigated under anaerobic conditions to heap up the basic knowledge on microbial Sb(V) reduction in aquatic environment, and also to find out the environmental sample suitable for screening/isolating effective Sb(V)-reducing bacteria.

In Chapter 3, three facultative-anaerobic Sb(V)-reducing bacterial strains were isolated from the sludge collected from a wastewater treatment facility in an antimony products plant. Two of the isolated strains were designated as *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3, and their ability to reduce Sb(V) and remove soluble Sb from water phase was investigated so as to evaluate their potential to be utilized in Sb-containing wastewater treatment.

In Chapter 4, possible factors affecting the Sb(V)-reducing capability of strains *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3 were investigated to further characterize these two Sb(V)-reducing bacteria from the viewpoints of the practical application to wastewater treatment.

Chapter 5 represented the summary and conclusions given from this study. Strains *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3, isolated as novel Sb(V)-reducing bacteria, were evaluated as the biocatalysts for treatment of Sb-containing wastewater, and necessary future studies on these two strains were discussed to realize the practical wastewater treatment technologies.

Chapter 2
Microbial antimonate reduction and removal potentials in aquatic sediments

2.1 Introduction

The objective of the study in this chapter was to evaluate the potential of Sb(V) reduction and Sb removal from the aqueous phase by native microbes in the aquatic environment so as to find out the environmental samples suitable for screening of effective Sb(V)-reducing bacteria. Sediment samples were collected from two rivers with and without the impact of Sb mining activities. They were tested for their Sb(V) reduction capabilities in the presence of high and minimum concentrations of sulfate to estimate the contribution of direct and indirect pathways. In addition, microbial consortia capable of Sb(V) reduction were enriched under minimum sulfate conditions using sediment samples with distinct characteristics of Sb(V) reduction, and microbial community compositions in two enrichment cultures were characterized.

2.2 Materials and methods

2.2.1 Sediment and water samples

Sediment samples and corresponding subsurface water samples were collected from the Ai River in Osaka, Japan, on January 9th, 2019, and the Yagi River in Hyogo, Japan, on April 9th, 2019 (Fig. 2-1). The Ai River flows through northern Osaka, and there has been no report on significant Sb pollution in this river. Eleven sampling points (A1 to A11, from upstream to downstream) were selected to cover upstream to downstream regions of the river. The Yagi River flows through an Sb mine area. Three sampling points (B1 to B3, from upstream to downstream) were selected on the Yagi River, among which sampling point B2 was located adjacent to the wastewater outlet of an antimony products plant (N35.3649, E134.6184). All collected samples were transported on ice to the laboratory, and the sediment samples were sieved through a 2.0 mm screen before storage at 4 °C.

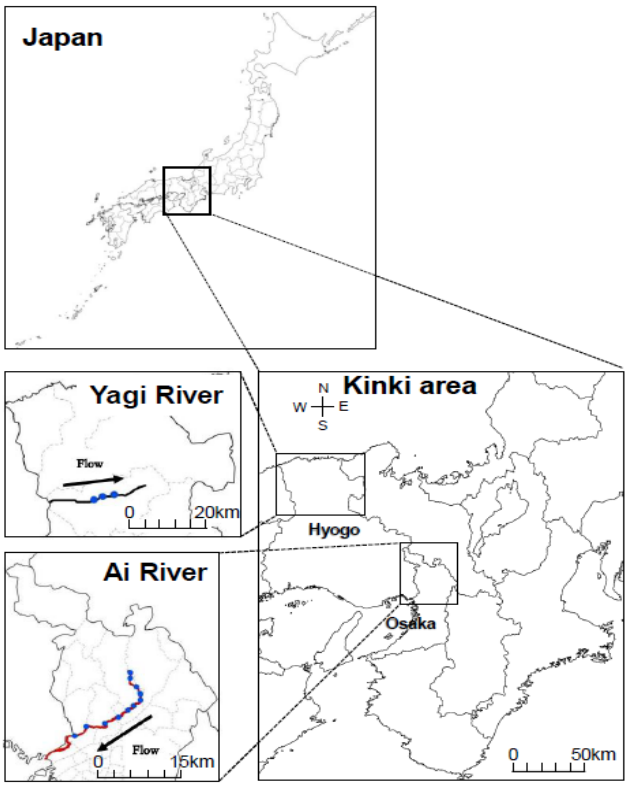


Fig. 2-1 The location of sampling points in the Ai River (Osaka, Japan) and Yagi River (Hyogo, Japan).

2.2.2 Culture media

The minimal salt medium containing sodium lactate and Sb(V) (L-Sb(V)-MSM) was composed of the following ingredients: NaCl 1.2 g/L, KCl 0.3 g/L, NH_4Cl 0.3 g/L, KH_2PO_4 0.2 g/L, Na_2SO_4 0.06 mM or 2.5 mM, $MgCl_2 \cdot 6H_2O$ 0.4 g/L, $CaCl_2 \cdot 2H_2O$ 0.15 g/L, H_3BO_3 1.2 mg/L, $CoCl_2 \cdot 6H_2O$ 0.34 mg/L, $CuCl_2 \cdot 2H_2O$ 0.18 mg/L, $MnCl_2 \cdot 4H_2O$ 0.2 mg/L, $ZnCl_2$ 0.44 mg/L, HEPES 20 mM, sodium lactate 5 mM, and $K[Sb(OH)_6]$ 1 mM, and used as the basic medium. Sodium lactate was used as a sole carbon source because it has been usually used as the electron donor for antimonate bio-reduction in previous studies, and $K[Sb(OH)_6]$ as the source of Sb(V) (Wang et al., 2013; Abin and Hollibaugh, 2014; Wang et al., 2018). The pH of the medium was adjusted to 7.0. Supplementation with Na_2SO_4 at 2.5 and 0.06 mM was intended to make high and minimum sulfate levels, respectively (hereinafter designated as the S_{high} and S_{low} conditions, respectively). The medium was added into glass serum bottles and sterilized by autoclaving (121 °C, 20 min).

2.2.3 Sb(V) removal experiments

Sediment suspension was prepared by adding 5 g-wet of each sediment sample into 50 mL of sterile inorganic salt solution (NaCl 1.2 g/L, KCl 0.3 g/L, NH_4Cl 0.3 g/L, KH_2PO_4 0.2 g/L, $MgCl_2 \cdot 6H_2O$ 0.4 g/L, and HEPES 20 mM) in a 100 mL serum bottle. To maintain the activity of anaerobic microorganisms, the serum bottle was closed with a rubber stopper and sealed with an aluminum crimp, and the sediment suspension was purged with nitrogen gas for 15 min and shaken at 120 rpm for 30 min. The sediment suspension (6 mL) was then inoculated into 54 mL of sterile L-Sb(V)-MSM in a 100 mL serum bottle. After purging with nitrogen gas for 15 min, the culture was incubated at 28 ℃ with rotary shaking at 120 rpm for 15 d. Aliquots (2 mL) of the supernatant of the cultures were collected every 5 d for chemical analysis. Sterilized control for each sediment sample was prepared by autoclaving before the incubation. All experiments

were performed in triplicates.

2.2.4 Enrichment experiments

The culture of samples A5, A7, A11 and B2 after 15 d of Sb(V) removal experiments under the S_{low} condition were used as the inoculum for enrichment of Sb(V)-reducing bacteria. Three milliliters of the culture was inoculated into 50 mL serum bottles containing 27 mL fresh L-Sb(V)-MSM under the S_{low} condition. The culture was then anaerobically incubated as described above. Every 7 d, 3 mL of the culture was subcultured into 27 mL fresh medium and cultivated in the same manner. The samples of the cultures were collected before and after the subcultures, for chemical analysis.

2.2.5 Microbial community analysis

The cultures were centrifuged (20000×*g*, 4 ℃, 10 min), and the pellets were washed twice with 5 mg/L sodium tripolyphosphate solution and resuspended in the same solution, which was then subjected to DNA extraction. Genomic DNA was extracted using Fast DNA SPIN Kit for soil (MP Biomedicals, Solon, OH, USA) following manufacturer's instructions, and stored at -20 ℃ until further use.

Amplicon sequencing targeting the V4 hypervariable region of 16S rRNA genes by Illumina Miseq was performed by the Bioengineering Lab (Kanagawa, Japan). Primers 515F and 806R (Peiffer et al., 2013) were used in a two-step tailed PCR to construct amplicon libraries. The procedures are provided in Table 2-1 and Table 2-2. The resulting amplicon was barcoded with the Index PCR primers (Illumina, San Diego, CA, USA) and sequenced on an llumina MiSeq platform (Illumina) with 2 × 300 bp paired end sequencing. Quality filtering of the raw sequencing data was conducted using QIIME v2.0 (Caporaso et al., 2010). The quality scores are provided in Table 2-3. The high-quality sequences were then clustered into

operational taxonomic units (OTUs) based on a 97% similarity threshold. The taxonomic assignments for the representative sequence from each OTU were conducted using QIIME v2.0 against the EzBioCloud 16S database (Yoon et al., 2017). Raw sequence reads were deposited in the DNA Data Bank of Japan (DDBJ) Sequence Archive database under the accession number DRA010123.

Table 2-1 Composition of PCR mixture

Ingredient	Volume (μL)
10× Ex Buffer	1.0
dNTPs (each 2.5 mM)	0.8
10 μM Forward primer*	0.5
10 μM Reverse primer*	0.5
Template DNA (1st step) or PCR products (5 ng/μL) (2nd step)	2.0
Ex Taq hot start version (Takara Bio, Shiga, Japan) (5U/μL)	0.1
Double distilled water	5.1

*Following primers were used for the 1st and 2nd PCR:

[1st PCR]

Forward primer: 5′-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNNN-GTGCCAGCMGCCGCGGTAA-3′

Reverse primer:

5′-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-NNNNN-GGACTACHVGGGTWTCTAAT-3′

[2nd PCR]

Forward primer: 5′-AATGATACGGCGACCACCGAGATCTACAC-Index2-ACACTCTTTCCCTACACGACGC-3′

Reverse primer:

5′-CAAGCAGAAGACGGCATACGAGAT-Index1-GTGACTGGAGTTCAGACGTGTG-3′

Table 2-2 Thermal cycling conditions

1st PCR			2nd PCR		
94 ℃	2 min	1 cycle	94 ℃	2 min	1 cycle
94 ℃	30 sec		94 ℃	30 sec	
50 ℃	30 sec	30 cycles	60 ℃	30 sec	10 cycles
72 ℃	30 sec		72 ℃	30 sec	
72 ℃	5 min	1 cycle	72 ℃	5 min	1 cycle

Table 2-3 The quality scores for the sequencings*

Enrichment culture	Q20 (%)	Q30 (%)
A11 on 7 d	93.6	85.9
A11 on 28 d	93.9	86.3
B2 on 7 d	93.7	86.2
B2 on 28 d	93.5	85.7

*The Q20 and Q30 values indicate the proportion with the accuracy of over 99.00% and 99.90% within the total read numbers, respectively.

2.2.6 Chemical analysis

The pH of the water samples was measured on site using a portable pH meter (LAQUA NAVI F-52S, HORIBA, Kyoto, Japan). The pH, volatile solids (VS), and water content of the sediment samples were determined according to the Method of Soil Analysis (JSF T 121, JSF T 221, 1990).

For analysis of solid Sb, the sediment samples were dried at 100 °C for 4 h and digested by aqua-regia at 100 °C for 1 h. After cooling to the room temperature, the suspension was filtered through a 0.45 μm cellulose acetate membrane filter (Advantec Toyo Kaisha, Ltd., Tokyo, Japan). Water samples and culture samples from the Sb(V) removal and enrichment experiments were also filtered through the same membrane filter prior to the analysis of soluble Sb, sulfate and sulfide. Inductively coupled plasma-atomic emission spectrometry (ICP-AES; SPS7800, SII Nano Technology, Tokyo, Japan) was used to determine the concentration of total Sb content in sediment and soluble Sb in water samples and culture solution. Speciation of Sb(V) and Sb(III) in the samples was performed by a Shimadzu LC-20A high performance liquid chromatography system (HPLC; Shimadzu, Kyoto, Japan) and a hydride generation-atomic fluorescence spectrometry (HGMillennium Excalibur System, P S analytical, Kent, UK). A PRP-X100 anion exchange HPLC column (Hamilton, Reno, NV, USA) and a mobile phase of 200 mM ammonium tartrate (pH 5) were used for the separation of Sb(V) and Sb(III). The separated Sb species were detected with a hollow cathode lamp for Sb (Super lamp P802SF;

Photron Pty Ltd., Victoria, Australia). The sulfate concentration was measured using an HIC SP ion chromatography system (Shimadzu) with a Dionex IonPac AS4A-SC column (Thermo Fisher Scientific Inc., Waltham, MA, USA). A mixture of 1.7 mM $NaHCO_3$ and 1.8 mM Na_2CO_3 was used as the mobile phase. The sulfide concentration was determined by the photometric method using a Spectroquant sulfide test kit (Merck KGaA, Darmstadt, Germany) and a UV-1850 UV-Vis spectrophotometer (Shimadzu).

2.3 Results

2.3.1 Characteristics of sediment and water in investigated rivers

Sb contents and other parameters of the sediment and water samples collected from the two rivers are presented in Table 2-4. The VS concentrations of the sediment samples varied largely, ranging from 6.6 to 177.6 mg g^{-1}. The pH of the sediment samples was almost neutral (6.5 to 8.1), whereas the pH of the water samples was neutral or weakly alkaline (7.1 to 9.0) and slightly higher than that of the sediment sample at all of the sampling points. The concentration of soluble Sb in all water samples was below the detection limit (<0.01 mM). Total Sb content in the sediment samples from both rivers ranged from 0.04 to 0.25 mmol kg^{-1}, except in sample B3, which contained a much higher level of Sb (1.46 mmol kg^{-1}) probably due to contribution from the upstream Sb mine area. The overall concentration of sulfate in water samples, which may affect the fate of Sb, was below 0.5 mM, except in samples A11 and B2 whose sulfate concentration was 2.2 and 2.3 mM, respectively.

2.3.2 Sb reduction and removal potential of sediment samples

Time courses of Sb concentrations under two different sulfate conditions and of sulfate concentrations under the S_{high} condition for all 14 sediment samples are shown in Fig. 2-3. Sb removal efficiency and sulfate consumptions during the 15-d experiments are summarized in Table 2-5. Microbial communities in 8 of the 14 samples had the potential of Sb removal under

the S_{high} condition accompanied with the decline of sulfate, reaching the highest Sb removal efficiency of 99.4%. Besides, 6 of the 8 samples could obviously remove Sb under the S_{low} condition with the highest removal efficiency of 89.0%. The concentration of sulfide increased slightly accompanied with the decline of sulfate under the S_{high} condition. However, the maximum concentration of sulfide on 15 d was 0.06 mM, and the accumulation of sulfide could not stoichiometrically account for the decline of sulfate (0.37-2.57 mM), far less than the anticipated values. Although the average Sb(V) concentrations after 5 d were slightly higher than those on 0 d in several cases, they were probably caused by the variations of triplicate experiments using natural microbial communities and the measurement errors. Under both S_{high} and S_{low} conditions, a significant decrease in soluble Sb was not observed during the experimental period in sterilized control experiments (data not shown), suggesting that the decrease in soluble Sb in the test systems was resulted from microbial activities in the sediment samples.

Sb removal property for the 14 sediment samples tested in this study were classified into four patterns (patterns I to IV) based on the detailed time courses of soluble Sb removal under both S_{high} and S_{low} conditions (Fig. 2-3). The patterns of Sb removal properties for each samples are shown Table 2.5. Six samples (A1, A2, A3, A8, B1, and B3) showed negligible Sb removal under both S_{high} and S_{low} conditions (pattern I; Fig. 2-3a). Both soluble Sb concentration and redox states of Sb did not significantly change during the 15-d experimental timeframe, except a slight reduction of Sb(V) into Sb(III) after 15 d in sample A8 (Fig. 2-3a).

In two samples, A4 and A5, soluble Sb and sulfate apparently decreased between 10 and 15 days under the S_{high} condition, whereas Sb removal did not occur under the S_{low} condition (pattern II; Fig. 2-3b).

The remaining six samples showed notable Sb removal ability under both S_{high} and S_{low} conditions. In five of the six samples (A6, A7, A9, A10, and A11), soluble Sb began to decrease

within 10 d and reached to less than 20% of the initial concentration after 15 d under both S_{high} and S_{low} conditions (pattern III; Fig. 2-3c). Under the S_{high} condition, sulfate concentration also decreased concomitantly with the decline in soluble Sb. Sb(III) was slightly detected along with the decline in Sb(V), suggesting that Sb(V) was reduced to Sb(III) and then removed from the liquid phase. In addition, the reduction in Sb(V) occurred faster under the S_{low} condition than under the S_{high} condition. Sample B2, which was collected at a place adjacent to the wastewater outlet of a Sb refinery plant, showed different Sb removal ability from the others. Soluble Sb removal was nearly completed within 10 d regardless of the sulfate concentration (pattern IV; Fig. 2-3d). Almost all the Sb(V) was reduced within 5 d under both S_{high} and S_{low} conditions. Sulfate reduction occurred only from 5 to 10 d.

Precipitates were formed in cases where soluble Sb was significantly removed. The precipitates formed under the S_{high} condition were colored rufous or orange red (Fig. 2-2a), suggesting the formation of Sb_2S_3 (Wang et al., 2018). In contrast, pale yellow precipitates were formed under the S_{low} condition (Fig. 2-2b). Since pure Sb_2O_3 forms white precipitates (Abin and Hollibaugh, 2014), the pale yellow precipitates observed in this study might be of Sb_2O_3 mixed with a small amount of Sb_2S_3 or other precipitates.

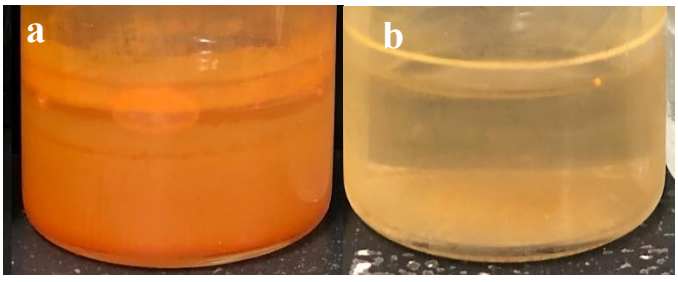


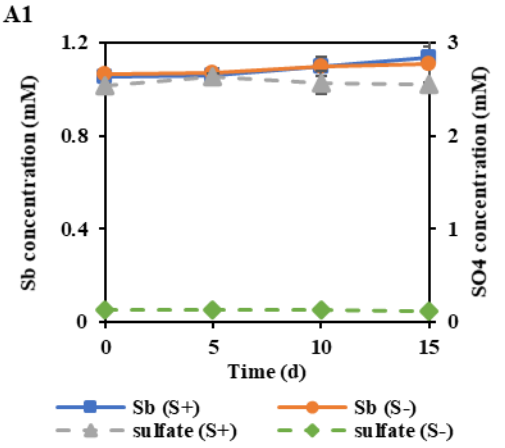
Fig. 2-2 Precipitates formed under the S_{high} (a) and S_{low} conditions (b) during Sb(V) removal experiments. Examples for sample B2 are shown.

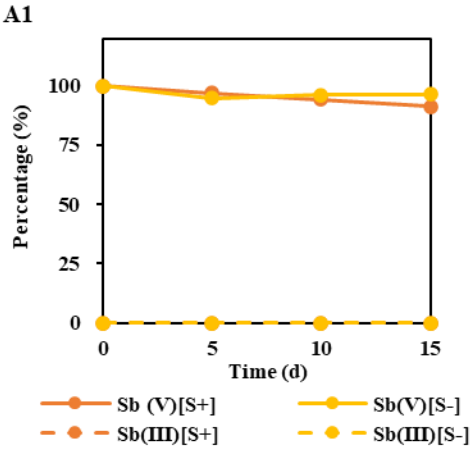
Table 2-4 Characteristics of sediment and subsurface water at sampling points

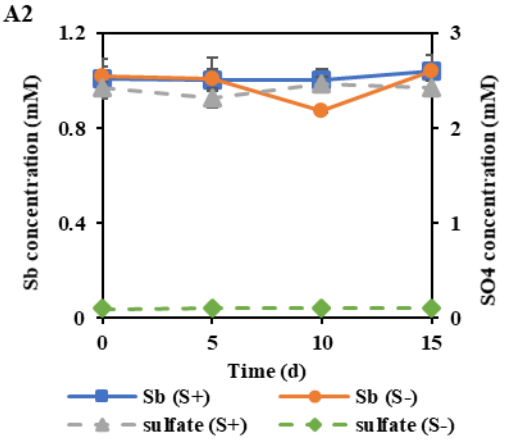
Sampling point	Sediment				Subsurface water	
	Water content (%)	VS (mg g^{-1})	pH	Sb content (mmol kg^{-1})	pH	Sulfate concentration (mM)
A1	24.0	20.6	7.0	0.06	8.7	0.4
A2	34.9	17.2	8.1	0.08	9.0	0.3
A3	25.5	15.1	7.1	0.06	9.0	0.3
A4	22.1	18.2	7.3	0.08	8.4	0.3
A5	59.0	49.6	7.6	0.08	8.4	0.2
A6	50.8	70.4	6.9	0.08	8.5	0.2
A7	31.6	22.8	6.7	0.04	7.3	0.4
A8	18.4	6.6	6.6	0.06	7.1	0.4
A9	25.5	23.5	6.7	0.08	7.4	0.3
A10	63.1	76.0	6.8	0.12	7.4	0.3
A11	69.6	177.6	6.5	0.25	7.2	2.2
B1	28.8	39.6	7.0	0.13	7.6	0.1
B2	42.3	47.2	7.0	0.12	7.4	2.3
B3	26.0	30.7	6.6	1.46	8.0	0.1

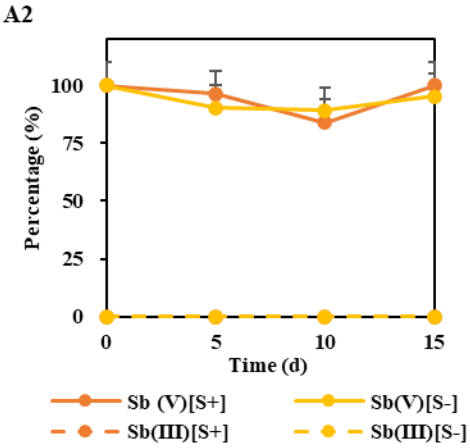
Table 2-5 Removal efficiency (%) of soluble Sb under high and minimum sulfate conditions (S_{high} and S_{low}, respectively) and depletion efficiency (%) of sulfate under the S_{high} condition by sediment microbial communities after 15 d

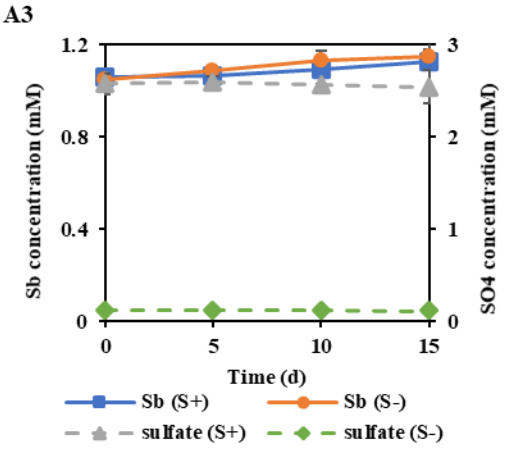
Sb/sulfate	Classification													
	Pattern I						Pattern II				Pattern III			Pattern IV
	A1	A2	A3	A8	B1	B3	A4	A5	A6	A7	A9	A10	A11	B2
Sb (S_{low})	0.0	0.0	0.0	0.0	0.0	0.0	4.7	6.1	87.3	89.0	88.5	81.2	83.2	84.3
Sb (S_{high})	0.0	0.0	0.0	0.0	0.0	0.0	89.6	24.7	98.0	99.4	87.9	97.0	86.9	98.4
Sulfate (S_{high})	0.0	0.0	0.0	0.0	0.0	0.0	71.3	14.2	85.4	84.4	28.9	97.7	16.4	39.6

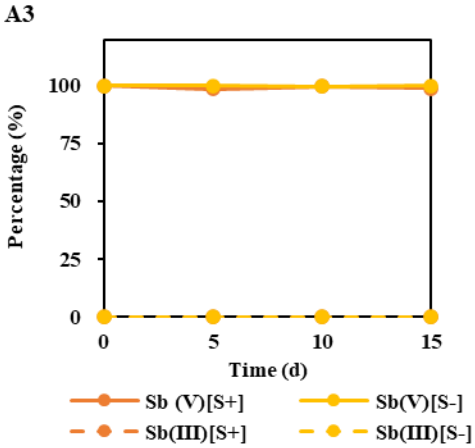

A1
Sb concentration (mM)
SO4 concentration (mM)
Time (d)
Sb (S+)
Sb (S-)
sulfate (S+)
sulfate (S-)


A1
Percentage (%)
Time (d)
Sb (V)[S+]
Sb(V)[S-]
Sb(III)[S+]
Sb(III)[S-]


A2
Sb concentration (mM)
SO4 concentration (mM)
Time (d)
Sb (S+)
Sb (S-)
sulfate (S+)
sulfate (S-)


A2
Percentage (%)
Time (d)
Sb (V)[S+]
Sb(V)[S-]
Sb(III)[S+]
Sb(III)[S-]


A3
Sb concentration (mM)
SO4 concentration (mM)
Time (d)
Sb (S+)
Sb (S-)
sulfate (S+)
sulfate (S-)


A3
Percentage (%)
Time (d)
Sb (V)[S+]
Sb(V)[S-]
Sb(III)[S+]
Sb(III)[S-]

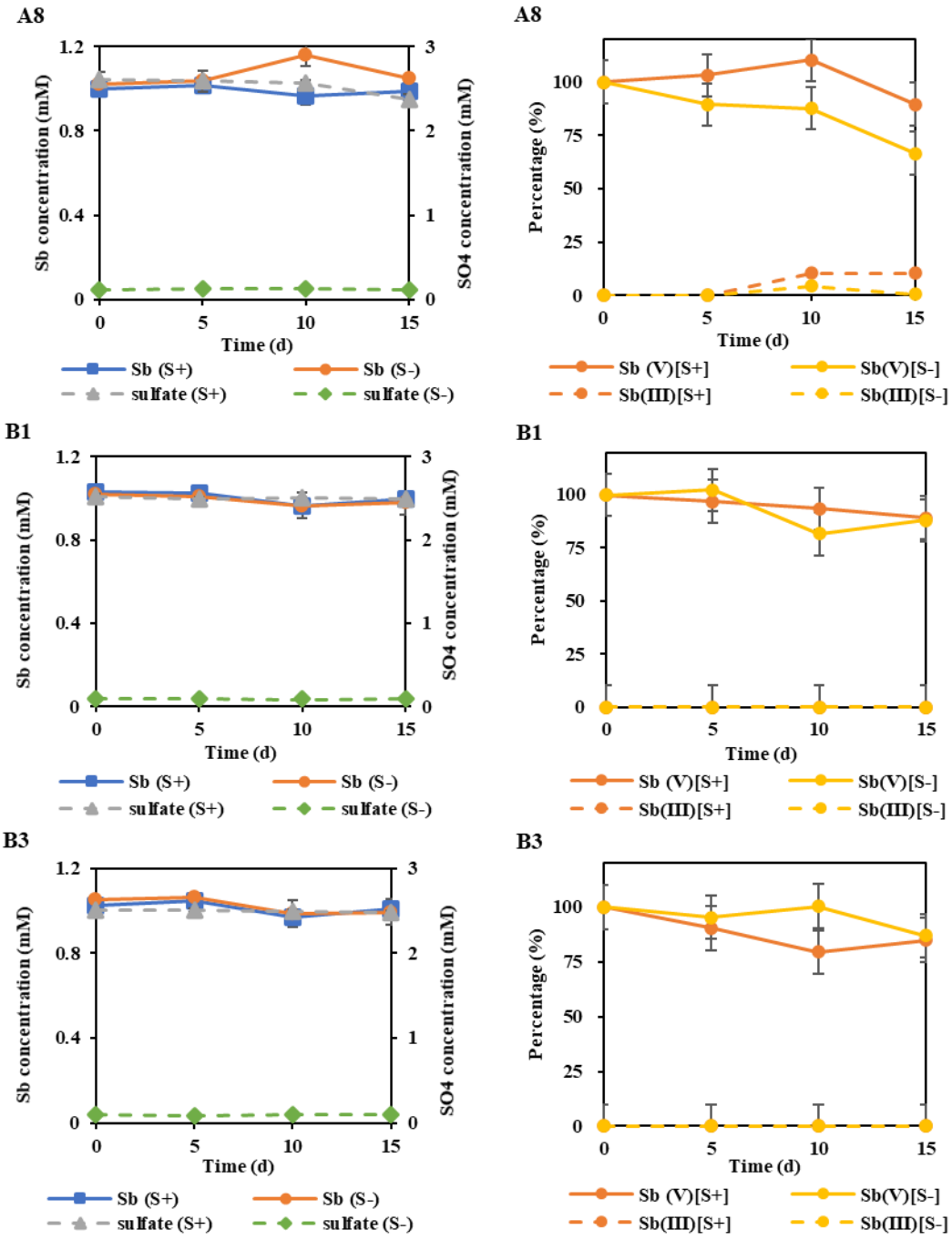


Fig. 2-3 (a) Temporal changes in soluble Sb and sulfate concentrations and valence states of soluble Sb during Sb(V) removal experiments for all the 14 samples under both sulfate conditions (S+: S_{high} condition; S-: S_{low} condition) (pattern I: A1, A2, A3, A8, B1, and B3)

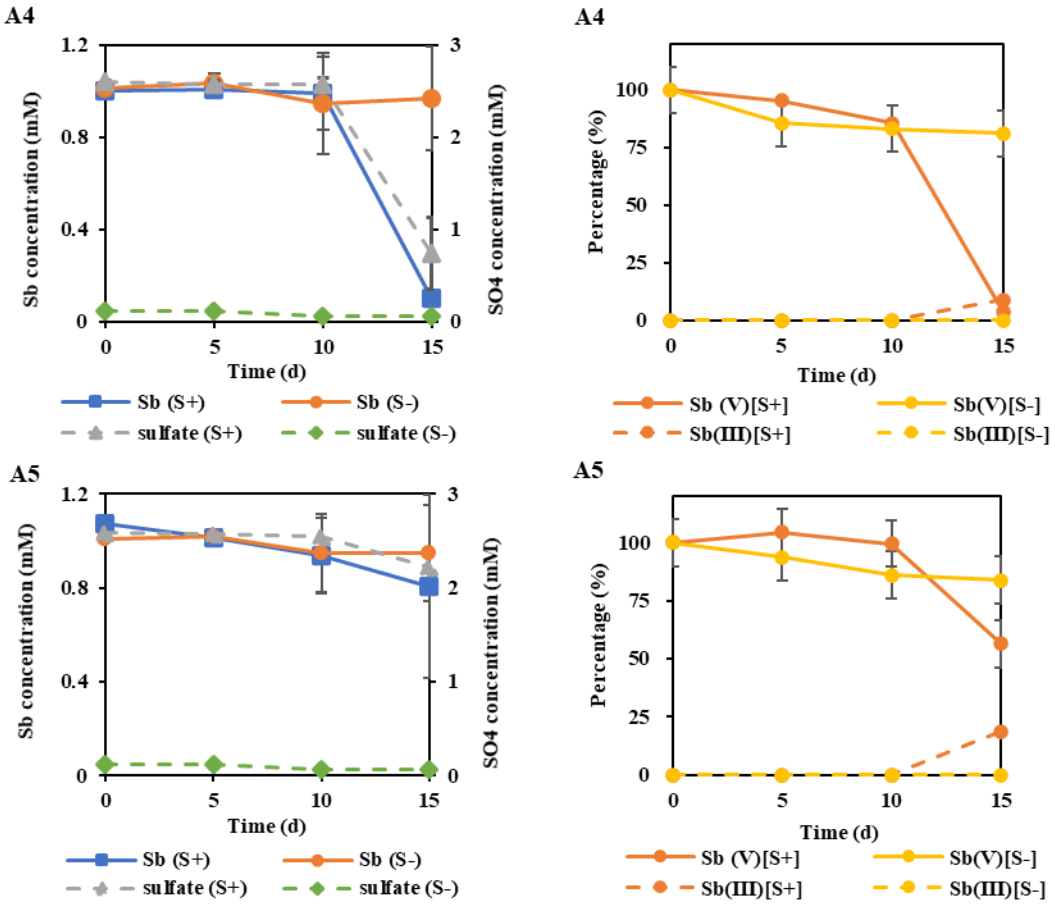
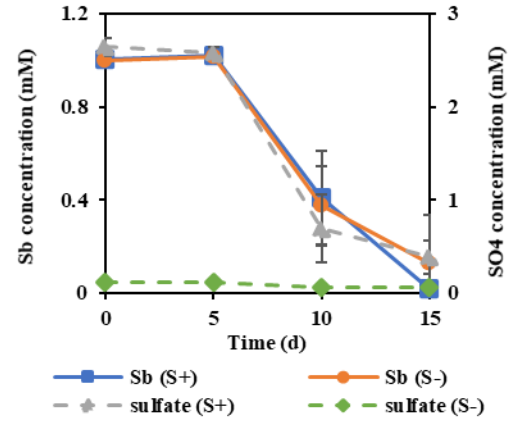
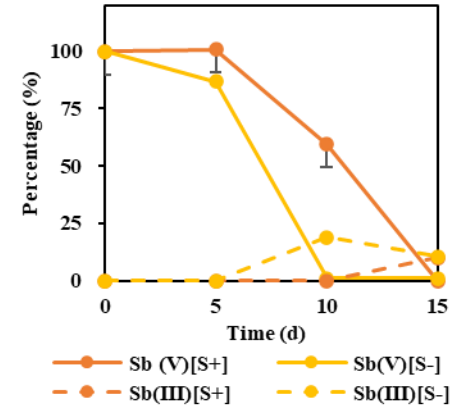


Fig. 2-3 (b) Temporal changes in soluble Sb and sulfate concentrations and valence states of soluble Sb during Sb(V) removal experiments for all the 14 samples under both sulfate conditions (S+: S_{high} condition; S-: S_{low} condition) (pattern II: A4 and A5).

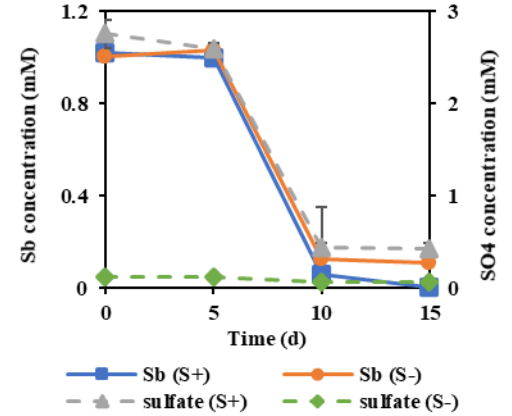
A6



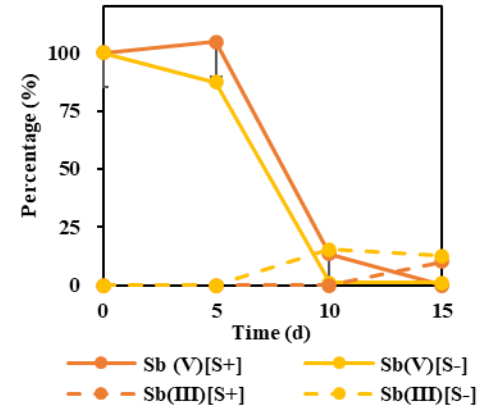
A6



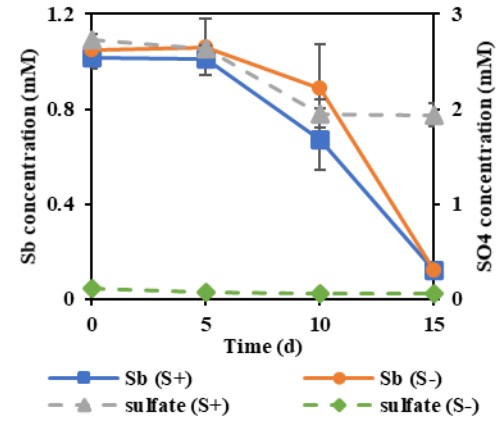
A7



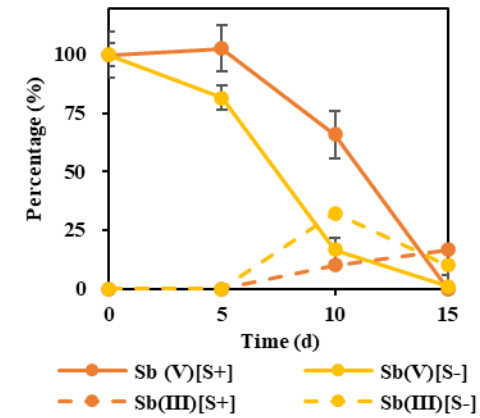
A7



A9



A9



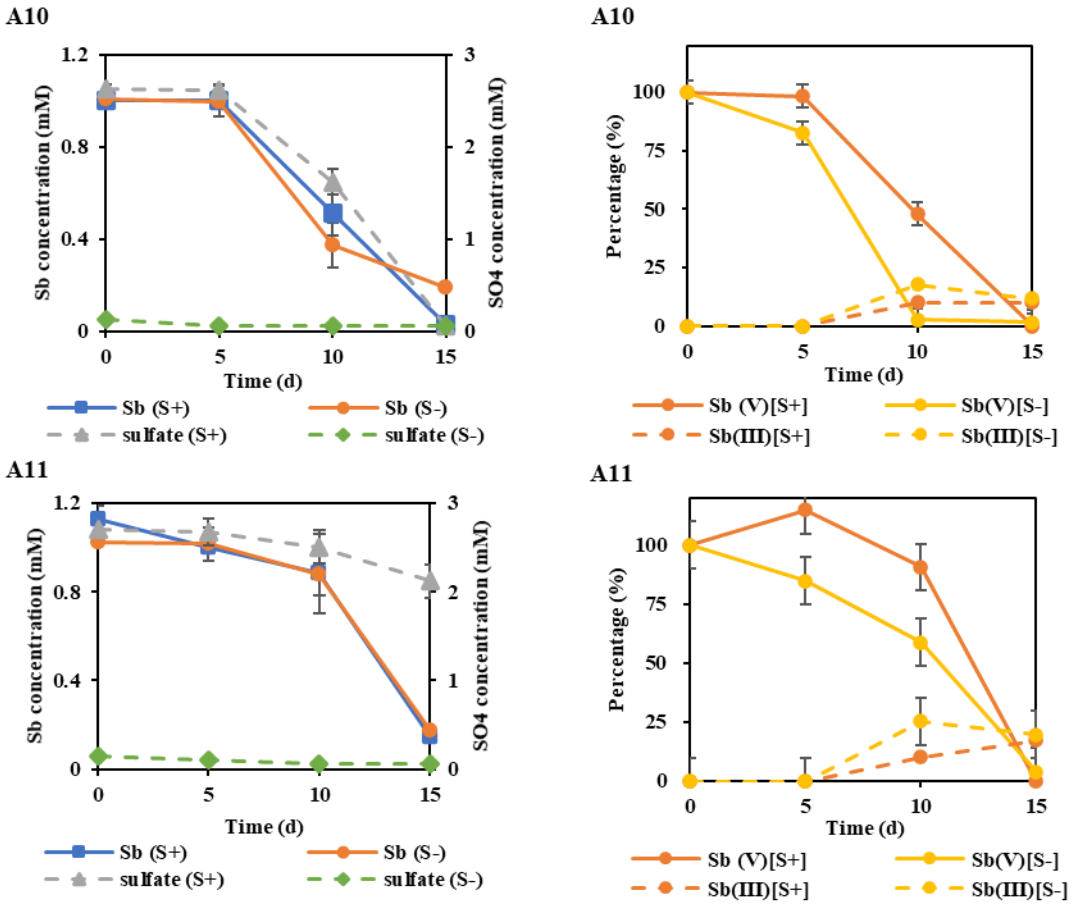


Fig. 2-3 (c) Temporal changes in soluble Sb and sulfate concentrations and valence states of soluble Sb during Sb(V) removal experiments for all the 14 samples under both sulfate conditions (S+: S_{high} condition; S-: S_{low} condition) (pattern III: A6, A7, A9, A10, and A11).

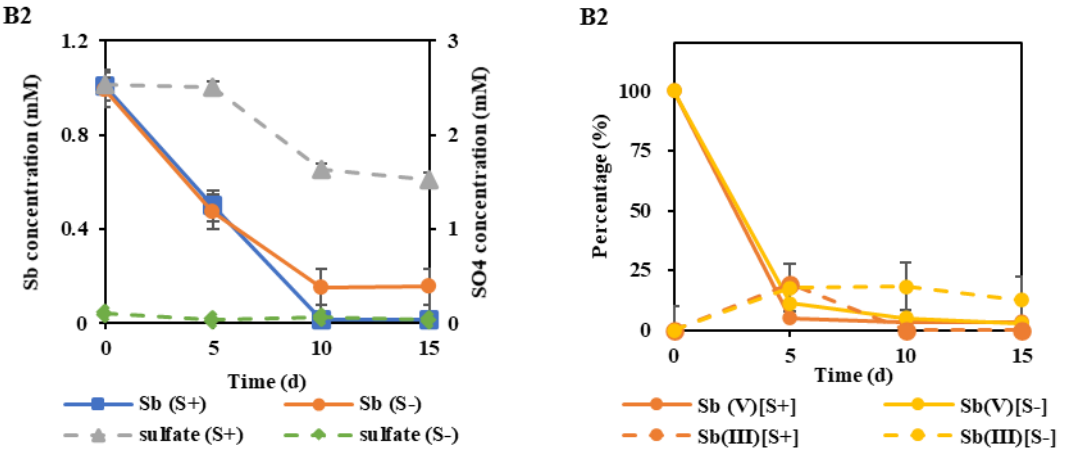


Fig. 2-3 (d) Temporal changes in soluble Sb and sulfate concentrations and valence states of soluble Sb during Sb(V) removal experiments for all the 14 samples under both sulfate conditions (S+: S_{high} condition; S-: S_{low} condition) (pattern IV: B2).

2.3.3 Attempt to Enrich Sb(V)-reducing bacteria

After the above Sb(V) removal experiments, the cultures of samples A5, A7, A11 and B2, whose Sb removal potentials were respectively assigned into patterns II, III, III and IV, were selected as the inoculum for the enrichment of Sb(V)-reducing bacteria. Fig. 2-4 shows the change in soluble Sb concentration during enrichment of the 4 cultures under the S_{low} condition. In the enrichment culture derived from sample A5, classified as pattern II, soluble Sb was stably removed from the 1st to 3rd batches. However, soluble Sb removal ability was lost in the 4th batch (Fig. 2-4a). Soluble Sb removal ability of the enrichment cultures from samples A7 and A11 (Sb removal pattern III) also disappeared in the 2nd and 4th batches, respectively (Figs. 2-4b, 2-4c). In contrast, the enrichment culture of sample B2 (Sb removal pattern IV) could steadily remove over 85% of soluble Sb within 7 d during 4 cycles of sequential batch cultivation (Fig. 2-4d).

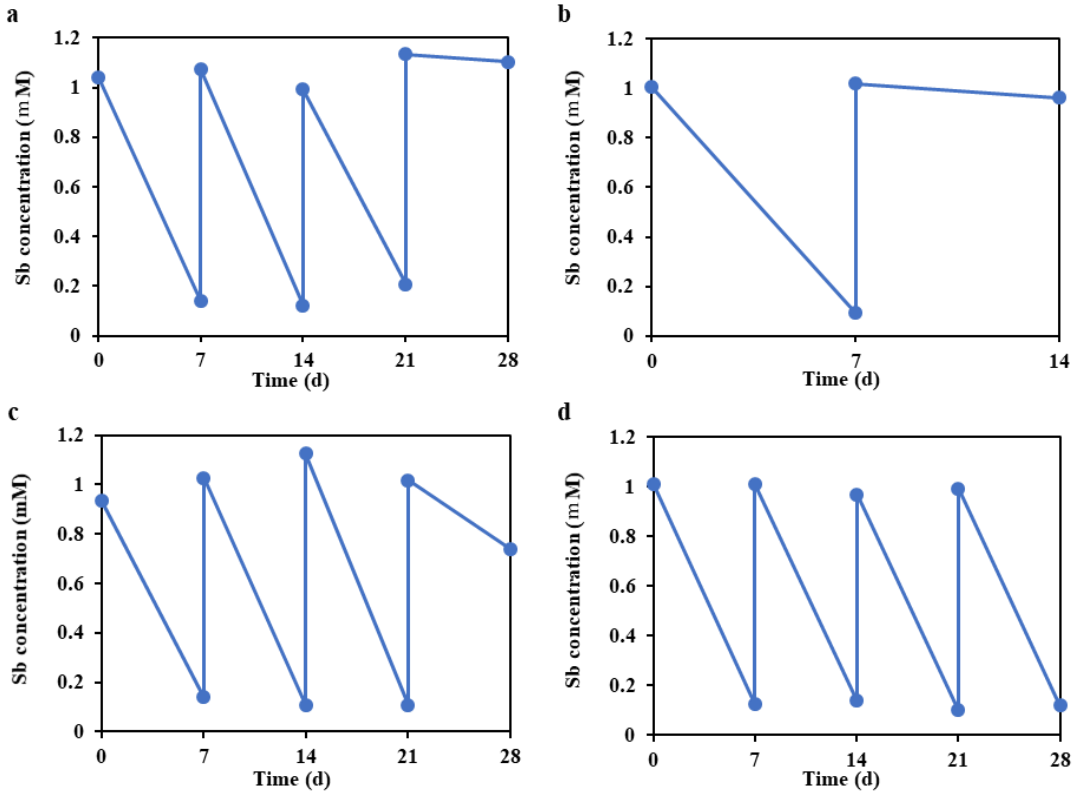


Fig. 2-4 Temporal changes in soluble Sb concentration during enrichment experiments under the S_{low} condition (a: A5; b: A7; c: A11; d: B2).

2.3.4 Microbial community compositions in enrichment cultures

Enrichment cultures derived from samples A11 and B2 were selected as representatives of the ones that lost Sb removal ability and maintained stable Sb removal ability during the enrichment process, respectively, for microbial community analysis. Fig. 2-5 shows the phylogenetic compositions at the genus level of the enrichment cultures at the of the 1st and 4th batches (7 and 28 d, respectively).

During the enrichment process of sample B2, overall bacterial composition did not greatly change and major genera were stably maintained in the culture, although several major genera enhanced their dominance (Fig. 2-5b). *Dendrosporobacter* was consistently dominant and increased its abundance from 29.79% on 7 d to 35.87% on 28 d. *Azospira* was the second major genus, and its abundance also increased from 15.37% on 7 d to 22.43% on 28 d. Additionally, the abundance of *Dechloromonas* and an uncultured genus classified within the order *Bacteroidales* was enhanced from 7.55% and 7.08% on 7 d to 14.02% and 11.86% on 28 d, respectively.

In contrast, the enrichment culture of sample A11 consisted of more diverse genera than sample B2, and its compositions changed drastically during the enrichment process (Fig. 2-5a). The enrichment culture was dominated by *Azospira* (18.02%), *Halodesulfovibrio* (14.49%), *Clostridium* (14.71%), and uncultured genera belonging to the families *Erysipelotrichaceae* (8.11%) and *Acholeplasmataceae* (7.70%) on 7 d. Although *Halodesulfovibrio* increased its abundance to 23.70%, *Azospira*, *Clostridium*, and two uncultured genera of the families *Erysipelotrichaceae* and *Acholeplasmataceae* largely decreased their abundance to 1.22%, 1.70%, and less than 1%, respectively.

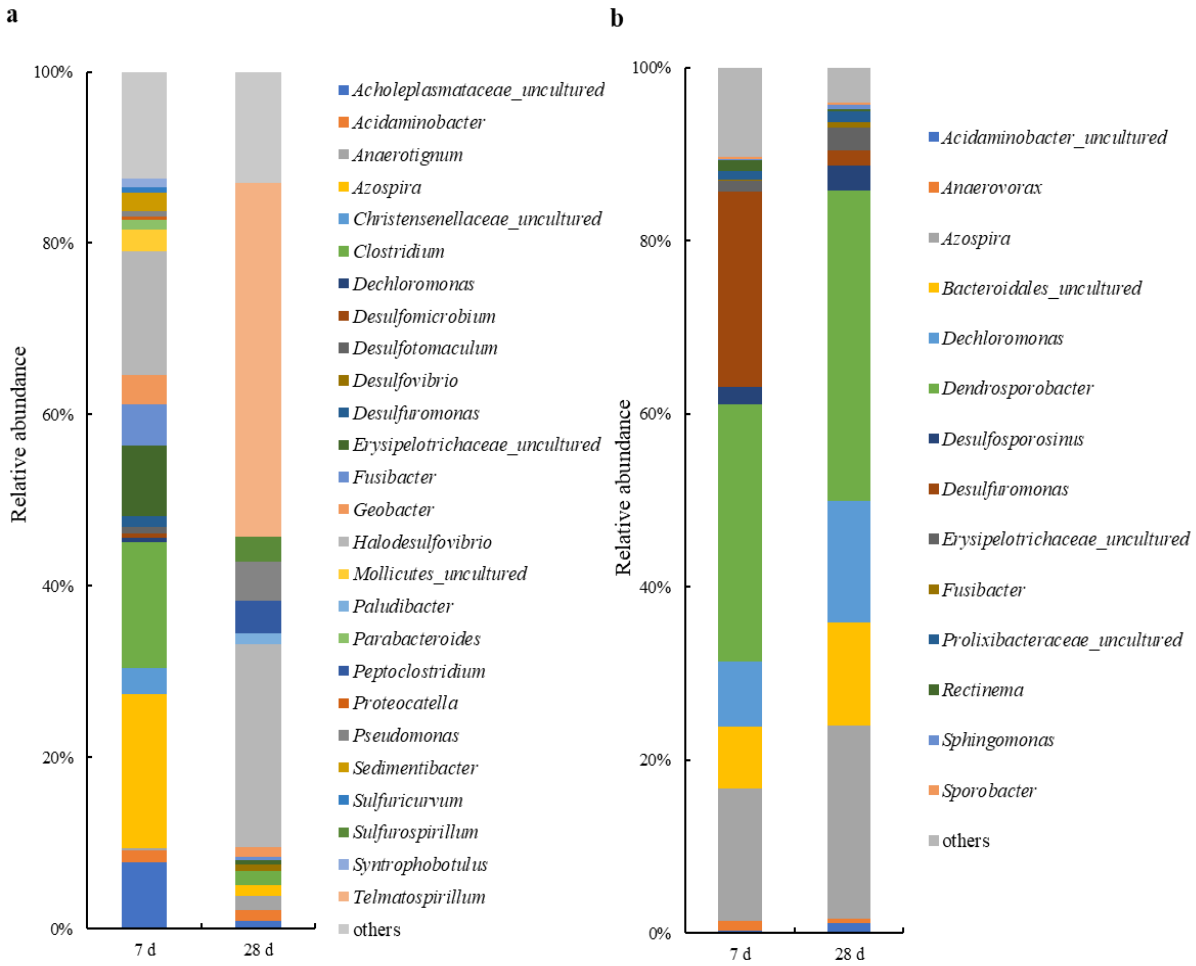


Fig. 2-5 Microbial community compositions at the genus level in enrichment cultures from samples A11 (a) and B2 (b) after 7 and 28 d.

2.4 Discussion

In this study, Sb(V) reduction and removal ability of sediment microbial communities in rivers with and without the impact of Sb mine activities was evaluated. Of the 14 samples investigated, six (pattern I: A1, A2, A3, A8, B1, and B3) did not show any Sb removal ability regardless of the sulfate level, whereas the other eight samples showed obvious Sb removal ability. These eight samples included those from the sampling points with and without anthropogenic Sb impact. This implies that microbial Sb reduction and removal potential is relatively widely distributed in river sediments. In addition, of the eight samples with Sb removal ability, two (pattern II: A4 and A5) removed soluble Sb only under the S_{high} condition,

while the other six (pattern III: A6, A7, A9, A10 and A11; pattern IV: B2) enabled Sb(V) reduction and soluble Sb removal regardless of the sulfate concentration. Furthermore, sample B2 (pattern IV) exhibited remarkably strong Sb reduction and removal ability compared with the other samples. These results suggest that the mechanism and strength of Sb(V) reduction and removal by microbial communities in river sediments are quite different from each other.

In all samples classified into patterns II to IV, soluble Sb was removed along with the reduction of sulfate under the S_{high} condition (Fig.2-3). The simultaneous formation of rufous or orange red precipitates (Fig. 2-2) suggested the formation of Sb_2S_3 precipitates (Wang et al., 2013). Thus, Sb removal under the S_{high} condition likely occurred primarily through the indirect pathway: Sulfate that was abundantly present in the culture was reduced to sulfide by SRB, which was followed by the chemical reduction of Sb(V) to Sb(III) coupled with sulfide oxidation. However, it is also possible that both direct and indirect Sb(V) reduction mechanisms proceed owing to the co-existence of SRB and Sb(V)-reducing bacteria in the sediment samples. Based on the temporal changes in Sb(V) and sulfate concentrations, Sb(V) and sulfate reductions occurring under the S_{high} condition may be further classified into three distinct groups. The first group is the one where Sb(V) reduction occurs more preferably than sulfate reduction, as represented by sample B2. The second group is the one where both Sb(V) and sulfate reductions are initiated almost simultaneously, but only sulfate reduction slows down later, as represented by sample A9. In the third group represented by sample A7, Sb(V) and sulfate reductions occur simultaneously. The well-known indirect Sb(V) reduction via sulfide generated by SRB activities was likely to occur exclusively in the third group, whereas in the first and second groups indirect Sb(V) reduction and Sb removal via sulfide provided by SRB and direct Sb(V) reduction by Sb(V)-reducing bacteria would occur in combination, which could not be confirmed in this study and requires further study.

On the other hand, under the S_{low} condition, we expected that the indirect Sb(V) reduction

pathway was restricted and the direct pathway should occur exclusively because of the deficiency of sulfate supplemented in the medium. We found transient generation of small amounts of Sb(III) along with Sb removal and the formation of pale yellow precipitates under the S_{low} condition in patterns III and IV (Fig. 2-3), indicating soluble Sb removal through the direct Sb(V) reduction pathway mediated by Sb-reducing bacteria in those sediment samples. The reason why not white (pure Sb_2O_3) but pale yellow precipitates were generated was likely because of unexpected minor contribution of the indirect pathway with sulfate that was provided from the sediment samples. If only the indirect pathway contributes to the Sb removal, the ratio of the amounts of the consumed sulfate to the amount of the removed Sb was theoretically 2.5:1 (Wang et al., 2013; Zhu et al., 2018). However, the observed ratios in our experiments (Table 2-5) were much lower than the theoretical one for samples A9, A11, and B2 even under the S_{high} condition. This indicates that direct Sb(V) reduction must have also greatly contributed to the Sb removal in these cases. Altogether, our results indicate the presence of multiple types of Sb(V) reduction ability in microbial communities in river sediments: the contribution of the indirect pathway by SRB and/or direct pathway by Sb(V)-reducing bacteria.

Sb(III) generated by microbial Sb(V) reduction can readily precipitate under the reducing environment (Li et al., 2016). Nevertheless, in most cases classified under patterns III and IV, even when Sb(V) disappeared, low concentrations of Sb(III) (less than 0.18 mM) persisted and consequently soluble Sb was not completely removed especially under the S_{low} condition (Fig. 2-3). These phenomena were probably due to the slight dissolution of Sb_2O_3 precipitates in the water phase as reported in Lemire et al. (1999). Besides, in most cases of pattern III, where Sb removal occurred under both S_{low} and S_{high} conditions, Sb(V) reduction was faster under the S_{low} condition than under the S_{high} condition (Fig. 2-3). This suggests that the efficiency of Sb(V) reduction to Sb(III) by Sb(V)-reducing bacteria (direct pathway) is higher than that by SRB (indirect pathway).

Although four sediment samples, all of which exhibited Sb removal ability under the S_{low} condition, were used for enrichment of Sb(V)-reducing bacteria, only sample B2 from the mine area could maintain Sb removal ability at the end of the 4 cycles 7-d batch enrichment (Fig. 2-4). The enrichment culture of sample B2 still maintained its efficient Sb removal thereafter (data not shown). The results of microbial community analysis revealed that although microbial community composition in the enrichment culture from sample B2 was stable, that from sample A11 changed drastically during enrichment (Fig. 2-5). These results suggested that a considerable decrease or loss of Sb(V)-reducing bacteria, which were present in the original sediment samples, would result in partial or complete loss of Sb removal ability during enrichment in three samples A5, A7 and A11.

To date, only two strains have been well characterized as Sb(V)-reducing bacteria: *Desulfribacillus stibiiarsenatis* MLFW-2 (Abin and Hollibaugh, 2014; 2017) and *Sinorhizobium* sp. JUK-1 (Nguyen and Lee, 2014). These genera were not detected in the enrichment cultures analyzed in this study (Fig. 2-5). Alternatively, *Azospira*, *Dechloromonas,* and *Dendrosporobacter* dominated continuously in the enrichment culture derived from sample B2, which stably maintained Sb(V) reducing ability (Fig. 2-5b). Among them, *Azospira* was predominated on 7 d in enrichment culture of sample A11 with high Sb removal ability. However, the abundance was largely decreased with worsening of Sb removal ability (Fig. 2-5a). The abundance of *Chlostridium* was also decreased with Sb removal ability in enrichment culture of sample A11 (Fig. 2-5a). Thus, some or all of *Azospira*, *Chlostridium*, *Dechloromonas*, and *Dendrosporobacter* probably contributed to Sb(V) reduction in enrichment cultures and their original sediment samples, although the contribution of other populations cannot be ruled out. Previous studies have found that *Azospira* can reduce (per)chlorate, nitrate and selenium oxyanions (Hunter 2007; Nam et al., 2016). Additionally, *Dechloromonas* has been reported to be capable of dissimilatory reduction of (per)chlorate, sulfate, nitrate, and nitrite (Achenbach

et al.,2001; Horn et al., 2005). Further, *Dendrosporobacter* was formerly classified in the genus *Chlostridium* (Strömpl et al., 2000), and has been detected in anaerobic sludge capable of reducing selenate to elemental selenium, although its function was not clarified (Lenz et al., 2008).

2.5 Summary

In this study, Sb(V) reduction and removal potential of microorganisms in river sediments was evaluated in the presence of high and minimum concentrations of sulfate. The results revealed that Sb(V) was reduced and removed from the aqueous phase by microorganisms in most tested sediment samples. This indicated that microorganisms capable of reducing Sb(V) and removing soluble Sb are widespread in the natural aquatic environment, irrespective of the historical impact of Sb mining activity. Our results also clarified that microbial communities in the environment possess multiple types of Sb(V) reduction and removal ability (i.e., direct and indirect pathways and their combinations). However, the Sb(V) reduction efficiency is comparatively higher under the S_{low} condition than under the S_{high} condition, where direct and indirect Sb(V) reduction pathways primarily occur, respectively, suggesting that the efficiency of Sb(V) reduction to Sb(III) by Sb(V)-reducing bacteria is higher than that by SRB. Further, the results of enrichment Sb(V)-reducing bacteria from selected samples suggested that the Sb(V)-reducing bacteria can be stably enriched from the sediment sample which has experienced significant Sb contamination. Therefore, to obtain effective biocatalysts for effective Sb removal, it is better to target the bacteria capable of Se(V) reduction via direct pathway, and to use Sb-contaminated environmental samples as the source for enrichment and isolation of Sb(V)-reducing bacteria.

Chapter 3
Isolation and characterization of facultative-anaerobic antimonate-reducing bacteria

3.1 Introduction

To date, two different types of dissimilatory Sb(V)-reducing bacteria, *Desulfuribacillus stibiiarsenatis* MLFW-2 and *Sinorhizobium* sp. JUK-1 (Abin and Hollibaugh, 2014; Nguyen and Lee, 2014) have been well-characterized for their Sb(V) reduction abilities. Sb(V) reduction by these strains generated insoluble precipitates, suggesting the feasibility of Sb(V) reduction for aqueous Sb removal. However, both strains are obligate anaerobes and require strictly anaerobic conditions for cultivation and Sb(V) reduction, thereby limiting the range of their application. To our knowledge, no Sb(V)-reducing strain capable of growing under the aerobic conditions or even reducing Sb(V) in the presence of oxygen has been found so far, despite the usefulness of such a strain in practical Sb-removal technology. In this chapter, Sb(V)-reducing bacterial strains capable of aerobic growth were newly isolated from sludge collected from a wastewater treatment facility in an antimony products plant. The isolated strains were phylogenetically identified and their growth and Sb(V) reduction capability were characterized.

3.2 Materials and methods

3.2.1 Culture media and cultivation conditions

The anoxic minimal medium (AMM, Macy et al., 1989) was used to enrich, cultivate, and characterize Sb(V)-reducing bacteria. AMM was prepared with ultrapure water and contained the following ingredients: NaCl 1.2 g/L, KCl 0.3 g/L, NH_4Cl 0.3 g/L, KH_2PO_4 0.2 g/L, Na_2SO_4 0.009 g/L, $MgCl_2 \cdot 6H_2O$ 0.4 g/L, $CaCl_2 \cdot 2H_2O$ 0.15 g/L, H_3BO_3 0.06 mg/L, $CoCl_2 \cdot 6H_2O$ 0.19 mg/L, $CuCl_2 \cdot 6H_2O$ 0.002 mg/L, $MnCl_2 \cdot 4H_2O$ 0.1 mg/L, $ZnCl_2$ 0.07 mg/L, $FeCl_2 \cdot 4H_2O$ 1.5 mg/L, $NaMoO_4 \cdot 6H_2O$ 0.036 mg/L, $NiCl_2 \cdot 6H_2O$ 0.024 mg/L, 25% HCl 0.01 mL/L, vitamin solution 10 mL/L and HEPES 20 mM. Vitamin solution contained *p*-aminobenzoric acid 5 mg/L, biotin 5 mg/L, folic acid 2 mg/L, thiotic acid 5 mg/L pyridoxine-HCl 1 mg/L, riboflavin-HCl 5 mg/L, niacinamide 5 mg/L, D-Ca-pantothenate 5 mg/L and cyanocobalamin (B12) 0.1 mg/L (Oremland et al., 1994). $K[Sb(OH)_6]$ was added at 5 mM as the source of Sb(V) to AMM. Sodium lactate or sodium acetate was added at 5 mM as the sole carbon source to AMM containing Sb(V), and these media were designated L-Sb(V)-AMM and A-Sb(V)-AMM, respectively. The pH of both media was adjusted to 7.0. To isolate and cultivate Sb(V)-reducing bacteria, 1/10 strength Tryptic Soy Broth (1/10 TSB; Becton, Dickinson and Company, Sparks, MD, USA) and LB broth (Lennox; Becton, Dickinson, and Company, Sparks, MD, USA) were also used. Agar was added at 1.5% (w/v) to make solid media. Unless otherwise noted, cultivation was conducted at 28 °C. For aerobic and microaerobic cultivation, the liquid culture was sealed with a silicone sponge plug; then, the culture was incubated with rotary shaking at 120 rpm for aerobic conditions, whereas it was incubated statically without shaking for microaerobic conditions. The creation of microaerobic condition with static cultivation has been verified previously (Somerville and Proctor, 2013). Anaerobic cultivation of the liquid culture was conducted with rotary shaking at 120 rpm after being sealed with a butyl rubber septum and an aluminum crimp, followed by purging nitrogen gas for 15 min.

3.2.2 Enrichment of the Sb(V)-reducing bacteria

The sludge used as the inoculum for the enrichment of Sb(V)-reducing bacteria was collected from a sedimentation basin in the wastewater treatment facility in an antimony product plant in Hyogo, Japan, considering the results obtained in Chapter 2. The sample was transported on ice to the laboratory and stored at 4 °C until use. The pH of the sludge sample was 7.3 and its aqueous phase contained Sb at 1.95 mg /L.

Before enrichment of the Sb(V)-reducing bacteria, the sludge sample (5 g-wet) was added to 50 mL of L-Sb(V)-AMM, excluding Sb(V), in a 100 mL glass vial. After creating anaerobic conditions as described above, the culture was shaken at 120 rpm and 28 °C for 30 min and homogenized using a VCX130PB ultrasonic processor (Sonics & Materials, Newtown, CT, USA) for 1 min to disperse the flocs. Four milliliters of the pretreated inoculum culture was inoculated into 36 mL of L-Sb(V)-AMM in a 100 mL glass vial. Then, the culture was incubated anaerobically. Aliquots (4 mL) of the culture were subcultured in 36 mL fresh medium at an interval of 10 d from the 1st to 4th batch cycles and 7 d after the 5th batch cycle.

3.2.3 Isolation of Sb(V)-reducing bacteria

The enrichment culture after the 6th batch cycle was appropriately diluted by 5 mg/L sodium tripolyphosphate solution. Then 100 μL was spread on 1/10 TSB and L-Sb(V)-AMM agar media and cultivated at 28 °C under aerobic and anaerobic conditions, respectively. Anaerobic incubation was carried out using Anaero Pack anaerobic cultivation sets (Mitsubishi gas chemical, Tokyo, Japan). Morphologically different colonies appearing on each medium were picked up, and repeatedly transferred onto agar plate to isolate those strains as possible Sb(V)-reducing bacteria. The isolated strains were cultured with LB broth under the aerobic condition, and the growth ability of them under aerobic condition was confirmed. The isolated

strains which showed aerobic growth was examined for Sb(V)-reducing ability in L-Sb(V)-AMM, and ones with positive results were isolated as Sb(V)-reducing bacteria.

3.2.4 Phylogenetic, physiological and biochemical characterization

The isolated Sb(V)-reducing strains were phylogenetically identified based on partial 16S rRNA gene sequences. Genomic DNA was extracted using the Cica Geneus DNA extraction reagent (Kanto Chemical, Tokyo, Japan). Partial sequences of the 16S rRNA genes were amplified using universal primers 27F and 1392R (Weisburg et al., 1994; Lane et al., 1985). The PCR products were purified using a NucleoSpin Gel and PCR Clean-up kit (Takara Bio, Shiga, Japan), and sequenced by Macrogen Japan (Kyoto, Japan). The obtained sequences were compared with sequences in the NCBI database using the BLAST search program (http://www.ncbi.gov/blast/). The partial 16S rRNA gene sequences of isolated strains were deposited in GenBank/EMBL/DDBJ databases under the accession numbers LC569773 to LC569775.

Physiological and biochemical characterization of isolated strains were entrusted to Techno Suruga Laboratory (Shizuoka, Japan). Microscopic observation of cell morphology, and catalase, oxidase, oxidation-fermentation (O/F), and organic compounds utilization tests were conducted by Techno Suruga Laboratory.

3.2.5 Characterization of growth ability

The isolated strains were inoculated into LB broth and cultivated under aerobic condition for 24–48 h. The cells were collected by centrifugation (6000 ×*g*, 4 °C, 10 min), inoculated into fresh LB broth, and cultured again for 7 h. Thereafter, the cells were collected in the same manner, washed with an inorganic solution (Table 3-1). The collected cells were inoculated into 5 mL of LB broth in L-shaped test tubes at an initial optical density at 600 nm (OD_{600}) of 0.05.

The effects of temperature on the growth of isolated strains were tested in LB broth (pH 7.8) at 20, 28, 37, and 45 °C. The effects of pH were examined at 28 °C using LB broth whose initial pH was adjusted to 5.0–8.5 using the appropriate buffer (Table 3-2). All cultivations used to examine the effects of temperature and pH on growth were conducted in a TVS062CA compact rocking incubator (Advantec) with shaking at 70 rpm, and the optical density of the culture at 660 nm was automatically recorded every 30 min. All experiments were run in triplicate.

Table 3-1 Composition of the inorganic solution

Component	Concentration
NaCl	1.2 g/L
KCl	0.3 g/L
NH_4Cl	0.3 g/L
KH_2PO_4	0.2 g/L
Na_2SO_4	0.009 g/L
$MgCl_2 \cdot 6H_2O$	0.4 g/L
HEPES (pH 7.0)	20 mM

Table 3-2 Buffers used to adjust the pH of LB broth in experiments to test the growth ability of strains AR-2 and AR-3

pH	Buffer
5.0	MES (2-(*N*-Morpholino)ethanesulfonic acid)
6.0	MES
7.0	HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid)
7.8	CHES (*N*-Cyclohexyl-2-aminoethanesulfonic acid)
8.5	CAPS (*N*-Cyclohexyl-3-aminopropanesulfonic acid)

3.2.6 Sb(V) reduction experiments

To characterize the ability of Sb(V) reduction, the isolated strains were precultivated as described above and inoculated into 20 mL of A-Sb(V)-AMM in a 50 mL glass vial at an initial OD_{600} of 0.05. The culture was incubated under aerobic, microaerobic, and anaerobic conditions as mentioned in 3.2.1. Controls without inoculation of the isolated strains or supplementation with acetate were also prepared. Multiple identical cultures for the test and control systems were prepared, and three vials were sacrificed periodically to monitor variations in Sb, acetate, and protein concentrations during the Sb(V) reduction experiments. Because the formation of precipitates might disturb the determination of OD_{600}, protein concentration, which has been confirmed to be positively correlated to cell concentration (Fig. 3-1), was used to represent the cell concentration of isolated strains during Sb(V) reduction experiments.

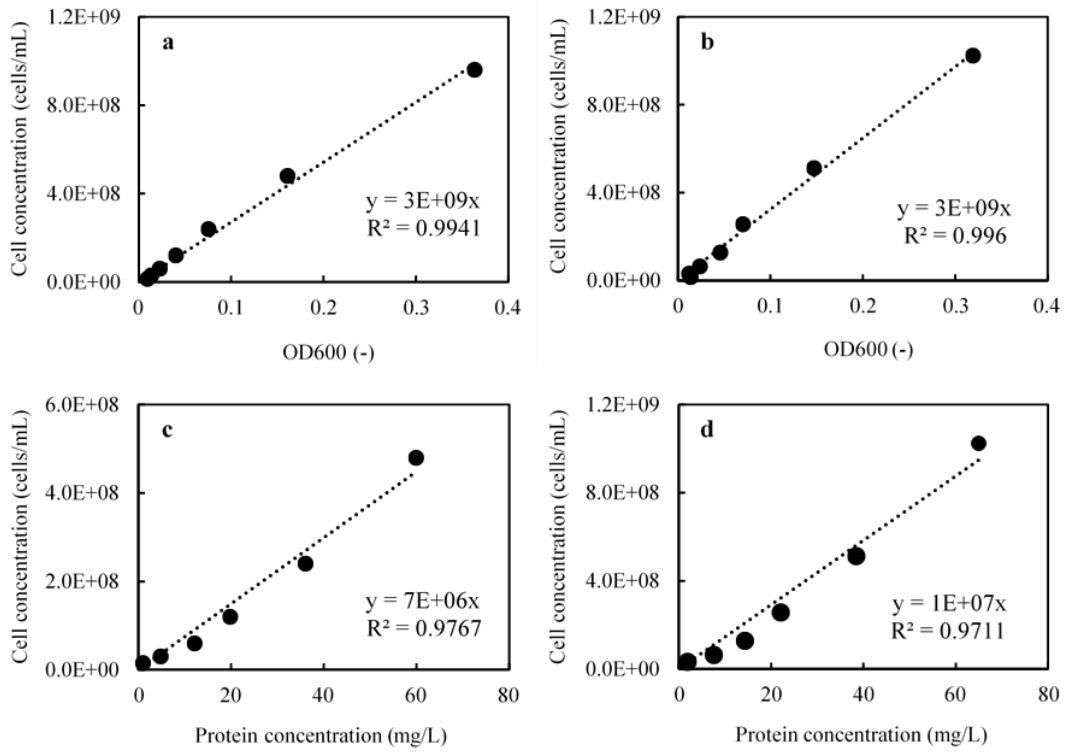


Fig. 3-1 Relationships between cell concentrations (y) and OD_{600} values (a and b) or protein concentrations (c and d) (x) for strains AR-2 (a and c, respectively) and AR-3 (b and d, respectively).

3.2.7 Analytical methods

Before analyses of soluble Sb and acetate, the collected samples were filtered through a 0.2 μm cellulose acetate membrane filter (Advantec). ICP-AES (SII Nano Technology) was used to determine the concentration of total soluble Sb. The speciation of Sb(V) and Sb(III) was performed using a HPLC-HG-AFS with a mobile phase of 200 mM ammonium tartrate (pH 5.0) as described in 2.2.6. The acetate concentration was determined with a Shimadzu LC-10A HPLC system equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA) using a mobile phase of 5.0 mM sulfuric acid.

The OD_{600} of the culture was measured with a UV-1850 UV–vis spectrophotometer (Shimadzu). The protein concentration was determined using a BCA Protein Assay kit (Takara Bio) and an Epoch 2 microplate spectrophotometer (BioTek, Winooski, VT, USA). In the preliminary study, cell concentration was determined using haemocytometer (Erma, Tokyo, Japan) for confirming its linear relationships with OD_{600} and protein concentration. The quantity of cells on haemocytometer were counted with Fluorescence Microscope (BX50, Olympus, Tokyo, Japan).

3.2.8 Solid analysis

For analysis of precipitates, the washed cells were inoculated into 60 mL of A-Sb(V)-AMM in a 100 mL glass vial at an initial OD_{600} of 0.05 and cultivated anaerobically for 7 or 35 d. The culture was centrifuged (2000 ×*g*, 24 °C, 1 min), and the recovered precipitates were rinsed successively with ultrapure water, 100% acetone, and ultrapure water. After removing the supernatant, the precipitates were vacuum-dried and stored in a desiccator until use.

The morphology and elemental distributions of the solids were analyzed through SEM-EDX (SU-70, Hitachi, Tokyo, Japan) at accelerating voltages of 10 or 15 kV. The crystal structure was analyzed by XRD using a Rigaku Ultima IV high-performance, multi-purpose

XRD system (Rigaku, Tokyo, Japan) at 40 kV and 40 mA.

3.3 Results and discussion

3.3.1 Isolation and identification of Sb(V) reducing bacteria

Enrichment of Sb(V)-reducing bacteria from the inoculum sludge was conducted with six sequential batch cultivations. In the 1st batch of the enrichment experiment, soluble Sb was removed sufficiently and yellowish precipitates were formed in the culture (Fig. 3-2). Thereafter, although Sb removal occurred stably, the color of precipitates gradually changed during the enrichment process and became white by the 6th batch (Fig. 3-2). According to the evidence provided in previous studies, the white precipitates were likely Sb_2O_3 formed by microbial Sb(V) reduction (Abin and Hollibaugh, 2014). These results indicated the enrichment of Sb(V)-reducing bacteria during the enrichment process.

Fig. 3-2 Appearance of the enrichment culture before the onset of enrichment (a) and after the 1st (b) and 6th batches (c).

Isolation of Sb(V)-reducing bacteria from the enrichment culture was attempted after the 6th batch with two distinct cultivation conditions. Two different types of bacterial colonies appeared on the 1/10 TSB solid media under aerobic condition, and one type appeared on L-Sb(V)-AMM solid media under anaerobic conditions. Consequently, two strains designated strains AR-1 and AR-2 and one strain designated strain AR-3 were isolated successfully by aerobic cultivation on 1/10 TSB agar medium and anaerobic cultivation on L-Sb(V)-AMM agar medium, respectively. These three strains were confirmed to possess Sb(V)-reducing abilities

(data not shown), i.e., three Sb(V)-reducing bacterial strains were obtained. Based on the partial 16S rRNA gene sequences, all three strains belonged to the order *Rhodocyclales* of the class β-*Proteobacteria*. The sequences of strains AR-1 and AR-3 (1292 and 1294 bp, respectively) shared identical sequences for 1292 bp (an additional nucleotide at both the 5′- and 3′-ends occurred in the sequence for strain AR-3) and had 99.7% and 99.8% nucleotide similarities with that of *Propionivibrio militaris* MP^T (accession No. NR_125528), respectively (Table 3-3). The 16S rRNA gene sequence of strain AR-2 (1290 bp) had 98.4% nucleotide similarity with that of *Dechloromonas agitata* CKB^T (accession No. NR_024884). Thus, the isolated strains were identified as *Propionivibrio* sp. AR-1, *Dechloromonas* sp. AR-2, and *Propionivibrio* sp. AR-3. Because of the identical 16S rRNA gene sequences, strain AR-3 was chosen from strains AR-1 and AR-3 as the representative for further characterization.

3.3.2 Physiological and biochemical characteristics of strains AR-2 and AR-3

Both strains AR-2 and AR-3 were gram-negative, rod-shaped, non-spore-forming motile bacteria, and positive for catalase, oxidase, nitrate reduction, and cytochrome oxidase tests (Table 3-3, Table 3-4). Both strains were capable of growing under both aerobic and anaerobic conditions; it should be noted that Sb(V)-reducing bacteria which can grow aerobically have never been reported before these strains. Additionally, they can grow in LB medium under the following temperature and initial pH conditions: 15–37 °C and pH 6–8.5 for strain AR-2 and 15–37 °C and pH 5–8.5 for strain AR-3. These fundamental physiological and biochemical characteristics of strains AR-2 and AR-3 were similar to those of *D. agitata* and *P. militaris* (Achenbach et al., 2001; Thrash et al., 2010), respectively. However, neither strains AR-2 nor AR-3 could assimilate most of the carbohydrates and fatty acids tested, including glucose and citrate, which required further study to explore the preferable substrates for their growth.

Table 3-3 Physiological and phylogenetic characteristics of strains AR-2 and AR-3

Strain	AR-2	AR-3
Cellular morphology	Rod (0.7–0.8 × 1.0–2.0 μm)	Rod (0.5–0.7 × 1.5–3.0 μm)
Gram stain	−	−
Spore	−	−
Motility	+	+
Catalase test	+	+
Oxidase test	+	+
O/F test	−	−
Growable temperature in LB medium	15–37 °C	15–37 °C
Growable pH in LB medium	6–8.5	5–8.5
Closest relative of 16S rRNA gene sequence	*Dechloromonas agitata* CKB^T (Similarity: 98.4%)	*Propionivibrio militaris* MP^T (Similarity: 99.8%)

+: Positive; −: Negative; O/F test: oxidation-fermentation test.

Table 3-4 Results of biochemical and assimilation tests of strains AR-2 and AR-3

Reaction/Enzyme		AR-2	AR-3
Biochemical tests	Nitrate reduction	+	+
	Indole production	-	-
	Glucose fermentation	-	-
	Arginine dihydrolase	-	-
	Urease	-	-
	Hydrolysis (esculin)	-	-
	Hydrolysis (gelatin)	-	-
	β-Galactosidase	-	-
	Cytochrome oxidase	+	+
Assimilation tests	Glucose	-	-
	L-Arabinose	-	-
	D-Mannose	-	-
	D-Mannitol	-	-
	N-acetyl-D-glucosamine	-	-
	Maltose	+	+
	Potassium gluconate	-	-
	n-Capric acid	-	-
	Adipic acid	-	-
	DL-Hydroxybutanedioic acid	-	-
	Sodium citrate	-	-
	Phenyl acetate	+	-

+: Positive, -: Negative

3.3.3 Anaerobic Sb(V) reduction by strains AR-2 and AR-3

Time courses of soluble Sb(V) and Sb(III) concentrations during anaerobic Sb reduction experiments are shown in Fig. 3-3. No significant Sb(V) reduction occurred in the abiotic control. When strains AR-2 and AR-3 were inoculated with the addition of acetate, Sb(V) concentration in the liquid phase declined concomitantly with acetate consumption after 2 d and reached 0.4–0.5 mM after 7 d (Fig. 3-3).

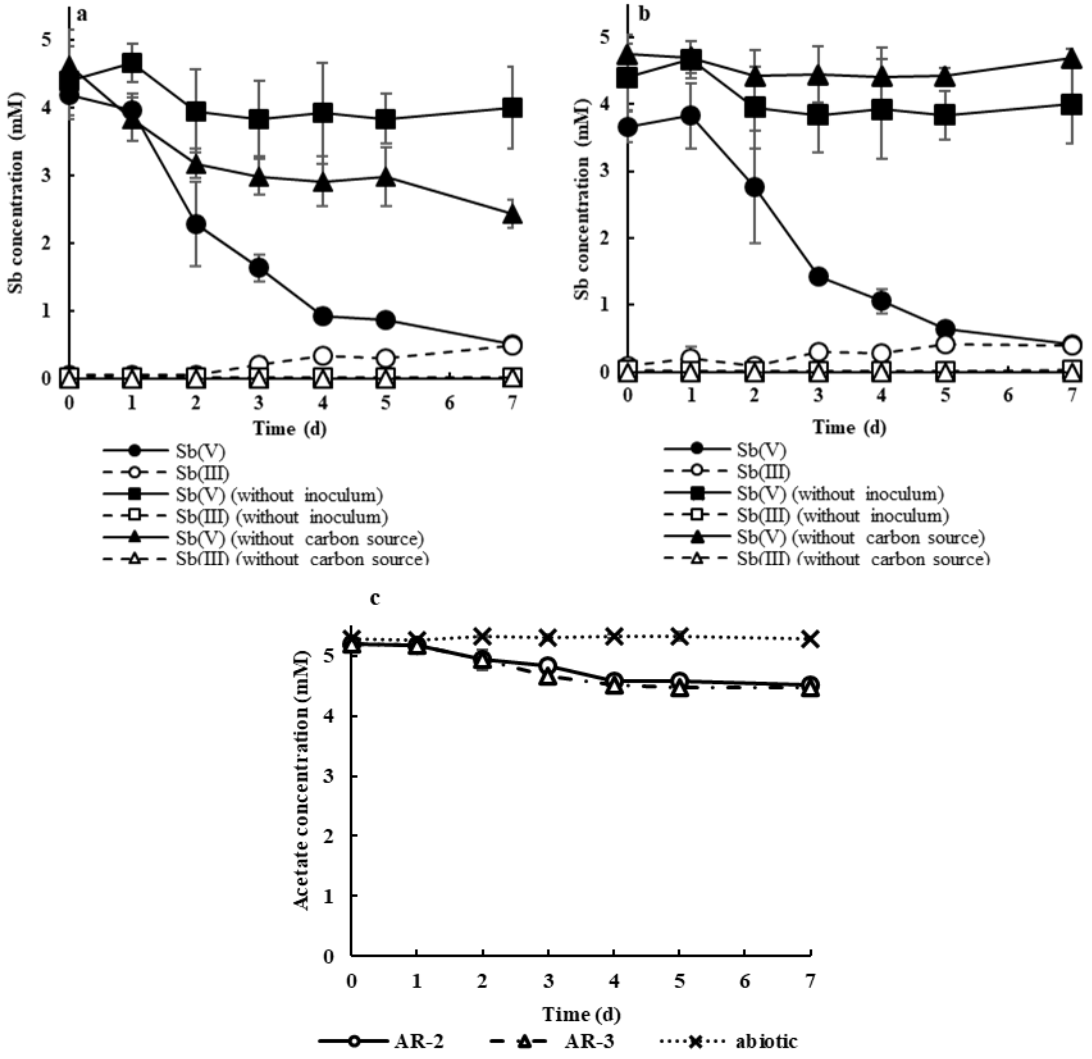


Fig. 3-3 Time course of soluble Sb(V) and Sb(III) during anaerobic Sb(V) reduction by strains AR-2 (a) and AR-3 (b) and acetate consumption (c). Error bars represent the standard deviation (n = 3).

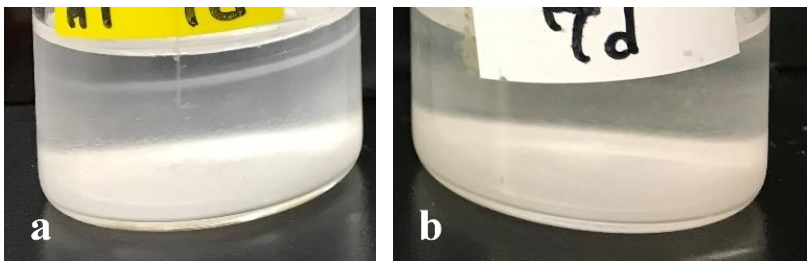


Fig. 3-4 Precipitates formed during Sb(V) reduction experiments under microaerobic (a) and anaerobic conditions (b).

Along with the decline of soluble Sb(V), Sb(III) was detected in the liquid phase and reached 0.4–0.5 mM after 7 d. Additionally, white precipitates were observed during the Sb(V) reduction (Fig. 3-4). These results indicate that both strains AR-2 and AR-3 were capable of respiratory Sb(V) reduction, and a large portion of Sb(III) formed by Sb(V) reduction was removed from the liquid phase as insoluble Sb compounds. Among the two previously isolated Sb(V)-reducing bacterial strains, *D. stibiiarsenatis* MLFW-2 could completely reduce 2 mM Sb(V) within approximately 80 h (Abin and Hollibaugh, 2014). Another Sb(V)-reducing strain, *Sinorhizobium* sp. JUK-1, reduced half of 5 mM Sb(V) after 100 h, and further reduced it to less than 0.5 mM after approximately 150 h, with the remaining of soluble Sb(III) being 3 mM (Nguyen and Lee, 2014). Thus, the Sb(V) reduction and removal abilities of strains AR-2 and AR-3 were comparable to or higher than those of previous isolates.

Anaerobic respiration using Sb(V) and acetate as the electron acceptor and donor, respectively, can be described with the following equation (Nguyen and Lee, 2014):

$$4Sb(OH)_6^- + CH_3COO^- + 3H^+ \rightarrow 4Sb(OH)_3 + 2HCO_3^- + 8H_2O$$

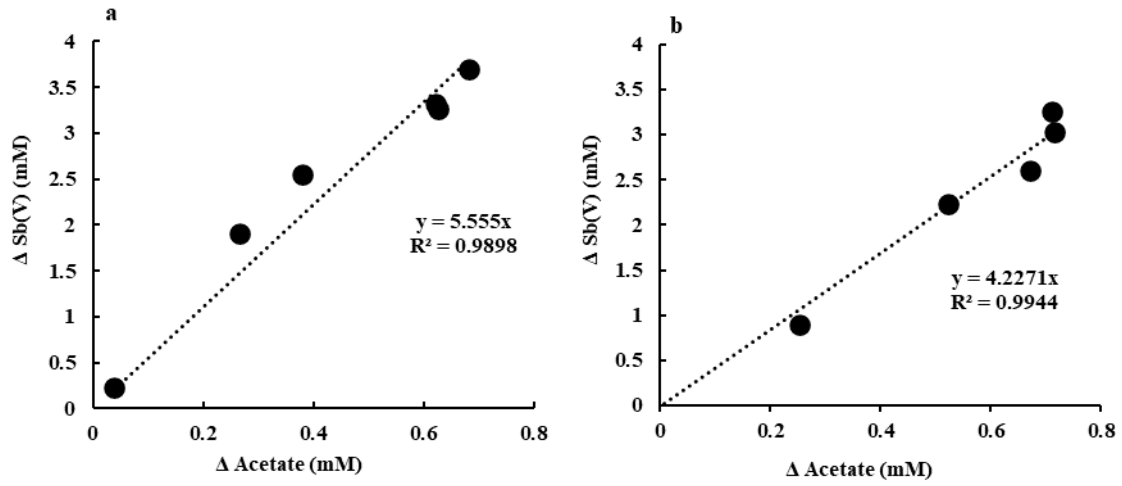


Fig. 3-5 The relationship between acetate consumption (ΔAcetate) and Sb(V) decline (ΔSb(V)) during anaerobic Sb(V) reduction by strains AR-2 (a) and AR-3 (b).

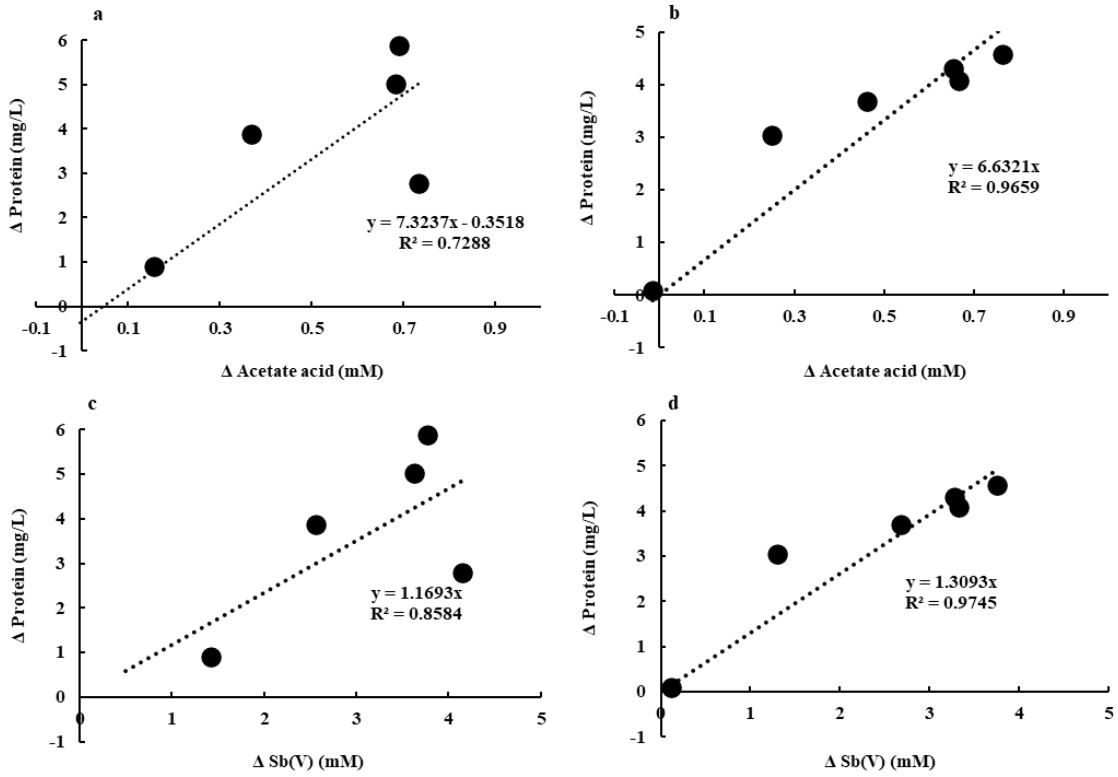


Fig. 3-6 Positive correlations between cell growth represented by the increase of protein concentration and acetate consumption (ΔAcetate) (a and b) and between cell growth and Sb(V) decline (ΔSb(V)) (c and d) during anaerobic Sb(V) reduction by strains AR-2 (a and c, respectively) and AR-3 (b and d, respectively).

According to the equation, the molar ratio of Sb(V) reduction (ΔSb(V)) to acetate consumption (ΔAcetate) during anaerobic respiration should be 4:1. As shown in Fig. 3-5, linear correlations between ΔSb(V) and ΔAcetate occurred during Sb(V) reduction with both strains. The cell growth of both strains was also correlated with each of ΔSb(V) and ΔAcetate (Fig. 3-6), which suggests the strains can obtain energy for proliferation from the reactions. The ratio of ΔSb(V) to ΔAcetate during Sb(V) reduction by strain AR-2 was 4.2:1, which was close to the theoretical value. On the other hand, the ΔSb(V)/ΔAcetate ratio during Sb(V) reduction by strain AR-2 was 5.6:1, which was higher than the theoretical value. Although Sb(V) reduction by strain AR-3 did not occur without the addition of acetate (Fig. 3-3 b), the slight Sb(V) reduction by strain AR-2 was observed even when acetate was not added (Fig. 3-3 a).

Dechloromonas sp., to which strain AR-2 belongs, has been known to accumulate polyhydroxyalkanoates (PHA) as intracellular carbon and energy storage compounds (Salineo *et al*., 2009). Thus, larger Sb(V) reduction by strain AR-2 than that estimated theoretically from acetate consumption was probably caused by utilizing PHA as an additional electron donor for anaerobic Sb(V) respiration.

3.3.4 Effects of oxygen on Sb(V) reduction by strains AR-2 and AR-3

In contrast to Sb(V)-reducing bacteria isolated in previous studies, which were obligate anaerobes (Abin and Hollibaugh, 2014; Nguyen and Lee, 2014), both stains AR-2 and AR-3 were facultative anaerobes. Therefore, the possibility of Sb(V) reduction by the strains not only under the anaerobic conditions but also under aerobic or microaerobic conditions was investigated. During the Sb(V) reduction experiment using strain AR-2 under aerobic conditions, Sb(V) reduction was not observed, although the cell density (protein concentration) increased sharply concomitant with rapid consumption of acetate (Fig. 3-7a, b and 3-8). When strain AR-3 was used, rapid growth with acetate consumption was also found and the Sb(V) concentration declined marginally from 1 to 2 d; however, Sb(III) was never detected (Fig. 3-7c) and the total soluble Sb concentration remained unchanged (data not shown).

Therefore, it is suggested that Sb(V) reduction by both strains is not possible under aerobic conditions. In contrast, obvious Sb(V) reduction by both strains was detected under the microaerobic conditions with the appearance of Sb(III) and relatively rapid cell growth (Fig. 3-7 and 3-8). Notably, Sb(V) reduction efficiency by strain AR-3 under microaerobic conditions was comparable to that under anaerobic conditions (Fig. 3-7c). Sb(V) reduction by strain AR-2 under microaerobic conditions was also similar to that under anaerobic conditions during the initial period, although it slowed during the latter period (Fig. 3-7a). These results indicated that both strains AR-2 and AR-3 were capable of Sb(V) reduction even under microaerobic

conditions (i.e., in the partial presence of dissolved oxygen). It was also worth noting that both strains were capable of growing more efficiently under microaerobic conditions than under anaerobic conditions even when Sb(V) was used as the electron acceptor.

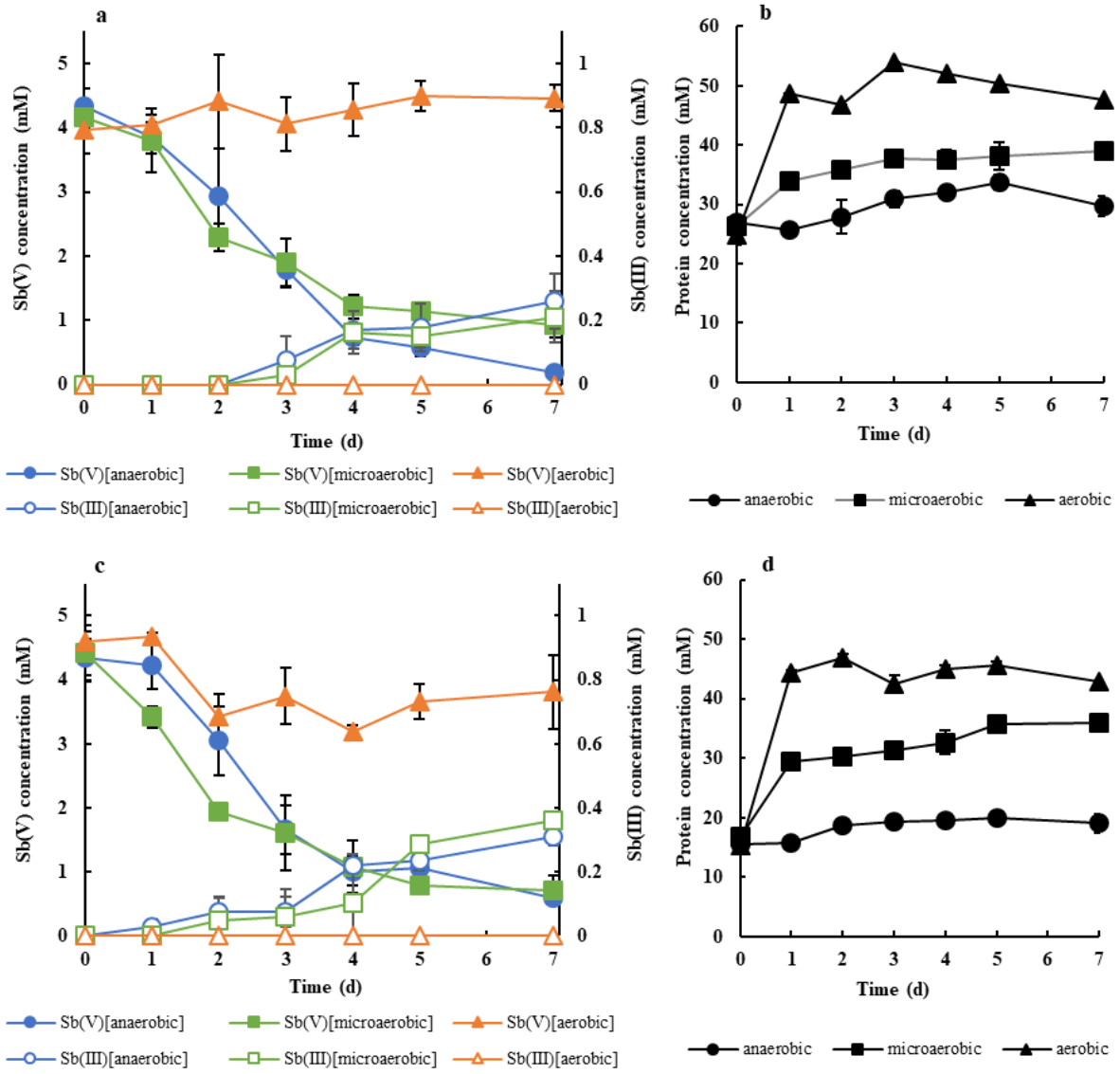


Fig. 3-7 Time course of soluble Sb(V) and Sb(III) (a,c) and protein concentrations (b,d) during Sb(V) reduction experiments under anaerobic, microaerobic, and aerobic conditions by strains AR-2 (a and b, respectively) and AR-3 (c and d, respectively). Error bars represent the standard deviation (n = 3).

To our knowledge, this was the first study indicating the possibility of microbial Sb(V) reduction without strict control of anaerobic conditions. Under microaerobic conditions, dissolved oxygen in the culture was consumed through the initial rapid growth of strains AR-2 and AR-3, which enabled the occurrence of their anaerobic respiration using Sb(V) as the electron acceptor. The Sb(V) reduction and growth abilities of the strains under microaerobic conditions present a great advantage for strains AR-2 and AR-3 in practical application for Sb(V) treatment.

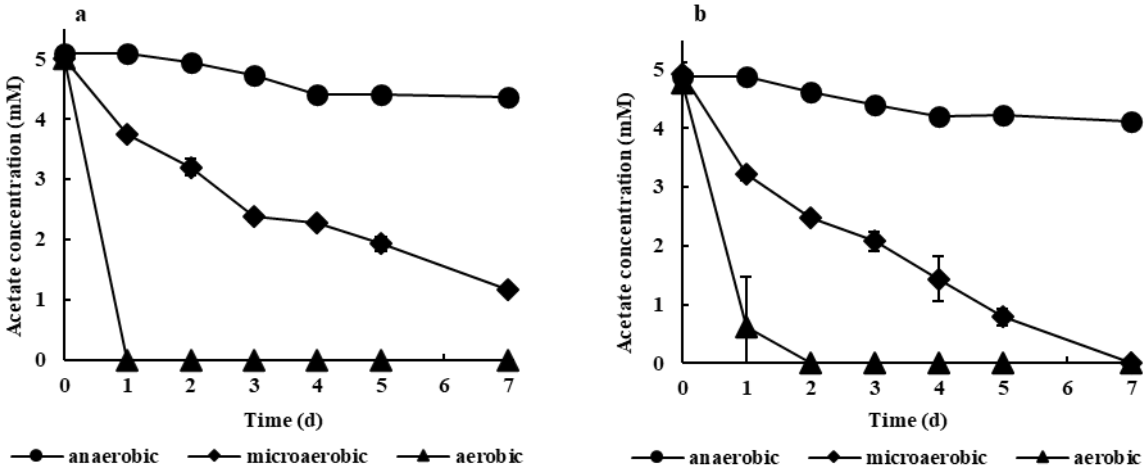


Fig. 3-8 Acetate consumption during anaerobic, microaerobic and aerobic Sb(V) reduction experiments by strains AR-2 (a) and AR-3 (b). Error bars indicate the standard deviation (n = 3).

3.3.5 Properties of precipitates formed during Sb(V) reduction

The scanning electron micrographs of white precipitates formed during anaerobic Sb(V) reduction by strains AR-2 and AR-3 are shown in Fig. 3a and b, respectively. The precipitates were produced extracellularly by both strains, and the aggregates of spherical particles measured 0.1–0.5 μm. EDX analysis showed that the particles produced by both strains comprised Sb and O (Fig. 3-9c, d), with an atomic ratio (Sb:O) of approximately 1:4 (Table 3-5). The results suggested that the precipitates were likely solid forms of Sb_2O_3 and/or $Sb(OH)_3$.

$Sb(OH)_3$ could be formed by biological reduction of $Sb(OH)_6^-$ and further dehydrated to form Sb_2O_3 (Zotov et al., 2003; Abin and Hollibaugh, 2014).

Earlier studies have reported that microbial Sb(V) reduction could form Sb_2O_3 precipitates as cubic (sénarmontite) and orthorhombic polymorphs (valentinite) or amorphous bio-minerals (Abin and Hollibaugh, 2014; Lai et al., 2016; Lai et al., 2018a; Nguyen et al., 2019). Nguyen et al. also reported the simultaneous formation of amorphous and crystallized Sb_2O_3 as products of microbial Sb(V) reduction. In contrast, Nguyen and Lee demonstrated the formation of $Sb(OH)_3$ precipitates as amorphous particles (Nguyen and Lee, 2014). Based on the XRD patterns registered in the Powder Diffraction File (PDF) database of the International Center for Diffraction Data, strong peaks of crystalline Sb compounds appeared to be detected at 2θ of 27.698, 32.077, and 45.985 (cubic Sb_2O_3; PDF#05-0534) or of 25.172, 28.382, and 28.605 (orthorhombic Sb_2O_3; PDF#11-0689) in XRD analysis. However, XRD analysis of the precipitates formed during Sb(V) reduction by strains AR-2 and AR-3 did not detect any significant peaks (Fig. 3-9e, f), which possibly resulted from the very small particle size and/or amorphous structure. Based on the similarity in the size and structure, the precipitates formed by strains AR-2 and AR-3 in this study were likely amorphous $Sb(OH)_3$ solids, as found by Nguyen and Lee (2014). Amorphous $Sb(OH)_3$ could develop into crystallized Sb_2O_3 by prolonged incubation time. Increased particle size and crystallization were observed when Sb(V) reduction experiments with strain AR-2 were extended to 35 d, whereas similar crystallization was not detected during Sb(V) reduction by strain AR-3 (Fig. 3-10), which requires further study.

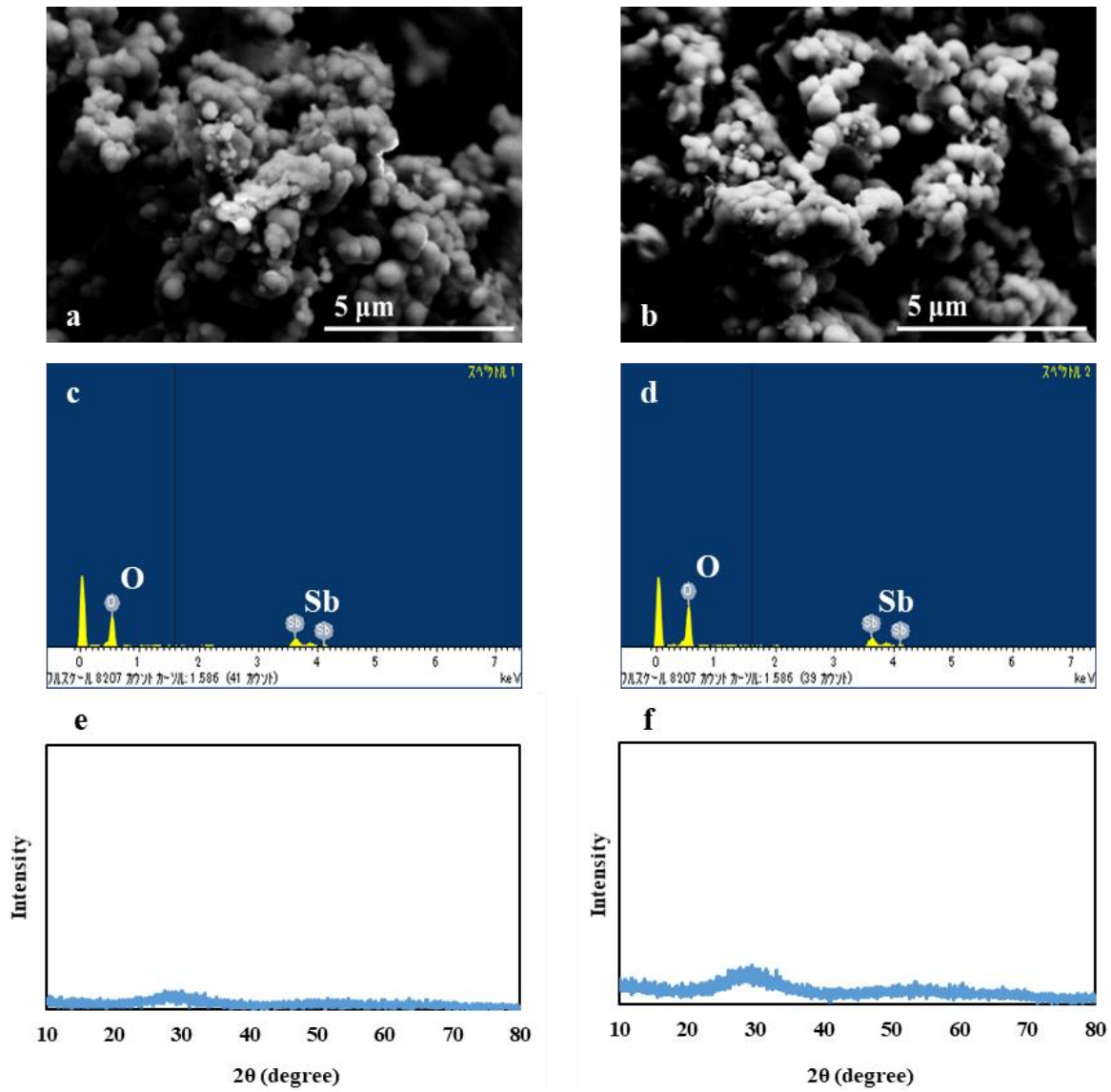


Fig. 3-9 Scanning electron micrographs (a, b), EDX spectra (c, d), and X-ray diffraction patterns (e and f) of precipitates formed during anaerobic Sb(V) reduction for 7 d by strains AR-2 (a, c and e, respectively) and AR-3 (b, d, and f, respectively).

Table 3-5 Elemental distribution in precipitates formed through anaerobic Sb(V) reduction by strains AR-2 and AR-3

Strain	Elemental Distribution in Precipitates (%)		
	O	S	Sb
AR-2	78.2	-	21.8
AR-3	82.4	0.1	17.5

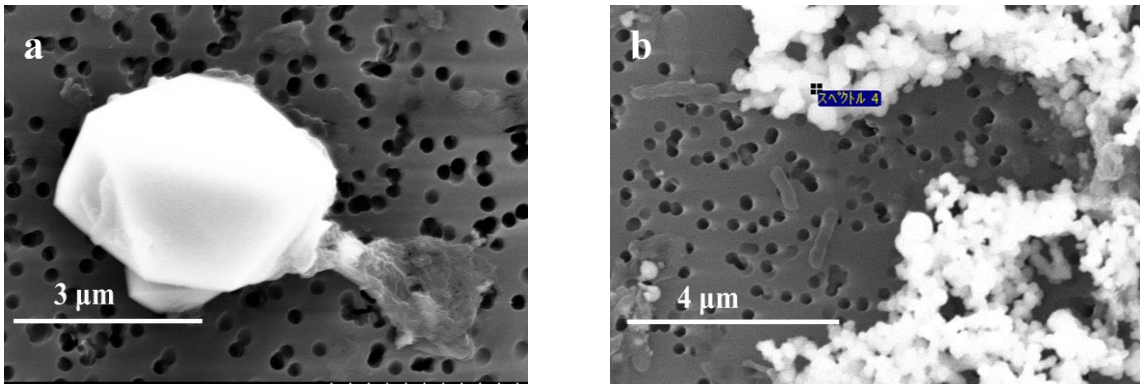


Fig. 3-10 Scanning electron micrographs of precipitates after 35 d in anaerobic Sb(V) reduction experiments using strains AR-2 (a) and AR-3 (b).

3.4 Summary

In this chapter, three novel Sb(V)-reducing bacterial strains, all of them can aerobically grow, were isolated successfully from the sludge collected from the wastewater treatment facility in an antimony products plant. Two of the isolated strains, designated *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3, were characterized for their Sb(V)-reducing ability. Both of the two strains can efficiently reduce Sb(V) and grow under anaerobic conditions, using Sb(V) and acetate as the electron accepter and donor, respectively. Meanwhile, most of the soluble Sb was removed from the aqueous phase as a result of the formation of white precipitate, which were likely amorphous $Sb(OH)_3$ solids. In addition, respiratory Sb(V) reduction by both strains occurred under not only anaerobic but also microaerobic conditions. The occurrence of Sb(V) reduction under microaerobic condition (not strict anaerobic condition) has never been reported before this study. The first (and the second) facultative anaerobic, Sb(V)-reducing bacteria, strains AR-2 and AR-3, are considered to have a great advantage in practical application to Sb(V)-containing wastewater treatment.

Chapter 4
Factors affecting antimonate bio-reduction by *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3

4.1 Introduction

In chapter 3, novel Sb(V)-reducing bacterial strains capable of growing both aerobically and anaerobically were isolated successfully. Two of the isolated strains AR-2 and AR-3 were found to respiratory reduce Sb(V) to Sb(III) not only under anaerobic conditions but also under microaerobic conditions. The evidence suggested the feasibility of the two strains to be used in practical Sb treatment and remediation. However, factors affecting their Sb(V) reduction and removal abilities were still unclarified, in spite that the knowledge is requisite for practical application.

The objective of this chapter was to investigate the effects of representative environmental factors on Sb(V) reduction by strains AR-2 and AR-3. Effects of temperature and pH and usability of different organic compounds as the electron donor were examined to clarify fundamental environmental settings for application of strains AR-2 and AR-3. In addition, their utilization of electron acceptors other than Sb(V) under anaerobic conditions was tested, and the possibility of other coexisting electron acceptors to inhibit their Sb(V) reduction were explored.

4.2 Materials and methods

4.2.1 Media

LB broth was used in the pre-cultivation of two Sb(V)-reducing bacteria strains. AMM was used for characterization of Sb(V)-reduction abilities of the strains. Unless otherwise stated, the pH of the media was adjusted to 7 with HEPES buffer, and $K[Sb(OH)_6]$ was added at 5 mM as Sb(V) source. Hereinafter, AMM supplemented with 5 mM $K[Sb(OH)_6]$ designated as Sb(V)-AMM. TSB supplemented with 5 mM $K[Sb(OH)_6]$ was also used in the investigation of effects of carbon sources on Sb(V) reduction. All cultivations were conducted in a 50 mL glass vial containing 20 mL medium with rotary shaking at 120 rpm. When cultivated under anaerobic conditions, the vial was sealed with a butyl rubber septum and an aluminum crimp and purged with nitrogen gas for 15 min.

4.2.2 Pre-cultivation

The strains were precultured and collected following the steps described in section 3.2.5. The collected cells were inoculated into the media used in each experiments to yield an $OD_{600}$of 0.05.

4.2.3 Effects of temperature and pH on Sb(V) reduction

The effects of temperature and pH on their Sb(V) reduction were examined using lactate, a common electron donor for anaerobic respiration, as the sole carbon source. The effects of temperature on Sb(V) reduction by the strains were tested at 15, 20, 25, 30 and 35 °C, temperatures at which the strains are capable of growth according to the result in section 3.3.2, with an initial pH of 7.0. The effect of pH was examined at 28 °C using Sb(V)-AMM, the pH of which was adjusted to 5.0 and 6.0 with MES buffer, 7.0 with HEPES buffer, and 8.0 with CHES buffer. An abiotic control was prepared in each experiment, and all experiments were

conducted in triplicate for 7 d.

4.2.4 Sb(V) reduction with various electron donors

The results in chapter 3 indicated the usable electron donor in the anaerobic respiration of strains AR-2 and AR-3 is considerably limited. Based on the evidence that simple fatty acids and carbohydrates can be used as electron donor in Sb(V) bio-reduction (Nguyen et al., 2018), reduction by the two strains was examined with lactate and acetate as the representative fatty acids and maltose as the utilizable carbohydrate according to the result in section 3.3.2. Each organic compound was added to Sb(V)-AMM at an initial concentration of 5 mM. TSB containing 5 mM Sb(V) was also prepared to investigate the ability of the strains to reduce Sb(V) with a complex carbon source. All cultivations were conducted at 28 °C.

4.2.5 Utilization of various electron acceptors and their effects on Sb(V) reduction

To evaluate the utilization of various electron acceptors by the isolated strains, the precultured strains were inoculated into AMM containing 5 mM of sodium acetate. Sb(V), $NaNO_3$ (nitrate), NaH_2AsO_4 (As(V)), Na_2SeO_3 (Se(VI)), Na_2SO_4 (sulfate), or $FeCl_3$ (Fe(III)) were added to the media at 1 mM, and their utilization as the electron acceptor was examined. An abiotic control was prepared for each experiment. Experiments were also conducted using Sb(V)-AMM supplemented with 1 mM of As(V) and nitrate to evaluate the effects of coexisting electron acceptors on Sb(V) reduction. All experiments were carried out in triplicate at 28 °C for 7 d.

4.2.6 Chemical analysis

The OD_{600} of the culture was determined as described in section 3.2.7.

Before the chemical analysis, the collected samples were filtered through a 0.2 μm cellulose acetate membrane filter (Advantec). Sb(V) concentration was determined by an HIC-SP ion chromatography system (Shimadzu) with Shim-pack IC-SA3 column (Shimadzu) using 3 mM Na_2CO_3 solution as the mobile phase or by HPLC-HG-AFS. Sb(III) was also determined by HPLC-HG-AFS. Concentrations of As(V), Se(VI), Se(IV), nitrate and sulfate were measured using a HIC-SP ion chromatography system with a Dionex IonPac AS4A-SC column (Thermo Fisher Scientific Inc) using 3 mM Na_2CO_3 solution as mobile phase. ICP-AES (SII Nano Technology) was used to determine the total concentration of soluble Sb, As, Se and Fe. The Fe(II) concentration was measured using UV–vis spectrophotometer (Shimadzu) at 510 nm using 1,10-phenanthroline according to the procedures of JIS K 0400-57-10. As(III) and Fe(III) concentrations were calculated as the difference between total As and As(V) concentrations and between total Fe and Fe(II) concentrations, respectively. The concentrations of organic compounds were determined with a Shimadzu LC-10A HPLC system (Shimadzu) equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories) using a mobile phase of 5.0 mM sulfuric acid.

4.3 Results and discussion

4.3.1 Effects of temperature on Sb(V) reduction

To clarify the effects of temperature on Sb(V) reduction by strains AR-2 and AR-3, Sb(V) reduction experiments were performed at various temperatures (15–35 °C). The time courses of Sb(V) and soluble Sb concentrations in all experiments are shown in Fig. 4-1 and Fig. 4-2. Temporal changes in Sb(V) reduction and Sb removal efficiency under different temperature conditions are depicted in Fig. 4-3.

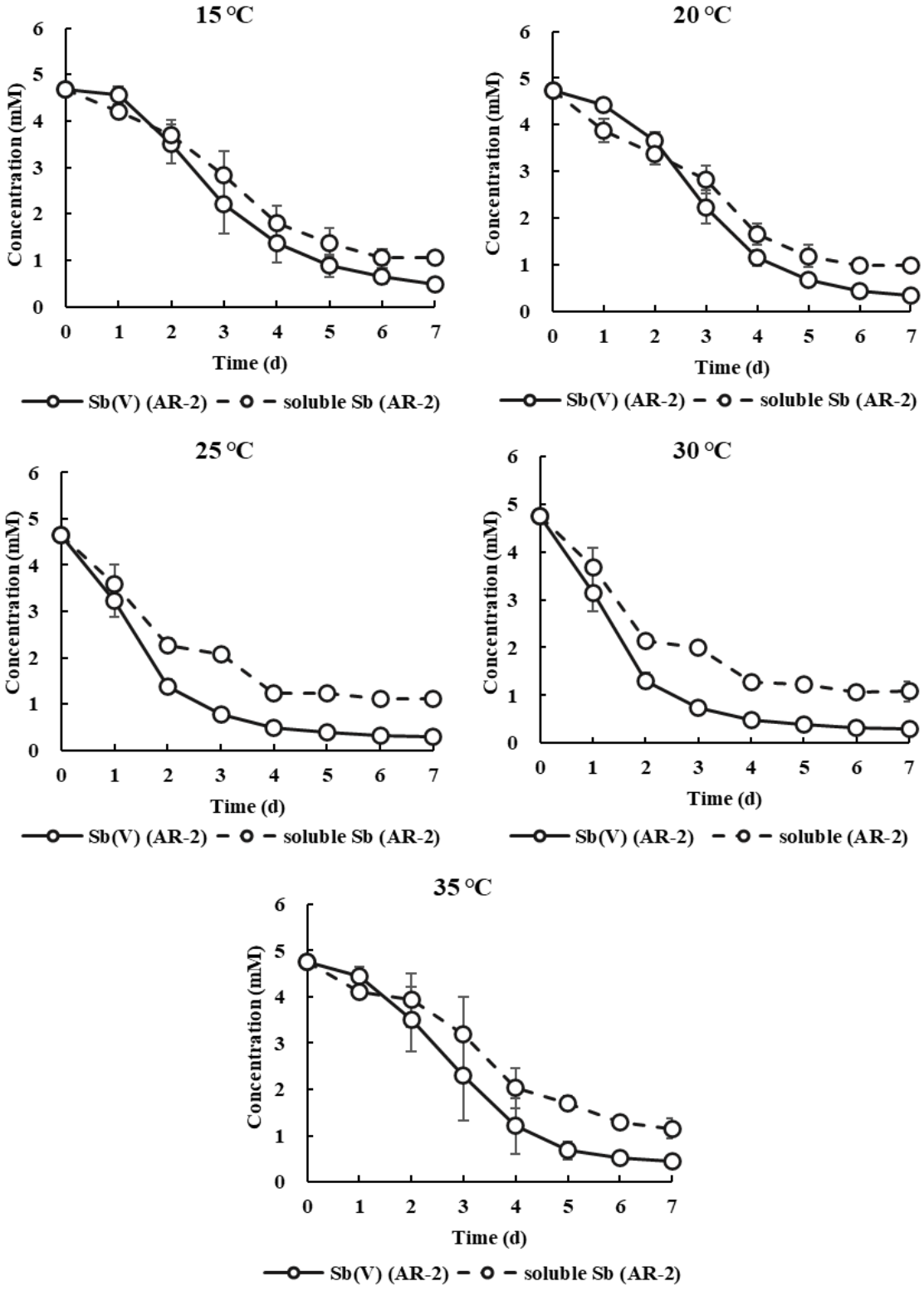


Fig. 4-1 Time course of Sb(V) and total Sb in aqueous phase under different temperature during Sb(V) reduction by AR-2.

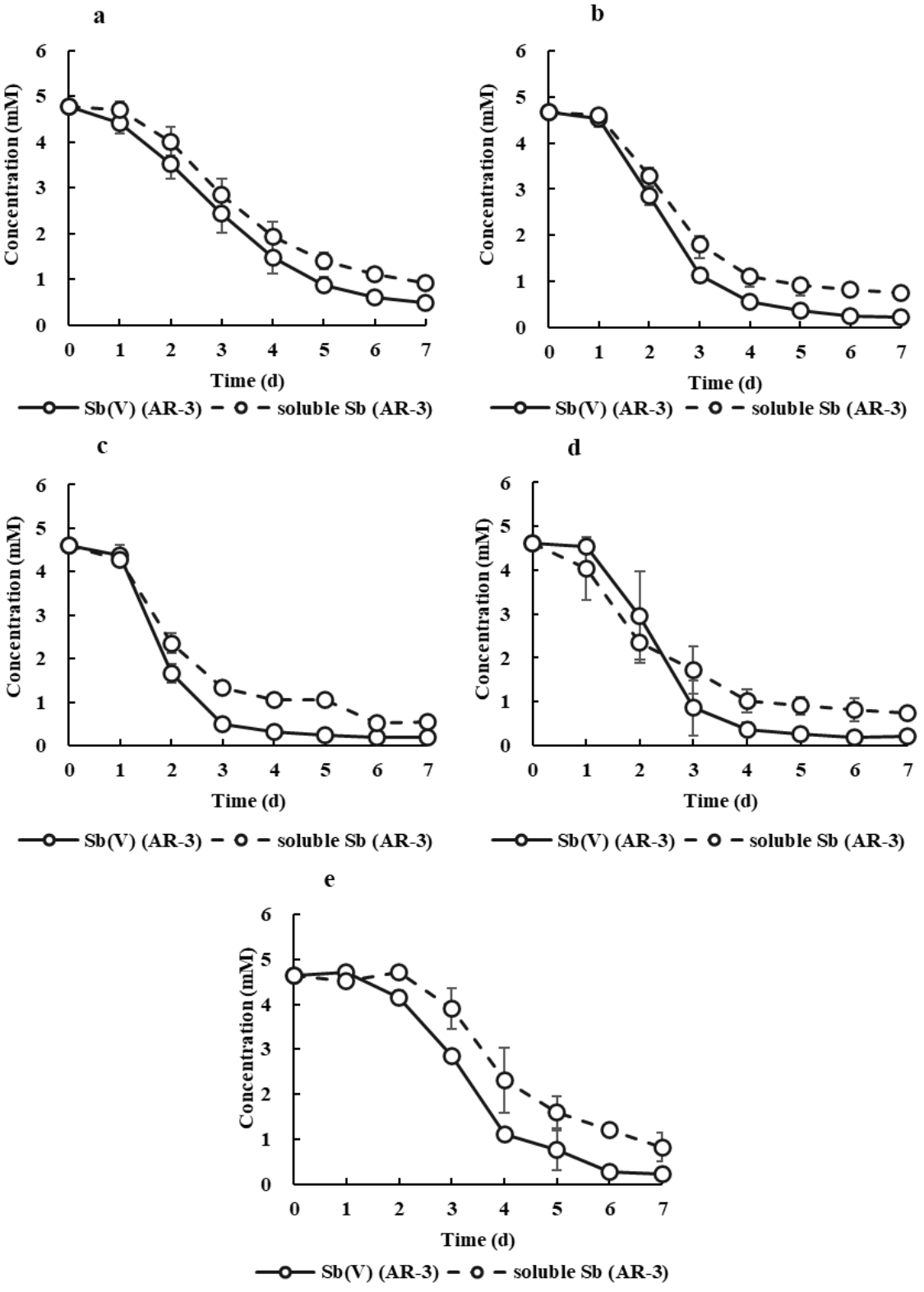


Fig. 4-2 Time course of Sb(V) and total Sb in aqueous phase under different temperature during Sb(V) reduction by AR-3.

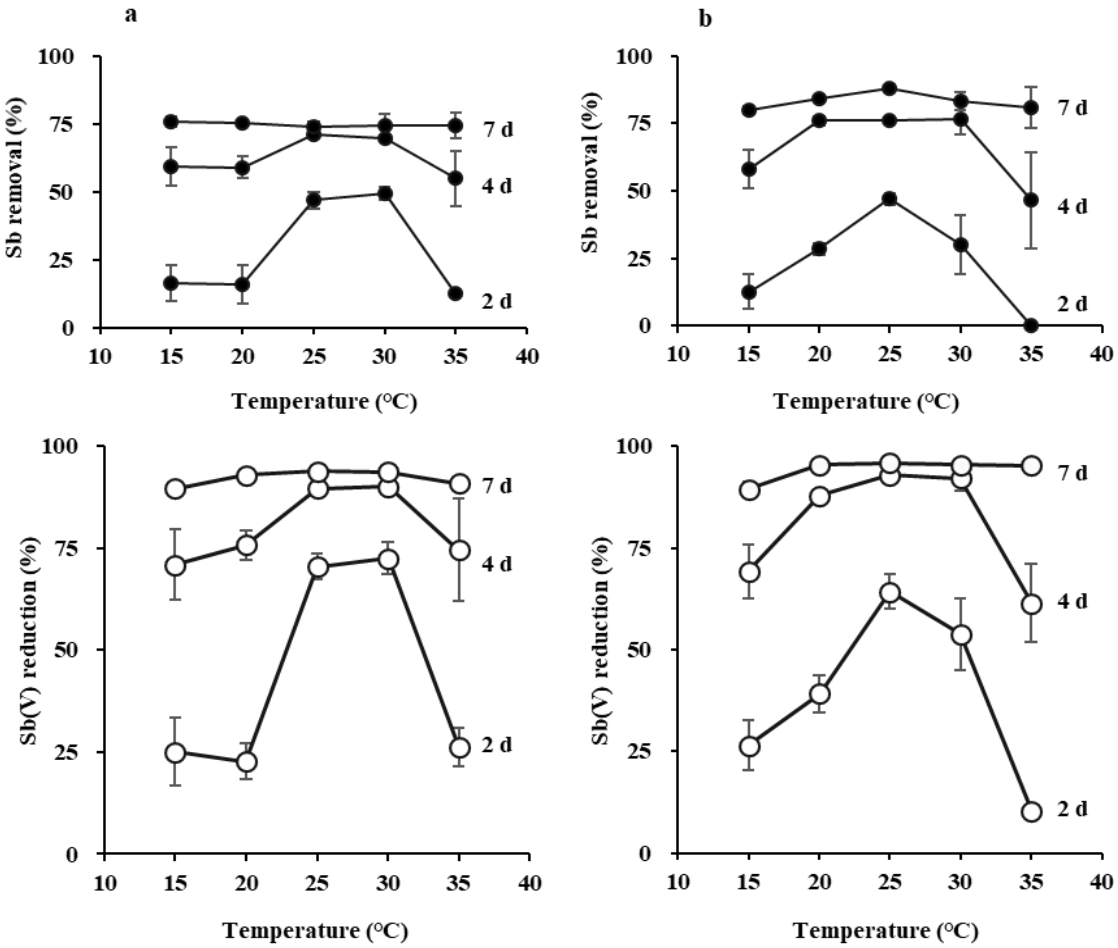


Fig. 4-3 Sb(V) reduction efficiency and Sb removal efficiency of strains AR-2 (a) and AR-3 (b) under different temperature conditions. Error bars represent the standard deviation (n = 3)

By day 7 of the experimental period, both strains AR-2 and AR-3 had reduced more than 89.6% and 89.5% of the initial Sb(V), respectively, and correspondingly removed more than 74.3% and 79.8% of the aqueous Sb, respectively, irrespective of the temperature conditions. The fact that Sb removal efficiency was slightly lower than Sb(V) reduction efficiency suggested the incomplete precipitation of generated Sb(III).

In strain AR-2, Sb(V) reduction occurred efficiently after 1 d when the temperature was set at 25–30 ℃ and reached over 70% within 2 d (Fig. 4-3a). However, Sb(V) reduction was considerably lower at lower (15 and 20 ℃) and higher (35 ℃) temperatures, with a lag of 1 d before the onset of reduction (Fig. 4-1). Regarding strain AR-3, Sb(V) reduction initiated after a lag of 1 d at all tested temperatures (Fig. 4-2). The Sb(V) reduction and removal abilities of

strain AR-3 were the highest at 25 °C, and Sb(V) reduction reached over 60 % within 2 d; they were slightly lower at 20 and 30 °C and notably lower at 15 and 35 °C (Fig. 4-3b).

From the experimental results, the optimal temperatures for Sb(V) reduction and Sb removal by strains AR-2 and AR-3 were found to be 25–30 °C and 25 °C, respectively. However, when the cultivation was prolonged to 7 d, both strains showed good performance in reducing Sb(V) and removing Sb within the tested temperature range (15–35 °C); the final Sb(V) reduction efficiencies were 89.6–93.8% and 89.5–95.9% and the final Sb removal efficiencies were 74.3–76.1% and 79.8–87.9% in strains AR-2 and AR-3, respectively. Therefore, the results suggest that both strains could be applied to treat Sb-containing wastewater under a relatively wide temperature range, that is, under normal ambient temperatures. However, as the reduction of Sb(V) at 15–20 °C and 35 °C required a relatively longer reaction time, the hydraulic retention time of the treatment process should be extended at temperatures outside of the optimal range.

4.3.2 Effects of pH on Sb(V) reduction

To clarify the effects of pH on Sb(V) reduction by strains AR-2 and AR-3, Sb(V) reduction experiments were performed using media with pH values between 5 and 8. The time courses of Sb(V) and soluble Sb concentrations in all experiments are shown in Fig. 4-4 and Fig. 4-5. Temporal variations in Sb(V) reduction and Sb removal efficiency under different pH conditions are shown in Fig. 4-6. During Sb(V) reduction experiments in which the initial pH was adjusted from 5 to 8, no notable variations in pH were observed (data not shown).

pH exerted a greater influence on Sb(V) reduction and Sb removal capabilities of both strains than did temperature, and the effects of pH on strains AR-2 and AR-3 were similar. When the pH was adjusted to 5, both strains reduced only appropriately 10% of Sb(V) and removed no aqueous Sb by day 7 of the experimental period. In contrast, efficient Sb(V) reduction and

resultant aqueous Sb removal by both strains occurred when the pH was adjusted to 6 or higher, with the highest Sb(V) reduction and removal abilities of over around 90% occurring at pH 7. At pH 6, the final Sb(V) reduction and aqueous Sb removal efficiencies after 7 d reached almost the same level as that at pH 7, although the reduction and removal speeds were slower. In contrast, at pH 8, in addition to the slowing of Sb(V) reduction and aqueous Sb removal, the final Sb(V) reduction and removal levels after 7 d were 16% to 27%, much lower than those at pH 6–7. Thus, alkaline conditions appeared to have more detrimental effects on Sb(V) reduction by both strains than did acidic conditions.

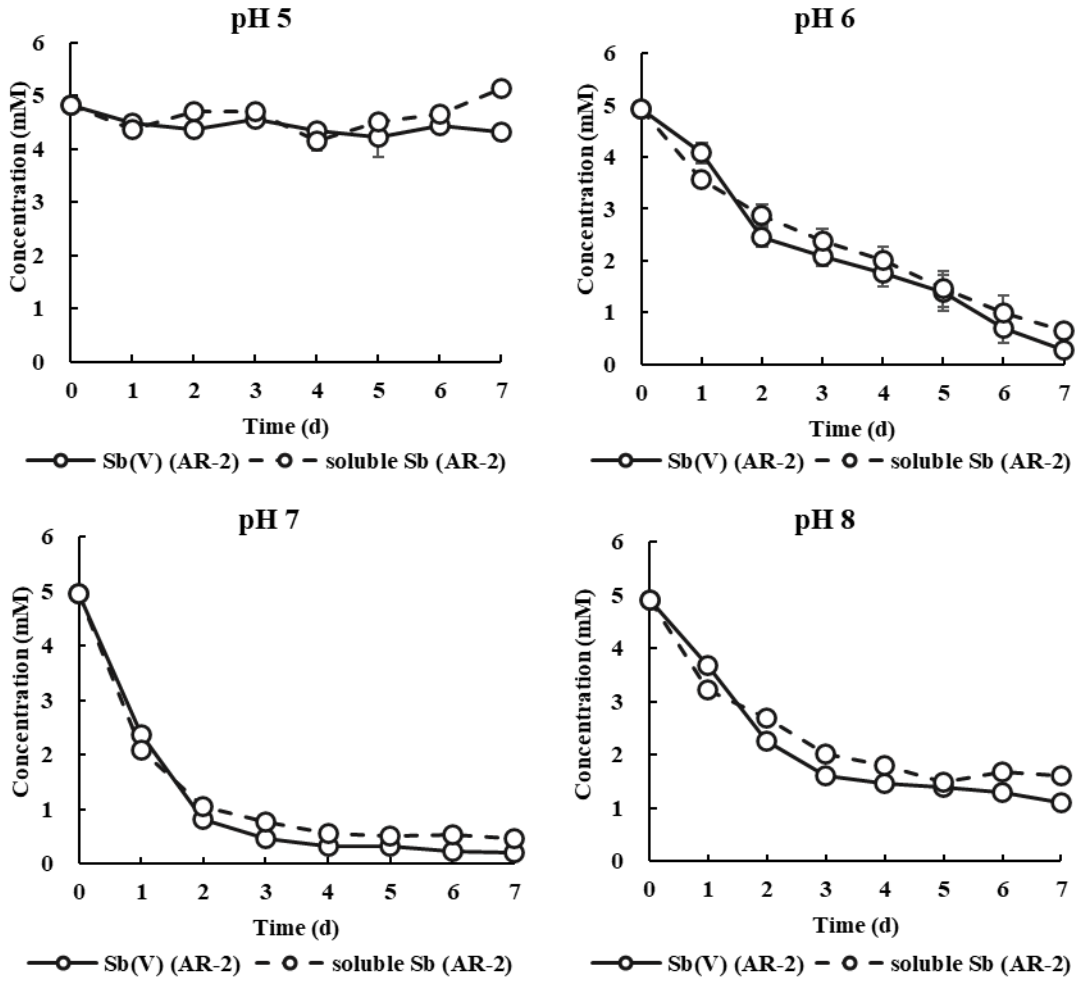


Fig. 4-4 Time course of Sb(V) and total Sb in aqueous phase with different initial pH during Sb(V) reduction by AR-2.

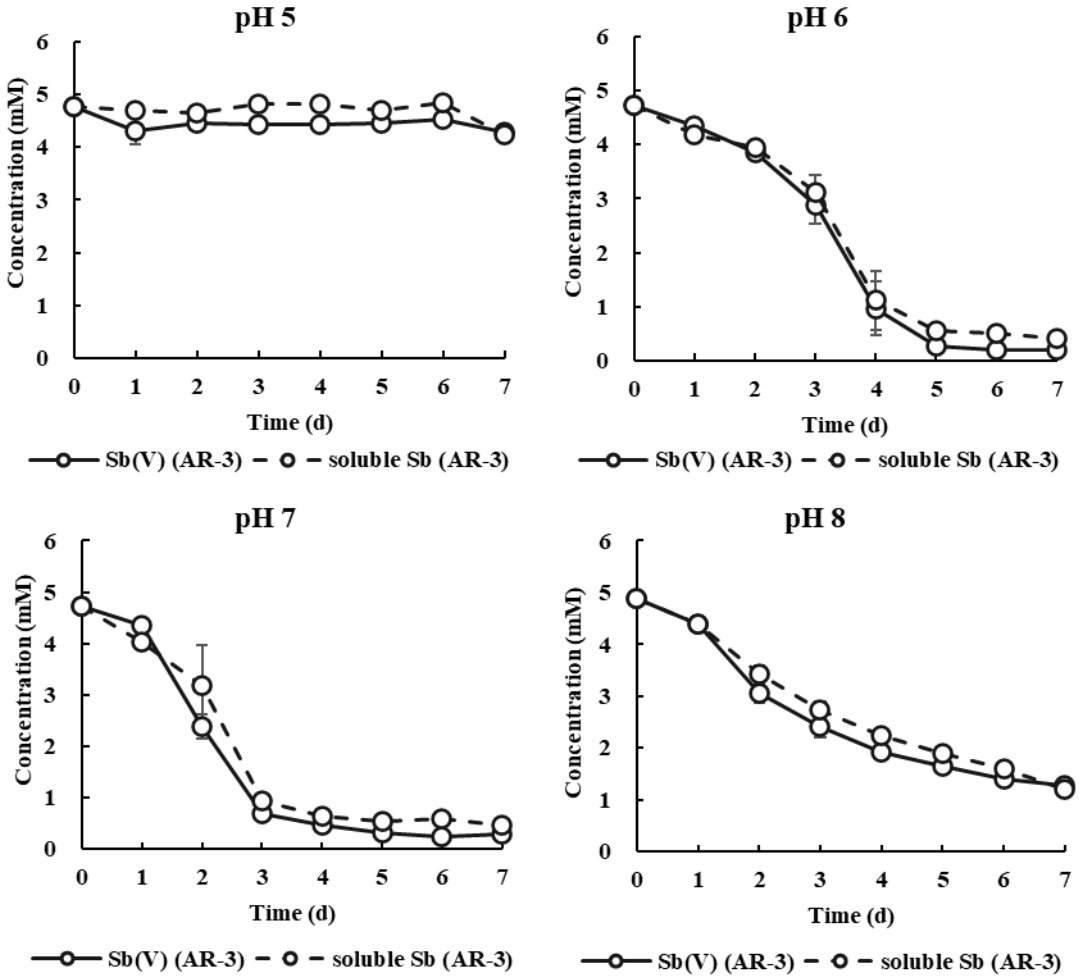


Fig. 4-5 Time course of Sb(V) and total Sb in aqueous phase with different initial pH during Sb(V) reduction by AR-3.

The results above indicated that the pH of Sb-containing wastewater should be adjusted to slightly acidic to neutral conditions before bio-treatment using strains AR-2 and AR-3. The pH of the water sample from the wastewater treatment facility where the sources of strains AR-2 and AR-3 were collected, was 6.7. In addition, previous studies showed that pH values of the wastewater and mine water from the Xikuangshan Sb mine area were within the range of 6–8 and 7.56–9.88, respectively (Zhu et al., 2011; Wen et al., 2016). Therefore, at least some actual Sb-containing wastewater can be treated without pH adjustment with strains AR-2 and AR-3, although alkaline wastewater should be neutralized before treatment.

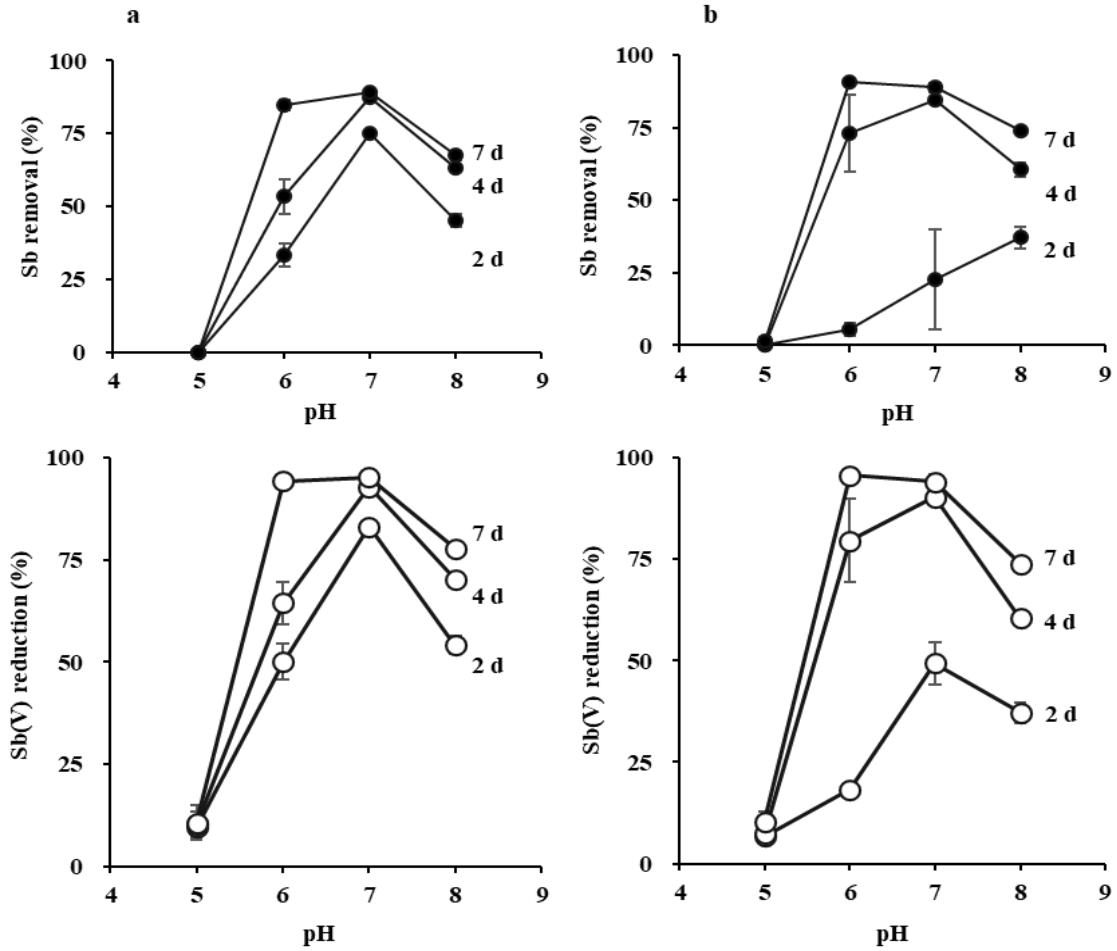


Fig. 4-6 Sb(V) reduction efficiency and Sb removal efficiency of strains AR-2 (a) and AR-3 (b) under different pH conditions. Error bars represent the standard deviation (n = 3)

4.3.3 Effects of carbon source on Sb(V) reduction

Sb(V) reduction via anaerobic respiration requires an electron donor, and the supplementation of appropriate organic compounds for use as electron donors is important for facilitating the biological treatment of Sb-containing wastewater, since such wastewater typically lacks utilizable organic compounds. Nguyen et al. (2018) investigated suitable electron donors for Sb(V) reduction by a soil microbial consortium among acetate, lactate, propionate, butyrate, glucose, inositol, and hydrogen and found that saccharides such as glucose were preferable. In contrast, optimal electron donors for Sb(V) reduction by pure Sb(V)-reducing bacteria have not been well described (Abin and Hollibaugh, 2014; Nguyen and Lee,

2014; Zhang and Hu, 2019; Wang et al., 2020). Therefore, this study selected lactate, acetate, and maltose as potential electron donors and examined their applicability for Sb(V) reduction by strains AR-2 and AR-3.

Efficient Sb(V) reduction by both strains was observed when acetate or lactate was applied, with Sb(V) reduction efficiencies of over 85% after 7 d, indicating that simple fatty acids such as acetate and lactate are preferable electron donors for Sb(V) reduction. Among the two fatty acids, lactate appeared to be a more efficient electron donor, allowing faster and higher Sb(V) reduction by both strains (Fig. 4-7). The occurrence of acetate was detected during lactate consumption by strain AR-2 (Fig. 4-7a), suggesting the transformation of lactate into acetate through anaerobic respiration as reported in previous studies (Abin and Hollibaugh, 2014; Wang et al., 2018). However, the same phenomenon was not observed in experiments employing strain AR-3 (Fig. 4-7b). Because of slightly more efficient utilization of acetate by strain AR-3 than that by strain AR-2 (Fig. 4-7c and d), strain AR-3 may quickly utilize acetate generated through lactate degradation for Sb(V) reduction. On the other hand, when maltose was supplemented, the two strains consumed very little; consumption by strain AR-3 was especially low. The resultant Sb(V) reduction efficiency of strain AR-2 within 7 d was 26.6%, and that of strain AR-3 was only 9.5%. In experiments using any of the three carbon sources (lactate, acetate, or maltose), major portions of Sb(III) generated by Sb(V) reduction were successfully removed from the aqueous phase by precipitation. When using lactate and acetate as electron donors, approximately 80% of Sb could be removed within 7 d.

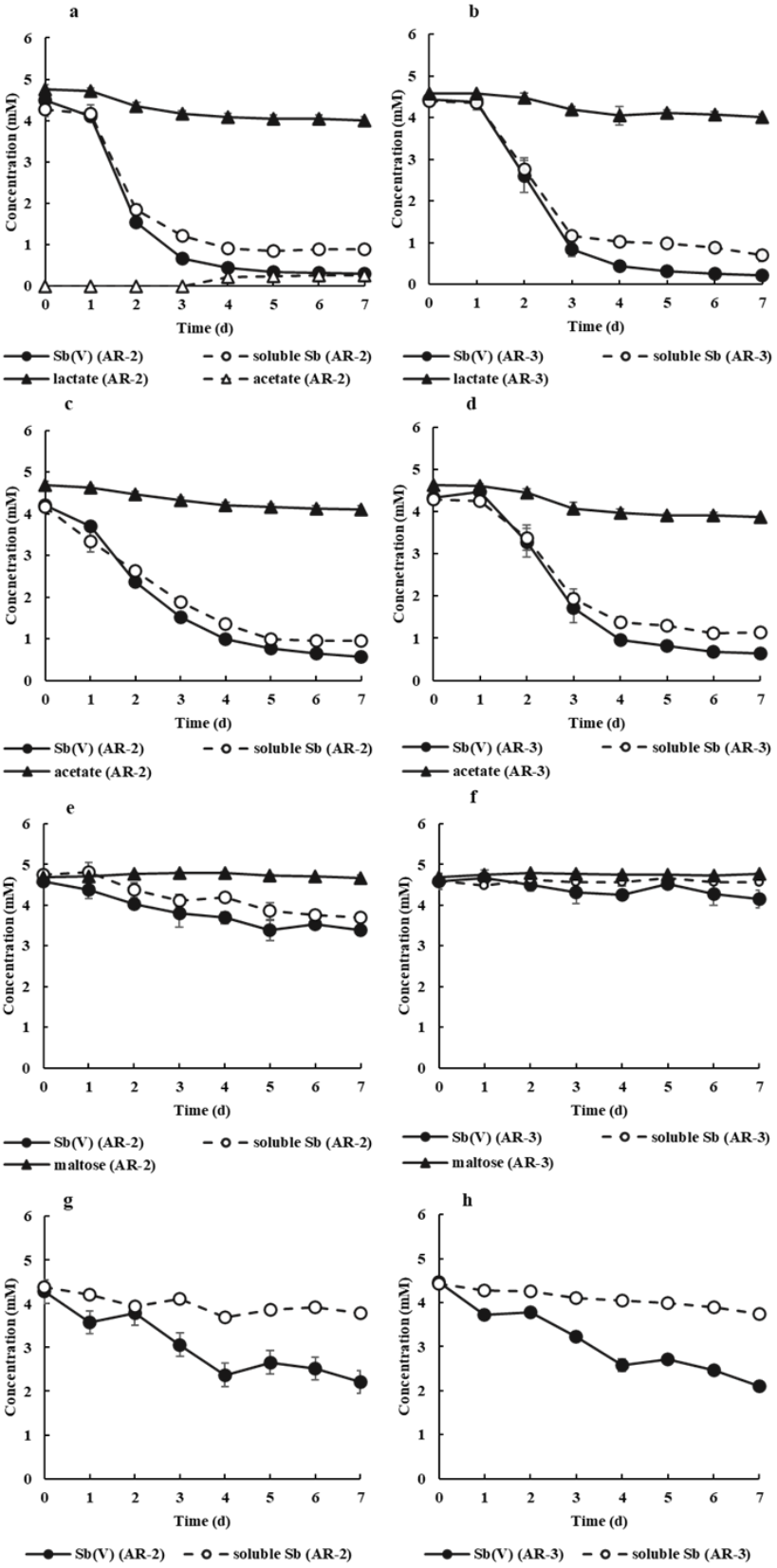


Fig. 4-7 Sb(V) reduction by the isolated strains with various carbon source. (a) AR-2 lactate; (b) AR-3 lactate; (c) AR-2 acetate; (d) AR-3 acetate; (e) AR-2 maltose; (f) AR-3 maltose; (g) AR-2 TSB; (h) AR-3 TSB.

In addition, TSB was used to investigate the use of complex proteinous organics (as a proxy for inexpensive organic wastes) as potential electron donors for Sb(V) reduction. TSB was found to support Sb(V) reduction by the two strains to a certain degree; Sb(V) reduction was about 50% after 7 d for both strains. However, soluble Sb removal was only around 15% after 7 d, probably due to inhibition of Sb(III) precipitation by some ingredients in TSB.

The obtained results suggested that the carbon sources available for effective Sb(V) reduction and removal by both strains are very limited, and only lactate and acetate are effective carbon sources. Since these simple fatty acids are relatively inexpensive and have been utilized in environmental applications as electron donors for the denitrification and dechlorination of solvents (Li et al., 2016a; Du et al., 2017a; Wang et al., 2017; Deshmukh et al., 2009), they are acceptable for practical use. However, it is still desirable to identify less costly electron donors for effective Sb(V) reduction by strains AR-2 and AR-3.

4.3.4 Utilization of other electron acceptors

Sb(V) reduction through anaerobic respiration is reported to be catalyzed by enzymes belonging to the DMSOR family (Abin and Hollibaugh, 2019). A previous study found that *D. stibiiarsenatis* MLFW-2 can reduce nitrate, nitrite, arsenate, selenate, selenite, and dimethylsulfoxide in addition to Sb(V) under anaerobic conditions owing to its DMSOR family enzymes (Abin and Hollibaug, 2019). Thus, if other electron acceptors that can be reduced by a Sb(V)-reducing strain coexist with Sb(V) in wastewater, Sb(V) reduction by the Sb(V)-reducing strain may be competitively inhibited, resulting in reduced Sb removal. In contrast, the range of electron acceptors reducible by a Sb(V)-reducing strain should vary depending on the substrate specificity of its DMSOR family enzymes. Therefore, the ability of strains AR-2 and AR-3 to reduce various electron acceptors (As(V), Se(VI), Fe(III), nitrate, and sulfate) in addition to Sb(V) was evaluated.

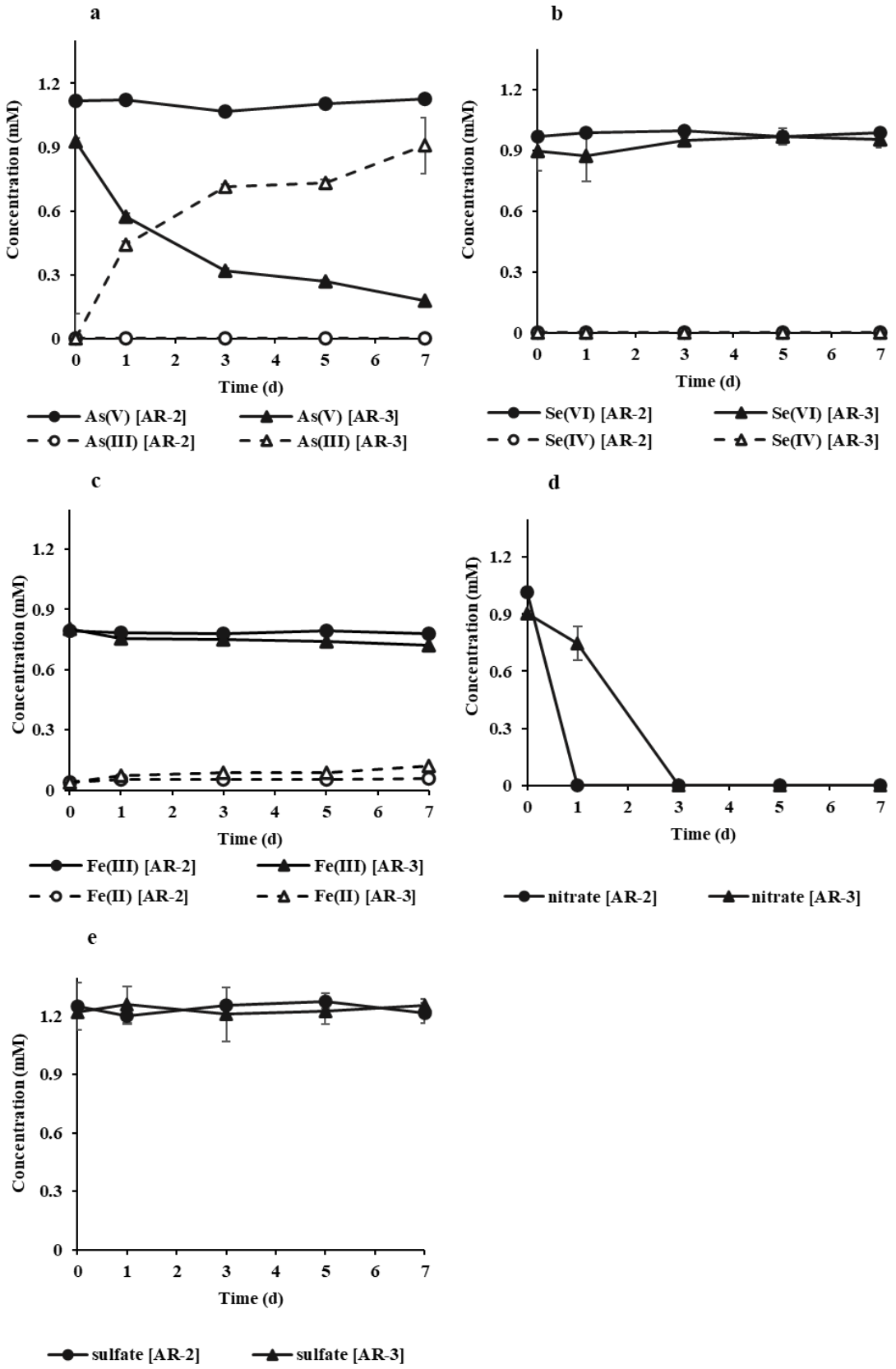


Fig. 4-8 The ability of strains AR-2 and AR-3 to utilize various electron accepters. (a) arsenate; (b) selenate; (c) Fe(III); (d) nitrate; (e) sulfate. Error bars represent the standard deviation (n = 3).

The results are shown in Fig. 4-8. Throughout the 7 d experimental period, no reduction of electron acceptors was observed in abiotic controls (data not shown). Therefore, a significant decrease in the concentration of tested electron acceptors is considered to reflect bio-reduction.

Strain AR-2 could reduce nitrate in addition to Sb(V), but did not significantly reduce As(V), Se(VI), Fe(III), and sulfate. Nitrate was rapidly reduced by strain AR-2 and was removed completely within 24 h, indicating that nitrate is an efficient electron acceptor (Fig. 4-8d).

Strain AR-3 could utilize As(V) and nitrate in addition to Sb(V) as the electron acceptor, but not Se(VI), Fe(III), or sulfate (Fig. 4-8). Unlike strain AR-2, nearly 80% of As(V) was reduced by AR-3 within 7 d with the generation of almost equimolar As(III) (Fig. 4-8a). Nitrate was reduced rapidly and removed completely within 3 d (Fig. 4-8d).

Compared with an obligate anaerobic Sb(V)-reducing bacterium, *D. stibiiarsenatis* MLFW-2, utilizable electron acceptors for strains AR-2 and AR-3 under anaerobic conditions are very limited, probably because these strains are facultative anaerobic bacteria. Because facultative anaerobic bacteria can utilize oxygen as a very efficient electron acceptor for energy acquisition, it may be possible that they have not developed versatile anaerobic respiration pathways using various electron acceptors.

4.3.5 Effects of coexisting electron acceptors on Sb(V) reduction

As Sb(V) reduction via anaerobic respiration is possibly inhibited by the presence of other electron acceptors, this study evaluated the effects of coexisting electron acceptors that can be reduced by AR-2 and AR-3 on Sb(V) reduction. Sb(V) reduction experiments in the absence and presence of electron acceptors utilizable by strains AR-2 and AR-3 were comparably performed. The results for strains AR-2 and AR-3 are shown in Figs. 4-9 and 4-10, respectively.

As shown in Fig. 4-9, coexisting nitrate did not have a significant effect on Sb(V) reduction

and soluble Sb removal by strain AR-2 overall, although Sb(V) reduction efficiency was slightly lower in the presence of nitrate at the end of the 7-d experiment. Soluble Sb(V) removal reached about 75% both in the presence and absence of nitrate. In addition, the reduction of nitrate was not inhibited by the coexistence of Sb(V) (Fig. 4-9b).

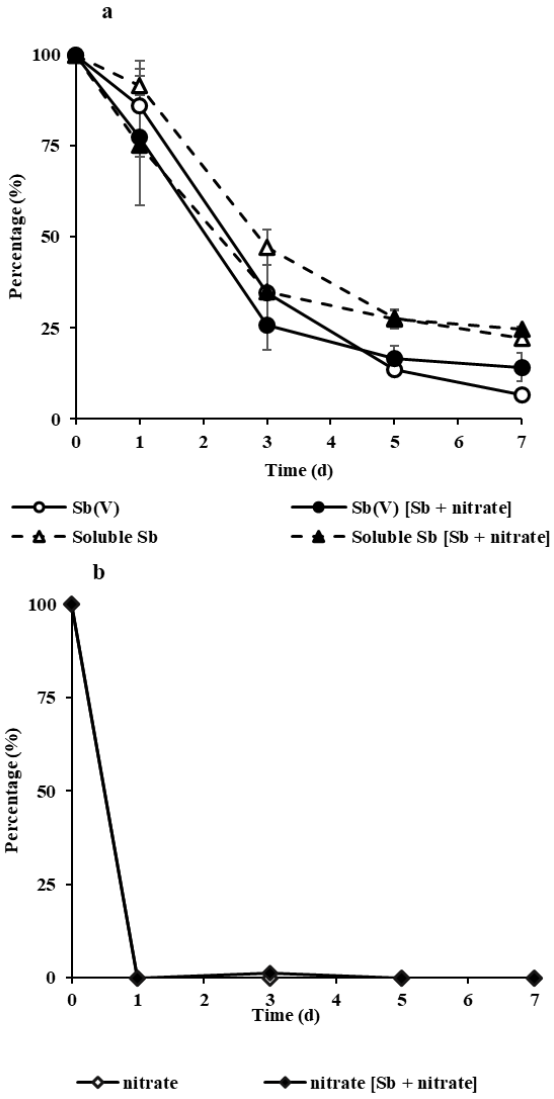


Fig. 4-9 (a) Sb(V) reduction and Sb removal by strain AR-2 in the coexistence of nitrate and (b) the change in coexisting nitrate during Sb(V) reduction. Error bars represent the standard deviation (n = 3)

The coexistence of nitrate showed a minor inhibitory effect on Sb(V) reduction by strain AR-3; Sb(V) reduction at 7 d dropped to 85% in the presence of nitrate compared with 95%

observed in its absence (Fig. 4-10c). On the other hand, the removal efficiency of soluble Sb was almost the same in the presence and absence of nitrate. In addition, nitrate reduction was not largely affected when coexisting with Sb(V) (Fig. 4-10d). The coexisting As(V) showed a slight inhibitory effect on Sb(V) reduction by strain AR-3 (Fig. 4-10a), and both Sb(V) reduction and soluble Sb removal efficiencies were slightly lower in the presence of As(V) compared with those in the absence of As(V). However, the final Sb removal efficiency was still relatively high (75%) even in the presence of As(V). As(V) reduction was not significantly influenced by the presence of Sb(V) (Fig. 4-10b).

Thus, the results indicate that the presence of other electron acceptors does not severely inhibit Sb(V) reduction and removal capability by strains AR-2 and AR-3. Therefore, although Sb(V) typically occurs with other possible electron acceptors such as As(V) and nitrate in wastewater (Zhu et al., 2011; Wen et al., 2016; Arnold et al., 2019), it seems feasible to reduce Sb(V) and remove soluble Sb from such wastewater using strains AR-2 and AR-3. Although a slight decline in Sb(V) reduction was observed in the presence of coexisting electron acceptors, this did not seem to reflect competitive inhibition. Therefore, in strains AR-2 and AR-3, the reduction of Sb(V) and the other electron acceptors (nitrate for strain AR-2 and nitrate and As(V) for strain AR-3) is likely catalyzed by distinct enzymes.

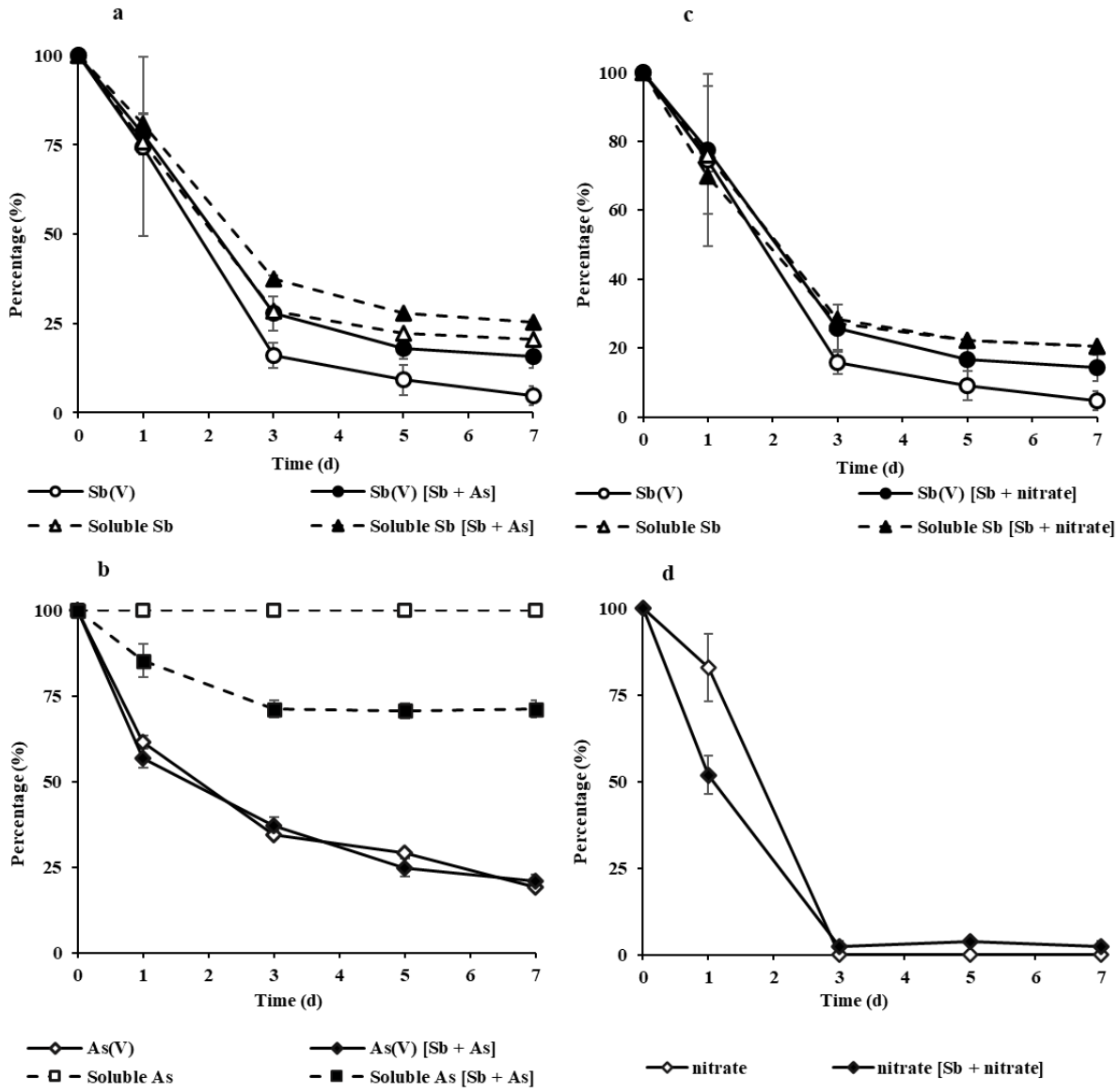


Fig. 4-10 Sb(V) reduction and Sb removal by strain AR-3 under the coexistence of other electron acceptors (a: As; c: nitrate) and the change in coexisting electron acceptors during Sb(V) reduction (b: As; d: nitrate). Error bars represent the standard deviation (n = 3)

4.4 Summary

In this chapter, the effects of temperature, pH, carbon sources (electron donors), and coexisting electron acceptors on Sb(V) reduction by strains AR-2 and AR-3 were evaluated. Efficient Sb(V) reduction and removal by both strains, with approximately 90% Sb(V) reduction and over 74% Sb removal from the aqueous phase, were observed over 7 d under a relatively wide temperature range of 15–35 ℃ and around neutral pH (6–7). Therein, optimal temperature for Sb(V) reduction by AR-2 and AR-3 was 25-30 ℃ and 25 ℃, respectively, and the highest Sb(V) reduction efficiencies of both strains were achieved when pH was 7. Carbon sources for the two strains to use in Sb(V) reduction and removal are limited, among which simple fatty acids such as lactate and acetate are more effective. In addition to Sb(V), AR-2 can also reduce nitrate, while As(V) and nitrate are available electron acceptors for AR-3. The copresence of those usable electron acceptors would not inhibit the Sb(V) reduction by the two strains. These results suggest that while work remains to identify suitable electron donors for effective Sb(V) reduction, strains AR-2 and AR-3 are useful for effective reduction and removal of soluble Sb(V) in wastewater. The limit of the application of Sb(V) bio-treatment by the two strains might be the usable carbon source. In consequence, searching for some cost-effective substrates for Sb(V) reduction should be an important subject in the next stage.

Chapter 5
Summary and conclusions

Although Sb is a kind of widely utilized metalloid, many countries and organizations have strict limits for its discharge into environment because of its high toxicity. Especially Sb pollution has become apparent recently, and the necessity of Sb removal from wastewater is emphasized. However, existing physico-chemical technologies for Sb-containing wastewater treatment are not always effective and have common problems of high-cost and huge energy-/resource-consumption. Instead, the biological treatment technology utilizing microbial Sb(V) reduction is attracting a significant concern as a promising alternative to remove soluble Sb(V), which can give the benefits of low-cost and environmental-friendliness. However, current knowledge about microbial Sb(V) reduction is still limited. Currently, there are only a few reports about the successful isolation of Sb(V)-reducing bacteria, and furthermore those isolated bacteria in previous reports seem not always effective to be utilized in wastewater treatment.

Therefore, the objective of this thesis was to isolate and characterize novel Sb(V)-reducing bacteria which can be effectively utilized in Sb wastewater treatment.

In Chapter 1, literature review was performed on the present situation of Sb pollution in aquatic environment and related regulations, existing technologies of Sb wastewater treatment, and microbial Sb(V) reduction and previously reported Sb(V)-reducing bacteria as the background of this study. Based on the review, the objective of this study was set as mentioned above.

In Chapter 2, the potential of Sb(V) reduction and removal from the aqueous phase by microbial communities existing in river sediments was evaluated to find out the environmental sample suitable for screening/isolating effective Sb(V)-reducing bacteria. Among the tested

sediment samples, microbial communities in most of the samples could reduce and remove Sb(V) in the presence/absence of high concentrations of sulfate, indicating the wide distribution of microbial Sb(V) reduction potential in the environment, irrespective of the anthropogenic impact. The Sb(V) reduction and removal abilities under different sulfate levels also suggested the presence of multiple types of Sb(V) reduction and removal pathways, including the direct Sb(V) reduction by anaerobic respiration, indirect (chemical) Sb(V) reduction by SRB, and their combination. It was also suggested that Sb(V)-reducing bacteria have higher Sb(V) reduction capability than SRB. Further it was clarified that Sb(V)-reducing bacteria can be more stably enriched from the sediment with the impact of Sb contamination than from those without significant Sb contamination. These indicated that it is recommended to use Sb-contaminated environmental samples as the source for enrichment and isolation of effective Sb(V)-reducing bacteria in the following section.

In Chapter 3, three facultative-anaerobic Sb(V)-reducing bacterial strains were isolated successfully and two of the isolated strains, designated *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3, were characterized based on their growth and Sb(V) reducing abilities in order to evaluate their potential to be utilized in Sb-containing wastewater treatment. Both of the strains can reduce 5.0 mM Sb(V) efficiently within 7 d under anaerobic conditions with acetate as electron donor. Along with Sb(V) reduction, major portion of the soluble Sb was removed from the aqueous phase as a result of the formation of white precipitates, which were likely amorphous $Sb(OH)_3$ solids. In addition, both strains could reduce Sb(V) under not only anaerobic but also microaerobic conditions, indicating that both strains possess significant potential in Sb wastewater treatment.

In Chapter 4, the factors that may affect the Sb(V) reduction by the strains AR-2 and AR-3 were investigated in order to clarify the appropriate conditions for the successful application of these strains. The effects of temperature, pH, carbon source and coexistence of other

utilizable electron acceptors were evaluated. Sb(V) was efficiently reduced and removed by both strains in 7 d at the temperature range of 15-35 ℃ and around neutral pH (6–7). In contrast, the carbon sources usable by these strains as electron donors for Sb respiration were limited to simple fatty acids such as acetate and lactate. Although strain AR-2 utilized nitrate and AR-3 utilized nitrate and arsenate as electron acceptors for anaerobic respiration in addition to Sb(V), the copresence of other electron acceptors did not inhibit Sb(V) reduction. These results suggest that strains AR-2 and AR-3 can be applied in the practical treatment of Sb(V)-containing wastewater.

In this study, novel Sb(V)-reducing bacterial strains have been successfully isolated, and *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3 were further characterized as the bio-catalysts to be utilized in Sb-containing wastewater treatment. It should be noted that strains AR-2 and AR-3 are the first (and second) Sb(V)-reducing bacteria which can aerobically grow; i.e., facultative anaerobic Sb(V)-reducing bacteria has never been reported before this study. Further it was clarified that strains AR-2 and AR-3 can efficiently reduce Sb(V) under not only anaerobic condition but also microaerobic condition for the first time. These novel, facultative anaerobic, Sb(V)-reducing bacteria strains AR-2 and AR-3, are considered to give a great advantage in Sb(V)-containing wastewater treatment. Large amounts of inoculum for bio-treatment processes can be easily obtained, because aerobic growth is much effective compared with anaerobic growth. Another significant advantage is that strict control of anaerobic condition for Sb(V) reduction is not required for utilizing these strains for wastewater treatment, which means that stable process control is easy to be achieved.

This study further investigated the environmental factors which can affect Sb(V) reduction ability of strains AR-2 and AR-3, aiming to basically evaluate the applicability of these strains to practical wastewater treatment conditions. The results indicated that Sb(V) reduction by these strains is not drastically inhibited under general wastewater treatment conditions as for

temperature, pH and co-presence of electron acceptors other than Sb(V). However, further studies are necessary to overcome clarified limitations of these strains for achieving efficient soluble Sb removal (Table 5-1), before strains AR-2 and AR-3 can be utilized in practical wastewater treatment.

Table 5-1 Further studies required for practical applications of AR-2 and AR-3

- To optimize the cultivation conditions in order to completely reduce Sb(V) and remove soluble Sb from water phase within shorter period, because a considerable portion of Sb(V) and soluble Sb remained even at the end of 7-d cultivation in this study. Possibly post-treatment technologies for removing remaining soluble Sb should be developed to achieve sufficient treatment performance like chemical coagulation and adsorption.
- To identify the possible factors which inhibit Sb(V) reduction and soluble Sb removal in real wastewaters other than low/high temperature and pH and electron acceptors (e.g., toxic metals, high salinity) and to develop pre-treatment methods to reduce their inhibitory effects.
- To find out cheaper carbon sources including organic wastes (electron donors) available for efficient Sb(V) reduction and soluble Sb removal other than lactate and acetate for cost-reduction of the treatment.
- To design the bioreactors suitable for utilizing the Sb(V)-reducing bacteria and to develop reactor operation strategies for efficient treatment. Possibly process configuration including pre-treatment and post-treatment and bioreactor should be considered.
- To test the long-term performance of treatment process(es) including the bioreactor for typical, real Sb-containing wastewater in lab- to bench-scale at first. Further step up to pilot-scale tests to upgrade the process to find out the real problem and improvement points.

References

Abin, C.A., Hollibaugh, T.J. 2014. Dissimilatory antimonate reduction and production of antimony trioxide microcrystals by a novel microorganism. Environ. Sci. Technol. 48: 681-688. https://doi.org/10.1021/es404098z

Abin, C.A., Hollibough, T.J. 2017. *Desulfuribacillus stibiiarsenatis* sp. nov., an obligately anaerobic, dissimilatory antimonate- and arsenate-reducing bacterium isolated from anoxic sediments, and emended description of the genus *Desulfuribacillus*. Int. J. Syst. Evol. Microbiol. 67: 1011-1017. https://doi.org/10.1099/ijsem.0.001732

Achenbach, L.A., Michaelidou, U., Bruce, R.A., Fryman, J., Coates, J.D. 2001. *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate reducing bacteria and their phylogenetic position. Int. J. Syst. Evol. Microbiol. 51: 527-533. https://doi.org/10.1099/00207713-51-2-527

Anderson, C.G. 2012. The metallurgy of antimony. Geochemistry 72 (Suppl. 4): 3-8 https://doi.org/10.1016/j.chemer.2012.04.001

Asaoka, S., Takahashi, Y., Araki, Y., Tanimizu, M. 2012. Comparison of antimony and arsenic behavior in an Ichinokawa River water–sediment system. Chem. Geol. 334: 1-8. https://doi.org/10.1016/j.chemgeo.2012.09.047

Asakura, K., Satoh, H., Chiba, M., Okamoto, M., Serizawa, K., Nakano, M., Omae, K. 2009. Genotoxicity studies of heavy metals: Lead, Bismuth, Indium, Silver and Antimony. J. Occup. Health 51: 498-512. https://doi.org/10.1539/joh.L9080

Arnold, M., Kangas, P., Makinen, A., Lakay, E., Isomaki, N., Laven, G., Gericke, M., Gericke, M., Pajuniemi, P., Kaartinen, T., Wendling, L. 2019. Mine water as a resource: selective removal and recovery of trace antimony from mine-impacted water. Mine Water Environ. 38: 431-446. https://doi.org/10.1007/s10230-019-00597-2

Bruce, R.A., Achenbach, L.A., Coates, J.D. 1999. Reduction of (Per)chlorate by a Novel Organism Isolated From Paper Mill Waste. Environ. Microbiol. 1: 319-329. https://doi.org/10.1046/j.1462-2920.1999.00042.x

BSR-sustainable water group. 2010. Sustainable water group water quality guidelines.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7: 335-336. https://doi.org/10.1038/nmeth.f.303

Carlin, J.F., Jr. 2000. Antimony. U.S. Geological Survey Mineral Commodity Summaries.

China. 2006. GB 5749-2006 Standards for drinking water quality.

Cidu, R., Dore, E., Biddau, R., Nordstrom, K. 2018. Fate of Antimony and Arsenic in Contaminated Waters at the Abandoned Su Suergiu Mine (Sardinia, Italy). Mine Water Environ. 37:151–165. https://doi.org/10.1007/s10230-017-0479-8

Culioli, J.L, Fouquoire, A., Calendini, S., Mori, C., Orsini, A. 2009. Trophic transfer of arsenic and antimony in a freshwater ecosystem: A field study Aquatic Toxicology. Aquat. Toxicol. 94: 286–293. https://doi.org/10.1016/j.aquatox.2009.07.016

Deshmukh, N.S, Lapsiya, K.L, Savant, D.V., Chiplonkar, S.A, Yeole, T.Y. 2009. Upflow anaerobic filter for the degradation of adsorbable organic halides (AOX) from bleach composite wastewater of pulp and paper industry. Chemosphere 75: 1179-1185. https://doi.org/10.1016/j.chemosphere.2009.02.042

Du, R., Cao, S., Li, B., Niu, M., Wang, S., Peng, Y. 2017a. Performance and microbial community analysis of a novel DEAMOX based on partial-denitrification and anammox treating ammonia and nitrate wastewaters. Water Res. 108: 46-56. http://

doi.org/10.1016/j.watres.2016.10.051

EU, 1976. Council Directive 76/464/EEC of 4 May 1976 on pollution caused by certain dangerous substances discharged into the aquatic environment of the Community. Official Journal L 129: 23–29

EU. 1998. Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption. Official Journal L 330: 0032–0054

Filella, M., Belzile, N., Chen, Y.W. 2002a. Antimony in the environment: a review focused on natural waters I. Occurrence. Earth-Sci. Rev. 57: 125–176. https://doi.org/10.1016/S0012-8252(01)00070-8

Filella, M., Belzile, N., Chen, Y.W. 2002b. Antimony in the environment: a review focused on natural waters II. Relevant solution chemistry. Earth-Sci. Rev. 59: 265–285. https://doi.org/10.1016/S0012-8252(02)00089-2

Fu, Z., Wu, F., Amarasiriwardena, D., Mo, C., Liu, B., Zhu, J., Deng, Q., Liao, H. 2010. Antimony, arsenic and mercury in the aquatic environment and fish in a large antimony mining area in Hunan, China. Sci. Total Environ. 408: 3403–3410. https://doi.org/10.1016/j.scitotenv.2010.04.031

Gagen, E.J., Zaugg, J., Tyson, G.W., Southam, G. 2019. Goethite reduction by a neutrophilic member of the alphaproteobacterial genus *Telmatospirillum*. Front. Microbiol. 10: 2938. https://doi.org/10.3389/fmicb.2019.02938

Gao, B., Lu, J., Zhou, H.D., Yin, S.H., Hao, H. 2012. The distribution, accumulation and potential source of seldom monitored trace elements in sediments of Beijiang River, South China. Water Sci. Technol. 65: 2118–2124. https://doi.org/10.2166/wst.2012.128

Government of Canada. 1997. Guidelines for Canadian drinking water quality.

Hamamura, N., Fukushima, K., Itai, T. 2013. Identification of antimony- and arsenic-oxidizing bacteria associated with antimony mine tailing. Microbes Environ. 28: 257–263.

https://doi.org/10.1264/jsme2.ME12217

Han, Y., Zhang, F., Wang, Q., Zheng, S., Guo, W., Feng, L., Wang, G. 2016. *Flavihumibacter stibioxidans* sp nov., an antimony-oxidizing bacterium isolated from antimony mine soil. Int. J. Syst. Evol. Microbiol. 66: 4676–4680. https://doi.org/10.1099/ijsem.0.001409

Hargreaves, A.J., Vale, P., Whelan, J., Constantino, C., Dotro, G., Cartmell, E. 2016. Mercury and antimony in wastewater: fate and treatment. Water Air Soil Pollut. 227: 89. https://doi.org/10.1007/s11270-016-2756-8

He, M., Wang, X., Wu, F., Fu, Z. 2012. Antimony pollution in China. Sci. Total Environ. 421: 41–50. https://doi.org/10.1016/j.scitotenv.2011.06.009

He, M., Wang, N., Long, X., Zhang, C., Ma, C., Zhong, Q., Wang, A., Wang, Y., Pervaiz, A., Shan, J. 2018. Antimony speciation in the environment: Recent advances in understanding the biogeochemical processes and ecological effects. J. Environ. Sci. 01547: 26. https://doi.org/10.1016/j.jes.2018.05.023

Herath, I., Vithanage, M., Bundschuh, J. 2017. Antimony as a global dilemma: Geochemistry, mobility, fate and transport. Environ. Pollut. 223: 545-559. https://doi.org/10.1016/j.envpol.2017.01.057

Horn, M.A., Ihssen, J., Matthies, C., Schramm, A., Acker, G., Drake, H.L. 2005. *Dechloromonas denitrificans* sp. nov., *Flavobacterium denitrificans* sp. nov., *Paenibacillus anaericanus* sp. nov. and *Paenibacillus terrae* strain MH72, N_2O-producing bacteria isolated from the gut of the earthworm *Aporrectodea caliginosa*. Int. J. Syst. Evol. Microbiol. 55: 1255-1265. https://doi.org/10.1099/ijs.0.63484-0

Hunter, W.J. 2007. An *Azospira oryzae* (syn *Dechlorosoma suillum*) strain that reduces selenate and selenite to elemental red selenium. Curr. Microbiol. 54: 376-381. https://doi.org/10.1007/s00284-006-0474-y

International Agency for Research on Cancer. 2019. Agents classified by the IARC Monographs,

Volumes 1–125. https://monographs.iarc.fr/list-of-classifications

International Finance Corporation, World Bank Group. 2007. Environmental, health and safety guidelines for glass manufacturing.

Kulp, T.R., Miller, L.G., Braiotta, F., Webb, S.M., Kocar, B.D., Blum, J.S., Oremland, R.S. 2014. Microbiological reduction of Sb(V) in anoxic freshwater sediments. Environ. Sci. Technol. 48: 218-226. https://doi.org/10.1021/es403312j

Lai, C.Y., Wen, L.L., Zhang, Y., Luo, S.S., Wang, Q.Y., Luo, Y.H., Chen, R., Yang, X., Rittmann, B.E., Zhao. H.P. 2016. Autotrophic antimonate bio-reduction using hydrogen as the electron donor. Water Res. 88: 467-474. https://doi.org/10.1016/j.watres.2015.10.042

Lai, C.Y., Dong, Q.Y., Rittmann, B.E. Zhao, H.P. 2018a. Bioreduction of Antimonate by Anaerobic Methane Oxidation in a Membrane Biofilm Batch Reactor. Environ. Sci. Technol. 52: 8693−8700. https://doi.org/10.1021/acs.est.8b02035

Lane, D.J., Pace, B., Olsen, G.J., Stahi, D.A., Sogin, M.L., Pace, N.R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. U. S. A. 82: 6955–6959. http:// doi.org/10.1073/pnas.82.20.6955

Lemire, R.J, Tosello, N.B, Halliday, J.D. 1999. Solubility behaviour of antimony(III) and antimony(V) solids in basic aqueous solutions to 300 ℃. AECL-12064.

Lenz, M., Hullebusch, E.D.V., Hommes, G., Corvini, P.F.X., Lens, P.N.L. 2008. Selenate removal in methanogenic and sulfate-reducing upflow anaerobic sludge bed reactors. Water Res. 42: 2184-2194. https://doi.org/10.1016/j.watres.2007.11.031

Li, J., Wang, Q., Oremland, R.S., Kulp, T.R., Rensing, C., Wang, G. 2016. Microbial antimony biogeochemistry: Enzymes, regulation, and related metabolic pathways. Appl. Environ. Microbiol. 82: 5482-5495. https://doi.org/10.1128/AEM.01375-16

Li, J., Zheng, B., He, Y., Zhou, Y., Chen, X., Ruan, S., Yang, Y., Dai, C., Tang, L. 2018. Antimony contamination, consequences and removal techniques: A review. Ecotoxicol.

Environ. Saf. 156: 125-134. https://doi.org/10.1016/j.ecoenv.2018.03.024

Lin, Q., Liu, E., Zhang, E., Nath, B., Shen, J., Yuan, H., Wang, R. 2018. Reconstruction of atmospheric trace metals pollution in Southwest China using sediments from a large and deep alpine lake: historical trends, sources and sediment focusing. Sci. Total Environ. 613: 331–341. https://doi.org/10.1016/j.scitotenv.2017.09.073

Macy, J.M., Thomas, A.M., Donald, G.K. 1989. Selenate reduction by a *Pseudomonas* species: A new mode of anaerobic respiration. FEMS Microbiol. Letter 61:195–198. https://doi.org/10.1111/j.1574-6968.1989.tb03577.x

Macy, J.M., Santini, J.M., Pauling, B.V., O'Neill, A.H., Sly, L.I. 2000. Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. Arch. Microbiol. 173: 49-57. https://doi.org/10.1007/s002030050007

Ministry of ecology and environment of China. 2014. Emission standards of pollutants for stannum, antimony and mercury industries.

Ministry of the environment of Japan. 2004. Monitoring substances.

Nam, J.H., Ventura, J.R., Yeom, I.T., Lee, Y., Jahng, D. 2016. A novel perchlorate- and nitrate-reducing bacterium, *Azospira* sp. PMJ. Appl. Microbiol. Biotechnol. 100: 6055-6068. https://doi.org/10.1007/s00253-016-7401-3

Nguyen, V. K., Lee, J. 2014. Isolation and characterization of antimony-reducing bacteria from sediments collected in the vicinity of an antimony factory. Geomicrobiol. J. 31: 855-861. https://doi.org/ 10.1080/01490451.2014.901440

Nguyen, V.K., Choi, W., Park, Y., Yu, J., Lee, T. 2018. Characterization of diversified Sb(V)-reducing bacterial communities by various organic or inorganic electron donors. Bioresour. Technol. 250: 239-246. https://doi.org/10.1016/j.biortech.2017.11.044

Nguyen, V. K., Park, Y. Lee T. 2019. Microbial antimonate reduction with a solid-state electrode as the sole electron donor: A novel approach for antimony bioremediation. J. Hazard.

Mater. 377: 179-185. https://doi.org/10.1016/j.jhazmat.2019.05.069

Oremland, R.S., Blum, J.S., Culbertson, C.W., Visscher, P.T., Miller, L.G., Dowdle, P., Strohmaier, F.E. 1994. Isolation, growth, and metabolism of an obligately anaerobic, selenite-respiring bacterium, strain SES-3. Appl. Environ. Microbiol. 60: 3022–3019. https://doi.org/10.1128/AEM.60.8.3011-3019.1994

Paton, G.R., Allison, A.C. 1972. Chromosome damage in human cell cultures induced by metal salts. Mutat. Res. 16: 332-336. https://doi.org/10.1016/0027-5107(72)90166-2

Peiffer, J.A., Spor, A., Koren, O., Zhao, J., Tringe, S.G., Dangl, J.L., Buckler, E.S., Ley, R.E. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. U.S.A. 110: 6548-6553. https://doi.org/10.1021/es403312j

Pierce, M., Moore, C. 1982. Adsorption of arsenite and arsenate on amorphous iron hydroxide. Water Res. 16: 1247-1253. https://doi.org/10.1016/0043-1354(82)90143-9

Resongles, E., Casiot, C., Elbaz-Poulichet, F., Freydier, R., Bruneel, O., Piot, C., Delpoux, S., Volant, A., Desoeuvre, A. 2013. Fate of Sb(V) and Sb(III) species along a gradient of pH and oxygen concentration in the Carnoul`es mine waters (Southern France). Environ. Sci. Process Impacts 15: 1536-1544. https://doi.org/10.1039/c3em00215b

Ritchie, V.J., Ilgen, A.G., Mueller, S.H., Trainor, T.P., Goldfarb, R.J. 2013. Mobility and chemical fate of antimony and arsenic in historic mining environments of the Kantishna Hills district, Denali National Park and Preserve, Alaska. Chem. Geol. 335: 172-188. https://doi.org/10.1016/j.chemgeo.2012.10.016

Salineo, K.K., Keller, K., Feil, W.S., Feil, H., Trong, S., Bartolo, G.D., Lapidus, A. 2009. Metabolic analysis of the soil microbe *Dechloromonas aromatica* str. RCB: indications of a surprisingly complex life-style and cryptic anaerobic pathways for aromatic degradation. BMC Genom. 10: 351. https://doi.org/10.1186/1471-2164-10-351

Scheinost, A.C., Rossberg, A., Vantelon, D., Xifra, I., Kretzschmar, R., Leuz, A., Funke, H., Johnson, C.A. 2006. Quantitative antimony speciation in shooting-range soils by EXAFS spectroscopy. Geochim. Cosmochim. Acta 70: 3299–3312. https://doi.org/10.1016/j.gca.2006.03.020

Sh, T., Liu, C.Q., Feng, C. 2012. Solubility, toxicity and sorption of antimony from smelter release. J. Geochem. Explor. 118: 14–18. https://doi.org/10.1016/j.gexplo.2012.03.007

Shi, L.D., Wang, M., Han, Y.L., Shapleigh, J.P., Zhao, H.P. 2019. Multi-omics reveal various potential antimonate reductases from phylogenetically diverse microorganisms. Appl. Microbiol. Biotechnol. 103: 9119-9129. https://doi.org/10.1007/s00253-019-10111-x

Shivani, Y., Subhash, Y., Sasikala, C., Ramana, C.V. 2017. *Halodesulfovibrio spirochaetisodalis* gen. nov. sp. nov. and reclassification of four *Desulfovibrio* spp. Int. J. Syst. Evol. Microbiol. 67: 87-93. https://doi.org/10.1099/ijsem.0.001574

Shtangeeva, I., Bali, R., Harris, A. 2011. Bioavailability and toxicity of antimony. J. Geochem. Explor. 110: 40–45. https://doi.org/10.1016/j.gexplo.2010.07.003

Sizova, M.V., Panikov, N.S., Spiridonova, E.M., Slobodova, N.V., Tourova, T.P. 2007. Novel facultative anaerobic acidotolerant *Telmatospirillum siberiense* gen. nov. sp. nov. isolated from mesotrophic fen. Syst. Appl. Microbiol. 30: 213–220. https://doi.org/10.1016/j.syapm.2006.06.003

Somerville, G.A., Proctor, R.A. 2013. Cultivation conditions and the diffusion of oxygen into culture media: The rationale for the flask-to-medium ratio in microbiology. BMC Microbiol. 13: 9. https://doi.org/ 10.1186/1471-2180-13-9

Strömpl, C., Tindall, B.J., Lünsdorf, H., Wong, T.Y., Moore, E.R.B., Hippe, H. 2000. Reclassification of *Clostridium quercicolum* as *Dendrosporobacter quercicolus* gen. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 50: 101-106. https://doi.org/10.1099/00207713-50-1-101

Sun, W., Xiao, E., Xiao, T., Krumins, V., Wang, Q., Haggblom, M., Dong, Y., Tang, S., Hu, M., Li, B., Xia, B., Liu, W. 2017. Response of soil microbial communities to elevated antimony and arsenic contamination indicates the relationship between the innate microbiota and contaminant fractions. Environ. Sci. Technol. 51: 9165–9195. https://doi.org/10.1021/acs.est.7b00294

Sundar, S., Chakravarty, J. 2010. Antimony Toxicity. Int. J. Environ. Health Res. 7: 4267-4277. https://doi.org/10.3390/ijerph7124267

Survey, U.S.G. 2012. Mineral commodity Summaries 2012, p. 198.

Survey, U.S.G. 2013. Mineral commodity Summaries 2013, p. 198.

Survey, U.S.G. 2014. Mineral commodity Summaries 2014, 2014, p. 196.

Survey, U.S.G. 2015. Mineral commodity Summaries 2015. U.S. Geological Survey, p. 196.

Survey, U.S.G. 2016. Mineral Commodity Summaries 2016. U.S. Department of the Interior, U.S. Geological Survey, p. 202.

Terry, L.R., Kulp, T.R., Wiatrowski, H., Miller, L.G., Oremland, R.S. 2015. Microbiological oxidation of antimony(III) with oxygen or nitrate by bacteria isolated from contaminated mine sediments. Appl. Environ. Microbiol. 81: 8478–8488. https://doi.org/10.1128/AEM.01970-15

Thrash, J.C., Pollock, J., Torok, T., Coates, J.D. 2010. Description of the novel perchlorate-reducing bacteria *Dechlorobacter hydrogenophilus* gen. nov., sp. nov. and *Propionivibrio militaris*, sp. nov. Appl. Microbiol. Biotechnol 86: 335–343. https://doi.org/10.1007/s00253-009-2336-6.

Tian, H., Zhao, D., Cheng, K., Lu, L., He, M., Hao, J. 2012. Anthropogenic atmospheric emissions of antimony and its spatial distribution characteristics in China. Environ. Sci. Technol. 46: 3973–3980. https://doi.org/10.1021/es2041465

Ungreanu, G., Santos, S., Boaventura, R., Botelho, C. 2015. Arsenic and antimony in water and

wastewater: Overview of removal techniques with special reference to latest advances in adsorption. J. Environ. Manage. 151: 326-342. https://doi.org/10.1021/es405817u

US EPA, 1979. Water related fate of the 129 priority pollutants(Vol.1). United State Environmental Protection Agency: Office of Water, Washington DC, USA

US EPA, 2009. National Primary Drinking Water Standards, Doc. 810-94-001. United State Environmental Protection Agency: Office of Water, Washington DC, USA.

Wang, H., Chen, F., Mu, S., Zhang, D., Pan, X., Lee, D., Chang, J. 2013. Removal of antimony (Sb(V)) from Sb mine drainage: Biological sulfate reduction and sulfide oxidation-precipitation. Bioresour. Technol. 146: 799-802. https://doi.org/10.1016/j.biortech.2013.08.002

Wang, L., Ye, L., Yu, Y., Jing, C. 2018. Antimony redox biotransformation in the subsurface: effect of indigenous Sb(V) respiring microbiota. Environ. Sci. Technol. 52: 1200-1207. https://doi.org/10.1021/acs.est.7b04624

Wang, J.; Gong, B.; Wang, Y.; Wen, Y.; Zhou, J.; He, Q. 2017. The potential multiple mechanisms and microbial communities in simultaneous nitrification and denitrification process treating high carbon and nitrogen concentration saline wastewater. Bioresour. Technol. 243: 708-715. http://doi.org/10.1016/j.biortech.2017.06.131

Wang, N., Zhang, S., He, M. 2018. Bacterial community profile of contaminated soils in a typical antimony mining site. Environ. Sci. Pollut. Res. 25: 141–152. https://doi.org/10.1007/s11356-016-8159-y

Wang, Q., He, M., Wang, Y. 2011b. Influence of combined pollution of antimony and arsenic on culturable soil microbial populations and enzyme activities. Ecotoxicology 20: 9–19. https://doi.org/10.1007/s10646-010-0551-7

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. 1991. 16S Ribosomal DNA Amplification for Phylogenetic Study. J. Bacteriol. 173: 697–703.

http://doi.org/10.1128/jb.173.2.697-703.1991

Wen, B., Zhou, J., Zhou, A., Liu, C., Xie, L. 2016. Sources, migration and transformation of antimony contamination in the water environment of Xikuangshan, China: Evidence from geochemical and stable isotope (S, Sr) signatures. Sci. Total Environ. 569-570: 114-122. http://dx.doi.org/10.1016/j.scitotenv.2016.05.124

WHO. 2003. Antimony in Drinking-water. Background document for development of WHO Guidelines for Drinking-water Quality. WHO Press, World Health Organization, Geneva, Switzerland.

Wilson, N., Webster-Brown, J. 2009. The fate of antimony in a major lowland river system, the Waikato River, New Zealand. Appl. Geochem. 24: 2283-2292. https://doi.org/10.1016/j.apgeochem.2009.09.016

Wu, X., Song, J., Li, X., Yuan, H., Li, N. 2011. Behaviors of dissolved antimony in the Yangtze River Estuary and its adjacent waters. J. Environ. Monit. 13: 2292-2303. https://doi.org/10.1039/c1em10239g

Xi, Y., Lan, S., Li, X., Wu, Y., Yuan, X., Zhang, C., Liu, Y., Huang, Y., Quan, B., Wu. S. 2020. Bioremediation of antimony from wastewater by sulfate-reducing bacteria: Effect of the coexisting ferrous ion. Int. Biodeterior. Biodegradation 148: 104912. https://doi.org/10.1016/j.ibiod.2020.104912

Xiao, E.Z., Krumins, V., Tang, S., Xiao, T.F., Ning, Z.P., Lan, X.L., Sun, W. 2016. Correlating microbial community profiles with geochemical conditions in a watershed heavily contaminated by an antimony tailing pond. Environ. Pollut. 215: 141–153. https://doi.org/10.1016/j.envpol.2016.04.087

Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. 2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA and whole genome assemblies. Int. J. Syst. Evol. Microbiol. 67: 1613-1617. https://doi.org/10.1099/ijsem.0.001755

Zhang, G., Liu, C. Q., Liu, H., Hu, J., Han, G., Li, L. 2009. Mobilisation and transport of arsenic and antimony in the adjacent environment of Yata gold mine, Guizhou province, China. J. Environ. Manage. 11: 1570-1578. https://doi.org/10.1039/b908612a

Zhang, G., Ouyang, X., Li, H., Fu, Z., Chen, J. 2016. Bioremoval of antimony from contaminated waters by a mixed batch culture of sulfate-reducing bacteria. Int. Biodeterior. Biodegrad: 115: 148–155. https://doi.org/10.1016/j.ibiod.2016.08.007

Zhang, H., Hu, X. 2019. Bioadsorption and microbe-mediated reduction of Sb(V) by a marine bacterium in the presence of sulfite/thiosulfate and the mechanism study. Chem. Eng. J. 359: 755-764. https://doi.org/10.1016/j.cej.2018.11.168

Zhou, J., Nyirenda, M.T., Xie, L., Li, Y., Zhou, B., Zhu, Y., Liu, H. 2017. Mine waste acidic potential and distribution of antimony and arsenic in waters of the Xikuangshan mine. China. Appl. Geochem. 77: 52–61. https://doi.org/10.1016/j.apgeochem.2016.04.010

Zhu, Y., Wu, M., Gao, N., Chu, W., An, N., Wang, Q., Wang, S. 2018. Removal of antimonate from wastewater by dissimilatory bacterial reduction: Role of the coexisting sulfate. J. Hazard. Mater. 341: 36-45. https://doi.org/10.1016/j.jhazmat.2017.07.042

Zhu, J., Wu, F., Pan, X., Guo, J., Wen, D. 2011. Removal of antimony from antimony mine flotation wastewater by electrocoagulation with aluminum electrodes. J. Environ. Sci. 23(7): 1066-1071. https://doi.org/10.1016/S1001-0742(10)60550-5

Zhang, H., Hu, X. 2019. Bioadsorption and microbe-mediated reduction of Sb(V) by a marine bacterium in the presence of sulfite/thiosulfate and the mechanism study. Chem. Eng. J. 359: 755-764. https://doi.org/10.1016/j.cej.2018.11.168

Zotov, A.V., Shikina, N.D., Akinfiev, N.N. 2003. Thermodynamic properties of the Sb(III) hydroxide complex $Sb(OH)_{3(aq)}$ at hydrothermal conditions. Geochimica et Cosmochimica Acta 67: 1821–1836. https://doi.org/10.1016/S0016-7037(00)01281-4.

Achievements

Publications

1. Yang Z., Hosokawa H., Sadakane T., Kuroda M., Inoue D., Nishikawa H., Ike M. (2020) Isolation and characterization of facultative-anaerobic antimonate-reducing bacteria. Microorganisms 8, 1435. https://doi.org/10.3390/microorganisms8091435
2. Yang Z., Hosokawa H., Kuroda M., Inoue D., Ike M. (2021) Microbial antimonate reduction and removal potentials in river sediments. Chemosphere 266, 129192. (in press) https://doi.org/10.1016/j.chemosphere.2020.129192
3. Yang Z., Sadakane T., Hosokawa H., Kuroda M., Inoue D., Ike M. Factors affecting antimonate bioreduction by *Dechloromonas* sp. AR-2 and *Propionivibrio* sp. AR-3. 3 Biotech. (under review)
4. Gan G., Liu J., Zhu Z., Yang Z., Zhang C., Hou X. (2017) A novel magnetic nanoscaled Fe_3O_4/CeO_2 composite prepared by oxidation-precipitation process and its application for degradation of orange G in aqueous solution as Fenton-like heterogeneous catalyst. Chemosphere 168, 254-263. https://doi.org/10.1016/j.chemosphere.2016.10.064

International and domestic conferences

1. Yang Z., Hosokawa H., Kuroda M., Inoue D., Ike M. (2019) Antimony-removing potential of microorganisms existing in natural water environments. Water and Environment Technology Conference 2019 (WET2019), Suita, Japan.
2. Yang Z., Hosokawa H., Kuroda M., Inoue D., Ike M. (2019) Antimony-removing potential of microorganisms existing in natural water environments. The 12th Joint Workshop Advanced Engineering Technology for Environment and Energy (AETEE), Koshu, Japan.

Acknowledgements

First of all, I would like to sincerely thank everyone who supported me to finish this research work and helped me in my daily life with kindness.

I would like to extend my sincere gratitude to my supervisor **Prof. Michihiko Ike**, for his patience, motivation and support throughout this 3 years. His instructive advice and useful suggestions on my thesis and my research work helped me a lot.

I would also express my great appreciate to **Associate Prof. Daisuke Inoue.** I am deeply grateful to his help in the revise of my thesis. Without his guidance, it would not be possible to finish those manuscripts so smoothly

I would like to gratefully thank **Assistant Professor Masashi Kuroda** for his helps and advices on my research and the publication.

I am also deeply indebted to all the other staffs in Ike Laboratory for their direct and indirect help to me.

Mrs. Maki Morita

Mrs. Yuriko Yamaoka

I gratefully acknowledge the work of past and present members of our research group for their encouragement and advice.

Mr. Hisaaki Hosokawa Mr. Takuya Sadakene

I also would like to thank other genial members in our laboratory, especially **Ms. Yu Ren** and **Ms. Jing Cai**. Thanks for their support and encouragement.

I would like to thank **Prof. Hiroshi Nishikawa,** for his help on SEM-EDS and XRD analysis in chapter 3.

I sincerely thank **NIHON SEIKO CO.,LTD.** for providing the sludge used in Chapter 3.

Finally, I would like to express my sincere gratitude to my family for their moral and financial support to me.